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Escherichia coli Peptide Methionine Sulfoxide Reductase Gene: Regulation of Expression and Role in Protecting Against Oxidative Damage

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The *Escherichia coli* peptide methionine sulfoxide reductase gene (*msrA*) encodes a single-subunit polypeptide of 212 amino acid residues (M. A. Rahman, H. Nelson, H. Weissbach, and N. Brot, J. Biol. Chem. 267:15549–15551, 1992). RNA blot analysis showed that the gene is transcribed into an mRNA of about 850 nucleotides. The promoter region was characterized, and the transcription initiation site was identified by primer extension. The synthesis of the MsrA protein increased about threefold in a growth-phase-dependent fashion. In an attempt to define the in vivo role of *msrA*, a chromosomal disruption was constructed. This mutant was more sensitive to oxidative stress, suggesting that oxidation of methionine in proteins plays an important role in oxidative damage.

The enzyme peptide methionine sulfoxide reductase (MsrA) catalyzes the reduction of methionine sulfoxide [Met(O)] residues in proteins to methionine (1, 5, 12). The presence of Met(O) residues in proteins may arise during aerobic metabolism as a consequence of the oxidation of methionine by such reagents as hydrogen peroxide, hydroxyl radicals, and hypochlorite and superoxide ions (reviewed in reference 4). It has also been reported that oxidized linolenic acid is capable of oxidizing methionine residues in proteins in vitro (20). In many cases, the oxidation of a specific methionine residue leads to the loss of biological activity (4), which can be restored upon incubation of the oxidized protein with MsrA (1, 5, 12, 17, 27).

The gene for the enzyme from *Escherichia coli* has recently been cloned and sequenced (24). The coding region of the gene is 636 nucleotides long and encodes a single-subunit protein with a calculated molecular weight of 23,316. In addition, the reductase has been overexpressed in *E. coli* and purified to homogeneity (23). In this report, we examine the regulation of expression of this gene and show that an *msrA* mutant is more sensitive to oxidative damage than wild-type cells.

MATERIALS AND METHODS

Materials. Restriction enzymes, T4 polynucleotide kinase, and T4 DNA ligase were purchased from New England Biolabs (Beverly, Mass.). Avian myeloblastosis virus reverse transcriptase was from Promega (Madison, Wis.). Radioisotopes were from either Amersham (Arlington Heights, Ill.) or Dupont/NEN (Boston, Mass.). Immobilon N transfer membranes were from Millipore (Bedford, Mass.). All chemicals were from Sigma (St. Louis, Mo.) or Boehringer Mannheim Biochemicals (Indianapolis, Ind.). Purified MsrA and antiserum against the protein were prepared as previously described (23).

Northern (RNA) analysis. Total RNA was isolated as described previously (3) from 100-ml cultures (A_{600} of 0.6) of either *E. coli* XL1-Blue or *E. coli* XL1-Blue/pAR100, which contains a plasmid bearing a genomic copy of the *msrA* gene (24). The coding region of the gene was amplified by PCR using two synthetic primers. The DNA was purified by agarose gel electrophoresis and the GeneClean (Bio 101, La Jolla, Calif.) procedure. Five hundred nanograms of the DNA was used for random primer labeling using [α-^{32}P]dCTP and Klenow polymerase. For Northern analysis, 25 μg of total *E. coli* RNA was denatured by heating at 65°C for 10 min in an RNA denaturing solution (50% formamide, 2.2 mM formaldehyde, 25% glycerol, 0.02% bromophenol blue, and 0.02% xylene cyanol in 1× 3-*N*-morpholinopropanesulfonic acid [MOPS] buffer). RNA was applied to a 1.2% agarose–2.2 mM formaldehyde gel, and electrophoresis was carried out in MOPS buffer (40 mM MOPS [pH 7.0], 10 mM sodium acetate, 1 mM EDTA). After electrophoresis, the gel was rinsed in 200 ml of 50 mM NaOH containing 0.15 M NaCl for 30 min and neutralized by soaking in 200 ml of 0.1 M Tris-HCl (pH 7.5)–0.15 M NaCl for 30 min. RNA was transferred by using 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 sodium citrate) onto Immobilon N membranes by pressure blotting (Stratagene, La Jolla, Calif.) at 75 lb/in^2 for 2 h. The RNA was then cross-linked to the membrane with a UV cross-linker and hybridized at 65°C for 16 h to the ^{32}P-labeled DNA probe described above. The membrane was washed twice with 2× SSC–0.2% sodium dodecyl sulfate (SDS) at 65°C and twice with 0.1× SSC–0.1% SDS at room temperature. The blot was autoradiographed for 16 h.

