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A Small, Stable RNA Induced by Oxidative Stress: Role as a Pleiotropic Regulator and Antimutator

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Summary

Exposure of E. coli to hydrogen peroxide induces the transcription of a small RNA denoted *oxyS*. The *oxyS* RNA is stable, abundant, and does not encode a protein. *oxyS* activates and represses the expression of numerous genes in E. coli, and eight targets, including genes encoding the transcriptional regulators FhlA and σ^s, were identified. *oxyS* expression also leads to a reduction in spontaneous and chemically-induced mutagenesis. Our results suggest that the *oxyS* RNA acts as a regulator that integrates adaptation to hydrogen peroxide with other cellular stress responses and helps to protect cells against oxidative damage.

Introduction

Small, stable RNA species have been shown to carry out diverse functions in both prokaryotes and eukaryotes. Some small RNAs act as antisense regulators (reviewed in Wagner and Simons, 1994; Delihas, 1995). The 93 nucleotide (nt) *micF* RNA in E. coli decreases the synthesis of the OmpF outer membrane protein by pairing with the 5′-UTR of the *ompF* transcript, thereby inhibiting translation and destabilizing the *ompF* message (Andersen et al., 1989). In C. elegans, the *lin-4* RNA downregulates expression of *lin-14*, a central gene in early development (Lee et al., 1993; Wightman et al. 1993). The *lin-4* RNA, which is possibly as short as 22 nt, is thought to inhibit protein synthesis by interacting with seven elements in the 3′-UTR of *lin-14* message. Recent studies have suggested other regulatory functions for small RNAs. The 362 nt E. coli 10Sa RNA has been proposed to encode a peptide tag that targets proteins for degradation; ribosomes appear to switch from damaged mRNAs to the 10Sa RNA, allowing the addition of a specific amino acid sequence which then is recognized by carboxy-terminal-specific proteases (Keiler et al., 1996). In human cells, small nucleolar RNAs have been shown to direct site-specific 2′-O-methylation of preribosomal RNA by pairing with the rRNAs (Kiss-László et al., 1996).

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While unique functions have been discovered for a subset of the small RNA population, the roles of other small RNAs have not been established. For example, the function of the 184 nt 6S RNA, one of the first E. coli RNAs to be sequenced (Brownlee, 1971), is still unknown. Numerous small nuclear, small nucleolar, and small cytoplasmic RNAs have been cataloged in yeast and human cells (reviewed in Reddy and Busch, 1988; Maxwell and Fournier, 1995). In several cases, these RNAs have been found to be associated with specific proteins, but the roles of many of the RNAs have not been elucidated.

Here, we report the discovery of a 109 nt RNA, denoted *oxyS*, whose synthesis is dramatically induced by oxidative stress in E. coli. We show that this small RNA is stable and abundant. The *oxyS* RNA regulates the expression of as many as 40 genes in E. coli and protects the cells against spontaneous and chemically induced DNA damage. Our results suggest that *oxyS* plays an important role in integrating the oxidative stress response with other cellular responses and helps to protect cells against oxidative damage.

Results

A Small RNA Induced by Treatment with Hydrogen Peroxide

Bacterial cells respond to the deleterious effects of reactive oxygen species by inducing the expression of antioxidant defense genes (reviewed in Jamieson and Storz, 1997). The synthesis of a number of hydrogen peroxide–inducible proteins in E. coli is controlled by the transcriptional activator OxyR. Previous studies showed that mutations that constitutively activate OxyR affect activity rather than OxyR protein levels (Christman et al., 1989), and accordingly, Northern blots showed that the levels of the *oxyR* message are similar in wild-type (K12) and *oxyR2* constitutive mutant strains (Figure 1A). Surprisingly, these Northern blots, which were probed with a fragment carrying the *oxyR* open reading frame and 200 bp of upstream sequence, also showed that the constitutive *oxyR2* mutant expressed a distinct small RNA species not seen for the K12 cells.

To determine whether this small RNA, denoted *oxyS*, is synthesized in response to oxidative stress in wild-type cells, an exponential culture of K12 cells was split and half of the culture was treated with hydrogen peroxide. The corresponding Northern blot in Figure 1B showed that the expression of the *oxyS* RNA is induced within 1 min after exposure to 60 mM hydrogen peroxide with the highest levels between 2.5 and 5 min. The induction by hydrogen peroxide is greater than 60-fold and is not observed in an *oxyR* deletion strain (data not shown). We also examined *oxyS* levels under other stress conditions such as heat shock, cold shock, acid shock, high and low osmolarity, and exposure to novobiocin, but observed little (<5-fold) or no induction, suggesting that hydrogen peroxide is the primary inducer of *oxyS* (data not shown).

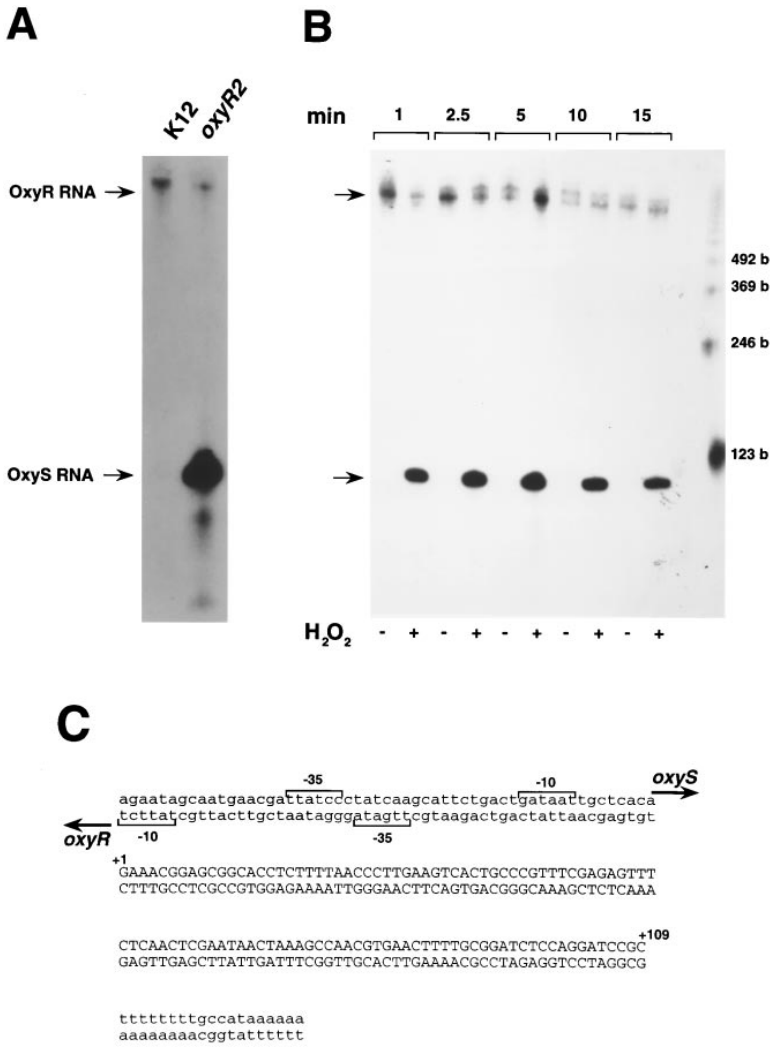

Figure 1. Induction of *oxyS* RNA by Hydrogen Peroxide

(A) Northern blot of total cellular RNA isolated from wild-type K12 and *oxyR2* mutant cells.

