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The *Escherichia coli* OxyS regulatory RNA represses *fhlA* translation by blocking ribosome binding

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OxyS is a small untranslated RNA which is induced in response to oxidative stress in *Escherichia coli*. This novel RNA acts as a global regulator to activate or repress the expression of as many as 40 genes, including the *fhlA*-encoded transcriptional activator and the *rpoS*-encoded σ^s subunit of RNA polymerase. Deletion analysis of OxyS showed that different domains of the small RNA are required for the regulation of *fhlA* and *rpoS*. We examined the mechanism of OxyS repression of *fhlA* and found that the OxyS RNA inhibits *fhlA* translation by pairing with a short sequence overlapping the Shine–Dalgarno sequence, thereby blocking ribosome binding/translation.

Keywords: antisense regulation/oxidative stress/OxyS RNA/ribosome binding/translation

Introduction

Small untranslated antisense RNA species have been shown to regulate gene expression in both bacterial and mammalian cells (reviewed in Wagner and Simons, 1994; Delihas, 1995). This control occurs at many levels, including transcription termination, RNA processing and decay, and translation initiation. In many cases, the small RNAs are encoded at the same loci as the target RNAs and regulate the expression of these specific target genes by base pairing at the regions of complementarity. One example is the 90-nucleotide CopA antisense RNA of the R1 plasmid. The CopA RNA is encoded on the strand opposite the transcript encoding RepA, a replication initiator protein. The CopA RNA inhibits *repA* translation by pairing with an upstream region of the mRNA (denoted CopT) (Blomberg *et al*., 1992). A second example is the 69-nucleotide RNA-OUT of the IS10 transposon. This antisense RNA is made from a promoter located within the coding region of the *IS*10 transposase (*tnp*) gene and blocks ribosome binding to the *tnp* mRNA by pairing across the ribosome-binding site (Ma and Simons, 1990). A few small regulatory RNAs encoded by genes located at genetic loci other than those of the target genes have been identified. The 93-nucleotide MicF RNA, which is encoded at 48 min on the *Escherichia coli* chromosome, decreases the synthesis of the OmpF outer membrane protein, which is encoded at 21 min. MicF acts by pairing with the 5′-untranslated region of the *ompF* transcript, thereby inhibiting translation and destabilizing the *ompF* message (Andersen *et al*., 1989; Andersen and Delihas, 1990).

We recently reported the discovery of OxyS, an abundant, 109 nucleotide, untranslated RNA which is induced by oxidative stress in *E.coli* (Altuvia *et al*., 1997). OxyS acts as a pleiotropic regulator, leading to increased and decreased expression of multiple genes, and as an antimutator, protecting the cells from spontaneous and chemically induced mutagenesis. Eight targets of OxyS regulation were identified, including *fhlA*, which encodes a transcriptional activator, and *rpoS*, which encodes the σ^s subunit of RNA polymerase. Both *fhlA* and *rpoS* are repressed by OxyS expressed constitutively from a multi-copy plasmid or from the chromosome. Conversely, the two genes are derepressed in an *oxyS* deletion strain treated with hydrogen peroxide (Altuvia *et al*., 1997).

We have initiated studies to examine the mechanism of OxyS regulation of its target genes. In this study, we carried out deletion analysis which showed that separate domains of the OxyS RNA are required for the regulation of *fhlA* and *rpoS*. We examined OxyS repression of *fhlA* and found that the RNA inhibits ribosome binding and translation by pairing with a short sequence overlapping the *fhlA* ribosome-binding site.

Results

Deletion analysis of OxyS

The secondary structure of OxyS was predicted to contain three stem–loops (**a**, **b** and **c**), of which the first two stem–loops were confirmed by *in vivo* structure probing (Altuvia *et al*., 1997). The computer folding programs also predicted the presence of a linker region of 27 nucleotides between stem–loops **b** and **c** (Figure 1). To learn more about the mechanism of OxyS action and to dissect the domains critical for OxyS activity, we constructed a series of deletion mutants. We then tested the ability of the truncated OxyS constructs to repress ϕ*fhlA–lacZ* and *rpoS742–lacZ* translational fusions (Table I). We found that OxyS derivatives which carry 5′-deletions of stem–loop **a** (poxy$S_{\Delta 1–47}$) and both stem–loops **a** and **b** (poxy$S_{\Delta 1–63}$) could still repress *fhlA* and *rpoS*. Surprisingly, an OxyS derivative carrying a 5′ deletion up to stem–loop **c** (poxy$S_{\Delta 1–90}$) was still able to repress the ϕ*fhlA–lacZ* fusion, indicating that stem–loop **c** is sufficient for *fhlA* repression. In contrast, OxyS repression of the *rpoS742–lacZ* fusion was abolished by this 90-nucleotide deletion suggesting that *rpoS* regulation by OxyS requires the 27-nucleotide region preceding stem–loop **c**. A 3′-deletion of stem–loop **c** (poxy$S_{\Delta 91–109}$) abolished all activity on both