Primer extension analysis. Primer extension was done essentially as previously described (3). Total RNA (20 μg) (extracted from cells in logarithmic growth) was hybridized at 55°C for 20 min to a 5′-end ^{32}P-labeled 20-mer complementary to nucleotides 18 to 37 of the coding region of the gene. After ethanol precipitation, the RNA-DNA hybrid was dissolved in 20 μl of primer extension mix and incubated at 42°C for 90 min. After phenol extraction, the reaction mixture was treated with ethanol, the precipitate was resuspended in 7 μl of loading buffer and heated at 70°C for 2 min, and 2.5 μl was loaded onto a gel alongside a sequencing reaction using the same primer.

DNA sequencing. Plasmid DNA was isolated from *E. coli* XL1-Blue/pAR100 cells (24) by alkaline lysis (3). Sequencing reactions were performed by the dideoxy-chain termination method (26), using the Sequenase version 2 kit (U.S. Biochemical) and [α-^{35}S]dATP.

Construction of an MsrA-lacZ fusion plasmid. The 5′ untranslated region of the *msrA* gene and the codons for the first 13 amino acids of the MsrA protein were fused in frame to the *lacZ* coding region in plasmid pMC1403 by obtaining a PCR product that incorporated a *Bam*HI site at amino acid codon 13 of the *msrA* gene and an engineered *Eco*RI site 271 bases upstream. The product was ligated with T4 DNA ligase (Boehringer Mannheim) into vector pMC1403 which had been digested with *Bam*HI and *Eco*RI (New England Biolabs) and then treated with calf intestinal alkaline phosphatase. Competent cells were transformed with an aliquot of the ligation mix, and positive clones were selected by the appearance of blue colonies on LB plates containing ampicillin (50 μg/ml) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal).

Construction of a chromosomal *msrA1*::*kan* mutant. Cells were grown in LB; for plates, 2% agar was added to the LB. Ampicillin (50 μg/ml), kanamycin (50 μg/ml), or chloramphenicol (20 μg/ml) was added as needed. pBluescriptII SK(+) containing the *msrA* gene (pAR100) on a 3-kb insert (24) was digested with *Sph*I and blunted with the Klenow fragment of DNA polymerase I. The kanamycin resistance gene from pUC-4K (Pharmacia) was isolated after restriction with *Eco*RI and was then blunt-end ligated to the blunt-end sites of pAR100. This resulted in the insertion of the kanamycin resistance (Km^r) gene at amino acid 30, causing disruption of the *msrA* gene. This plasmid was then transformed into competent *E. coli* MC1061 cells. [F$^-$ *araD139* Δ(*ara-leu*)*7697* Δ(*lac*)*X74*

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galU galK strA]. Plasmid from these cells was obtained by alkaline lysis and digested with *Xba*I and *Kpn*I. The fragment containing the disrupted *msrA* gene was isolated by gel electrophoresis and was ligated to the *Xba*I and *Kpn*I sites of pMAK705 (15), creating plasmid pJM100. This new plasmid also contains both the gene for chloramphenicol resistance (Cm^r) as well as the temperature-sensitive pSC101 origin of replication which were originally present on pMAK705.

The *msrA1*::*kan* disruption mutant constructed in plasmid pJM100 was moved into the *E. coli* chromosome by using a modification of the gene replacement technique of Hamilton et al. (15). pJM100 was transformed into MC1061, and Cm^r transformants were selected at 30°C. One Cm^r transformant was grown at 30°C to a cell density of 8×10^8/ml and then plated at 44°C at various dilutions on prewarmed L-agar plates containing chloramphenicol. At the elevated temperature, Cm^r colonies can arise only through integration of the plasmid into the chromosome. The number of cointegrates observed was 0.03%. Fifteen cointegrates were pooled and inoculated into 100 ml of L broth (50 μg of chloramphenicol per ml) and grown overnight with shaking at 30°C. The next day the culture was diluted 1/1,000 into fresh medium and allowed to grow again overnight. This procedure was repeated twice. Subsequently, individual colonies were obtained by plating the culture on L-agar plates at 30°C. Resolution of cointegrates was determined by streaking individual colonies on L-agar plates containing chloramphenicol at both 30 and 44°C.