(B) Northern blot of total cellular RNA isolated from wild-type K12 cells at 1, 2.5, 5, 10, and 15 min after half of the culture was treated with 60 μM hydrogen peroxide. A 123 bp DNA ladder was electrophoresed alongside the RNA to determine the approximate size of the RNA species.

(C) Sequence of E. coli *oxyS* denoted in capital letters. The −10 and −35 sequences for the *oxyS* promoter are indicated by brackets above the sequence, and the promoter sequences for *oxyR* are indicated by brackets below the sequence.

Characterization of the *oxyS* RNA

By probing Northern blots with specific primers and subfragments of the original 1.5 kb probe, we found that the gene encoding the small RNA was located just upstream of and transcribed divergently from the *oxyR* gene (Figure 1C). The 5′ end of *oxyS* RNA was mapped by primer extension analysis of total RNA isolated from uninduced wild-type K12 and *oxyR2* constitutive mutant cells (data not shown). The extension product observed for the *oxyR2* cells but not for the wild-type cells allowed the start of the small RNA to be mapped to a guanosine residue. Corresponding −10 and −35 promoter sequences that contain 5 of the 6 residues found in canonical σ^{70} promoters are indicated in Figure 1C.

The 3′ end of the *oxyS* transcript was determined by S1 nuclease analysis using a 3′ end-labeled oligonucleotide probe that was annealed to total RNA isolated from uninduced wild-type K12 and *oxyR2* constitutive mutant strains. The products remaining after S1 digestion were separated on a sequencing gel and compared to 3′ end-labeled oligonucleotides run in a neighboring lane (data not shown). Two digestion products were observed for the RNA isolated from *oxyR2* mutant but not wild-type cells. The predominant 32 nt fragment mapped the 3′ end to a cytidine residue (C+107), while the second 33 nt fragment mapped the 3′ end to a guanosine residue (G+108). We suggest that the 3′ end of *oxyS* is actually the cytidine residue at position C+109, since G+108 and C+109 could basepair with G+91 and C+92 (see Figure 2A). The observed S1 nuclease products are likely to be the result of breathing between the probe and the last few nucleotides of the *oxyS* transcript. The 5′ and 3′ end mapping data showed that the total length of the *oxyS*-encoded RNA is 107–109 nt. The lack of an AUG codon, the absence of translation initiation sites detected by several computer algorithms (T. D. Schneider, personal communication), and mutagenesis of the two UUG sequences (data not shown) indicate that the small RNA is not translated into a protein product.

***oxyS* RNA Structure**

The secondary structure of the E. coli *oxyS* RNA predicted by folding programs contains three stem-loop structures designated (a), (b), and (c) (Figure 2A). To examine the *oxyS* conformation in vivo, we treated cells with the chemical probe dimethyl sulfate (DMS), which

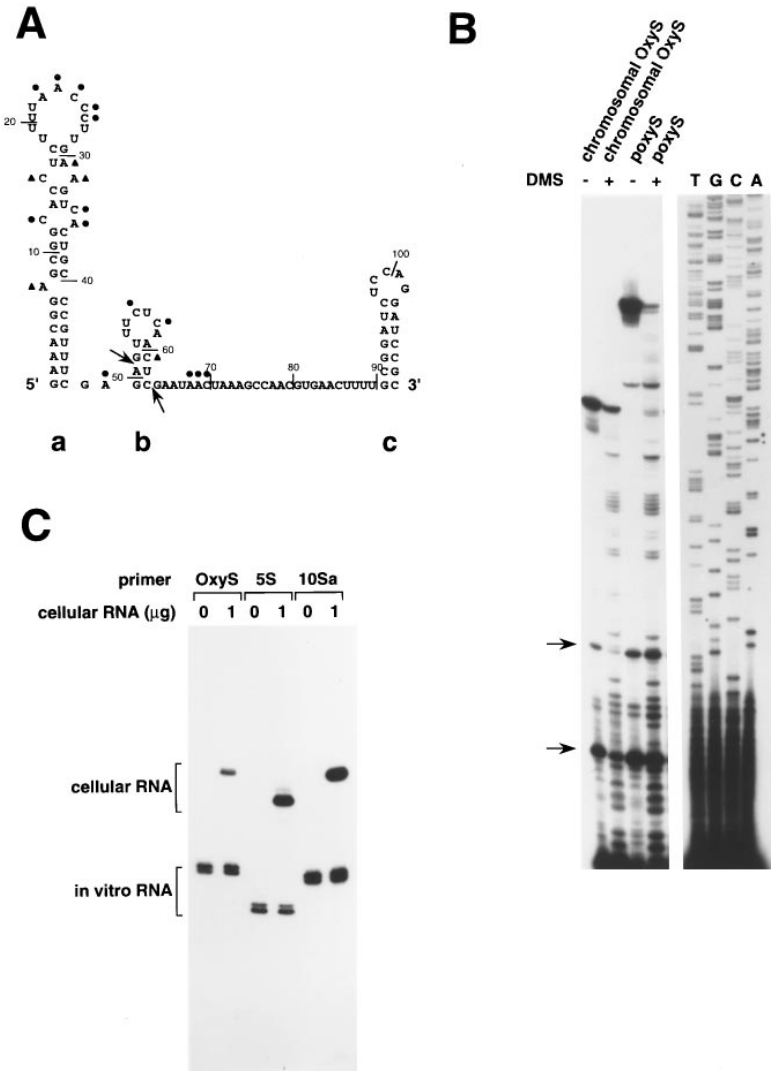

Figure 2. *oxyS* RNA Structure and Levels

(A) Structure of the *oxyS* RNA. The *oxyS* secondary structure was predicted by the Genetics Computer Group program FOLD, using default parameters. The stabilities of the three stem loop structures are predicted to be: stem (a), (G+1 to C+47) $\Delta G = -14.9$ kcal/mol; stem (b), (G+50 to C+63) $\Delta G = -1.8$ kcal/mol; and stem (c), (G+91 to C+109), a *rho*-independent transcription terminator, $\Delta G = -9.4$ kcal/mol. The closed circles indicate strong and closed triangles weak dimethyl sulfate modification sites, and the arrows denote the two processing sites.

(B) In vivo structure determination of the *oxyS* RNA. Hydrogen peroxide–induced K12 cells and uninduced K12/poxyS cells were incubated with and without dimethyl sulfate for 5 min. Total RNA (10 μg) from the samples was then subjected to primer extension analysis. The arrows denote the two processing sites.

(C) Determination of cellular levels of *oxyS*. In vitro synthesized control *oxyS* (0.02 pmol), 5S (0.05 pmol), or 10Sa (0.02 pmole) RNA was mixed with yeast tRNA (1 μg) or total cellular RNA (1 μg) isolated from hydrogen peroxide–treated cells. Labeled primers specific to the three RNAs were incubated with the samples and extended with reverse transcriptase. The cellular levels of the *oxyS*, 5S, and 10Sa RNAs were determined from the ratio of the cellular RNA extention product to the in vitro RNA extension product. The in vitro RNAs were designed to be shorter than the cellular RNAs (described in Experimental Procedures).

methylates unpaired adenosine and cytidine residues. Methylated bases lead to the termination of elongation by reverse transcriptase, and therefore can be detected in a primer extension assay. Exponentially growing wild-type K12 cells were induced with hydrogen peroxide and then treated with DMS for 5 min. Total RNA isolated from these cells was subjected to primer extension analysis with an end-labeled oligonucleotide complementary to the sequence between stem loop (b) and stem loop (c). As shown in Figure 2B, multiple termination products were detected for the DMS-treated samples. The positions of these products are in agreement with the conformation predicted for stem loops (a) and (b). We also probed the in vivo structure of a plasmid-encoded *oxyS* RNA (poxyS) that is transcribed from the *tac* promoter and has a different transcription start (described in Experimental Procedures), and found that the modification patterns obtained for the plasmid-encoded RNA were virtually identical to those obtained for the chromosomally encoded *oxyS* RNA (Figure 2B).