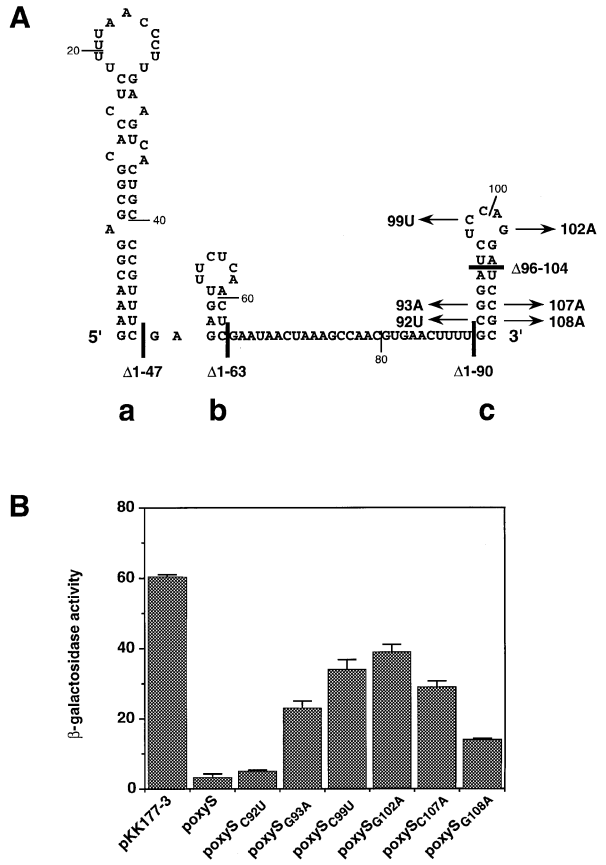

Fig. 1. Mutational analysis of *oxyS*. (**A**) Secondary structure of the OxyS RNA. The bars indicate the extent of the deletion mutations and the arrows indicate the point mutations generated by random mutagenesis. Based on the FOLD (Genetics Computer Group) program, the secondary structures of the mutants are similar to wild-type OxyS. (**B**) Effect of mutations on OxyS repression of ϕ*fhlA–lacZ*. The β-galactosidase activity (in Miller units) of stationary phase cells was assayed. Average (in Miller units) of two independent β-galactosidase assays is shown. Single colonies were grown overnight in LB with amp.

Table I. Effect of OxyS deletion mutants on *fhlA–lacZ* and *rpoS–lacZ* fusions

Construct[b]	β-galactosidase activity[a]	
	ϕ*fhlA–lacZ*	*rpoS742–lacZ*
pKK177-3	52 ± 5	386 ± 94
poxyS	3 ± 2	54 ± 15
poxyS$_{\Delta 1–47}$	3 ± 1	107 ± 45
poxyS$_{\Delta 1–63}$	4 ± 2	138 ± 29
poxyS$_{\Delta 1–90}$	5 ± 2	522 ± 102
poxyS$_{\Delta 91–109}$	51 ± 2	341 ± 58
poxyS$_{\Delta 96–104}$	36 ± 4	189 ± 54

[a]Average (in Miller units) of four independent experiments. Cells were diluted 1:100 and grown in LB medium with cm for 12 h.
[b]All plasmids carry cm^r. Based on the FOLD (Genetics Computer Group) and *mfold* (http://www.ibc.wustl.edu/~zuker/rna) programs, the secondary structures of the 3′-ends of OxyS$_{\Delta 1–47}$, OxyS$_{\Delta 1–63}$, and OxyS$_{\Delta 1–90}$, and the 5′-ends of OxyS$_{\Delta 91–109}$ and OxyS$_{\Delta 96–104}$ are similar to those of the wild-type.

genes. We also constructed a deletion of the loop sequence of stem–loop **c** (poxyS$_{\Delta 96–104}$) and found that this derivative was unable to repress *fhlA* but still showed some repression of *rpoS*. Northern blot analysis confirmed that the 5′-deletion derivatives had the expected sizes and that the mutant RNA levels were similar to wild-type levels (poxyS$_{\Delta 1–47}$) or slightly reduced (2-fold for poxyS$_{\Delta 1–63}$ and 2.5-fold for poxyS$_{\Delta 1–90}$) (data not shown). The majority of the OxyS transcripts from poxyS$_{\Delta 91–109}$ and some of the transcripts from poxyS$_{\Delta 96–104}$ were longer than expected, confirming that stem–loop **c** is a transcription terminator. Stem–loop **c** is also likely to confer stability to OxyS since the RNA levels from poxyS$_{\Delta 91–109}$ and poxyS$_{\Delta 96–104}$ were reduced (6- and 4-fold, respectively). Together, our results show that different regions of the small RNA are required for repression of *fhlA* and *rpoS*: OxyS repression of *fhlA* requires stem–loop **c** while OxyS repression of *rpoS* requires the 27-nucleotide linker region together with stem **c**. The findings also suggest that the OxyS RNA may act by different mechanisms at different genes. Here we examine the mechanism of OxyS regulation of *fhlA*. The regulatory action of OxyS on *rpoS* expression has been described recently (Zhang *et al*., 1998).