Plasmid DNA was isolated from eight resolved cointegrates and digested with *Hin*dIII. Two of the eight plasmids carried the wild-type *msrA* sequence, indicating that the *msrA1*::*kan* disruption was in the chromosome. One of these isolates was grown at 44°C in L broth for approximately 200 generations. When 40 individual colonies were tested at 30°C for both Km^r and Cm^r, all of them had lost the plasmid and retained Km^r. A bacteriophage P1 lysate was made on one of these isolates (SK8778) and was used to transduce (29) MC1061 to Km^r. One of the Km^r transductants (SK8779, *msrA1*::*kan*) was used in the experiments in these studies.

Immunoblot analysis. *E. coli* MC1061 and SK8779 (*msrA1*::*kan*) were grown in LB medium to the mid-log stage of growth, and the cells were harvested and suspended in a buffer containing 50 mM Tris-Cl (pH 7.4) and 1 mM dithiothreitol. The cells were sonicated and centrifuged, and 20 μg of protein was subjected to SDS-polyacrylamide gel electrophoresis using a 12.5% gel. Proteins were transferred to Hybond-ECL (Amersham) and probed for MsrA protein, using a rabbit polyclonal anti-MsrA serum (23). Immunoreactive bands were detected with the Amersham ECL system.

Disk inhibition assay. Cells were grown to either log phase or stationary phase in LB medium and then diluted into LB agar and poured (10^8 cells) into plates. A 12.6-mm^2 filter disk was placed in the middle of the plate and impregnated with either 10 μl of H_2O or 10 μl of 250 mM H_2O_2. The plates were incubated overnight at 37°C.

RESULTS

Regulation of MsrA. In an attempt to investigate whether there is any regulation of MsrA synthesis in vivo, *E. coli* XL-1 Blue cells (see Materials and Methods) were grown in LB medium for various periods of time and then assayed for MsrA activity. Figure 1 shows that there is about a threefold increase in enzyme activity as the cells grow from the mid-log phase to the stationary phase of growth. This increased activity remains elevated even after 24 h of growth. Northern blot analysis also showed a parallel increase in the *msrA* mRNA, and immunoblot analysis of cell extracts by using an MsrA antiserum verified that the MsrA protein showed a similar increase (data not shown). The addition of oxidizing agents, such as H_2O_2 or paraquat, to the growth medium did not affect the expression of the *msrA* gene.

***msrA* mRNA.** To determine the transcript size of the *msrA* gene, total RNA from either XL1-Blue or XL1-Blue/pAR100 cells was subjected to Northern analysis (see Materials and Methods). The RNAs were hybridized to a DNA probe (labeled with [α-^{32}P]dCTP) which contained only the coding region of the gene. As shown in Fig. 2, an RNA transcript of approximately 850 nucleotides was found from both cell types. The increased level found in the cells transformed with a plasmid containing the *msrA* gene is consistent with an increase in the MsrA enzyme level previously observed (24).

To determine the transcriptional start site, a 5′-end primer labeled with [γ-^{32}P]ATP and complementary to nucleotides 18 to 37 of the coding region of the gene was hybridized to total RNA from cells grown to mid-log phase and extended with

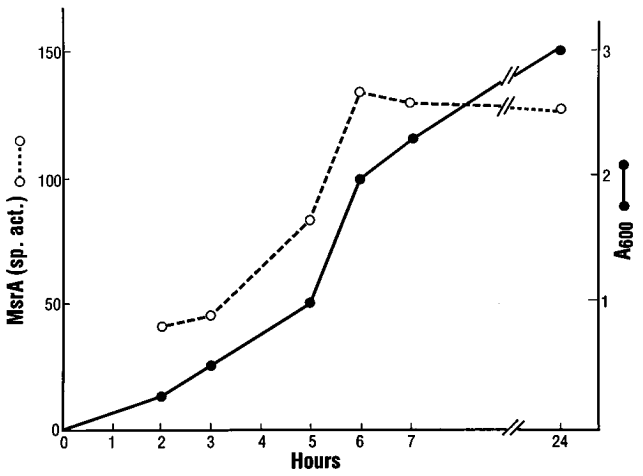

FIG. 1. Effect of growth on expression of the *msrA* gene. *E. coli* XL-1 Blue cells were grown to stationary phase in LB medium at 37°C and then diluted to an A_{600} of 0.05 in LB medium. They were then grown and harvested at various stages of growth, disrupted by sonication, and centrifuged, and the supernatant was assayed for MsrA activity (4). Specific activity is defined as picomoles of *N*-acetyl Met(O) reduced per hour per milligram of protein.