Two termination products (indicated by arrows in Figure 2B) observed in the absence of DMS treatment suggested that the *oxyS* RNA may undergo processing at positions G+52 and G+64. To establish whether the termination sites were due to cleavage of *oxyS* or due to reverse-transcriptase termination products, we probed a Northern blot with the same end-labeled oligonucleotide used for the primer extension reactions. In addition to the full-length *oxyS* RNA, the Northern blot showed the presence of two shorter RNA species that corresponded to the termination products seen in the primer extension assays (data not shown, also detected in long exposures of Figures 1A and 1B). These results indicate that *oxyS* undergoes a cleavage process; however, it is not clear whether the cleavage is critical for function.

oxyS Is Stable and Abundant

To determine the in vivo stability of the *oxyS* RNA, exponentially growing wild-type K12 cells were exposed to hydrogen peroxide. After 5 min, the culture was treated with rifampicin to block any further initiation of transcription. Samples were then taken at 5, 20, 40, and 60 min time intervals, and the purified RNA was subjected to primer extension analysis (data not shown). From densitometric analysis of the corresponding autoradiograms,

Table 1. *oxyS*-Regulated Genes

Gene[a,b]	Function[c]	β-Galactosidase Activity[d] pKK177-3	poxyS	psyxO
fhlA	Activator of the *hyp* operon (61.4′, *hypABCDE fhlA*)	61	0.3	60
*yhiV**	Metal resistance/nodulation/cell division–type transporter (78.8′, *yhiUV*)	217	0	213
*yhiM**	Possible transporter (78.3′, *yhiM*)	1,044	1	979
uhpT	Hexose phosphate transporter (82.8′, *uhpABC T*)	0	15	0.1
pqqL	Homology to endopeptidases (33.8′, *yddABpqqLM*)	0	7	0
*dps**	Nonspecific DNA-binding protein (18.3′, *dps*)	43,794	5,937	40,053
*gadB**	Glutamate decarboxylase (33.8′, *gadB*)	18,534	69	19,964
rpoS	σ^s (61.7′, *nlpDrpoS*)	737[e]	106[e]	580[e]

[a] Corresponding GenBank accession numbers: *fhlA* (M58504, X52227), *uhpT* (M89479), *pqqL* (X71917), *dps* (X69337), *gadB* (M84025), *rpoS* (X16400).

[b] Genes also regulated by σ^s are indicated with an asterisk. Only *oxyS* regulation of *dps* is completely dependent on σ^s; *yhiV*, *yhiM*, and *gadB* show partial dependence.

[c] Map locations according to Berlyn et al. (1996), and position in operon are given in parenthesis.

[d] Average (in Miller units) of three independent assays. Cells were diluted 1:100 in LB and then grown for 12 hours.

[e] Due to *amp*^{r} on the chromosome of RO91, *cm*^{r} was inserted into the unique PvuI site of pKK177-3, poxyS, and psxyO.

oxyS half-life in the presence of rifampicin was calculated to be between 12 and 15 min, indicating that the *oxyS* RNA is relatively stable compared to most E. coli mRNAs, which have a 2–4 min half-life (Belasco, 1993). *oxyS* stability was also examined in MC4100 cells (used in experiments below), and a half life of approximately 30 min was calculated (data not shown).

To assess the abundance of *oxyS*, we compared the level of *oxyS* present after treatment with hydrogen peroxide to the levels of the 5S rRNA and the 10Sa RNA. Samples of total RNA isolated from wild-type K12 cells were mixed with defined amounts of in vitro synthesized *oxyS*, 5S, or 10Sa RNAs. The relative levels of the cellular RNA compared to the in vitro synthesized species were then determined by primer extension analysis. Quantitation of the bands in Figure 2C showed that the levels of the 10Sa and *oxyS* RNAs are 26% and 9% of the 5S rRNA level, respectively. Assuming that there are approximately 50,000 ribosomes/cell in log-phase E. coli cells with a doubling time of 20–30 min (Bremer and Dennis, 1987), this corresponds to approximately 50,000 molecules of 5S rRNA, 13,000 molecules of 10Sa RNA, and 4,500 molecules of *oxyS* RNA per hydrogen peroxide–treated cell. Similar levels of *oxyS* were observed in MC4100 cells (data not shown).

Effect of *oxyS* on the Levels of E. coli Proteins

The high level of *oxyS* induced by hydrogen peroxide suggested that the small RNA has a physiological role. Hence, we examined the consequences of deleting and constitutively expressing *oxyS*. Since the effects of constitutive expression were more dramatic than the effects of the *oxyS* deletion, we first characterized the constitutive phenotype. In these experiments, the *oxyS* RNA (poxyS) and a control RNA complementary to *oxyS* (psyxO) were transcribed from the *tac* promoter of the pKK177-3 vector. The levels of *oxyS* expressed from poxyS, even in the absence of IPTG, were comparable (2-fold higher) to the levels seen in *oxyR2* mutants and in wild-type cells after treatment with hydrogen peroxide (Figure 2B and data not shown). Constitutive *oxyS* expression had a dramatic effect on the protein synthesis pattern for K12 cells grown in rich medium (Figure 3A). The levels of more than ten proteins were visibly affected; some protein levels were reduced and others were elevated in the presence of *oxyS*. Changes in protein synthesis were also observed for cells grown in minimal medium (data not shown).

Two proteins whose synthesis was repressed by constitutive *oxyS* expression in rich media (indicated by arrows in Figure 3A) were identified by N-terminal sequence analysis. The 19 kDa protein was unambiguously identified as Dps, a nonspecific DNA-binding protein abundant in stationary phase (Almirón et al., 1992), and the 50 kDa protein was found to be GadB, one of two glutamate decarboxylases in E. coli (Smith et al., 1992). Assays of *lacZ* fusions to the promoters of the *dps* and *gadB* genes (Table 1) and immunoblots probed with antibodies directed against the Dps and the two homologous glutamate decarboxylase proteins (GAD) (Figure 3B) confirmed that *dps* and *gadB* are repressed by the *oxyS* RNA but not the complementary control RNA.

Identification of *oxyS*-Regulated Genes

The dramatic change in protein synthesis pattern observed with constitutive *oxyS* expression suggested that *oxyS* may be a regulator of gene expression and prompted us to undertake a screen for E. coli genes whose expression is modulated by *oxyS*. We used a bacteriophage λ*plac*Mu carrying a *lacZ* gene that is deleted for its promoter and translational signals and is flanked by Mu sequences that allow random insertion in the bacterial genome (Bremer et al., 1985). When the Mu-*lac* construct is inserted in the correct orientation

and reading frame, a gene fusion is created in which *lacZ* is expressed from the promoter and translation start site of the target gene. The phage λ*plac*Mu was used to infect MC4100 (Δ*lac*) cells carrying a poxyS derivative (poxyS-lacI) in which the expression of *oxyS* is under tight control of the LacI repressor. This allowed us to screen for fusions whose expression was increased or decreased upon *oxyS* induction with IPTG. In a screen of approximately 2500 in-frame gene fusions, more than 20 were found to be affected by *oxyS*, and five were selected for further study. The fragments carrying the corresponding Mu-*lac* insertions were identified on Southern blots using a labeled *lacZ* probe and were subsequently subcloned and sequenced.