Table II. Effect of OxyS on *fhlA7*::*lacZ* transcriptional and translational fusions

Fusion	β-galactosidase activity[a]		
	pKK177–3[b]	poxyS[b]	Fold repression
fhlA–lacZ (transcriptional fusion)	234 ± 9	90 ± 11	3×
fhlA7–lacZ (translational fusion)	37 ± 4	2 ± 1	19×

[a]Average (in Miller units) of two independent assays. Single colonies were grown in LB medium with amp for 18 h.
[b]amp^r.

OxyS inhibits fhlA expression at a post-transcriptional level

The initial OxyS-regulated ϕ*fhlA–lacZ* fusion was isolated as a Mu–*lac* insertion in the *E.coli* chromosome. To delineate the *fhlA* sequences required for OxyS repression of this target gene, we constructed a series of *fhlA–lacZ* translational fusions carrying 1426, 1177, 816 and 514 bp of *fhlA* 5′ upstream sequence. These fusions were then recombined onto λ and integrated into the *att* site. All the constructs showed OxyS regulation similar to the chromosomal insertion at the *fhlA* gene (data not shown). Therefore, 514 bp of the 5′ upstream sequence appear to include all the elements necessary for OxyS regulation of *fhlA*.

OxyS could act to regulate transcription, mRNA stability, translation or protein stability. To determine the level of OxyS regulation of *fhlA*, the fragment carrying 514 bp of 5′ upstream sequence was subcloned upstream of *lacZ* gene in expression vectors carrying the *lacZ* gene which is deleted either for its promoter (pRS551) or for both the promoter and the translational signals (pRS552) (Simons *et al*., 1987). Again the fusions were recombined onto λ and inserted into the chromosome at the *att* site. We then compared the effect of OxyS on the *fhlA7–lacZ* transcriptional fusion to its effect on the *fhlA7–lacZ* translational fusion (Table II). Expression of the transcriptional fusion was only slightly repressed. Endogenous *fhlA* mRNA levels were also only decreased 2- to 3-fold in

strains carrying poxyS (data not shown). Thus neither transcription initiation nor mRNA stability is significantly affected by OxyS. In contrast, OxyS strongly regulates the translational fusion. This finding suggests that OxyS affects translation of the *fhlA* mRNA. OxyS could also be affecting FhlA stability; however, the *fhlA7–lacZ* translational fusion carries only seven amino acids of the FhlA protein sequence.

oxyS mutations

To further characterize OxyS domains involved in the regulation of *fhlA*, we mutagenized the plasmid expressing OxyS using hydroxylamine and screened for OxyS mutants that were no longer able to repress the translation of ϕ*fhlA–lacZ*. All 10 mutants selected were found to carry single point mutations in the *oxyS* gene, and four different mutations were obtained (C92U, G93A, C99U, G102A). Two additional mutations were isolated in a screen following PCR amplification of the *oxyS* gene (G108A, C107A). All the mutations mapped to stem–loop **c**, the domain found to be important for *fhlA* regulation by deletion analysis (Figure 1A). The effect of the six OxyS mutants on *fhlA* expression is shown in Figure 1B. The mutations could be subdivided into two subgroups; nucleotide changes that disrupt the stem and changes that are located in the loop. The stem mutations with the greatest effect on stem stability are also associated with the greatest decrease in activity. Accordingly, the C92U mutant, which is still capable of forming a G–U base pair at the base of stem **c**, is a fairly efficient repressor. An OxyS mutant in which a C–G base pair in stem **c** (C92 and G108) was replaced by a G–C base pair (92G and 108C), to change the sequence while retaining stem stability, was still active for *fhlA* repression (data not shown). The point mutations in the loop **c** almost completely abolished OxyS repression of *fhlA*, in agreement with the results obtained with the loop **c** deletion. A Northern blot showed that none of the mutations affected OxyS RNA levels, suggesting that the lack of repression is not due to decreased amounts of OxyS (data not shown), but as expected, the transcripts from the stem mutants were extended relative to wild-type OxyS.

fhlA mutations

To define elements in *fhlA* that respond to regulation by OxyS, we mutagenized the *fhlA* promoter sequences and screened for *fhlA6–lacZ* fusions that could no longer be repressed by OxyS. Phage λ carrying a *fhlA6–lacZ* translational fusion were mutagenized with nitrosoguanidine. Given that λ *fhlA6–lacZ* forms white plaques when plated on MC4100 carrying poxyS and blue plaques on MC4100 carrying the pKK177-3 control plasmid, we plated the mutagenized lysate on cells expressing OxyS and screened for blue plaques. After an initial characterization of ~20 mutants, two were selected for further analysis. The 5′ upstream region of the *fhlA* mutants was recloned into the *lacZ* expression vector, and the constructs were recombined onto λ and integrated into the *att* site of MC4100. We compared the effect of OxyS on wild-type *fhlA* to its effect on *fhlA* mutants (Figure 2B). The basal levels of *lacZ* expression from both mutants were not greatly affected, but repression by OxyS was impaired. Sequence analysis mapped the mutations directly adjacent

