OxyS small RNA induces cell cycle arrest to allow DNA damage repair

Shir Barshishat1,†, Maya Elgrably-Weiss1,†, Jonathan Edelstein1,†, Jens Georg2,†, SutharsanGovindarajan1, Meytal Haviv1, Patrick R Wright3, Wolfgang R Hess2,* & Shoshy Altuvia1,*,†

Abstract

To maintain genome integrity, organisms employ DNA damage response, the underlying principles of which are conserved from bacteria to humans. The bacterial small RNA OxyS of Escherichia coli is induced upon oxidative stress and has been implicated in protecting cells from DNA damage; however, the mechanism by which OxyS confers genome stability remained unknown. Here, we revealed an OxyS-induced molecular checkpoint relay, leading to temporary cell cycle arrest to allow damage repair. By repressing the expression of the essential transcription termination factor nusG, OxyS enables read-through transcription into a cryptic prophage encoding kilR. The KilR protein interferes with the function of the major cell division protein FtsZ, thus imposing growth arrest. This transient growth inhibition facilitates DNA damage repair, enabling cellular recovery, thereby increasing viability following stress. The OxyS-mediated growth arrest represents a novel tier of defense, introducing a new regulatory concept into bacterial stress response.

Keywords cell cycle arrest; checkpoint; Escherichia coli; prophage; small RNA

Subject Categories Cell Cycle; Microbiology, Virology & Host Pathogen Interaction; RNA Biology

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Introduction

The oxidative stress-induced OxyS small RNA (sRNA) was one of the first characterized sRNAs (Altuvia et al., 1997). A global regulatory role for OxyS was indicated by the substantial changes in the protein synthesis patterns observed with constitutive OxyS expression in Escherichia coli (Altuvia et al., 1997). Over the years, several approaches have been employed to identify genes controlled by OxyS including genetic screens, computational target prediction, and transcriptome analysis (Altuvia et al., 1997; Tjaden et al., 2006; De Lay & Gottesman, 2012). OxyS was shown to negatively regulate the mRNAs of the transcription factors FhlA, RpoS, and FlhDC, as well as a number of additional proteins (Altuvia et al., 1997; Tjaden et al., 2006; De Lay & Gottesman, 2012). Whereas negative regulation of rpoS (stationary-phase sigma factor) appears to be indirect, that is, via titration of Hfq, regulation of fhlA and flhDC by OxyS results from direct base pairing with their mRNAs (Altuvia et al., 1998; Zhang et al., 1998; Argaman & Altuvia, 2000; Moon & Gottesman, 2011; De Lay & Gottesman, 2012).

Despite these findings, an intriguing aspect of OxyS remained enigmatic ever since its discovery; the RNA has been proposed to play a key role in protecting cells against the damaging effects of spontaneous and induced mutagenesis (Altuvia et al., 1997). However, attempts to reveal targets responsible for the sRNA antimutagenic phenotype failed. DNA can undergo various forms of damage by exposure to environmental stresses from various sources, or as a result of normal metabolism, producing genotoxic products. To prevent mutagenesis and maintain genome stability, DNA damage responses have evolved that control multiple processes including DNA repair, cell cycle checkpoints, and cell death (Spampinato, 2016). Here, we show that the OxyS antimutagenic phenotype is intricately linked with transient induction of cell growth arrest. By lowering the expression of the transcription termination factor NusG, OxyS enables expression of a rfac prophage kilR gene encoding an inhibitor of cell division. We propose that the transient inhibition of cell division induced by OxyS facilitates DNA repair and recovery from oxidative stress.

NusG is a highly conserved protein regulator of RNA polymerase (RNAP). Through its N-terminal domain, it associates with RNAP, cell cycle checkpoints, and cell death (Spampinato, 2016). Here, we show that the OxyS antimutagenic phenotype is intricately linked with transient induction of cell growth arrest. By lowering the expression of the transcription termination factor NusG, OxyS enables expression of a rfac prophage kilR gene encoding an inhibitor of cell division. We propose that the transient inhibition of cell division induced by OxyS facilitates DNA repair and recovery from oxidative stress.