Table 1 summarizes some of the characteristics of the eight *oxyS* target genes we have characterized; the five genes that were identified by the Mu-*lac* fusions, the two genes identified by N-terminal sequence analysis (Figure 3), and *rpoS* (discussed below). Three subcloned Mu-*lac* fusions were found to correspond to previously identified genes: *fhlA*, a transcriptional activator (Maupin and Shanmugam, 1990; Schlensog and Böck, 1990), *uhpT*, a hexose phosphate transporter (Island et al., 1992), and *pqqL*, a gene that shows homology to a family of endopeptidases and can complement a defect in pyrroloquinoline quinone synthesis in Methylobacterium organophilum (Springer et al., 1996, Turlin et al., 1996). The two other fusions were in sequences identified as open reading frames in the E. coli sequence database: one of the ORFs, *yhiV*, showed homology to E. coli *acrB* and *acrF,* which encode members of the heavy metal resistance/nodulation/cell division family of bacterial transporters (reviewed in Dinh et al., 1994; Ma et al., 1994). Based on hydrophobicity predictions and partial sequence homologies, the second ORF, *yhiM*, is also likely to encode a transporter or permease. Three of the genes (*fhlA*, *yhiV*, and *yhiM*) are repressed while two (*uhpT* and *pqqL*) are activated by *oxyS* (Table 1). Immunoblot assays confirmed that the levels of the endogenous FhlA protein were also repressed in wild-type K12 cells constitutively expressing the *oxyS* RNA (Figure 3B).

Intriguingly, Figure 3B showed that some of the *oxyS*-regulated genes, such as *dps* and *gadB*, are induced upon entry into stationary phase, and we observed that *yhiV*, *yhiM*, *dps*, and *gadB* are regulated by the stationary phase–specific sigma factor σ^s/RpoS (A. Z., S. A., and G. S., unpublished data). This prompted us to examine the levels of σ^s in *oxyS*-overexpressing cells. Immunoblots (Figure 3B) probed with antiserum against σ^s and assays (Table 1) of a *rpoS-lacZ* translational fusion (RO91, Lange and Hengge-Aronis, 1994) both showed that σ^s synthesis was repressed by *oxyS*. Although σ^s itself acts as a global regulator, we still observed significant *oxyS* effects on the protein synthesis pattern in

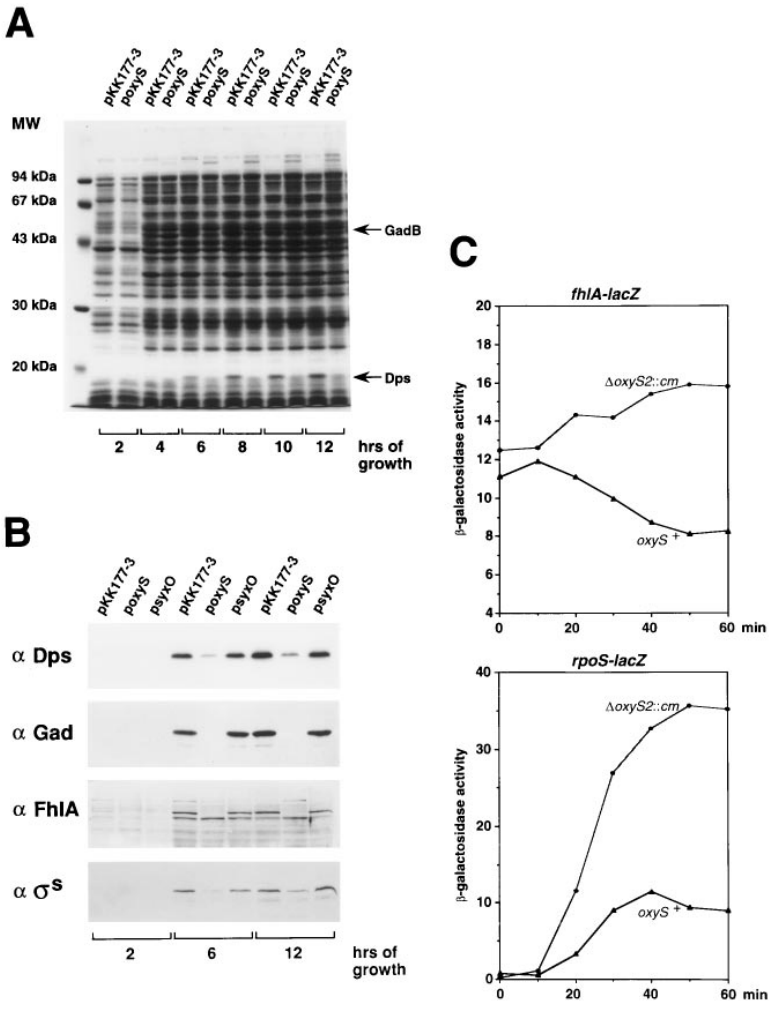

Figure 3. Changes in Protein Levels Due to Constitutive *oxyS* Expression

(A) Protein synthesis pattern of K12 cells carrying pKK177-3 and poxyS. The proteins from equal amounts of cells grown in LB medium for 2, 4, 6, 8, 10, and 12 hr were separated by SDS–PAGE.

(B) Immunoblot of Dps, GAD, FhlA, and σ^s levels in K12 cells carrying pKK177-3, poxyS, and psyxO. The proteins from equal amounts of cells grown in LB medium for 2, 6, and 12 hr were separated by SDS–PAGE and probed with α-Dps, α-Gad, α-FhlA, and α-σ^s polyclonal antibodies.

(C) *fhlA-lacZ* (top) and *rpoS-lacZ* (bottom) expression in *oxyS*$^+$ and Δ*oxyS2*::*cm* mutant cells after treatment with hydrogen peroxide. β-galactosidase activity (in Miller units) was assayed at 10 min intervals after exponential cultures were treated with 200 μM hydrogen peroxide. Experiments were repeated a minimum of three times; a typical data set is shown (the average basal levels for *fhlA-lacZ* and *rpoS-lacZ* were 9.9 ± 1.9 and 1.0 ± 0.4, respectively).

rpoS$^-$ mutant strains, suggesting that only a subset of the *oxyS* targets are part of the σ^s regulon (data not shown). The finding that two *oxyS*-repressed genes (*fhlA* and *rpoS*) encode transcriptional regulators indicated that the *oxyS* RNA might function to integrate the response to hydrogen peroxide with other regulatory networks.

To confirm that *oxyS* transcribed from its natural promoter has the same effects as *oxyS* transcribed from the *tac* promoter of poxyS, we examined the expression of the *oxyS* target genes in *oxyR2* constitutive mutant strains. The repression and induction in *oxyR2* constitutive mutant strains was the same as that observed for the strains carrying poxyS (data not shown). We also examined the effects of deleting *oxyS* and found that the *fhlA*- and *rpoS-lacZ* fusions were consistently derepressed (2- to 4-fold) in Δ*oxyS2*::*cm* strains treated with hydrogen peroxide (Figures 3C). The repression by *oxyS* induced by hydrogen peroxide is less than the repression by constitutively expressed *oxyS*, but this is likely to be due to the transient nature of the induction. Despite differences in the magnitude of the regulation, the results of deleting *oxyS* demonstrate that the RNA has a regulatory role under physiological conditions. Finally, to eliminate the possibility that the *oxyS* encodes a small peptide, we also mutated the two UUG codons to UAG but still observed regulation by *oxyS* (data not shown).