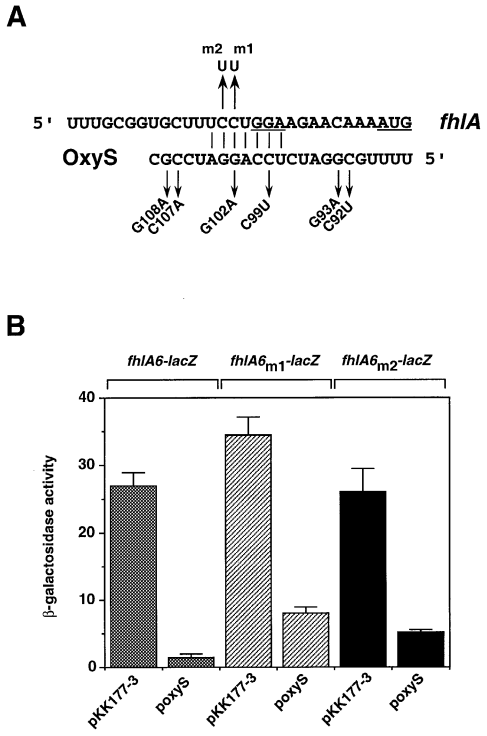

Fig. 2. Mutational analysis of *fhlA*. (**A**) Potential base pairing between the OxyS RNA and the *fhlA* mRNA. The large arrows indicate *fhlA* mutations generated by random mutagenesis. The small arrows indicate *oxyS* mutations described in Figure 1. The Shine–Dalgarno sequence and initiating AUG codon of *fhlA* are underlined. (**B**) Effect of *fhlA* mutations on OxyS repression. The extent of repression is 19-fold for *fhlA6–lacZ*, 4-fold for f*hlA6*$_{m1}$*–lacZ* and 5-fold for *fhlA6*$_{m2}$*–lacZ*. The average (in Miller units) of three independent β-galactosidase assays is shown. Single colonies were grown overnight in LB with amp.

to the putative Shine–Dalgarno sequence of *fhlA* (Figure 2A) suggesting that OxyS might interfere with ribosome binding.

fhlA and OxyS duplex formation

In a search for complementary or identical sequence elements between the OxyS RNA and the *fhlA* mRNA, we found that seven nucleotides in loop **c** of OxyS were complementary to part of the ribosome-binding site of *fhlA* (Figure 2A). The two OxyS loop mutants and both *fhlA* mutants map to this region. This observation led us to suggest that the regulation of *fhlA* translation by OxyS is mediated via duplex formation between OxyS loop **c** and a response element in the *fhlA* ribosome-binding site.

To learn more about this putative interaction and to determine whether base pairing is required for the regulation, we modified both OxyS and *fhlA* to carry mutations in the putative duplex site (Figure 3A). The nucleotide G102 in OxyS was replaced by C (*oxyS*$_{G\rightarrow C}$), and the corresponding complementary nucleotide C in *fhlA* was replaced by G (*fhlA7*$_{C\rightarrow G}$*–lacZ*). The results in Figure 3B show that OxyS$_{G\rightarrow C}$ is unable to repress the wild-type *fhlA7–lacZ* fusion and wild-type OxyS cannot repress the *fhlA7*$_{C\rightarrow G}$*–lacZ* fusion. *fhlA* repression by OxyS is restored when both genes carry the corresponding suppressor mutations (*fhlA7*$_{C\rightarrow G}$*–lacZ*, *oxyS*$_{G\rightarrow C}$). These results support the conclusion that loop **c** in OxyS represses *fhlA*

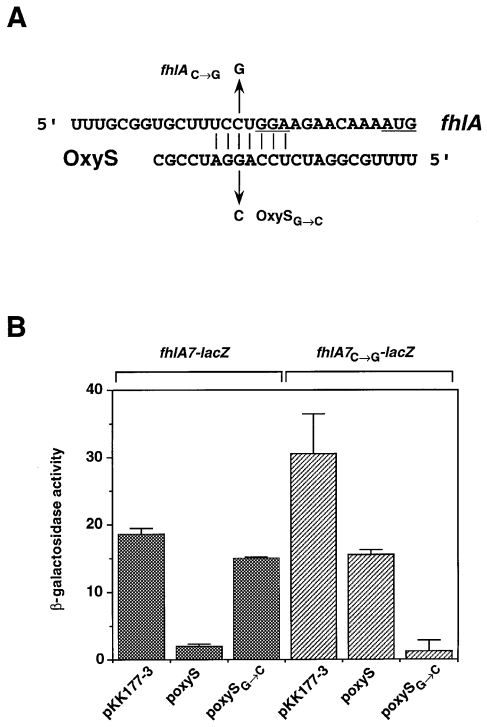

Fig. 3. Compensatory mutations. (**A**) Potential base pairing between the OxyS RNA and the *fhlA* mRNA. The arrows indicate specific nucleotide changes in *oxyS* and *fhlA* generated by site-directed mutagenesis. (**B**) Effect of $oxyS_{G\rightarrow C}$ and $fhlA7_{C\rightarrow G}$–*lacZ* mutations on OxyS repression of *fhlA*. The average (in Miller units) of three independent β-galactosidase assays is shown. Single colonies were grown overnight in LB with amp.

