Bacterial peptide methionine sulfoxide reductase: co-induction with glutathione S-transferase during chemical stress conditions

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Peptide methionine sulfoxide reductase (MsrA; EC 1.8.4.6) is a ubiquitous enzyme catalysing the reduction of methionine sulfoxide to methionine in proteins, while the glutathione S-transferases (GSTs) are a major family of detoxification enzymes. A gene homologous to *MsrA* was identified in a chromosomal fragment from the bacterium *Ochrobactrum anthropi*, and this gene is located just downstream of a GST gene identified previously (*OaGST*) [Favaloro, Tamburro, Angelucci, De Luca, Melino, Di Ilio and Rotilio (1998) *Biochem. J.* **335**, 573–579]. This raises the question of whether the products of these two genes may be involved in a common cellular protection function. To test this hypothesis, the hypothetical MsrA protein has been overexpressed in *Escherichia coli* as a functional 51 kDa GST fusion protein. Following cleavage with thrombin and purification, the soluble 24 kDa protein showed MsrA activity with *N*-acetylmethionine sulfoxide as substrate, as well as with other sulfoxide compounds. Therefore polyclonal antibodies were

raised against the recombinant protein, and the modulation of MsrA in this bacterium, grown in the presence of different stimulants simulating several stress conditions, was investigated. The level of expression of MsrA was detected both by measuring the mRNA level and by immunoblotting experiments, in addition to measuring its catalytic activity. MsrA is a constitutive enzyme which is also inducible by chemical stress involving phenolic compounds such as phenol and 4-chlorophenol. Recently we reported that the GST of this bacterium, like MsrA, is only modulated by toxic chemical compounds [Favaloro, Tamburro, Trofino, Bologna, Rotilio and Heipieper (2000) *Biochem. J.* **346**, 553–559]; therefore this is the first indication of a co-induction of the MsrA and GST enzymes during chemical stress.

Key words: chemical stress, co-induction, GST, MsrA, *Ochrobactrum anthropi*.

INTRODUCTION

The oxidation of methionine residues in proteins is considered to be one of the consequences of oxidative damage to cells, which in many cases leads to the loss of biological activity [1]. The enzyme peptide methionine sulfoxide reductase (MsrA; EC 1.8.4.6) catalyses the reduction of methionine sulfoxide [Met(O)] residues in proteins to methionine [2–4], restoring specific biological properties [2–6].

The oxidation of proteins is important in many pathological conditions, such as Alzheimer disease [7], cardiovascular dysfunction [8,9] and other aging-related phenomena [10–14]. MsrA is an ubiquitous enzyme which is found in a wide variety of organisms and animal tissues [3,15,16]. The first MsrA gene was isolated from *Escherichia coli* [17]; moreover, an *E. coli* MsrA mutant seemed to be more sensitive to oxidative damage than the parent strain [18], suggesting a possible involvement of the MsrA enzyme in protecting cells from oxidative damage. MsrA has been found to be involved in many bacterial pathological conditions: MsrA is a virulence determinant for the plant pathogen *Erwinia chrysanthemi* [19], and it was found to be important for virulence in other mammalian pathogens, e.g. *Streptococcus pneumoniae* and uropathogenic *E. coli* [20].

Until now, no evidence has been presented to suggest an involvement of MsrA in the detoxification system. However, such an involvement has been proved for the glutathione S-transferases (GSTs). In fact, these latter proteins represent a major group of detoxification enzymes, and previous studies in

eukaryotic organisms have suggested that the level of expression of GSTs is an important factor in determining the sensitivity of cells to a broad spectrum of toxic chemicals [21]. To date, modulation studies on MsrA under chemical stress conditions, and possible co-regulation with GST in both prokaryotic and eukaryotic organisms, have not been reported. The present paper aims to elucidate this important aspect. In this study we use as a prokaryotic model *Ochrobactrum anthropi*, a bacterium isolated from soil contaminated by xenobiotic pollutants [22]. This bacterium contains a GST enzyme [23], a phase II enzyme of the detoxification system, that is modulated by xenobiotics [24]. A gene homologous to MsrA was identified, located just downstream of the GST gene. In the present study we describe the overexpression and characterization of the recombinant *O. anthropi* MsrA enzyme. Moreover, evidence is presented that the MsrA gene product is expressed *in vivo*, and that its expression is modulated by phenolic compounds simulating chemical stress conditions.

MATERIALS AND METHODS

Identification of the *MsrA* gene

The chromosomal DNA region of *O. anthropi* of approx. 5 kb, cloned previously [25] in pBluescript (pB-OaGST), was sequenced by using a *Taq* DyeDeoxy terminator cycle sequencing kit (Applied Biosystems). The *MsrA* gene was identified using the TFasta program [26].

Abbreviations used: as-PCR, asymmetric PCR; GST, glutathione S-transferase; Met(O), methionine sulfoxide; MsrA, peptide methionine sulfoxide reductase; ROS, reactive oxygen species; TBS, Tris-buffered saline.