Effect of *oxyS* on Hydrogen Peroxide–Induced Killing and Mutagenesis

Since the *oxyS* RNA is primarily induced by hydrogen peroxide, we tested whether poxyS or *oxyS* deletion strains showed altered sensitivity to peroxides. Exponentially growing cultures were split, and half of the cells were pretreated with 200 μM hydrogen peroxide for 15 min. Both naive and pretreated cells were then challenged with 1, 2.5, 5, and 10 mM hydrogen peroxide. We found that neither the K12 cells constitutively expressing *oxyS* (Figure 4A) nor cells carrying a deletion of the *oxyS* gene (Figure 4B) showed increased or decreased killing by hydrogen peroxide when compared to the isogenic parent. Both the constitutive strains and the deletion strains also displayed wild-type adaptation to hydrogen peroxide. Similar experiments were carried out in minimal media and during later stages of growth, but no significant differences in hydrogen peroxide sensitivity were observed under any of the conditions tested (data not shown).

Reactive oxygen species are known to contribute to DNA damage. Therefore, we also investigated whether *oxyS* had an effect on mutagenesis. The pretreated and peroxide-challenged K12 cultures monitored in the killing curves above were grown overnight and plated on rifampicin-containing media. The numbers of rifampicin-resistant mutants were then normalized to the numbers of viable cells. Interestingly, the K12/poxyS strain showed reduced mutagenesis by hydrogen peroxide compared to the K12/pKK177-3 control strain (Figure 4C). Conversely, cells carrying a deletion of the *oxyS* locus showed a higher level of mutagenesis than wild-type cells (Figure 4D). These observations indicate that *oxyS* has a protective effect against hydrogen peroxide–induced mutagenesis in E. coli K12. Similar results were observed in a different strain background (Table 2A and data not shown). In these assays, we also found that cells carrying poxyS consistently had lower frequencies of spontaneous mutagenesis than cells carrying psyxO (Table 2B).

Characterization of *oxyS* Protection against Mutagenesis

Given the effects of *oxyS* on hydrogen peroxide–induced mutagenesis, we examined the role of *oxyS* in protecting against other mutagens. For some of these assays, we used the *lacZ*$^-$ strain CC102, which allows for the rapid detection of base substitutions by monitoring the number of *lacZ*$^+$ revertant colonies (Cupples and Miller, 1989). CC102 cells carrying pKK177-3, poxyS, and psyxO were treated with 100 μg/ml of 1-methyl-3-nitro-1-nitrosoguanidine (MNNG) and assayed for mutagenesis. The reversion frequency of *oxyS*-expressing cells was substantially lower than the reversion frequency of cells carrying either the pKK177-3 (data not shown) or the control psyxO plasmid (Table 2C). Consistent with these results, the reduced frequency of MNNG mutagenesis observed for CC102 *oxyR2*/pKK177-3 (1,029 revertants compared to 7,971 revertants for CC102 *oxyR*$^+$/pKK177-3) could partially be reversed by the psyxO construct (2,697 revertants).

To determine whether *oxyS* also protected against UV mutagenesis, the *his4* mutant strain AB1157 carrying either poxyS or psyxO was irradiated with UV, and the number of His$^+$ revertants was monitored on low histidine agar plates. In contrast to the results seen with hydrogen peroxide and MNNG, AB1157 carrying the sense plasmid had a higher number of revertants than AB1157 carrying the antisense plasmid (Table 2D). The lack of protection against UV suggests that the antimutator phenotype of *oxyS* does not involve the SOS DNA repair system. This conclusion was supported by assays in which we plated poxyS- and psyxO-carrying cells on

Table 2. Rates of Spontaneous and Chemical-Induced Mutagenesis

(A) Rif^r Mutants Induced by 5 mM Hydrogen Peroxide

Strain	Rif^r Mutants per 10^8 Cells[a]
CC102/psyxO	28.8 ± 11.5
CC102/poxyS	8.5 ± 7.5

(B) Spontaneous Rif^r Mutants

Strain	Rif^r Mutants per 10^8 Cells[a]
CC102/psyxO	5.9 ± 4.8
CC102/poxyS	2.9 ± 3.8

(C) Lac$^+$ Revertants Induced by 100 μg/ml MNNG

Strain	Lac$^+$ Revertants per 10^8 Cells[a]
CC102/psyxO (78%)[b]	7224 ± 1886
CC102/poxyS (43%)[b]	128 ± 40

(D) His$^+$ Revertants Induced by UV

Strain	His$^+$ Revertants per 10^8 Cells[a]
AB1157/psyxO (69%)[b]	142 ± 25
AB1157/poxyS (18%)[b]	333 ± 27

[a] Average of at least two independent experiments, each carried out with 3–4 cultures of each strain.
[b] Survival.

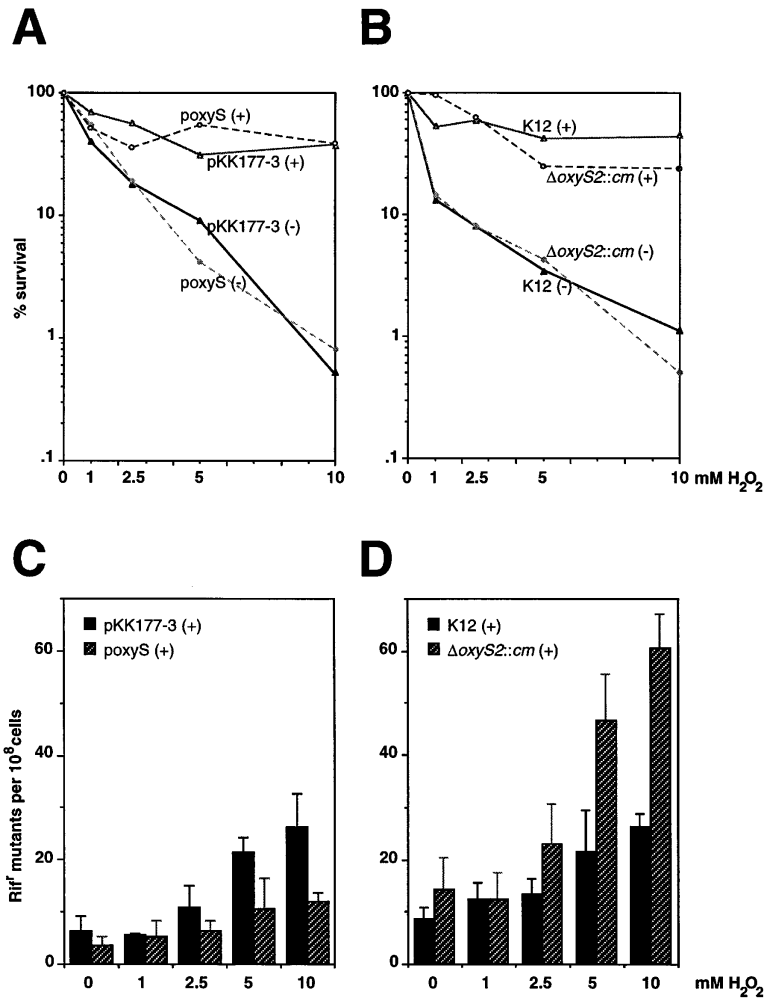

Figure 4. *oxyS* Protection against Oxidative DNA Damage

(A) Sensitivity of naive (−) and pretreated (+) K12/pKK177-3 and K12/poxyS cells to hydrogen peroxide.

