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 \(\sigma^\prime\) 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

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**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 IS10 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 anti-mutator, 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 \(\sigma^\prime\) 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 \(\phi fhlA–lacZ\) and \(rpoS742–lacZ\) translational fusions (Table 1). We found that OxyS derivatives which carry 5′-deletions of stem–loop a (poxyS\(\Delta1-47\)) and both stem–loops a and b (poxyS\(\Delta1-63\)) could still repress *fhlA* and *rpoS*. Surprisingly, an OxyS derivative carrying a 5′ deletion up to stem–loop c (poxyS\(\Delta1-98\)) was still able to repress the \(\phi fhlA–lacZ\) 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* repression by OxyS requires the 27-nucleotide region preceding stem–loop c. A 3′-deletion of stem–loop c (poxyS\(\Delta91-109\)) 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 \( \phi fhlA-lacZ \). The \( \beta \)-galactosidase activity (in Miller units) of stationary phase cells was assayed. Average (in Miller units) of two independent experiments. Cells were grown in LB medium with amp for 18 h.

Table I. Effect of OxyS deletion mutants on \( \phi fhlA-lacZ \) and \( rpoS-lacZ \) fusions

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<th>Construct</th>
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<th>( rpoS742-lacZ )</th>
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<td>pKK177-3</td>
<td>52 ± 1</td>
<td>386 ± 94</td>
</tr>
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<td>poxyS</td>
<td>3 ± 1</td>
<td>54 ± 15</td>
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<tr>
<td>poxyS(_{\Delta1-47})</td>
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<td>107 ± 45</td>
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<td>poxyS(_{\Delta1-63})</td>
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<td>138 ± 29</td>
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<tr>
<td>poxyS(_{\Delta1-90})</td>
<td>5 ± 2</td>
<td>522 ± 102</td>
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<tr>
<td>poxyS(_{\Delta91-109})</td>
<td>51 ± 2</td>
<td>341 ± 58</td>
</tr>
<tr>
<td>poxyS(_{\Delta96-104})</td>
<td>36 ± 4</td>
<td>189 ± 54</td>
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</tbody>
</table>

\(^a\)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. Based on the FOLD (Genetics Computer Group) and mfold (http://www.ibt.wustl.edu/~zuker/rna) programs, the secondary structures of the 5'-ends of OxyS\(_{\Delta1-47}\), OxyS\(_{\Delta1-63}\), and OxyS\(_{\Delta1-90}\), and the 5'-ends of OxyS\(_{\Delta91-109}\) and OxyS\(_{\Delta96-104}\) are similar to those of the wild-type.

genes. We also constructed a deletion of the loop sequence of stem–loop \( \epsilon \) (poxyS\(_{\Delta96-104}\)) 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\(_{\Delta1-47}\)) or slightly reduced (2-fold for poxyS\(_{\Delta1-63}\) and 2.5-fold for poxyS\(_{\Delta1-90}\)) (data not shown). The majority of the OxyS transcripts from poxyS\(_{\Delta91-109}\) and some of the transcripts from poxyS\(_{\Delta96-104}\) were longer than expected, confirming that stem–loop \( \epsilon \) is a transcription terminator. Stem–loop \( \epsilon \) is also likely to confer stability to OxyS since the RNA levels from poxyS\(_{\Delta91-109}\) and poxyS\(_{\Delta96-104}\) 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 \( \epsilon \) while OxyS repression of \( rpoS \) requires the 27-nucleotide linker region together with stem \( \epsilon \). 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).

**OxyS inhibits fhlA expression at a post-transcriptional level**

The initial OxyS-regulated \( \phi fhlA-lacZ \) 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 \( \lambda \) 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 \( \lambda \) and inserted into the chromosome at the att site. We then compared the effect of OxyS on the \( fhlA-lacZ \) translational fusion to its effect on the \( fhlA-lacZ \) translational fusion (Table II). Expression of the translational 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 ψfhlA-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 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 (oxyS6→C), and the corresponding complementary nucleotide C in fhlA was replaced by G (fhlA7C→G-lacZ). The results in Figure 3B show that OxyS6→C is unable to repress the wild-type fhlA7-lacZ fusion and wild-type OxyS cannot repress the fhlA7C→G-lacZ fusion. fhlA repression by OxyS is restored when both genes carry the corresponding suppressor mutations (fhlA7C→G-lacZ, oxyS6→C). These results support the conclusion that loop c in OxyS represses fhlA
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A

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 oxySΔ96–104 and fhlA7C→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.