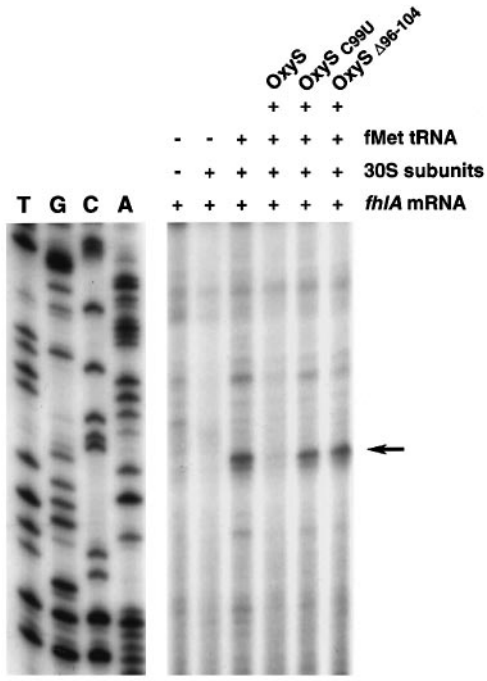

Fig. 4. Toeprinting analysis of 30S ribosomal subunit binding to *fhlA* mRNA. The arrow indicates the termination site found 15 nucleotides downstream of the AUG start codon. The DNA sequencing reactions were carried out with the same end-labeled oligonucleotide used in the toeprinting assays.

translation by interacting with a sequence element at the ribosome-binding site of *fhlA*.

OxyS prevents ribosome binding

Our genetic studies indicated that the OxyS RNA represses *fhlA* mRNA translation by pairing with the ribosome-binding site. To assess the effect of OxyS on ribosome binding *in vitro*, we carried out toeprinting assays. In these assays, also denoted primer extension inhibition assays, the binding of 30S ribosomes to the mRNA blocks the elongation of a cDNA primer by reverse transcriptase (Hartz *et al.*, 1988). The blockage usually occurs 15 nucleotides from the first nucleotide of the initiation codon. Toeprinting assays have been used to examine the formation of translation initiation complexes, to define the level at which translation is regulated and to examine which factors impact on ribosome binding.

In vitro synthesized *fhlA* mRNA was annealed with a labeled primer complementary to a region 77 base pairs downstream of the *fhlA* translation start site. This complex was then incubated with 30S ribosome subunits in the presence or absence of uncharged fMet-tRNA. Analysis of the extension products revealed one ribosome-induced, fMet-tRNA-dependent termination site at the G residue 15 nucleotides downstream of the AUG (Figure 4). The termination site was not detected when *in vitro* synthesized OxyS RNA was added prior to the incubation with the 30S subunit and the uncharged fMet-tRNA. In contrast, OxyS mutant RNAs carrying a deletion (Δ96–104) or a point mutation (C99U) in loop **c** were unable to repress 30S binding. These assays demonstrate that OxyS inhibits the formation of *fhlA* mRNA–30S initiation complex.

Discussion

The *E.coli oxyS* gene encodes a 109 nucleotide, untranslated RNA. This small RNA is expressed in response to oxidative stress and acts as a pleiotropic regulator to activate and repress as many as 40 genes. Among the OxyS targets are the *fhlA*-encoded transcriptional activator and the *rpoS*-encoded σ^s subunit of RNA polymerase. The analysis of OxyS deletion mutants showed that different regions of the small RNA are required for repression of *fhlA* and *rpoS*. Here we report that OxyS represses *fhlA* translation by pairing with a complementary sequence overlapping the ribosome-binding site of the *fhlA* mRNA. Toeprinting experiments with 30S ribosomal subunits indicated that the formation of the duplex interferes with ribosome binding. A recent report shows that OxyS repression of *rpoS* translation is dependent on the *hfq*-encoded RNA-binding protein (Zhang *et al.*, 1998). Co-immunoprecipitation and gel-mobility shift experiments revealed that OxyS binds the Hfq protein and prevents Hfq from activating *rpoS* mRNA translation.

The observation that different domains of OxyS are required for *fhlA* and *rpoS* repression suggests that OxyS may regulate the expression of these genes by separate mechanisms. Since RNA molecules are extremely flexible, it is plausible that RNAs have more than one mechanism of action. In fact, recent mutational studies of DsrA suggest that this small RNA may have multiple functions (Sledjeski and Gottesman, 1995; Sledjeski *et al.*, 1996; Majdalani *et al.*, 1998). However, additional studies are required to determine whether the modes of OxyS action

at *fhlA* and *rpoS* are completely different. Given some sequence complementarity between OxyS and the *rpoS* mRNA, OxyS repression of *rpoS* could also involve antisense pairing.