avian myeloblastosis virus reverse transcriptase. Figure 3 shows that a single transcription start site corresponding to an A residue is located 86 nucleotides upstream of the translational start site (ATG). Figure 4 shows the sequence of the 5′ untranslated region and the positions of the transcriptional start site as well as the −10 and −35 promoter regions. Attempts were made to delete or alter the promoter region in order to identify any regulatory region responsible for the growth-induced expression of *msrA*. For these experiments, an *msrA* promoter–β-galactosidase fusion was constructed. Deletion of the 5′ region upstream of nucleotide −61 had no effect on either the basal expression or the induction of β-galactosidase from the *msrA-lac* fusion during growth. In addition, base substitution of the inverted repeat seen at positions −43 to

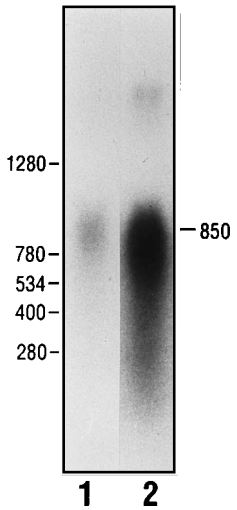

FIG. 2. Northern blot analysis of *msrA* mRNA. RNA was isolated from *E. coli* XL1-Blue cells and XL-1 Blue cells transformed with plasmid pAR100 [pBluescriptII SK(+) *msrA*$^+$]. The RNA blot was hybridized to a ^{32}P-labeled DNA probe complementary to the *msrA* coding region. Details are described in the text. Lane 1, XL-1 Blue; lane 2, XL-1 Blue/pAR100. The approximate size of the transcript is indicated in nucleotides.

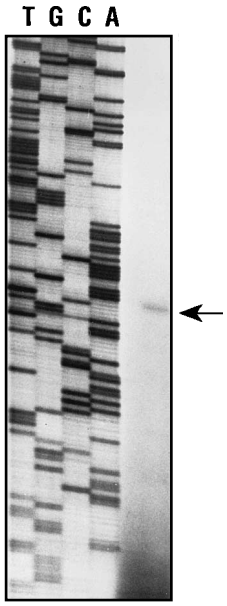

FIG. 3. Primer extension analysis. Total RNA was isolated from *E. coli* XL-1-Blue as described in Materials and Methods, hybridized to a 5′-end ^{32}P-labeled oligonucleotide, and extended with avian myeloblastosis virus reverse transcriptase. After ethanol precipitation and phenol extraction, an aliquot was loaded onto a gel along with a sequencing reaction. After electrophoresis, the gel was exposed to X-ray film.

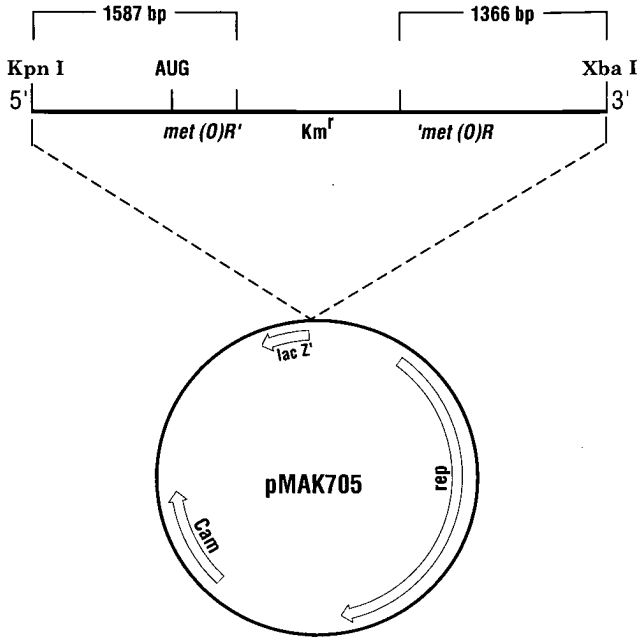

FIG. 5. Scheme for the construction of plasmid pJM100 containing *msrA1*::*kan*.