1 Department of Microbiology and Molecular Genetics, IMRIC, The Hebrew University-Hadassah Medical School, Jerusalem, Israel
2 Faculty of Biology, Genetics and Experimental Bioinformatics, University of Freiburg, Freiburg, Germany
3 Bioinformatics Group, Department of Computer Science, University of Freiburg, Freiburg, Germany
*Corresponding author. Tel: +49 761 2032796; E-mail: wolfgang.hess@biologie.uni-freiburg.de
†Corresponding author. Tel: +972 54 882 0623; E-mail: shoshy.altuvia@mail.huji.ac.il
‡These authors contributed equally to this work
ribosome and provides the physical framework for the coupling of transcription and translation in bacteria (Burmann et al., 2010).

Transcription termination is essential for cell viability and so are Rho and its cofactor, NusG (Downing et al., 1990). Studies of RNP distribution and mapping of transcription elongation and termination in E. coli have provided an explanation for the essentiality of NusG. Rho activity clusters in horizontally acquired DNA fragments including prophages, indicating that Rho and NusG play an important role in silencing potentially harmful foreign genes (Cardinale et al., 2008; Mooney et al., 2009; Peters et al., 2009, 2012). Moreover, elimination of such foreign DNA elements permits deletion of nusG, although Rho itself remains essential (Cardinale et al., 2008). Here, we provide evidence that inhibition of cell division due to transient repression of nusG by OxyS protects cells from DNA damage.

**Results**

**OxyS expression inhibits cellular growth**

Studying OxyS, we noticed that plasmid-borne, unregulated expression of OxyS is detrimental. Moreover, we identified an oxyS point mutation (oxySA69C) that promoted toxicity beyond wild-type levels. Intrigued by this phenotype, we set to randomly mutagenize OxyS wild type and OxySA69C and screened for OxyS suppressor mutants that suppressed toxicity. In light of our observation that OxyS toxicity was more pronounced in strains deficient for RelA, the stringent response major regulator, we used this genetic background to select for suppressor mutations. To eliminate mutations rendering OxyS completely inactive, for example, by decreasing sRNA stability, we focused on mutants that are no longer toxic, but capable of repressing fliA-lacZ, a previously characterized target of OxyS (Argaman & Altuvia, 2000). We isolated and sequenced 25 mutants including some carrying identical mutations. Mutations that rendered OxyS harmless clustered in two sites: the loop sequence of hairpin B (OxySC56U; C58U) and the single-stranded region located between hairpins B and C (OxySC70U; C77U and OxySC70U) (Fig 1A). The nontoxic mutant OxySC76G; C77G was constructed by site-directed mutagenesis based on the screen above. For Plac-controlled expression, the mutations were transferred to pBR-Plac plasmid (Guillier & Gottesman, 2006), and the mutants were examined for their effect on cellular growth upon induction with IPTG. Growth curves and survival assays showed that whereas wild-type OxyS and OxySA69C inhibited growth, forming only few CFU, the growth arrest phenotype was no longer detectable in the OxySC56U; C58U and OxySC70U; C77U mutants (Fig 1B and Appendix Fig S1A). The revertant of OxyS that suppressed the translation of fliA-lacZ (Appendix Fig S1B), indicating that the OxyS mutants were active regulators, though not toxic.

**OxyS represses the nusG mRNA**

The availability of highly toxic and nontoxic OxyS mutants prompted us to search for targets whose putative complementary sites match the observed changes in OxyS mutants. We performed a whole genome IntaRNA (Wright et al., 2014) search in E. coli with wild-type OxyS and its suppressor mutant versions. Of 4,301 investigated genes, 66 matched the predicted pattern. To further pin down the critical target(s), this list of genes was compared with the list of E. coli essential genes (Baba et al., 2006), yielding three candidates (nusG, pyrG, and orn). Here, we present the physiological consequences of OxyS-nusG interaction.