Our genetic studies showed that the OxyS RNA pairs with a short sequence overlapping the *fhlA* Shine–Dalgarno sequence. The point mutants that affect OxyS repression of *fhlA* could be subdivided into two subgroups; four mutations are located in the stem **c**, and two mutations are located in the loop **c**. Since the effects of the stem mutations on OxyS activity vary with their effects on stem stability, the thermodynamic stability of stem **c**, rather than the nucleotide sequence, is probably critical for OxyS function. This conclusion is supported by the finding that a mutant in which a C–G base pair in stem **c** is replaced by a G–C base pair is still active. The stem could be required for protein binding and/or to ensure a specific structure of OxyS and loop **c**. It is likely that loop **c** is interacting directly with the *fhlA* ribosome-binding site. *fhlA* repression by OxyS drops significantly when loop **c** carries single-point mutations or an internal (poxyS$_{\Delta 96–104}$) deletion, and a loop mutation can be complemented by corresponding suppressor mutations in *fhlA*. The remaining activity of the loop mutants could result from possible alternative interactions such as an interaction between 3′-GCCU of the OxyS RNA with 5′-UGGA of the *fhlA* mRNA. The interaction between OxyS and *fhlA* may also be influenced by the secondary structure of the *fhlA* transcript which has not been characterized.

The short interaction between the OxyS RNA and the *fhlA* transcript bears some resemblance to steps during mRNA translation. The translation process depends on short RNA–RNA recognition events, such as the base pairing between the Shine–Dalgarno sequence to a hexanucleotide sequence at the 3′-end of the 16S rRNA and the anticodon–codon duplex formation. We propose that OxyS binds to the *fhlA* response element and thereby competes with and prevents the binding of 16S rRNA. Given that the duplex site is larger than the Shine–Dalgarno site, the binding of OxyS could be advantageous.

The ΔG value of duplex formation between OxyS and *fhlA* mRNA is –13.9 kcal/mol, whereas the ΔG value of stem–loop **c** of OxyS is only –9.4 kcal/mol, suggesting that the formation of the OxyS–*fhlA* duplex would be favored. In addition, the interaction between many antisense RNAs and their targets begins with a few reversible base pairs formed between complementary loops or unstructured single-stranded regions (reviewed in Wagner and Simons, 1994). This initial interaction, also known as the kissing complex, subsequently leads to complete duplex formation. In a few cases, it has been suggested that complete duplex formation between the antisense RNA and its target may not even be necessary for regulation (Tomizawa, 1990; Brantl and Wagner, 1994; Siemering *et al*., 1994). In the case of CopA/CopT of plasmid R1, the kissing complex between CopT and truncated CopA (CopI), which is unable to form a full duplex with CopT, is sufficient to inhibit ribosome binding (Malmgren *et al*., 1996). For wild-type CopA, the initial CopA–CopT loop–loop interaction is subsequently stabilized by intermolecular base pairing involving the 5′ proximal 30 nucleotides of CopA and the complementary region of CopT (Malmgren *et al*., 1997). Although the formation of the OxyS–*fhlA* duplex should be favored, other sequences in OxyS and *fhlA* could aid in the stabilization of this critical seven-nucleotide RNA–RNA duplex.

It is possible that additional proteins are required for efficient OxyS repression of *fhlA*; however, they have not been identified in any of our genetic screens. Current studies are directed at dissecting the mechanism of OxyS action at *fhlA*. Since the seven-nucleotide interaction between OxyS and *fhlA* leads to extremely efficient repression of *fhlA* translation, we speculate that insight gained from these studies could be exploited for experimental and therapeutic antisense technology (Delihas *et al*., 1997).

Finally, it will also be important to understand further the physiological role of OxyS repression of *fhlA*. In the presence of formate, FhlA activates the transcription of a regulon required to assemble the formate-hydrogenlyase complex (Maupin and Shanmugam, 1990; Schlensog and Böck, 1990). The complex is primarily synthesized under anaerobic conditions and contains several metal co-factors. We proposed that OxyS repression of *fhlA*, and consequently the levels of the formate-hydrogenlyase complex, would reduce hydrogen peroxide-induced damage that would otherwise occur in the presence of the metal co-factors (Altuvia *et al*., 1997); however, other roles for OxyS repression of *fhlA* need to be considered.

Materials and methods

Bacterial strains and growth conditions

The bacterial strains and the plasmids used in this study are listed in Table III. Strains were routinely grown at 37°C in Luria-Bertani (LB) medium (Silhavy *et al*., 1984). Ampicillin (amp, 50–100 μg/ml), chloramphenicol (cm, 25 μg/ml), kanamycin (kan, 25 μg/ml) or 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal, 40 μg/ml) was added where appropriate. P1 transductions were carried out as described (Silhavy *et al*., 1984).