−48 and −56 to −60 had no effect on β-galactosidase expression.

Studies with an *msrA* mutant. Although peptide Met(O) reductase has been shown to reduce Met(O) residues in proteins and peptides in vitro, there has been no evidence that this enzyme has a similar function in vivo. It was hoped that an *msrA* mutant would provide a clue to the in vivo role of this protein.

An *msrA* disruption mutant was constructed as described in Materials and Methods (Fig. 5). Southern blot analysis of SK8779 (*msrA1*::*kan*) chromosomal DNA and plasmid pJM100 DNA, using a ^{32}P-labeled Km^r gene as a probe, revealed the presence of identical molecular weight bands after restriction with *Ssp*I, *Hin*dIII, and *Hae*III (data not shown), indicating that the mutant had a defective *msrA* gene. No MsrA enzymatic activity was detected in the *msrA* mutant. To obtain more definitive evidence for the absence of the *msrA* gene product, immunoblot analysis was performed. Figure 6 (lane 1) shows that the MsrA protein is readily detected in extracts from wild-type MC1061 cells but is absent in extracts from strain SK8779 (*msrA1*::*kan*) (lane 2).

Effect of *msrA* mutation on growth and sensitivity to oxidative stress. The growth rates of SK8779 (*msrA1*::*kan*) and MC1061 cells were determined under aerobic conditions in both a minimal salts-glucose medium and LB medium at 37°C. Under these conditions, the growth rates of both organisms were essentially identical (data not shown). In addition the growth rates and viabilities of the mutant and parent were identical when the cells were grown in the presence of 1 mM H_2O_2 or 100 μM methyl viologen (paraquat). In other experiments, cultures of SK8779 or MC1061 cells grown overnight were incubated in the absence or presence of 1 mM H_2O_2 for 1 h at 37°C and then diluted 100-fold into fresh medium. It was found that again there was no difference in growth rates of the mutant and wild type under these conditions.

In contrast, a difference was observed when growth was carried out on solid medium, using an H_2O_2 disk inhibition assay (see Materials and Methods). Figures 7A and B show that when the disk was impregnated with water, there was a complete lawn of growth around the disk. However, when the disk contained 10 μl of 250 mM H_2O_2, there was some growth inhibition of the wild-type strain (MC1061) (Fig. 7C) but much

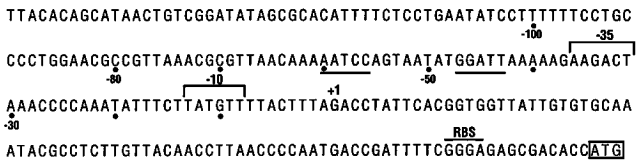

FIG. 4. Nucleotide sequence of the 5′ untranslated region of the *msrA* gene. The start of transcription is indicated by +1, the −10 and −35 sequences are overlined, and the inverted repeat is underlined. Also shown are the ribosome binding site (RBS) and the start of translation.

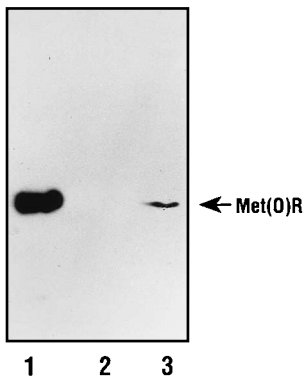

FIG. 6. Immunoblot analysis of the MsrA protein. Immunoblot analysis was carried out as described in the text. Lanes: 1, strain MC1061; 2, strain SK8779 (*msrA1*::*kan*); 3, MsrA protein (10 ng).