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Overexpression and purification of MsrA protein

On the basis of the open reading frame coding for MsrA, two oligonucleotide primers were designed as follows: primer F (containing a *Bam*HI site; underlined), 5'-CGGGATCCATGAGTTTCTTGGACAGC-3' (sense); primer R (containing an *Eco*RI site), 5'-CGGAATTCTTATTGTGCCAGCGGGAT-3' (antisense). PCR was performed to obtain the *MsrA* gene; in addition to the template (pB-OaGST) and the primers (F and R), the 50 µl reaction mixture contained 2 mM MgCl₂, 0.2 mM of each dNTP, *Pfu* DNA polymerase buffer and 2.5 units of *Pfu* DNA polymerase (Stratagene). The PCR reaction was subjected to six cycles of amplification (60 s at 94 °C, 60 s at 48 °C, 25 s at 55 °C and 60 s at 68 °C) followed by 30 cycles of amplification (60 s at 94 °C, 60 s at 55 °C and 60 s at 68 °C). Both the amplified product and the pGEX-4T-1 expression vector (Pharmacia) were digested with *Bam*HI and *Eco*RI. The PCR fragment encompassing the complete *O. anthropi* MsrA coding region was ligated into the restricted pGEX-4T-1 by using T4 DNA ligase (Boehringer Mannheim); the resulting plasmid was designated pGEX-MsrA. An overnight culture of *E. coli* DH5α cells transformed with pGEX-MsrA was diluted 1:10 and grown until the *A*₆₀₀ reached 0.4. To induce gene transcription, isopropyl β-D-thiogalactoside was added to a final concentration of 1 mM and the incubation was prolonged for a further 5 h. The cells were collected by centrifugation (10000 g for 15 min), suspended in PBS and disrupted by cold sonication. The lysate was centrifuged (100000 g for 30 min), and the supernatant was loaded on to a glutathione-Sepharose 4B column (Pharmacia). The MsrA recombinant protein was cleaved by treatment with thrombin as described by the manufacturer (Pharmacia).

The purity of the protein was analysed using by SDS/PAGE (12.5% polyacrylamide gel) [27], and proteins were detected by silver staining.

Preparation of anti-MsrA antibodies

PBS containing 100 µg of recombinant MsrA was emulsified with Freund's complete adjuvant (1:9, v/v) and subsequently injected intraperitoneally into a Balb/c mouse. After primary immunization, the animal was given 500 µl of emulsion containing 100 µg of antigen on days 7, 14 and 21. Abdominal swelling occurred after the third or fourth injection. The ascitic fluid was then collected and used for immunoblotting.

O. anthropi cell culture conditions

The strain was cultivated in either Luria-Bertani broth or M9 minimal medium supplemented with 20 mM sodium succinate as the sole carbon source. When grown in M9 medium, 0.3 g of yeast extract/l (Difco) was added to stimulate growth. Cells were grown in 150 ml Erlenmeyer flasks containing 40 ml of medium in a horizontal shaker at 30 °C. To avoid the toxic effects of stimulant compounds (phenol, 4-chlorophenol, H₂O₂, NaCl), concentrations causing 50% growth inhibition (LD₅₀) were chosen. These were estimated previously in growth tests on *O. anthropi* according to methods described by Heipieper et al. [28].

Western blot analysis

O. anthropi cells cultivated in the presence of various stimulants (as described above) were harvested by centrifugation (10000 g for 15 min), resuspended in 10 mM potassium phosphate buffer, pH 7, and disrupted by cold sonication. The particulate material was removed by centrifugation at 100000 g for 30 min, and the

protein concentration of the supernatant was determined using the bicinchoninic acid assay (BCA; Pierce), using BSA as standard. Crude extracts (25 µg) were analysed by SDS/PAGE on 12.5% (w/v) polyacrylamide gels [27]. Proteins were transferred electrophoretically to nitrocellulose (for 3 h at 1 A) using an immunoblot transfer apparatus (Hoefer). After transfer, the nitrocellulose was incubated for 1 h at room temperature in 3% (w/v) BSA in Tris-buffered saline (TBS; 500 mM NaCl and 20 mM Tris/HCl, pH 7.5) to block non-specific binding. The blot was incubated overnight at 4 °C with 3% (w/v) BSA in TBS containing antiserum at a dilution of 1:500. After three 15 min washes with TBS containing 0.1% (w/v) BSA and 0.2% (v/v) Nonidet P40, the blot was incubated for 1 h at room temperature with peroxidase-conjugated goat anti-(mouse immunoglobulin) (Sigma) diluted at 1:1000 in 3% (w/v) BSA in TBS. The blot was again washed three times with TBS containing 0.1% (w/v) BSA and 0.2% (v/v) Nonidet P40. Antibodies were visualized using a chemiluminescence detection system (ECL[®]; Amersham).

RNA isolation

Total RNA was isolated from late-exponential-phase cells grown under specific conditions as described above. Samples (10 ml) were centrifuged (15000 g for 15 min), and the cell pellet was then resuspended in 800 µl of buffer A (20 mM sodium acetate, pH 5.5, and 1 mM EDTA) on ice. After addition of 40 µl of 10% SDS and 800 µl of hot acidic phenol (pH 5.5, 65 °C), the samples were mixed for 1 min and incubated for 10 min at 65 °C. The aqueous phase was extracted with phenol/dichloromethane (1:1, v/v; pH 7) and dichloromethane. RNA was precipitated with 3 M sodium acetate/ethanol (1:10, v/v).