B

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 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 hsf-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 ε 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 ε, 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 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 ε. It is likely that loop ε is interacting directly with the fhlA ribosome-binding site. fhlA repression by OxyS drops significantly when loop ε carries single-point mutations or an internal (poxySΔ96–104) 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 ε 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; Siemerling 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 (Delilas 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 Shammugam, 1990; Schlesnog 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: poxySΔ1–4 [5′-CTT GAA TTC GAG AGT TTC TCA ACT CTG-3′ (5′-TGA GCC AAC CTT ATC GCC GGG)], poxySΔ1–6 [5′-CTT GAA TTC GAG AGT TTC TCA ACT CTG-3′ (5′-CTT GAA TTC GAG AGT TTC TCA ACT CTG) and #86], poxySΔ1–6 [5′-CTT GAA TTC GAG AGT TTC TCA ACT CTG-3′ (5′-CTT GAA TTC GAG AGT TTC TCA ACT CTG) and #86], and poxySΔ1–104 #60 [5′-CTT GAG TAC GTA GAT GGC GAA C-3′ (5′-CTT GAG TAC GTA GAT GGC GAA C) and #625 (5′-CTG GAG AAT TCG GGG CTG GAG TGC C)]. The PCR fragments were digested with EcoRI and HindIII and then cloned into pKK177–3 carrying cm (pGSO84, Altuvia et al., 1997). To construct the Δ96–104 derivative, the BamHI site in the tac promoter of poxyS (pGSO5) was eliminated and the plasmid was digested with BamHI and religated (pSA1). We found that digestion with BamHI occasionally generates an internal deletion of nine nucleotides at stem ε, presumably due to star activity of BamHI. The EcoRI–HindIII fragment of pSA1 was subsequently subcloned into pGSO4. 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 in LB medium (Silhavy et al., 1984). Ampicillin (amp, 50–100 μg/ml), chloramphenicol (cm, 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 lacZ fusions

To construct MC4100 [R S545 fhlA−/lacZ (transcriptional fusion)] and MC4100 [R S545 fhlA−/lacZ (transcriptional fusion)], the fhlA promoter fragment was amplified from MC4100 chromosomal DNA by PCR [139 (5′-GAG AAT TCG GGG CTT GAT GGA C) and #314 (5′-CGT AGA TCG CTC ATC GGT GAC AT)] and subcloned into the unique EcoRI and BamHI sites of both pRS551 and pRS552.
Table III. Bacterial strains and plasmids

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<td>Altuvia et al., 1997</td>
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Plasmid | Relevant Genotype | Source or reference |
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Site-directed mutagenesis

Site-directed mutagenesis was carried out with PCR using oligonucleotides that contained the desired mutations. The mutant oxyS_G–gac fragment was amplified from K12 chromosomal DNA by PCR [860 and #317 (5’–GCC AAC TCT ATG GCC GGG CTT TTG TAT GCC AAC AAA AAG CCG ATC GTC GAG ATC CGC)], digested with EcoRI and HindIII, and cloned into the corresponding sites of pKK177-3. The mutant fhlA6–gac fragment was amplified from K12 chromosomal DNA by PCR [8139 and #316 (5’–CCG GGA TCC ATC ATG GAT ATC CGC)] and #316 (5’–CCG GGA TCC ATC ATG GAT ATC CGC)], digested with EcoRI and BamHI, and cloned into the corresponding sites of pPRS552.

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 NH4Cl, 0.5 mM EDTA, and 2.5 mM β-mercaptoethanol. After preincubation for 5 min at 37°C, uncharged Met–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 ACC CCG T and 5’–GAA GGA TCC TCA CAC AGC GAG GCC]. The PCR fragment was digested with EcoRI and BamHI and...
OxyS RNA repression of fhlA translation

cloned into the corresponding sites of pGEM-3 (Promega). To construct pGEM–oxyS, pgoxS (pGSO5) was digested with HindIII (filled-in) and EcoRI, and the oxyS fragment was cloned into the SaI (filled-in) and EcoRI sites of pGEM-3. To construct pGEM–oxyS96–104 and pGEM–oxyS96–104 (pSA1) and pgoxS96–104 were digested with EcoRI and HindIII, 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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