Construction of deletion mutants

All DNA manipulations were carried out using standard procedures. The OxyS mutants with 5′ or 3′ deletions were constructed by PCR: poxyS$_{\Delta 1–47}$ [#200 (5′-CTT GAA TTC GAG AGT TTC TCA ACT CG) and #86 (5′-TGA GCC AAG CTT ATC GCC GGG)], poxyS$_{\Delta 1–63}$ [#601 (5′-CTT GAA TTC GAA TAA CTA AAG CCA AC) and #86], poxyS$_{\Delta 1–90}$ [#602 (5′-CTT GAA TTC GCG GAT CTC CAG GAT CC) and #86], and poxyS$_{\Delta 91–109}$ #60 [(5′-TCT GAC TGA GAA TTC CTC ACA GAA) and #625 (5′-CTG GAG AAG CTT AAA AGT TCA CGT TGG)]. The PCR fragments were digested with *Eco*RI and *Hin*dIII and then cloned into pKK177–3 carrying cm^r (pGSO84, Altuvia *et al*., 1997). To construct the Δ96–104 derivative, the *Bam*HI site in the *tac* promoter of poxyS (pGSO5) was eliminated and the plasmid was digested with *Bam*HI and religated (pSA1). We found that digestion with *Bam*HI occasionally generates an internal deletion of nine nucleotides at stem **c**, presumably due to star activity of *Bam*HI. The *Eco*RI–*Hin*dIII fragment of pSA1 was subsequently subcloned into pGSO84. The sequences of all constructs were confirmed. The levels of the mutant RNAs were examined by Northern blot analysis: total RNA isolated from cells grown for 12 h was separated on a 6% polyacrylamide–urea gel, transferred to a nylon membrane by electroblotting, and probed with a γ-^{32}P-end labeled primer (5′-CCTGTGTGAAATTCTTATCC, corresponding to pKK177-3 sequence upstream of the *oxyS* coding sequence).

Construction of lacZ fusions

To construct MC4100 [λRS45 *fhlA7–lacZ* (transcriptional fusion)] and MC4100 [λRS45 *fhlA7–lacZ* (translational fusion)], the *fhlA* promoter fragment was amplified from MC4100 chromosomal DNA by PCR [#139 (5′-GAG AAT TCG GGG CTG GAT GGC GAA C) and #314 (5′-CCG GGA TCC CTC ATC GGT GTA TAT GAC AT)] and subcloned into the unique *Eco*RI and *Bam*HI sites of both pRS551 and

Table III. Bacterial strains and plasmids

Strain	Relevant genotype	Source or reference
MC4100	Δ(arg–lac)u169	N.Trun
GSO38	MC4100 ϕ*fhlA–lacZ* (kan^r)	Altuvia *et al*., 1997
RO91	MC4100 [λRZ5 *rpoS742–lacZ* (hybr.)] (amp^r)	Lange and Hengge-Aronis, 1994
GSO53	MC4100 [λRS45 *fhlA7–lacZ* (transc.)] (kan^r)	This study
GSO54	MC4100 [λRS45 *fhlA7–lacZ* (transl.)] (kan^r)	This study
GSO55	MC4100 [λRS45 *fhlA6–lacZ* (transl.)] (kan^r)	This study
GSO56	MC4100 [λRS45 $fhlA6_{m1}$–*lacZ* (transl.)] (kan^r)	This study
GSO57	MC4100 [λRS45 $fhlA6_{m2}$–*lacZ* (transl.)] (kan^r)	This study
GSO58	MC4100 [λRS45 $fhlA7_{C\rightarrow G}$–*lacZ* (transl.)] (kan^r)	This study
Plasmid	**Relevant Genotype**	**Source or reference**
pGSO5	poxyS (amp^r)	Altuvia *et al*., 1997
pGSO85	poxyS (cm^r)	Altuvia *et al*., 1997
pGSO86	$poxyS_{\Delta 1-47}$ (cm^r)	This study
pGSO87	$poxyS_{\Delta 1-63}$ (cm^r)	This study
pGSO88	$poxyS_{\Delta 1-90}$ (cm^r)	This study
pGSO89	$poxyS_{\Delta 91-109}$ (cm^r)	This study
pGSO90	$poxyS_{\Delta 96-104}$ (cm^r)	This study
pSA1	$poxyS_{\Delta 96-104}$ (amp^r)	This study
pGSO91	$poxyS_{C92U}$ (amp^r)	This study
pGSO92	$poxyS_{G93A}$ (amp^r)	This study
pGSO93	$poxyS_{C99U}$ (amp^r)	This study
pGSO94	$poxyS_{G102A}$ (amp^r)	This study
pSA2	$poxyS_{C107A}$ (amp^r)	This study
pSA3	$poxyS_{G108A}$ (amp^r)	This study
pGSO95	$poxyS_{G\rightarrow C}$ (amp^r)	This study
pSA4	pGEM–fhlA (amp^r)	This study
pSA5	pGEM–oxyS (amp^r)	This study
pSA6	pGEM–$oxyS_{C92U}$ (amp^r)	This study
pSA7	pGEM–$oxyS_{\Delta 96-104}$ (amp^r)	This study

pRS552 (Simons *et al*., 1987). The *lacZ* fusions were then recombined onto λRS45 and integrated into the attachment site of MC4100 as described (Simons *et al*., 1987). To construct MC4100 [λRS45 *fhlA6–lacZ* (translational fusion)], the *fhlA* promoter fragment was PCR amplified from GSO38 chromosomal DNA by PCR [#139 and #141 (5′-GCA GGA GCT CGT TAT CGC TAT GAC G)] and subcloned into the unique *Eco*RI (in polylinker) and *Sst*I (in *lacZ*) sites of pRS551. The *fhlA6–lacZ* translational fusion was then recombined onto λRS45 and integrated into the attachment site of MC4100.

oxyS mutagenesis

Random mutagenesis of the *oxyS* gene with hydroxylamine was carried out using a modification of the method described by Humphreys *et al*. (1976). Ten micrograms of poxyS (pGSO5) plasmid DNA (20 μl) was incubated with 100 μl of 0.1 M sodium phosphate (pH 6) containing 1 mM EDTA and 80 μl of 1 M hydroxylamine (pH 6) for 30 min at 65°C. The mutagenized DNA was then extensively dialyzed against Tris–EDTA buffer, and 0.7 μl was used to transform cells by electroporation.