Northern blot analysis

Total isolated RNA (15 µg) was separated on a 1.3% (w/v) formaldehyde/agarose gel and transferred to a nitrocellulose membrane by capillary blotting. The membranes were dried at 80 °C for 2 h. Filters were pre-hybridized at 42 °C for 3–5 h, then hybridized at the same temperature overnight in 50% (v/v) formamide, 5 × SSC (1 × SSC is 0.15 M NaCl/0.015 M sodium citrate), 0.1 M sodium phosphate and 1 × Denhardt's solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone and 0.02% BSA). The probe was radiolabelled by asymmetric PCR (as-PCR). The *MsrA* gene was obtained by digestion of pGEX-MsrA using *Bam*HI and *Eco*RI enzymes; the gene was recovered from a low-melting agarose gel and used as template for as-PCR. In addition to the template (100 ng) and the antisense R primer (500 ng), the 10 µl reaction mixture contained 1 mM MgCl₂, dNTPs (dATP, dTTP and dGTP at a concentration of 20 µM; dCTP at 10 µM), 25 µCi of [α-³²P]dCTP (3000 Ci/mmol; Amersham), *Taq* DNA polymerase buffer and 2.5 units of *Taq* DNA polymerase (Promega). The as-PCR reaction was subjected to 30 cycles of amplification (60 s at 94 °C, 120 s at 54 °C, 120 s at 72 °C). Blots were exposed to Kodak X-Omat film at –80 °C. Quantitative densitometric assays of chromatographic blot analysis of protein and mRNA were performed using laser scanning (Umax Data System Inc.) by NIH Image. Each blot was repeated at least three times, and the data shown represent typical results.

Determination of MsrA activity in *O. anthropi* cells

Crude extracts of *O. anthropi* cells were obtained as described above, and the MsrA activity of each sample was assayed using *N*-acetyl-[³H]Met(O) as substrate, as described elsewhere [29].

Activity of recombinant MsrA with various substrates

The activity of recombinant MsrA with Met(O), *N*-acetyl-Met(O) or other sulphoxide-containing compounds was measured by incubating the enzyme (20 µg) in 50 mM Tris/HCl (pH 7.4) containing 100 µmol of NADPH, 2 µg of thioredoxin reductase, 25 nmol of thioredoxin and 50 µM substrate in a final volume of 1 ml. One unit of activity is defined as 1 nmol of NADPH oxidized/min, as determined by the change in absorbance at 340 nm.

RESULTS

Identification of the *MsrA* gene, and overexpression and purification of the recombinant enzyme

Figure 1 shows the schematic map of the 5.0 kb chromosomal DNA fragment isolated previously from *O. anthropi* [25]. An open reading frame located downstream of the *OaGST* gene was identified by sequencing the whole DNA fragment. The open reading frame of 654 bp and a putative Shine–Dalgarno sequence upstream from the initiation codon are shown (Figure 1). The calculated molecular mass of the encoded protein was 24 185 Da.

Comparison of the deduced primary structure of MsrA with those of genes present in the GenBank database indicated that the greatest similarity was with those encoding prokaryotic and

eukaryotic MsrA proteins. The highest score of 60 % identity was found with *Pseudomonas aeruginosa* MsrA. Multiple alignments of the deduced amino acid sequences of the MsrA from *O. anthropi* and other MsrAs showed many conserved amino acids, including the three cysteine residues that are involved in the catalytic mechanism of the enzyme (Figure 2) [30]. These data indicated that this gene indeed codes for a MsrA protein.

In order to study the physicochemical properties, confirming the MsrA activity of this protein, and to assess its regulation in response to chemical stress, the gene was amplified by PCR from pB-*OaGST* [25], and subcloned into the pGEX-4T-1 expression vector to obtain a fusion protein with GST. Induced *E. coli* DH5α cells bearing pGEX-MsrA showed enhanced expression of a 51 kDa fusion protein (Figure 3, lane 2). Following glutathione–Sepharose affinity chromatography and thrombin treatment, the recombinant MsrA protein was purified (Figure 3, lane 3).

Characterization of the *O. anthropi* MsrA protein

To assess the ability of the recombinant MsrA to reduce protein-bound Met(O), *N*-acetyl-[³H]Met(O) was used as substrate. Crude extracts containing MsrA–GST fusion protein, as well as the purified protein, were able to reduce Met(O) to Met, confirming the presence of MsrA activity.