(B) Sensitivity of naive (−) and pretreated (+) K12 and Δ*oxyS2*::*cm* cells to hydrogen peroxide. Exponential cultures were split and half of the culture was treated with 200 μM hydrogen peroxide. After 30 min, both the naive and pretreated cells were challenged with 0, 1, 2.5, 5, and 10 mM hydrogen peroxide. Viability was assayed after 20 min by plating on LB medium. Experiments were repeated a minimum of three times; a typical data set is shown.

(C) Number of Rif^r mutants for pretreated (+) K12/pKK177-3 and K12/poxyS cells challenged with hydrogen peroxide.

(D) Number of Rif^r mutants for pretreated (+) K12 and Δ*oxyS2*::*cm* challenged with hydrogen peroxide. The pretreated cultures from above were grown for 24 hr. Aliquots were then plated on LB plates containing 100 μg/ml rifampicin to test for Rif^r mutants and on LB plates to determine the number of cells. The results correspond to the average of three independent experiments, and the error bars represent the standard deviation of the mean.

limiting histidine agar plates, placed disks impregnated with different mutagens in the centers of the plates, and monitored the ring of His$^+$ revertant colonies surrounding the killing zone. In these experiments, we found that while *oxyS* protected against mutagenesis caused by the alkylating agents MNNG and ethyl methanesulfonate (EMS), which are SOS independent, *oxyS* did not protect against methyl methane sulfonate (MMS), which is SOS dependent (data not shown).

Our results implied that *oxyS* directly or indirectly regulates one of the DNA repair systems of the cell. Therefore, we examined the effects of constitutive *oxyS* expression in strains defective in different repair activities. The antimutator phenotype of *oxyS* was significantly reduced in *dam*$^-$, *mutS*$^-$, and *mutL*$^-$ mutant backgrounds (Table 3 and data not shown), showing that DNA methylation and mismatch repair are required to see the effects of *oxyS*. In contrast, *oxyS* was able to suppress the elevated frequencies of MNNG mutagenesis in *mutM*$^-$ and *mutY*$^-$ strains (Table 3), suggesting that the small RNA is not modulating the *mutM* and *mutY* DNA glycosylases. We also found that mutations in SOS response genes such as *uvrA* and *umuDC* did not affect the ability of *oxyS* to protect against MNNG (data not shown), indicating that *oxyS* is not acting through the SOS or excision repair systems. In addition, since *oxyS* still had a protective effect on mutagenesis in an *rpoS* mutant background (data not shown), the antimutator phenotype is not dependent on RpoS. Given that the decrease in MNNG mutation frequency was not accompanied by an increase in survival, *oxyS* is also not acting through the Ada regulator (Table 2C). Although it

Table 3. Rates of MNNG-Induced Mutagenesis in DNA Repair Mutant Backgrounds

	His$^+$ Revertants per 5 × 10^7 Cells[a]				
	AB1157	*mutS*::Tn*10*[b]	*mutL*::*kan*[b]	*mutM*::Tn*10*[b]	*mutY*::mini-Tn*10*[b]
psyxO	250 ± 25	502 ± 32	315 ± 52	204 ± 65	254 ± 30
poxyS	31 ± 21	292 ± 91	177 ± 1	41 ± 20	41 ± 19

[a] Average of at least two independent experiments, each carried out with two cultures of each strain.
[b] Mutations were moved into AB1157 by P1 transduction.

is possible that a defect in a major repair system may mask the protective role of *oxyS* in another pathway, this genetic survey of DNA repair systems in E. coli suggests that mismatch repair is the most likely target for *oxyS*.

Discussion

In this paper, we report the discovery of *oxyS*, a small, stable RNA induced by oxidative stress. The *oxyS* RNA is synthesized rapidly, within 1 min after treatment with hydrogen peroxide, and is stable, with a 10–30 min half-life. The induced level of *oxyS* is as high as 4500 copies per cell. The RNA contains at least two stem-loop structures and does not encode a protein. Given the stability and abundance of the *oxyS* RNA, we investigated its physiological function. We found that *oxyS* activates and represses the expression of numerous genes in E. coli and protects against spontaneous and chemically induced mutagenesis.

oxyS as a Pleiotropic Regulator

A regulatory role for *oxyS* was indicated by the substantial changes in the protein synthesis pattern observed with constitutive *oxyS* expression. Peptide sequencing of two down-regulated polypeptides and genetic screens of randomly inserted *lacZ* translational fusions allowed us to identify eight genes whose expression is modulated by *oxyS*. Based on the number of *oxyS*-regulated *lacZ* fusions identified thus far, we estimate that *oxyS* may regulate the expression of as many as 40 genes (∼1% of all genes) in E. coli. This regulation appears to be complex; it involves the activation of some genes (*uhpT*, *pqqL*) and the repression of others (*fhlA*, *yhiV*, *yhiM*, *dps*, *gadB*, *rpoS*). *oxyS* could act at multiple levels including transcription, message stability, translation, or even protein stability. A comparison of the promoter and 5′-untranslated regions of the *oxyS*-regulated genes did not reveal a conserved sequence that might be a common target for the *oxyS* RNA. This observation and other preliminary results suggest that *oxyS* may regulate different target genes by different mechanisms (S. A., A. Z., and G. S., unpublished data). *oxyS* may also act indirectly through other genetic regulators. For a subset of the *oxyS* targets, the effects of *oxyS* are mediated by the stationary phase sigma factor σ^s, but *oxyS* regulation of other target genes is independent of σ^s.

Although we do not fully understand the physiological significance of the *oxyS* targets in the response to oxidative stress, it is intriguing that two of the genes, *fhlA* and *rpoS*, encode transcriptional regulators. FhlA is an activator of genes required for the synthesis of the formate hydrogenlyase system in E. coli (Maupin and Shanmugam, 1990; Schlensog and Böck, 1990). We suggest that *oxyS* repression of *fhlA* is important for the survival against hydrogen peroxide because the formate hydrogenlyase complex, which is primarily synthesized under anaerobic conditions, contains several metal cofactors that could be detrimental during oxidative stress. *oxyS* repression of *rpoS* may help to prevent redundant utilization of transcriptional regulators. We and others have observed that several genes such as *katG*, *dps*, and *gorA*, are regulated by both OxyR and σ^s (Altuvia et al., 1994; Ivanova et al., 1994; Becker-Hapak and Eisenstark, 1995). Since OxyR is strongly activated by hydrogen peroxide during log-phase growth, σ^s is not needed, and σ^s repression by *oxyS* prevents redundancy. In stationary phase, OxyR, for unknown reasons, is not activated by hydrogen peroxide (Altuvia et al., 1994; González-Flecha and Demple, 1995). Under these conditions, σ^s induction of *katG*, *dps*, and *gorA* is critical for cell survival of oxidative stress. Therefore, we propose that one function of the *oxyS* regulator is to integrate the response to oxidative stress with other regulatory networks.

oxyS as an Antimutator

The antimutator effect of the *oxyS* RNA was found by assays of spontaneous as well as hydrogen peroxide–, MNNG-, and UV-induced mutagenesis. Protection from the mutagenic effects of hydrogen peroxide is a logical response to oxidative stress, since most of the toxicity of this reactive oxygen intermediate in E. coli is attributed to DNA damage (reviewed in Imlay and Linn, 1988). Many of the lesions caused by hydrogen peroxide are repaired by the RecA-dependent SOS pathway; however, other cellular mechanisms clearly contribute to the repair of oxidative DNA damage (reviewed in Imlay and Linn, 1988; Demple and Harrison, 1994). We propose that a defense system regulated by the *oxyS* RNA, possibly methyl-directed mismatch repair, is another significant contributor to the protection against oxidative DNA damage.