The nusG gene is second in the operon see:nusG, and OxyS is predicted to base-pair with the ribosome-binding site of nusG (Fig 2A). The mutation OxySNusG is expected to extend the interaction with the Shine-Dalgarno sequence and to increase the stability of oxyS-nusG hybrid. Northern Blot analysis detected a 2.5-fold to 3.5-fold reduction in nusG mRNA levels upon exposure to wild-type OxyS or OxySA69C pulse expression compared to OxyS suppressor mutants, indicating that expression of nusG is negatively regulated by OxyS (Fig 2B). To examine the effect of OxyS on nusG at the post-transcriptional level, we constructed a Ptac-nusG-lacZ translational fusion. Levels of nusG-lacZ were reduced twofold to threefold in the presence of OxyS or OxySA69C, whereas the isolated OxyS suppressor mutants had no effect on nusG-lacZ expression (Fig 2C). Given that stable RNA hybrids may block the elongation by reverse transcriptase, we examined the interaction of in vitro-synthesized OxyS and nusG RNAs using primer extension assays. These assays showed that the interaction between OxyS and nusG results in a strong termination signal (Fig 2D). The termination site mapped to the Shine–Dalgarno sequence of nusG that is the center of complementarity between OxyS and nusG. The termination signal produced by OxySA69C was stronger than that of wild type, while the suppressor mutants exhibited a weaker signal. No termination was detected using OxySC76G, c77G. An RNase protection assay further confirmed these findings. In this assay, base-pairing RNAs are protected while unpaired nucleotides are cleaved by single-stranded specific ribonucleases. As the binding between OxyS and nusG is discontinuous, base-pairing efficiency between nusG and OxyS mutant RNAs was estimated based on the formation of the full-length hybrid (Appendix Fig S2A). The levels of the full-length hybrid formed with OxySA69C were higher than those formed with wild-type OxyS, whereas OxySC76G, c77G showed decreased levels of hybrid protection.

To affirm base pairing, we replaced the GG nucleotides at positions −15 and −16 in nusG mRNA by CC. This change was predicted to complement OxySC76G, c77G. The Ptac-nusG-lacZ translational fusion was slightly repressed by wild-type OxyS; however OxySC76G, c77G failed to restore repression by binding its complementary mutant nusG-lacZ, indicating that repression may involve more than one interaction site (Fig 3A and B, left panel). Searching the sequence nearby, we identified another site for OxyS binding slightly upstream of the site overlapping the RBS (Fig 3A). This site of nusG was predicted to bind a sequence at the 3’ end of OxyS from nucleotide G75 to U87, suggesting that nusG mRNA can be bound by two different OxyS molecules. The Ptac-nusG-lacZ was less prominent (Fig 3B). Expression of NusG quadruple mutant (G-15C; G-16C and G-31C; G-32C) carrying mutations at the two sites predicted to bind the same sequence in OxyS was repressed by OxySC76G, c77G and unaffected by wild-type OxyS (Fig 3B right panel). Together, the data indicate that two molecules of OxyS can simultaneously bind two different sites in nusG.
Analysis of RNA interactions by EMSA further showed that nusG quadruple mutant could be bound by its complementary OxySC76G;C77G mutant as opposed to wild-type OxyS (Appendix Fig S2B).

The toxic phenotype of OxyS results from nusG repression

NusG has been implicated in the suppression of toxic activities by horizontally acquired genes (Cardinale et al., 2008). Furthermore, deletion of the cryptic rac prophage in wild-type E. coli permits deletion of nusG. The rac prophage carries the kilR gene encoding an inhibitor of cell division (Conter et al., 1996; Burke et al., 2013). To determine whether the toxic phenotype of OxyS is due to activation of kilR, we transformed plasmid Plac-oxyS into an E. coli strain lacking the kilR gene. Wild-type E. coli failed to yield any colonies when transformed with Plac-oxyS or Plac-oxyS
to[69C], whereas mutation of kilR supported colony formation (Fig 1C). The suppressor nontoxic OxyS mutant formed normal size colonies in both wild type and ΔkilR. Similarly, growth of wild-type cells expressing OxyS and OxySA69C was inhibited, whereas ΔkilR::cat carrying plasmids were treated with 1 mM IPTG at dilution, OD was measured as indicated. Both strains are also relA::kan, lacIq. Although the plating of OxySΔ69C in ΔkilR seems less efficient than that of OxyS, their growth curves are very similar, indicating that neither OxyS nor OxySA69C are toxic in ΔkilR.