OANTHR	-----MSFLDSYR-----KKTLMPTDE-----	18
PSEAE	-----MVLRSSEILT-----KXSELTDPQ-----	19
BOVINE	MLSVTRRA--LQLFHSFPPIPRMGDSAAKIVSPQE-----	33
HUMAN	MLSATRRACQLLLHSLFPVPRMGNSASINVS PQE-----	35
ECOLI	-----SLF-----DKKHLVSPAD-----	13
ARATH	-MQVLVVS PPLIAAASLSKPLNLSKAALSFSRAKPICFPPQTSRRPISVYKSPMNNLFN	59
YEAST	-----	
↓		
OANTHR	--ALPGRAQPIPTSATHFVNSRPLKEPWPEGYKQVLFMGCFWGAERLFWQVP--GVYVT	74
PSEAE	--ALPGRESAMPVPEAHFVNGRPLTAPFPAGLQQVLFMGCFWGAERRLWQQP--GVVVT	75
BOVINE	--ALPGRKEPLVVAAKHHVNGNRTVEPFPEGTQMAVFGMGCFWGAERKFWTLK--GVYST	89
HUMAN	--ALPGRKEQTPVAAKHHVNGNRTVEPFPEGTQMAVFGMGCFWGAERKFWVLK--GVYST	91
ECOLI	--ALPGRNTMPVATLHAVNG-HSMTNVPDGMIAIFAMGCFWGVRLFWQLP--GVYST	68
ARATH	RLGFGSRPQAQADPSAAIAQGPDDVPSSGQQFAQFGAGCFWGVLAYQRVP--GVTKT	117
YEAST	-----MSSLISKTIKYDPAKD-----KLITLACGCFWCTEHMYRKYLNDRIVDC	44
	: : : . * * * * . *	
OANTHR	AVGYAGG-----VTPNPTYEETCTGLTGHAEVVLVVYDPKVVSLEDELLTLFWEEDHPTQ	128
PSEAE	AVGYAGG-----YTPNPTYDEVCSGLTGHSEVVLVVYNPQETSFEQLLKVFWEAHDPTQ	129
BOVINE	QVGFAGG-----YTPNPTYKEVCSGKTGHAEVVRVVFQPEHISFEELLKVFWENHDPTQ	143
HUMAN	QVGFAGG-----YTSNPTYKEVCSGKTGHAEVVRVVFQPEHMSFEELLKVFWENHDPTQ	145
ECOLI	AAGYTGG-----YTPNPTYREVCSGDTGHAEVRIVYDPSVISYEQLLQVFWENHDPAQ	122
ARATH	EVGYSHG-----IVHNPSEYEDVCTGTHNEVVRVQYDPKECSFESLLDVFWNRHDPTT	171
YEAST	KVGYANGEESKKDSSSVSYKRVCGGDTDEAEVLQVSYNPKVITLRELTDFEFRIHDPTT	104
	. * : : * : : * : * : : : : : : : : * : * : : * : * : * : *	
OANTHR	GMRQNDIGTYSRVIYTFDKADRDVAEKSREAYSQ-ALAGRGLGPITTEIEDAPELYYA	187
PSEAE	GMRQGGDIGTQYRSVIYTFDAAQKAAMASRESFQA-ELAKAGYDRITTEIADVPFFYYA	188
BOVINE	GMRQNDHGSQYRSIYPTSAEHVGAALKSKEDYQK-VLSEHGFLITTDIREGQTFYYA	202
HUMAN	GMRQNDHGTQYRSIYPTSAKQMEALSSKENYQK-VLSEHGFGPITTDIREGQTFYYA	204
ECOLI	GMRQNDHGTQYRSIYPLTPEQDAAARASLERFQAAMLAADDDRHITEIANATPFYYA	182
ARATH	LNRQGGDVGTQYRSIYTYTDEQERIAEAEEKQKQK-ILNKR----IVTEILPATKFYRA	226
YEAST	SNSQGPDKGTQYRSGLFAHSDADLKLAKIKEEWQF----KWGNKIATVIEPIKNFYDA	159
	* * * * : * : : . * . * * * : * *	
↓ ↓		
OANTHR	EDYHQYLAQN-PNGYCYLGRGTGVSCPIPLAQ	218
PSEAE	EAYHQYLAQN-PNGYCYLGGTGVCLPA----	215
BOVINE	EDYHQYLSKD-PDGYCYLGGTGVSCPLGIKK	233
HUMAN	EDYHQYLSKN-PNGYCYLGGTGVSCPVGIKK	235
ECOLI	EDDHQYLAHN-PYGYCYGIGGIGVCLPPEA--	211
ARATH	ENYHQYLAKGGRMGLRQSAEKCKDPIRCYG	258
YEAST	EEYHQLYLDKN-PQGYACPTHYLRREM-----	184
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Figure 2 Amino acid sequence alignment of *O. anthropi* MsrA with prokaryotic and eukaryotic MsrAs

The amino acid sequences of MsrAs from several organisms are shown. The locations of the cysteine residues involved in catalysis in other MsrAs are indicated by arrows. Abbreviations: OANTHR, *O. anthropi* (the present study); PSEAE, *Pseudomonas aeruginosa* (accession no. NP253705); BOVINE, *Bos taurus* (P54149); HUMAN, *Homo sapiens* (CAB59628); ECOLI, *Escherichia coli* (P27110); ARATH, *Arabidopsis thaliana* (P54150); YEAST, *Saccharomyces cerevisiae* (P40029). The asterisks indicate identical residues, colons indicate conserved substitutions, and periods (.) indicate semi-conservative substitutions.

Figure 3 SDS/PAGE analysis of fractions obtained during the purification of recombinant MsrA protein

Proteins were detected by silver staining. Lane 1, total cellular extracts of *E. coli* DH 5 α (pGEX-MsrA) before induction (3 μ g); lane 2, same as lane 1, but after induction with isopropyl β -D-thiogalactoside (the arrow indicates the expression of the GST-MsrA fusion protein); lane 3, MsrA protein (0.5 μ g) eluted from the GSH-agarose affinity resin after treatment with thrombin; lane 4, molecular mass standards (from top to bottom): BSA (69 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa) and trypsin inhibitor (21.5 kDa).

A more detailed study of substrate specificity showed that the enzyme was also able to reduce free D- and L-Met(O), as well as a wide variety of sulphoxide derivatives, as shown in Table 1. These results agree with previous studies on the substrate specificities of *E. coli* MsrA [31].