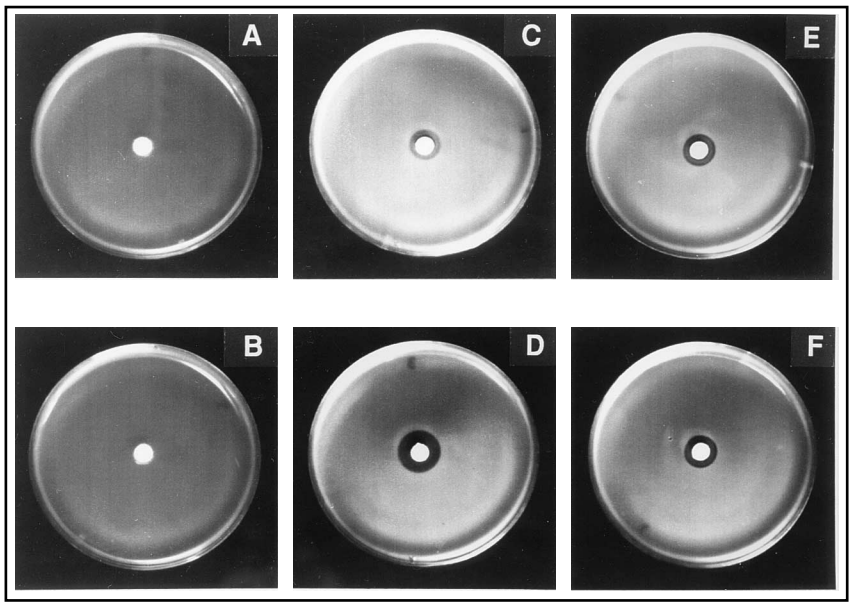

FIG. 7. Effect of H_2O_2 on the growth of wild-type and *msrA* mutant cells in a disk assay. Cells were poured into plates, and a filter disk containing either H_2O or H_2O_2 was placed in the middle of the plate. The amount of growth inhibition was observed after incubation at 37°C overnight. Details are described in the text. (A, C, and E) MC1061; (B, D, and F) SK8779 (Δ*msrA*); (A and B) disks containing H_2O; (C to F) disks containing H_2O_2 (10 μl, 250 mM); (E and F) cells transformed with an *msrA* plasmid.

less than observed with SK8779, the *msrA*-disrupted strain (Fig. 7D and Table 1). To determine whether this increased growth inhibition was due to the absence of the MsrA protein, both SK8779 (*msrA1*::*kan*) and MC1061 were transformed with a plasmid (pAR100) containing the $msrA^+$ gene. Although this transformation had no effect on the growth of MC1061 in the presence of H_2O_2 (Fig. 7C and E), the amount of growth inhibition by H_2O_2 of the transformed *msrA1*::*kan* mutant (SK8779) decreased to that observed with the wild-type (Fig. 7E and F). Table 1 summarizes and quantitates the results of the filter disk experiments shown in Fig. 7. Although not shown, when SK8779 was transformed with a plasmid containing a disrupted *msrA* gene, no decrease in its growth inhibition by H_2O_2 was observed. These results indicate that a lack of expression of the *msrA* gene makes *E. coli* cells more sensitive to H_2O_2 stress.

DISCUSSION

Despite the abundance of data on the characteristics of the *E. coli* peptide Met(O) reductase, there has been no in vivo information on the role of this protein. The ability of this protein to reduce Met(O) residues in proteins suggested that it might be involved in protecting cells against oxidative damage. It was thus somewhat surprising that there was no effect of oxidizing agents such as H_2O_2 or paraquat on *msrA* expression. In contrast, Met(O) reductase activity increased about threefold as the cells grew from the mid-log to the stationary phase of growth.

TABLE 1. Effect of H_2O_2 treatment on the growth of an *msrA1*::*kan* mutant

Genotype	H_2O_2	Area of growth inhibition[a] (mm^2)
$msrA^+$	−	0
msrA1::*kan*	−	0
$msrA^+$	+	26
msrA1::*kan*	+	59
$msrA^+$ (pAR100 $msrA^+$)	+	26
msrA1::*kan* (pAR100 $msrA^+$)	+	29

[a] The amount of growth inhibition (see Fig. 7) was calculated as the area of the clear zone minus the disk area, since there was no growth under the disk under the control conditions. The same results were obtained in three different experiments. Details are described in the text and the legend to Fig. 7.

The expression of genes during the late log and stationary phases of growth has been the subject of considerable interest. Because of the limited availability of nutrients in their natural environment, bacteria, for the most part, spend only a small fraction of their existence in rapid growth, spending the remainder in a nongrowing state. Bacteria have developed a variety of mechanisms to survive starvation for prolonged periods of time (14, 19), and it has been speculated that the proteins synthesized during the later stages of growth may be important for the survival of the organism. Examples of substances and proteins whose synthesis is known to be increased in a growth-dependent fashion include phosphatases (9), catalase (18), microcins (11, 16), glycogen (22), and polyphosphates (25). In some cases, the increase in the synthesis of certain proteins during the late stages of growth of the organism may be related to the synthesis of a new sigma factor, encoded by *rpoS* (2, 8, 21), which is required for the transcription of the genes of proteins expressed during late log and stationary phases.