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Figure 1. Toxic and nontoxic OxyS.

A Toxic (purple) and nontoxic suppressor mutations (green) in OxyS. The loops of hairpins A and C were found previously to interact with the fhlA ribosome-binding site. Hairpin C is Rho-independent transcription terminator.

B Growth curves of cells (MG1655 relA::cat, lacIq) with OxyS plasmids. Cultures carrying plasmids were treated with 1 mM IPTG at dilution, OD was measured as indicated.

C OxyS is not toxic in kilR-deficient cells. Wild-type and ΔkilR::cat cells were transformed with plasmids expressing OxyS, wild type and mutants, as indicated.

D Growth curves of wild-type and ΔkilR mutant cells with OxyS plasmids. Cultures of wild-type and ΔkilR mutant cells carrying plasmids were treated with 1 mM IPTG at dilution, OD was measured as indicated. Both strains are also relA::kan, lacIq. Although the plating of OxySΔ69C in ΔkilR seems less efficient than that of OxyS, their growth curves are very similar, indicating that neither OxyS nor OxySA69C are toxic in ΔkilR.
These results indicate that an increase in the expression levels of nusG can negate oxyS toxicity, whereas a concomitant increase in secE has no effect on oxyS toxicity. Therefore, oxyS is toxic because it decreases nusG expression levels.

The toxic phenotype of OxyS correlates with bacterial recovery from stress

Expression of OxyS is induced upon exposure to hydrogen peroxide (Altuvia et al., 1997). To determine whether OxyS expression influences recovery of E. coli exposed to oxidative stress, and to investigate whether OxyS toxicity is required for efficient recovery, we transformed Plac-oxyS (toxic) and Plac-oxyS_C58U; C58U into an oxyS mutant in which only the regulatory elements relevant for OxyS toxicity were deleted. In this mutant, the overlapping oxyR-oxyS promoters, the 5’ end hairpin of OxyS, and its 3’ end rho-independent terminator were left intact to avoid polar effects (∆oxySli). Cultures carrying control and OxyS expressing plasmids were treated with IPTG at OD_{600} = 0.1. β-Galactosidase activity was measured 120 min after treatment. Results are displayed as mean of two to eight biological experiments ± standard deviation.

Source data are available online for this figure.

Figure 2. OxyS represses nusG expression.

A Predicted base pairing between nusG and OxyS RNAs. The initiation codon and the Shine–Dalgarno sequence of nusG are marked in red. Suppressor nontoxic OxyS mutants are in green, the highly toxic mutant is in purple.

B Northern analysis of RNA extracted from cells carrying control and OxyS plasmids. Total RNA was extracted from cultures treated at dilution with 3 mM IPTG for 90 min. Samples were analyzed by 1.4% agarose–formaldehyde gel electrophoresis to detect the full-length transcript of secE-nusG and tmRNA or 6% urea–PAGE to detect OxyS and tmRNA. The tmRNA served as loading control. The membranes were probed with 5’ end-labeled OxyS and tmRNA-specific primers and antisense riboprobe to detect secE-nusG mRNA. Relative intensity (RI) of secE-nusG in the presence of control, wild-type, and OxyS plasmids.

C Cultures (relA::cat, ΔoxySli::frt, lacZ::Tn10, lacI) carrying Plac-nusG-lacZ (pSC101*, single copy) translational fusion and OxyS plasmids were treated with IPTG (1 mM) at OD_{600} = 0.1. β-Galactosidase activity was measured 120 min after treatment. Results are displayed as mean of two to eight biological experiments ± standard deviation.