Transcriptional analysis of the *MsrA* gene

To evaluate the possible co-induction of *MsrA* with the *GST* gene, which has been demonstrated previously to be mono-cistronically transcribed and induced only by chemical stress [24], we determined the transcript size of the *MsrA* gene and the modulation of its mRNA. Total RNA from *O. anthropi* cultivated in minimal medium with succinate as carbon source and in the presence of various stimulants (e.g. NaCl for osmotic stress, hydrogen peroxide for oxidative stress and phenol for chemical stress) was subjected to Northern analysis. The RNAs were hybridized to a specific antisense probe obtained by as-PCR (see

Table 1 Specific activity of MsrA from *O. anthropi* towards various substratesValues are means $\pm$ S.D., calculated from three replicates.

Substrate	Specific activity (nmol/min per mg)
<i>N</i> -Acetyl-Met(O)	21.8 $\pm$ 1.05
<i>S</i> -($-$)-Methyl <i>p</i> -tolyl sulphoxide	25.2 $\pm$ 1.11
Tetramethylene sulphoxide	15.5 $\pm$ 0.91
<i>D</i> -Met(O)	24.1 $\pm$ 1.20
<i>L</i> -Met(O)	23.8 $\pm$ 1.12
DMSO	9.6 $\pm$ 0.43
Diphenyl sulphoxide	8.9 $\pm$ 0.39
<i>L</i> -Ethionine sulphoxide	9.4 $\pm$ 0.48

Figure 5 Immunochemical analysis of the cytosolic fraction of *O. anthropi*

Samples of 25 μ g of crude extract were loaded per lane for Western blot analysis. C, control; 4-CP, 4-chlorophenol; RES, resorcinol; CAT, catechol; PHE, phenol; r-MsrA, recombinant MsrA of *O. anthropi*.

Table 2 Relative MsrA activities in *O. anthropi* after treatment with various chemicals

MsrA activities were measured in cell extracts prepared as described in the Materials and methods section. Data are expressed as the mean percentage variability ($\pm$ S.D.) from at least three independent experiments. Results were compared using Student's *t* test: **P* < 0.0005, ***P* < 0.0001.

Treatment	Activity (%)
Control	100 $\pm$ 1.2
NaCl	98 $\pm$ 4.7
Hydrogen peroxide	101 $\pm$ 3.7
4-Chlorophenol	263 $\pm$ 6.2*
Phenol	250 $\pm$ 3.8**

Figure 4 Northern blot analysis of total RNA from *O. anthropi* exposed to chemical shock (phenol) or other stresses

(A) Modulation of the MsrA transcript. Cells were exposed to 5 mM phenol (PHE), 4 mM H_2O_2 or 0.4 M NaCl, or left untreated (lane C). (B) Ethidium bromide staining of 23 S and 16 S rRNAs.

the Materials and methods section). As reported in Figure 4, the probe hybridized with an RNA transcript of approx. 850 nucleotides. The size of the MsrA mRNA was in agreement with the length of the open reading frame, indicating that the *MsrA* gene, like the *GST* gene, appears to be monocistronically transcribed. These results confirmed that the gene was transcribed under physiological conditions; moreover, its transcription was only induced by chemical stress conditions, such as exposure to phenol. On the other hand, the level of MsrA mRNA did not seem to be influenced by general stress conditions, such as osmotic or oxidative stress (Figure 4). It is remarkable that *MsrA* gene induction seems to be activated by the same kinds of stimuli that induce the expression of OaGST. This strongly suggests that a possible common regulator could be involved in the expression of these two genes in *O. anthropi*.

Immunochemical analysis of the cytosolic fraction of *O. anthropi*

The expression of MsrA was also measured by immunoblot analysis using an anti-MsrA serum prepared using recombinant MsrA, as described in the Materials and methods section. The increased level of the transcript found in *O. anthropi* cells grown

in the presence of phenol was consistent with the increase observed by Western blot analysis. The protein appeared to be present in *O. anthropi* under normal growth conditions, and thus appears to be constitutive (Figure 5, lane C). To quantify the effects of the different stress conditions on the regulation of MsrA expression, the various compounds were added to exponentially growing cells of *O. anthropi*. The chemicals (phenol, 4-chlorophenol, H_2O_2 , NaCl, etc.) were added at concentrations that reduced cell growth by up to 50%. Figure 5 is representative of an immunoblot analysis of the MsrA level in crude extracts of cells grown in the presence either of phenolic compounds simulating chemical stress or of other compounds that result in various stress conditions, such as osmotic (NaCl) or oxidative (H_2O_2) stress. Compounds leading to chemical stress seem to modulate MsrA expression: 4-chlorophenol and phenol led to 5-fold and 4-fold increases respectively in MsrA level.

To explore whether induction of MsrA might correlate with the oxidative metabolism of phenol, *O. anthropi* cells were grown in the presence of either catechol or resorcinol. In the phenol-degrading bacteria, the first metabolic step is the formation of catechol. As shown in Figure 5, the presence of this compound contributed to the induction of MsrA, whereas no significant induction was observed using resorcinol. These results suggest a possible protective role of MsrA during the action of either monooxygenase or dioxygenase enzymes that are involved in the degradation of phenols. On the other hand, general stress factors, such as salt and H_2O_2 , did not affect the level of MsrA.