Our current results together with previous findings (Storz et al., 1990) suggest that E. coli cells possess two levels of defense against oxidative stress. The first line of defense is the scavenging enzymes such as hydroperoxidase I and the alkyl hydroperoxide reductase, which act to eliminate the reactive oxygen species. The synthesis of these antioxidant activities is regulated directly by the redox-sensitive transcription factor OxyR. The second line of defense is activities that repair the damage caused by oxidative stress and activities that allow the cells to resume growth. We propose that the small *oxyS* RNA acts to control this second line of defense by modulating the levels of DNA repair activities. In addition, *oxyS* coordination of the hydrogen peroxide response with the responses controlled by other regulators such as σ^s prevents redundancy and facilitates an expedient return to normal growth.

Small, Stable RNAs

It is worth noting that two other small RNA species have been connected to stress responses in E. coli. Transcription of the 93 nt *micF* RNA, an antisense regulator of OmpF outer member protein synthesis, is controlled by numerous factors including the superoxide-response regulator SoxS (Chou et al., 1993). The level of the 85 nt *dsrA* RNA is increased at low temperature, and the RNA has recently been shown to be required for low temperature synthesis of σ^s (Sledjeski and Gottesman, 1995; Sledjeski et al., 1996). Together, these

findings raise the intriguing question of why RNA species have been recruited to be regulators of stress responses. One attractive hypothesis is that the relatively low input of energy and the short time required to synthesize small RNAs make them ideal regulators of rapid responses to changing environmental conditions. Secondly and perhaps more importantly, the ability of RNA species to act at posttranscriptional levels provides greater regulatory flexibility by permitting the repression or activation of genes that have already been transcribed.

It is also intriguing to speculate how many small RNA species are yet to be identified. Small RNAs are poor targets for mutational screens, are difficult to detect with biochemical assays, and are hard to identify by sequence analysis. Many open reading frames have been cataloged based on the sequences of whole genomes, but the genes encoding functional RNA species are likely to be missed. The region encoding *oxyS* was thought to be devoid of genes (Gustafsson and Warne, 1992), because the distance between the neighboring *argH* and *oxyR* genes is only 266 nt. As we have found for *oxyS*, other small RNA species may only be synthesized transiently yet may play critical roles in cellular metabolism.

Experimental Procedures

Strain and Plasmid Construction

Strains were grown at 37°C in Luria-Bertani (LB), MacConkey lactose, VBC glucose, or M63 glucose medium unless otherwise indicated (Vogel and Bonner, 1956; Silhavy et al., 1984). Ampicillin (50–100 μg/ml), kanamycin (25 μg/ml), or chloramphenicol (25 μg/ml) was added where appropriate. All DNA manipulations were carried out using standard procedures. P1 transductions were carried out as described (Silhavy et al., 1984).

poxyS (pGSO4)

To construct poxyS, the *oxyS* gene of pAQ26 (Altuvia et al., 1994) was amplified by PCR using primers (5′-TCTGACTGAGAATTCCTCACAGAA and 5′-TGAGCCAAGCTTATCGCCGGG). The fragment was then digested with EcoRI and HindIII and cloned into the corresponding sites of pKK177-3. The *oxyS* RNA derivative transcribed from the *tac* promoter of poxyS carries 48 additional 5′ nucleotides (5′-GAATTGTGAGCGGATAACAATTTCAGAGAGGAAACAGAATTCCTCACA). pKK177-3 is the same as pKK223-3 except for the deletion of a BamHI–PvuII fragment (Brosius and Holy, 1984).

psyxO (pGSO5)

To construct psyxO, the *oxyS* gene of pAQ26 was amplified by PCR using primers (5′-GAATTCTGGCTTATCGCCGGG and 5′-TCTGACTGAAGCTTGCTCACAGAA). The fragment was then digested with EcoRI and HindIII and cloned into the corresponding sites of pKK177-3. The *oxyS* RNA derivative transcribed from the *tac* promoter of psyxO carries 77 additional 5′ nucleotides (5′-GAATTGTGAGCGGATAACAATTTCAGAGAGGAAACAGAATTCTGGCTTATCGCCGGGCTTTTTTATGGCAAAAAAAA). Cm^r derivatives were constructed by amplifying the *cm^r* gene of pACYC184 by PCR and subcloning the fragment into the unique PvuI site of pKK177-3, poxyS, and psyxO.

poxyS-lacI (pGSO6)

To construct poxyS-lacI, the *lacI* gene was amplified from K12 chromosomal DNA by PCR using primers (5′-GACACCATCGAATGGCGC and 5′-CTCACAATTCCACACAAC). The *lacI* fragment was cloned into the unique HindIII site (filled-in) of pGSO4.

ΔoxyS2::cm

An *oxyS* deletion-insertion was generated as follows: the 2.2 kb BanI (filled-in)–EcoRI fragment of pAQ26 was cloned into the SstI (filled-in)–EcoRI sites of pUC13. The 2.2 kb BamHI–EcoRI fragment of this intermediate was then used to replace the BamHI–EcoRI fragment of pAQ26. The *cm^r* gene (amplified by PCR) was then inserted into the unique BamHI site of this pAQ26 derivative. Plasmids carrying *cm^r* in the same orientation as the original *oxyS* gene were linearized with HindIII and transformed into *recD1014* mutant strain V355. Finally, Δ*oxyS2*::*cm* was moved into K12 (GSO35), MC4100 Φ*fhlA-lacZ* (GSO45), RO91 (GSO46) by P1 transduction. The absence of *oxyS* was verified by Southern blot analysis.

Isolation of Mud*-lac *Fusions

To isolate *oxyS*-regulated *lacZ* fusions, 5 ml of an overnight culture of MC4100/poxyS-lacI (GSO32) was concentrated to 1 ml. Aliquots (100 μl) of λpMu507 and λ*plac*Mu9H (Bremer et al., 1985) were added to the concentrated culture and incubated at 37°C for 30 min. After LB and sodium citrate were added to give a final volume of 5 ml and a sodium citrate concentration of 30 mM, the cells were spun down and resuspended in 1 ml of LB containing 30 mM sodium citrate. Aliquots (67–100 μl) were spread on LB plates containing kanamycin, ampicillin, 7.5 mM sodium citrate, and 62.5 μg/ml of 5-bromo-4-chloro-3-indolyl-β-D-galactoside. The plates were incubated overnight at 37°C. All blue colonies (approximately 2500 total colonies) were then streaked onto selective LB plates with and without 18.75 μg/ml of isopropropyl-β-D-thiogalactoside. Insertions that showed different levels of expression with and without *oxyS* expression were transduced into MC4100 and assayed for β-galactosidase activity. Five fusions (MC4100 Φ*fhlA-lacZ* [GSO38], MC4100 Φ*yhiV-lacZ* [GSO39], MC4100 Φ*yhiM-lacZ* [GSO40], MC4100 Φ*uhpT-lacZ* [GSO41], and MC4100 Φ*pqqL-lacZ* [GSO42]) were chosen for further study. To identify the locations of the Mud-*lacZ* insertions, Southern blots of chromosomal DNA from the fusion strains were digested with EcoRI in conjunction with a series of enzymes and were probed with a 1.1 kb ClaI–SstI *lacZ* fragment of pRS415. Minilibraries were generated and probed with the same *lacZ* fragment to isolate subclones carrying hybridizing fragments of greater than 3 kb. Sequences upstream of the site of the *lacZ* insertion were then determined and compared to sequences in the GenBank database using the Blast program (Altschul et al., 1990).