D Primer extension of in vitro-synthesized nusG mRNA in the absence or presence of synthesized OxyS RNAs. Full-length cDNA (FL). Arrow denotes termination signal. The site of Shine–Dalgarno sequence (GGAC) is denoted in red. Relative intensity (RI) denotes the ratio of the termination signal per full-length compared to wild-type oxyS-nusG interaction, which was used as a 100% reference.

Source data are available online for this figure.
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induce oxidative stress. After 30 min of treatment, the cultures were washed and resuspended in fresh medium. Recovery was determined after 60 min of growth in nonstressful medium (after wash). Although the survival of cultures expressing wild-type OxyS decreased dramatically (Appendix Fig S1A), it was higher than cultures carrying a control plasmid after exposure to hydrogen peroxide. The recovery rate of the nontoxic OxyS mutant was increased after 60 min of growth in nonstressful medium (after wash). Although the survival of cultures expressing wild-type OxyS and to determine the role of kilR in oxyS-mediated recovery, cultures of wild-type ΔoxyS and ΔkilR exposed to hydrogen peroxide for 30 min were examined at 60 min after H₂O₂ removal. Survival of cells carrying an intact chromosomally located oxyS allele increased by ~4-fold, whereas survival of ΔoxyS and ΔkilR mutants decreased by ~1.5-fold (Fig 4B), indicating that oxyS-mediated recovery required kilR. Furthermore, quantification of kilR RNA level using real-time PCR demonstrated that upon exposure to H₂O₂, kilR mRNA levels increased by ~3-fold in wild-type cells carrying an intact oxyS allele compared to oxyS-deficient cells (Fig 4C). The increase in kilR levels followed a decrease in nusG mRNA stability; the half-life of nusG mRNA in wild-type cells exposed to hydrogen peroxide was reduced by 1.5-fold compared to its half-life in oxyS mutant (Fig 4D and E). Likewise, NusG:SPA protein levels carrying the sequential peptide affinity (SPA) tag at the NusG C-terminal end decreased upon exposure to H₂O₂ in wild type but not in an oxyS-deficient mutant (Fig 4F). Together, the results demonstrate that kilR expression was controlled by nusG and that expression of the latter was inhibited by OxyS.

OxyS promotes recovery from stress by interfering with cell division

Assembly of the essential, tubulin-like FtsZ protein into a ring-shaped structure at the nascent division site serves as a scaffold for recruitment of the cell division machinery. Kil proteins prevent cell division by interfering with FtsZ function (Conter et al., 1996; Burke et al., 2013; Haeusser et al., 2014; Hernandez-Rocamora et al., 2015). To determine whether recovery is due to interference with cell division, we examined growth resumption in cells with an intact oxyS allele and mild overexpression of ftsQAZ operon encoding ftsQ, ftsA,
and ftsZ genes from a low-copy plasmid (Bernhardt & de Boer, 2004). The presence of ftsQAZ abrogated bacterial recovery from stress (Appendix Fig S5A). Together, the data indicate that OxyS-mediated cell division inhibition is crucial for bacterial growth resumption following stress.

We visualized the effect of OxyS on cell morphology by fluorescence microscopy. Images of cells expressing OxyS or OxyS<sub>G69C</sub> displayed elongated filaments due to lack of cell division. In contrast, cells expressing the nontoxic mutant OxyS<sub>C56U; C58U</sub> formed short rod-shaped cells very similar to control cells (Fig 5A).
The ZapA protein interacts with FtsZ at an early stage of FtsZ-ring assembly and co-localizes with it (Gueiros-Filho & Losick, 2002). Images of cells with zapA-gfp fusions showed that ring assembly was impaired in cells expressing OxyS (Fig 5A). The OxyS-mediated filamentation was prevented in the ΔkilR mutant and by overexpression of FtsQAZ (Fig 5B and Appendix Fig S5B). Statistical analysis of cell length distribution of cultures expressing OxyS in wild type or ΔkilR and of wild-type cultures expressing both OxyS and FtsQAZ further confirmed the conclusion that OxyS-mediated filamentation requires active KilR and inactive FtsZ (Appendix Fig S5B). Moreover, images of cells expressing a plasmid-encoded KilR protein displayed elongated filaments and impaired ring assembly (Fig 5A).