Effect of phenolic compounds on MsrA enzyme activity

To assess whether an increase in MsrA expression, as seen in Figure 4, corresponds to an increase in MsrA activity, *O. anthropi* cell extracts were assayed for MsrA activity. The enzymic activity, using *N*-acetyl-[3H]Met(O) as substrate, was consistent with the

MsrA protein level detected by immunoblot experiments. As shown in Table 2, MsrA activity was found to be essentially doubled in the crude extracts of *O. anthropi* grown in the presence of either 4-chlorophenol or phenol.

DISCUSSION

In this paper we describe the expression of the *MsrA* gene isolated from the bacterium *O. anthropi*, and also provide evidence that this enzyme could be considered as part of the cellular detoxification system. Previously, in order to clone the *GST* gene from this bacterium, we isolated a 5 kb DNA chromosomal fragment [25]. The nucleotide sequence of the whole DNA fragment has been determined, and by a homology search the *MsrA* gene was located approx. 1 kb downstream of the *GST* gene identified. Comparison of the deduced amino acid sequence revealed significant identity with prokaryotic and eukaryotic MsrA proteins. Moreover, the protein contains the three cysteine residues and the consensus amino acid sequence, Gly-Cys-Phe-Trp-Gly, that are involved in the catalytic mechanism of MsrA [30,32].

The MsrA enzyme of *O. anthropi* has been overexpressed as an MsrA-GST fusion protein. The yield of thrombin-cleaved and purified recombinant MsrA was approx. 12 mg/litre of culture, allowing the biochemical characterization of the enzyme.

The substrate specificity of MsrA from *O. anthropi* was very similar to that of mammalian MsrA characterized previously [31]. In fact, it was found that this enzyme can reduce Met(O), both free and in peptide linkage, to Met using as substrate *N*-acetyl-Met(O), L-Met(O) or D-Met(O). Moreover, the enzyme showed activity against a variety of sulphoxide-containing compounds.

Western blot experiments indicated that this protein is constitutively expressed in *O. anthropi* cells under normal growth conditions (Luria-Bertani broth or minimal medium), suggesting a physiological role for the protein. Previous studies have demonstrated that MsrA is present in many prokaryotic and eukaryotic organisms, and its function seems to be related to the protection of the cells against oxidative stress [18,33]. It has been suggested that methionine in proteins could function to destroy reactive oxygen species (ROS) in a coupled reaction in which the oxidized Met residues would be reduced by MsrA [34].

In aerobic organisms, ROS are generated continually. During oxidative stress caused by stimuli such as exposure to xenobiotics, the overproduction of ROS could result in various deleterious effects. In order to protect against these harmful ROS, aerobic organisms have developed a number of cellular defences [35].

In mammalian cells, GSTs, which belong to a gene family of phase II detoxification enzymes, are involved in the conjugation of a wide range of xenobiotics to the endogenous nucleophile glutathione [21,36]. The GST in *O. anthropi* is modulated only by chemical stress conditions, in particular by aromatic compounds such as phenol and chlorophenols, and it was postulated that this enzyme could function as a detoxifying agent within the catabolism of xenobiotics [24].

To our knowledge, this is the first time that an *MsrA* gene has been found to be located near to a *GST* gene. An *MsrA* gene has been located previously on the *E. coli* chromosome between the *cysQ* gene and that coding for a pyrophosphatase [37]. RNA blot analysis indicated that the *MsrA* gene from *O. anthropi*, like the *GST* gene, appears to be monocistronically transcribed, and the transcript level increased during growth of the bacteria in the presence of phenolic compounds. This modulation was confirmed by increases both in the protein level and in enzyme activity. MsrA seems to be regulated by mechanisms very similar

to those involved in the regulation of *O. anthropi* GST. Indeed, like the GST, the level of the MsrA protein is not affected by general stress conditions, such as starvation, heat shock or osmotic stress, thus indicating that the global regulator sigma factor (σ^S) [38] is not involved in the induction of MsrA.

Moskovitz et al. [18] reported that an *E. coli* MsrA mutant was more sensitive to hydrogen peroxide than the parental strain, and similar results were reported in higher organisms using a *Saccharomyces cerevisiae* null MsrA mutant as a eukaryotic model [33]. Oxidative stress was applied to *O. anthropi* cells using a range of agents, such as hydrogen peroxide, hydrogen peroxide plus ferric chloride, or paraquat. None of these compounds affected MsrA, suggesting that the basal level of expression of the protein could be enough to protect the cells against this type of oxidative stress. In contrast, chemical stress conditions produced by phenolic compounds such as phenol or chlorophenol induced the *MsrA* gene. This activation could be associated with the oxidative metabolism of the phenol, since *O. anthropi* cells grown in the presence of catechol showed an increase in the MsrA level. On the other hand, this compound seems to work by redox-cycling and generation of free radicals [39,40], so it appears very difficult to explain the absence of induction of MsrA levels in cells grown in the presence of stimulants causing ROS production.

The co-induction of an MsrA enzyme with an enzyme of the phase II detoxification system has not been reported previously, and this finding suggests that MsrA enzymes could be involved in the detoxification of xenobiotic compounds. This hypothesis is supported by the fact that the recombinant MsrA protein showed reductase activity against methyl sulphoxide xenobiotic compounds, such as *S*-(–)-methyl *p*-tolyl sulphoxide and L-ethionine sulphoxide.