λRS45 fhlA6–lacZ mutagenesis

λRS45 *fhlA6–lacZ* (translational fusion) was mutagenized as described (Kornitzer *et al*., 1989). MC4100 cells were grown to early exponential phase in LB medium containing 10 mM $MgSO_4$. The cells were then infected with one fresh plaque of λRS45 *fhlA6–lacZ* phage. Cells were collected 20 min after infection, resuspended in half of the initial volume of 0.1 M sodium citrate (pH 5.5), and treated with nitrosoguanidine (50 μg/ml) for 20 min at 37°C with shaking. The cells were collected again and resuspended in LB containing 10 mM potassium phosphate. The culture was then allowed to complete lysis at 37°C. Subsequently, the lysates were plated on MC4100 carrying poxyS and screened for blue plaques. Lysates were generated from single blue plaques, and λ DNA was isolated as described previously (Silhavy *et al*., 1984). The *fhlA* promoter region of wild-type and two mutants was PCR amplified using primers #139 and #141, and subcloned into the unique *Eco*RI (in polylinker) and *Sst*I (in *lacZ*) sites of pRS551. The fusions were sequenced and then recombined onto λRS45. The effect of constitutive OxyS expression was assayed for single lysogens of the wild-type fusion and the two mutants.

Site-directed mutagenesis

Site-directed mutagenesis was carried out with PCR using oligonucleotides that contained the desired mutations. The mutant $oxyS_{G\rightarrow C}$ fragment was amplified from K12 chromosomal DNA by PCR [#60 and #317 (5′-GCC AAG CTT ATC GCC GGG CTT TTT TAT GGC AAA AAA AAG CGG ATC GTG GAG ATC CGC)], digested with *Eco*RI and *Hin*dIII, and cloned into the corresponding sites of pKK177-3. The mutant $fhlA_{C\rightarrow G}$ fragment was amplified from K12 chromosomal DNA by PCR [#139 and #316 (5′-CCG GGA TCC CTC ATC GGT GTA TAT GAC ATT TTG TTC TTC CAC GAA AGC ACC GC)], digested with *Eco*RI and *Bam*HI, and cloned into the corresponding sites of pRS552. The $fhlA_{C\rightarrow G}$–*lacZ* gene fusion was further recombined onto λRS45 and integrated into the attachment site of MC4100 to generate MC4100 [λRS45 $fhlA7_{C\rightarrow G}$|–*lacZ* (transl.)].

β-galactosidase assays

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

Toeprinting assays

The toeprinting assays were carried out essentially as described previously (Altuvia *et al*., 1989). Annealing mixtures containing 0.2 pmol of *fhlA* RNA, 0.6 pmol of 5′-end labeled oligonucleotide (5′-GAA GGA TCC TCA CAC AGC GAG GCC) complementary to *fhlA* RNA, and 12 pmol of OxyS RNA (wild-type or mutant) in DEPC-treated water were heated for 3 min at 65°C and then chilled in ice-water for 15 min. The extension inhibition reactions contained the annealing mixtures, 0.5 mM of each dNTP, 3 pmol of preactivated (30 min at 37°C) 30S ribosomal subunits (kindly provided by Steven Ringquist), 20 mM Tris–HCl, 10 mM Mg-acetate, 0.1 M NH_4Cl, 0.5 mM EDTA, and 2.5 mM β-mercaptoethanol. After preincubation for 5 min at 37°C, uncharged fMet-tRNA (12 pmol) was added and the reactions were incubated for an additional 10 min. Reverse transcriptase (1 U) was added, and cDNA synthesis was allowed to proceed for 15 min. To construct pGEM–fhlA for the *in vitro* synthesis of *fhlA* RNA, the 5′ end of the gene (412 nucleotides) was amplified from MC4100 chromosomal DNA by PCR (5′-GCG AAT TCC TGG GAC TGG ACG CCC T and 5′-GAA GGA TCC TCA CAC AGC GAG GCC). The PCR fragment was digested with *Eco*RI and *Bam*HI and

cloned into the corresponding sites of pGEM-3 (Promega). To construct pGEM–oxyS, poxyS (pGSO5) was digested with *Hin*dIII (filled-in) and *Eco*RI, and the *oxyS* fragment was cloned into the *Sal*I (filled-in) and *Eco*RI sites of pGEM-3. To construct pGEM–oxy$S_{\Delta 96–104}$ and pGEM–oxyS_{C99U}, poxy$S_{\Delta 96–104}$ (pSA1) and poxyS_{C99U} were digested with *Eco*RI and *Hin*dIII, and the *oxyS* containing fragments were cloned into the corresponding sites of pGEM-3. All plasmids were linearized with HindIII and the RNAs were synthesized with T7 RNA polymerase.

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