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Significantly, we find that the presence of an intact oxyS allele affects cell length in response to oxidative stress. The length of wild-type ΔoxyS and ΔkilR cells is comparable prior to exposure to H$_2$O$_2$ (Fig 5C), increasing after treatment. Importantly, cells expressing OxyS are longer than ΔoxyS or ΔkilR mutants, indicating that in response to oxidative stress, expression of chromosomally encoded oxyS impairs cell division.

The antimutator phenotype of OxyS is due to nusG repression by OxyS

In our previous study, we showed that OxyS protects cells against DNA damage, decreasing the rate of mutations of spontaneous or induced mutagenesis (Altuvia et al, 1997). To determine whether the reduced rate of mutations is due to the regulatory cascade that begins with OxyS-mediated repression of nusG, we examined the number of rifampicin-resistant mutants following exposure to hydrogen peroxide in ΔoxyS cells carrying plasmids expressing OxyS or OxyS$_{C56U, C58U}$. The number of rifampicin-resistant mutants

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Table 1. OxyS protects against DNA damage

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Rif mutants per 10⁶ cells</th>
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<tr>
<td>ΔoxyS::frt/Plac</td>
<td>68.3 ± 36.0</td>
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<tr>
<td>ΔoxyS::frt/Plac-oxYS</td>
<td>2.2 ± 1.8</td>
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<tr>
<td>ΔoxyS::frt/Plac-oxYS_C6U;C6U</td>
<td>37.4 ± 20.0</td>
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<tr>
<td>in ΔkilR mutant</td>
<td>Rif mutants per 10⁶ cells</td>
</tr>
<tr>
<td>ΔoxyS::frt, ΔkilR::cat/Plac</td>
<td>41.3 ± 12</td>
</tr>
<tr>
<td>ΔoxyS::frt, ΔkilR::cat/Plac-oxYS</td>
<td>62.3 ± 51</td>
</tr>
<tr>
<td>ΔoxyS::frt, ΔkilR::cat/Plac-kilR</td>
<td>2.0 ± 1.0</td>
</tr>
<tr>
<td>in the presence of pftsQAZ</td>
<td>Rif mutants per 10⁶ cells</td>
</tr>
<tr>
<td>ΔoxyS::frt/pftsQAZ, Plac</td>
<td>65.3 ± 2.4</td>
</tr>
<tr>
<td>ΔoxyS::frt/pftsQAZ, Plac-oxYS</td>
<td>49.7 ± 229.3</td>
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<tr>
<th>Rif mutants induced by 1 mM H₂O₂</th>
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<tr>
<td>Chromosomal oxyS</td>
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<tr>
<td>Wild type</td>
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<td>ΔoxyS</td>
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Results are displayed as mean of four to five biological experiments ± standard deviation.

normalized to the number of viable cells demonstrated that OxyS toxicity correlated with a reduced number of mutants; the nontoxic OxyS suppressor mutant exhibits a higher fraction of Rif⁰ mutants, similar to the numbers determined with the control cells (Table 1). The reduction in mutation rates was no longer detected in the ΔkilR mutant, or when FtsQAZ was overexpressed, further confirming that OxyS acts an antimutator by interfering with cell division. Moreover, cells with in trans expression of rac KilR from a plasmid exhibited a low number of Rif⁰ mutants similar to cells with OxyS expression (Table 1). This indicates that NusG signaling can be bypassed by expressing KilR, the last component in the oxyS-nusG-kilR cascade.

Importantly, we demonstrate that hydrogen peroxide-dependent induction of OxyS results in a dramatic reduction in the number of rifampicin-resistant mutants (Table 1).