In eukaryotic organisms the detoxification process includes two major phases: phase I is catalysed by the cytochrome P450 system, and phase II by GST enzymes. The oxidation reactions carried out by phase I enzymes are similar to the monooxygenase-catalysed oxidations of phenols performed by several bacteria, and, as in higher organisms, *O. anthropi* GST is induced by conditions of chemical stress. Our data represent a first description of a microbial MsrA that can be regulated by phenols. The two genes, *MsrA* and *GST*, are monocistronically transcribed, so they do not belong to the same operon system. However, they are located close to each other on the bacterial chromosome, and are induced only by compounds resulting in chemical stress. This co-induction is probably mediated by a common regulation factor, triggered by phenols. These findings suggest that MsrA, like GST, could be considered as part of the detoxification system, and may be involved in protecting cells from the damage caused by hydrophobic membrane-active compounds (e.g. phenols and chlorophenols). Thus these data suggest that the bacterial detoxification system may be similar to the eukaryotic one, thus opening up new areas of research into the involvement of MsrA in chemical detoxification, not only in bacteria but also in eukaryotic organisms.

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REFERENCES

1. Brot, N. and Weissbach, H. (1988) Methionine sulfoxide: chemistry and biochemistry. In *The Chemistry of Sulfones and Sulfoxides* (Patai, S. and Rappoport, Z., eds.), pp. 851–872, Wiley, New York.