The in vivo experiments reported here suggest that with respect to expression, the *msrA* gene is also in the class of genes which are expressed in a growth-dependent manner. However, the *rpoS*(KatF) gene product does not appear to be involved, since the same induction of *msrA* was observed in an *rpoS* mutant (data not shown). Whether this increase in *msrA* expression is in response to an increase in the amount of Met(O) residues in the cellular proteins at this late stage of growth is not known but, if so, would be an interesting regulatory signal. Other experiments have shown that the activity of

MsrA increases three- to fourfold when the growth of *E. coli* is limited due to the depletion of either glucose or a nitrogen source (data not shown).

The transcriptional start site and size of the *msrA* mRNA were also determined. The mRNA initiates 86 nucleotides upstream from the ATG initiation codon and contains about 850 nucleotides. Since the coding region accounts for 636 bases, this would leave about 135 bases on the 3′ untranslated region of the mRNA. The area around −10 has the sequence TATGTT, which is similar to the sequence of the −10 region of the *lac* promoter, and the −35 region is 50% homologous to the −35 consensus sequence (TTGACA). We have attempted to delete and alter the 5′ end of the gene to determine whether any upstream region was essential for either the basal level of expression or the growth-dependent increase of expression. It was found that there was no effect on the expression of β-galactosidase from an *msrA* promoter–β-galactosidase fusion gene when the nucleotides upstream from base −61 were deleted or an inverted repeat at positions −43 to −48 and −56 to −60 was disrupted.

It was hoped that the *msrA* disruption mutant constructed in this study would provide information on the function of this enzyme in reducing Met(O) residues in proteins exposed to oxidative stress. To study this, the growth characteristics of both the mutant and the wild type were compared in liquid culture in the absence and presence of various oxidizing agents. In some experiments, the oxidizing agent was added directly to the growing cells, while in others, stationary-phase cells were first pretreated with the oxidizing agent, diluted, and then regrown. Under these conditions, there were no differences in the growth rates of the mutant and the wild type. On the other hand, by using a filter disk assay, it has been possible to show that the *msrA1*::*kan* mutant is more sensitive to H_2O_2 than wild-type cells. That this was due to inactivation of MsrA was shown by reversal of the effect when SK8779 (*msrA1*::*kan*) was transformed with a plasmid containing the wild-type gene. In contrast, transformation of the wild type with the plasmid containing the *msrA* gene had no effect on the level of growth inhibition by hydrogen peroxide. This finding suggests that this growth inhibition might be due to DNA damage or some other oxidation, but not the presence of methionine sulfoxide in proteins. It is not clear why the mutant cells in liquid culture failed to show the same growth inhibition in the presence of peroxide as was observed in the disk assay. It might be related to a critical concentration of peroxide that is achieved only as the peroxide diffuses off of the disk, or perhaps the disk assay, which uses a solid growth support, is a much more sensitive assay than a liquid culture assay. Nevertheless, the results of the disk assay are the first in vivo demonstration that the MsrA enzyme functions to reverse damage due to methionine oxidations.

It is well documented that enzymes such as catalase, peroxidase, and superoxide dismutase function to destroy potential oxidizing agents before they can damage various biological molecules such as DNA, lipids, and proteins. Although oxidized DNA can be repaired (10), the oxidation of lipids and various amino acid residues in proteins, including lysine, histidine, and tryptophan, is essentially irreversible. In contrast, Met(O), the product of the oxidation of methionine, can be enzymatically reduced to methionine by MsrA. α-1-Proteinase inhibitor containing Met(O) (which inactivates the protein) has been found in patients suffering from emphysema (6) as well as adult respiratory distress syndrome (7) and rheumatoid arthritis (30). Met(O) residues have also been found in human cataractous lenses (13, 28). In addition, a large number of other proteins can be inactivated in vitro by the oxidation of methionine residues (4).

The present results suggest that methionine in proteins can be a primary target for H_2O_2 oxidation, since the growth inhibition of the *msrA1*::*kan* mutant by H_2O_2 was specifically reversed by transformation of these cells with the wild-type *msrA* gene. Since the only known function of MsrA is to reduce Met(O) residues in proteins, its ability to reverse the H_2O_2-induced growth inhibition is very likely due to its ability to catalyze the reduction of critical Met(O) residues in proteins that are essential for the growth of the organism.

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