Discussion

Our previous study demonstrated that OxyS protects cells from DNA damage (Altuvia et al., 1997). Here, we show that OxyS is toxic and that the protection from DNA damage is intricately linked with OxyS toxicity. Acting as a base-pairing RNA, OxyS decreases the expression of the essential transcription termination factor NusG. This decrease in transcription termination leads to an increase in the expression of the prophage-encoded kilR gene. The KilR protein interferes with FtsZ function and thus inhibits cell division. The OxyS-induced regulatory cascade results in bacterial growth arrest, thus facilitating DNA damage repair following stress.

To protect genomic integrity and to promote survival of cells exposed to genotoxic agents, prokaryotic and eukaryotic cells activate DNA damage response (DDR) pathways. In eukaryotes, the DDR is a signal transduction pathway that upon sensing of DNA damages promotes multiple physiological processes including apoptosis, senescence, activation of immune surveillance, and DNA repair. Many of these responses depend on post-translational modifications such as phosphorylation. Some are controlled by slowed transcription. An intriguing example is p53, the potent tumor suppressor protein and core transducer of the DDR. Exposure to DNA damage leads to rapid activation of p53, which in turn induces cell cycle arrest, apoptosis, or senescence. The activation of p53 in response to damage is transient and thus provides the cell with a mechanism that connects DNA repair with cell cycle progression (Ciccia & Elledge, 2010). Here, we establish a link between two enigmatic OxyS phenotypes: the toxic phenotype and the antimutagenic phenotype. We show that a cellular programming mediated by OxyS toxicity is at the heart of its antimutagenic phenotype; OxyS is toxic because it interferes with FtsZ function; however, by inhibiting cell division, OxyS enables the bacterial repair systems to repair damage for a longer period of time, thus leading to an increase in the rate of recovery. The observed reduction in DNA damage and greater recovery are both mediated by the rac prophage-encoded kilR gene.

The E. coli genome contains nine cryptic prophage elements. Their expression provides protection from sublethal concentrations of quinolone and β-lactam antibiotics, primarily through small proteins that inhibit cell division such as kilR of Rac prophage and dicB of Qin prophage. Furthermore, these prophages help bacteria to cope with adverse environmental conditions providing increased resistance to osmotic, oxidative, and acid stress (Wang et al., 2010). Although λ kil and rac kilR share no sequence similarities, their function is similar; λ kil prevents proper FtsZ assembly, producing shorter oligomers by disrupting FtsZ protofilaments while sequestering the protein subunits (Haeussser et al., 2014; Hernandez-Rocamora et al., 2015). Consequently, λ kil expression results in cell division inhibition and filamentation. Likewise, although no details on the mechanism have been reported, kilR of rac phage prevents E. coli from dividing, causing cells to grow into long filaments that become nonviable. kilR-mediated inhibition of cell division is relieved by excess FtsZ (Conter et al., 1996; Burke et al., 2013). Cell division is also inhibited following DNA damage, as part of the SOS response. Cell survival requires coordination of division with other processes, and SulA of the SOS system inhibits cell division until genetic errors are corrected (Huisman et al., 1984; Bi & Lutkenhaus, 1993; Chen et al., 2012). SOS-mediated inhibition of cell division involves sequestration of FtsZ subunits similar to that described for kil expression. This mechanism is different from that employed by division site selection antagonists, such as SlmA that binds specific regions on the chromosome preventing FtsZ from assembling over unregulated nucleoids, or MinC that prevents assembly of FtsZ in DNA-free regions of cell poles (Haeussser et al., 2014). In any case, as OxyS is toxic in SOS off-cells, the effect of OxyS is independent of SOS response involving SulA (Appendix Fig S6).

Here, we revealed an OxyS-induced molecular checkpoint relay, leading to temporary cell cycle arrest to allow damage repair. We propose that in addition to a first tier of defense that is aimed at elimination of reactive oxygen species and repair of cellular damages, E. coli employs a second tier of defense targeting cell division by modulating NusG levels. The transient growth inhibition caused by interference with cell division enables the bacterial repair systems to repair damage for a longer period of time, thus allowing
a higher number of cells to resume growth. We show that by decreasing nusG expression, the oxidative stress-induced OxyS sRNA leads to an increase in KilR and subsequently to inhibition of cell division (Fig 6). As the production of OxyS in response to oxidative stress is transient, decreasing dramatically by 60 min of exposure, and the levels of kilR reach a plateau at about the same time, the oxyS/nusG/kilR-dependent regulatory cascade is transient, leading to temporary cell cycle arrest.