- 2 Abrams, W. R., Weinbaum, G., Weissbach, L., Weissbach, H. and Brot, N. (1981) Enzymatic reduction of oxidized alpha-1-proteinase inhibitor restores biological activity. *Proc. Natl. Acad. Sci. U.S.A.* **78**, 7483–7486
- 3 Brot, N., Weissbach, L., Werth, J. and Weissbach, H. (1981) Enzymatic reduction of protein-bound methionine sulfoxide. *Proc. Natl. Acad. Sci. U.S.A.* **78**, 2155–2158
- 4 Fliss, H., Vasanthakumar, G., Schifflman, E., Weissbach, H. and Brot, N. (1982) Enzymatic reduction of oxidized chemotactic peptide *N*-formyl-L-methionyl-sulfoxide-L-leucyl-L-phenylalanine. *Biochem. Biophys. Res. Commun.* **109**, 194–201
- 5 Lawrence, D. A. and Loskutoff, D. J. (1986) Inactivation of plasminogen activator inhibitor by oxidants. *Biochemistry* **25**, 6351–6355
- 6 Tobler, P. H., Tschopp, F. A., Dembacher, M. A., Born, W. and Ficher, J. A. (1983) Identification and characterization of calcitonin forms in plasma and urine of normal subjects and medullary carcinoma patients. *J. Clin. Endocrinol.* **57**, 749–754
- 7 Good, P. F., Werner, P., Hsu, A., Olanow, C. W. and Perl, D. P. (1996) Evidence of neuronal oxidative damage in Alzheimer's disease. *Am. J. Pathol.* **149**, 21–28
- 8 Jackson, C. V., Mickelson, J. K., Pope, T. K., Rao, P. S. and Lucchesi, B. R. (1986) O₂ free radical-mediated myocardial and vascular dysfunction. *Am. J. Physiol.* **251**, H1225–H1231
- 9 Kukreja, R. C. and Hess, M. L. (1992) The oxygen free radical system: from equations through membrane-protein interactions to cardiovascular injury and protection. *Cardiovasc. Res.* **26**, 641–655
- 10 Stadtman, E. R. (1992) Protein oxidation and aging. *Science* **257**, 1220–1224
- 11 Rattan, S. I., Derventzi, A. and Clark, B. F. (1992) Protein synthesis, posttranslational modifications and aging. *Ann. N. Y. Acad. Sci.* **663**, 48–62
- 12 Gibson, G., Martins, R., Blass, J. and Gandy, S. (1996) Altered oxidation and signal transduction systems in fibroblasts from Alzheimer patients. *Life Sci.* **59**, 477–489
- 13 Yu, B. P. (1994) Cellular defenses against damage from reactive oxygen species. *Physiol. Rev.* **74**, 139–162
- 14 Yu, B. P. and Yang, R. (1996) Critical evaluation of the free radical theory of aging. A proposal for the oxidative stress hypothesis. *Ann. N.Y. Acad. Sci.* **786**, 1–11
- 15 Kuschel, L., Hansel, A., Schonherr, R., Weissbach, H., Brot, N., Hoshi, T. and Heinemann, S. H. (1999) Molecular cloning and functional expression of a human peptide methionine sulfoxide reductase (hMsrA). *FEBS Lett.* **456**, 17–21
- 16 Moskovitz, J., Jenkins, N. A., Gilbert, D. J., Copeland, N. J., Jursky, F., Weissbach, H. and Brot, N. (1996) Chromosomal localization of the mammalian peptide-methionine sulfoxide reductase gene and its differential expression in various tissues. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 3205–3208
- 17 Rahman, M. A., Nelson, H., Weissbach, H. and Brot, N. (1992) Cloning, sequencing, and expression of the *Escherichia coli* peptide methionine sulfoxide reductase gene. *J. Biol. Chem.* **267**, 15549–15551
- 18 Moskovitz, J., Rahman, A. T., Strassman, J., Yanacey, S. O., Kushner, S. R., Brot, N. and Weissbach, H. (1995) *Escherichia coli* peptide methionine sulfoxide reductase gene: regulation of expression and role in protecting against oxidative damage. *J. Bacteriol.* **177**, 502–507
- 19 Hassouni, M., Chambost, J. P., Expert, D., Van Gijsegem, F. and Barras, F. (1999) The minimal gene set member msrA, encoding peptide methionine sulfoxide reductase, is a virulence determinant of the plant pathogen *Erwinia chrysanthemi*. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 887–892
- 20 Wizemann, T. M., Moskovitz, J., Pearce, B. J., Cundell, D., Arvidson, C. G., So, M., Weissbach, H., Brot, N. and Masure, R. (1996) Peptide methionine sulfoxide reductase contributes to the maintenance of adhesins in three major pathogens. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 7985–7990
- 21 Hayes, J. D. and Pulford, D. J. (1995) The glutathione S-transferase supergene family: regulation of GST and the contribution of the isoenzymes to cancer chemoprotection and drug resistance. *Crit. Rev. Biochem. Mol. Biol.* **30**, 445–600
- 22 Laura, D., De Socio, G., Frassanito, R. and Rotilio, D. (1996) Effects of atrazine on *Ochrobactrum anthropi* membrane fatty acids. *Appl. Environ. Microbiol.* **62**, 2644–2646
- 23 Favalaro, B., Melino, S., Petruzzelli, R., Di Ilio, C. and Rotilio, D. (1998) Purification and characterization of a novel glutathione transferase from *Ochrobactrum anthropi*. *FEMS Microbiol. Lett.* **160**, 81–86
- 24 Favalaro, B., Tamburro, A., Trofino, M. A., Bologna, L., Rotilio, D. and Heipieper, H. J. (2000) Modulation of the glutathione S-transferase in *Ochrobactrum anthropi*: function of xenobiotic substrates and other forms of stress. *Biochem. J.* **346**, 553–559
- 25 Favalaro, B., Tamburro, A., Angelucci, S., De Luca, A., Melino, S., Di Ilio, C. and Rotilio, D. (1998) Molecular cloning, expression and site-directed mutagenesis of glutathione S-transferase from *Ochrobactrum anthropi*. *Biochem. J.* **335**, 573–579
- 26 Devereux, J., Haeblerli, P. and Smithies, P. (1984) A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res.* **12**, 387–395
- 27 Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**, 680–685
- 28 Heipieper, H. J., Löffeld, B., Kewelow, H. and de Bont, J. A. M. (1995) The *cis/trans* isomerisation of unsaturated fatty acids in *Pseudomonas putida* S12: an indicator for environmental stress due to organic compounds. *Chemosphere* **30**, 1041–1051
- 29 Brot, N., Werth, J., Koster, K. and Weissbach, H. (1982) Reduction of N-acetyl methionine sulfoxide: a simple assay for peptide methionine sulfoxide reductase. *Anal. Biochem.* **122**, 291–294
- 30 Lowther, W. T., Brot, N., Weissbach, H., Honek, J. F. and Matthews, B. W. (2000) Thiol-disulfide exchange is involved in the catalytic mechanism of peptide methionine sulfoxide reductase. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 6463–6468
- 31 Moskovitz, J., Weissbach, H. and Brot, N. (1996) Cloning the expression of a mammalian gene involved in the reduction of methionine sulfoxide residues in proteins. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 2095–2099
- 32 Moskovitz, J., Poston, M. J., Berlett, B. S., Nosworthy, N. J., Szczepanowski, R. and Stadtman, E. R. (2000) Identification and characterization of a putative active site for peptide methionine sulfoxide reductase (MsrA) and its substrate stereospecificity. *J. Biol. Chem.* **275**, 14167–14172
- 33 Moskovitz, J., Berlett, B. S., Poston, M. and Stadtman, E. R. (1997) The yeast peptide-methionine sulfoxide reductase functions as an antioxidant *in vivo*. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 9585–9589
- 34 Levine, R. L., Mosoni, L., Berlett, B. S. and Stadtman, E. R. (1996) Methionine residues as endogenous antioxidants in proteins. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 15036–15040
- 35 Halliwell, B. and Gutteridge, J. M. C. (1989) *Free Radicals in Biology and Medicine*, 2nd edn. Clarendon Press, Oxford
- 36 Mannervik, B. and Danielson, U. H. (1988) Glutathione transferases: structure and catalytic activity. *Crit. Rev. Biochem.* **23**, 283–337
- 37 Rahman, M. A., Moskovitz, J., Strassman, J., Weissbach, H. and Brot, N. (1994) Physical map location of the peptide methionine sulfoxide reductase gene on the *Escherichia coli* chromosome. *J. Bacteriol.* **176**, 1548–1549
- 38 Hengge-Aronis, R. (1996) Back to log phase: sigma S as a global regulator in the osmotic control of gene expression in *Escherichia coli*. *Mol. Microbiol.* **21**, 887–893
- 39 Rushmore, T. H., Morton, M. R. and Pickett, C. B. (1991) The antioxidant responsive element. Activation by oxidative stress and identification of the DNA consensus sequence required for functional activity. *J. Biol. Chem.* **266**, 11632–11639
- 40 Prestera, T., Holtzclaw, W. D., Zhang, Y. and Talalay, P. (1993) Chemical and molecular regulation of enzymes that detoxify carcinogens. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 2965–2969

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