Construction of lacZ Fusions

The *dps* and *gadB* promoter fagments were amplified using primers (5′-GAATTCGCTACTTTTCCTCTACACCG, 5′-GCGGGGATCCAGCAGATTGGTCGCTTTTG, 5′-GCAGAATTCGCTATTTTTATGT, 5′-GCGGATCCTCTTCTTTGTCGATCCAGTT) and subcloned into the unique BamHI and EcoRI sites of pRS552. The *lacZ* fusions were then recombined onto λRS45 and integrated into the attachment site of MC4100 as described (Simons et al., 1987) to generate MC4100 (λRS45 *dps-lacZ*) (GSO43) and MC4100 (λRS45 *gadB-lacZ*) (GSO44).

RNA Analysis

Total cellular RNA was isolated by three different methods: by buffered phenol extractions followed by DNAase I digestion, by acid-phenol extractions, or by lysozyme treatment and freeze-thawing followed by phenol extractions (Salser et al., 1967; McKenney et al., 1981; Gilman and Chamberlin, 1983; Storz and Altuvia, 1994).

Northern Blots

Total RNA was isolated from K12 and *oxyR2* (TA4110, Christman et al., 1985) cells grown to late exponential phase in VBC glucose medium. The RNA samples (10 μg) were then denatured at 65°C in 0.5 × TBE buffer, 70% formamide, separated on 6% urea–polyacrylamide gels, and transferred to nylon membranes by electroblotting. The membranes were hybridized with a random primer–labeled 1.5 kb BamHI–EcoRI fragment of pAQ17 (Christman et al., 1989).

In Vivo Structure Probing

The in vivo structure was determined as described (Altuvia et al., 1991). K12 cells were grown to midexponential phase in LB media, induced with 200 μM hydrogen peroxide for 5 min, and then treated with 50 mM DMS for 5 min. K12 cells carrying poxyS were treated with DMS for 5 min without prior hydrogen peroxide induction. Total RNA was extracted, and primer extension reactions to detect the modified bases were carried out using an end-labeled *oxyS* oligonucleotide (5′-GCAAAAGTTCACGTTGG). The extension products together with sequencing reactions primed with the same end-labeled *oxyS* primer were separated on an 8% sequencing gel.

Levels Determination

K12 and MC4100 cells were grown to midexponential phase in LB media and treated with 200 μM hydrogen peroxide for 5 min. Total

RNA was extracted, and in vitro synthesized control *oxyS* (0.02 pmol), 5S (0.05 pmol), or 10Sa (0.02 pmol) RNA was mixed with yeast tRNA (1 μg) or total cellular RNA (1 μg). These samples were then subjected to primer extension assays using primers specific to *oxyS* (5′-GCAAAAGTTCACGTTGG), 5S (5′-GAGACCCCACACTA CCATC), and 10Sa (5′-TTGCGACTATTTTTTGCGGC). The samples were separated on an 8% acrylamide gel, and the products were quantitated on a phosphorimager (Molecular Dynamics).

To synthesize the control RNA, fragments of *oxyS* (5′-CTTGAA TTCACTGCCCGTTC and 5′-TGAGCCAAGCTTATCGCCGGG), the 5S gene (5′-CTTGAATTCATGCCGAACTCAG and 5′-TTTGAAGCTT GGCAGTTCCCTACT), and the 10Sa gene (5′-GCGGAATTCAAGGTG CATGCCG and 5′-CTAAAGCTTAGTTTTCGTCGTTTG) were amplified by PCR and cloned into the EcoRI and HindIII sites of pGEM-1. The resulting plasmids were linearized with HindIII, and the control RNAs were synthesized with T7 RNA polymerase.

Protein and Enzyme Analysis

Protein Gels

Overnight cultures were diluted 1:100 and grown for the indicated times. Aliquots were centrifuged, and equal absorbance units were suspended in Laemmli buffer and separated by SDS–PAGE (12% acrylamide).

Immunoblot Assays

Proteins were transferred to nitrocellulose filters by electroblotting, and the filters were probed with a 1:250 dilution of α-Dps antibody (Almirón et al., 1992), a 1:2000 dilution of α-GAD antibody (Dean Smith and John Elliott, unpublished data), a 1:1000 dilution of α-FhlA (Korsa and Böck, 1997), or a 1:4000 dilution of α-σ^s antibody (Lange and Hengge-Aronis, 1994). The bound antibodies were visualized by incubating with ^{125}I-labeled protein A or were detected using the ECL Western blotting reagent kit (Amersham Life Sciences).

β-Galactosidase Assays

The β-galactosidase assays were carried out as described (Miller, 1972).

Mutagenesis and Killing Assays

Hydrogen Peroxide

Overnight cultures were subcultured and grown to A_{600} = 0.2 in LB. The cultures were split and half of the culture was pretreated with 200 μM hydrogen peroxide. After 30 min, aliquots of both the naive and pretreated cultures were treated with 0, 1, 2.5, 5, and 10 mM hydrogen peroxide. To determine viability, aliquots were taken after 20 min, diluted in M63 salts, and plated onto LB plates. To determine frequencies of mutagenesis, aliquots were taken after 24 hr and plated on LB plates containing 100 μg/ml of rifampicin. The numbers of Rif^r mutants were normalized to the numbers of viable cells at the 24 hr time point. For the assays with CC102, naive cells were treated with 5 mM hydrogen peroxide for 15 min at 22°C. Thereupon, 0.5 ml of cells were mixed with 2 ml LB, grown overnight, and plated as above.

Nitrosoguanidine

Overnight cultures were subcultured, grown to A_{600} = 0.5, washed in 100 mM sodium citrate (pH 5.5), and treated with 100 μg/ml MNNG for 10 min as described (Cupples and Miller, 1989). After mutagenesis, the cultures were washed twice in 100 mM potassium phosphate (pH 7), resuspended in the same buffer, and plated for viable cells. Aliquots (0.5 ml) of the washed suspension were also mixed with 2 ml LB and grown overnight. The overnight cultures were then plated on MacConkey lactose plates for mutants and on LB for viable cells. In the assays with AB1157, AB1157 *mutS215*::Tn*10* (SA14), AB1157 *mutL*::*kan* (SA15), AB1157 *mutM*::Tn*10* (SA16), and AB1157 *mutY*::mini-Tn*10* (SA17), aliquots (5×10^7 cells) of an overnight culture grown in M9 medium containing the appropriate supplements and high histidine levels (100 μg/ml) were plated onto M9 plates with limiting histidine (2 μg/ml). A disk impregnated with 2.5 μg MNNG was then placed in the center of each plate. The number of His$^+$ revertants was counted after 48 hr.

UV

UV-induced mutagenesis was assayed as described (Tadmor et al., 1992). Overnight cultures, grown in M9 medium containing the appropriate supplements and high histidine levels (100 μg/ml) were subcultured and grown to A_{600} = 0.4. The cells were collected, resuspended in 0.2 volumes of cold 10 mM Tris–HCl (pH 7.5) containing 150 mM NaCl, and irradiated in a small petri dish for 6 s at a distance of 20 cm from 254 mm bulbs. To determine viability, cells were diluted and plated onto LB. To determine mutation frequencies, 0.2 ml aliquots were plated directly onto M9 plates containing limiting histidine (2 μg/ml).

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GenBank Accession Number

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