The presence of an intact RelA allele seems to have an effect on the toxic phenotype of OxyS. In the relA mutant strain, both wild type and OxyS A69C exhibit a similar growth arrest phenotype, whereas in wild-type cells carrying an intact RelA allele, toxicity of OxySA69C is more pronounced than that of wild-type OxyS (Appendix Fig S7). The difference in toxicity of OxyS mutants could be attributed to the observation that RelA-mediated synthesis of ppGpp in LB medium increases FtsZ protein levels, possibly through transcriptional regulation (Powell & Court, 1998). The increase in FtsZ protein levels could possibly compensate for the interference with FtsZ function.

Intriguingly, our experiments indicate that two molecules of OxyS can bind one nusG molecule at the same time. The first binding site spans nucleotides –40 to –29 with respect to the first nucleotide of the start codon. The second experimentally verified interaction site in nusG spans from nucleotide –20 to +13 with respect to the AUG. Given that OxySc76G; c77G fails to repress its complementary mutant nusGC15G; c16G, it suggests that the upstream site aids OxyS in binding of the downstream site overlapping the ribosome-binding region. Expression of NusG quadruple mutant (G-15C; G-16C and G-31C; G-32C) carrying mutations at the two sites predicted to bind the same sequence in OxyS was repressed by OxySc76G; c77G and unaffected by wild-type OxyS. Together, the data indicate that two molecules of OxyS can simultaneously bind two different sites in nusG. sRNAs interacting with multiple sites within an mRNA have been described before. In most cases, pairing with either site was found to be sufficient for full regulation. For example, the RybB sRNA can base-pair with either one of two mutually exclusive pairing sites within the translated portion of OmpD mRNA. However, pairing with either site is sufficient for OmpD regulation (Balbontin et al, 2010). Sgrs sRNA binds two different sites in manXYZ operon, each capable of translation repression of different genes of the operon, although both sites are needed for RNase E-dependent degradation of the full-length transcript (Rice et al, 2012). The long lrp leader is bound by GevB at two independent sites (Lee & Gottesman, 2016). Pairing with either site was sufficient for significant repression of lrp expression, although pairing at both sites gave the best repression (Lee & Gottesman, 2016). Spot 42 interacts with a number of targets via two different sites. Both sites were shown to contribute to regulation; however, whether Spot 42 base pairs with both sites simultaneously is unclear (Beisel et al, 2012). Further biochemical analyses will be required to fully understand the OxyS dual binding of nusG and the involvement of Hfq in this unique base-pairing regulatory mechanism in which two molecules of OxyS repress one molecule of nusG mRNA.

The small RNA OxyS is highly conserved. An OxyS homology search based on iterative blasting and structural filtering implemented in the GLASSgo web server (http://rna.informatik.uni-freiburg.de/GLASSgo/Input.jsp) detected 603 potential OxyS homologs, which are widely distributed in the enterobacterial clade as they belong to 10 different genera representing at least 27 different species and more than 500 different strains (Appendix Fig S8). All homologs are located in the 5′ intergenic region of the oxyR gene, sharing a widely conserved gene synteny (Appendix Fig S9). Multiple alignments of 36 randomly selected OxyS homologs exhibited

Figure 6. The transient growth arrest caused by cell division inhibition allows a greater number of cells to resume growth.

By decreasing nusG expression, the oxidative stress-induced OxyS sRNA leads to an increase in KilR. KilR prevents proper FtsZ assembly by sequestering the protein subunits. The inhibition of cell division allows for greater repair leading to a decrease in mutation rates concomitant with an increase in the rate of recovery.
significant nucleotide conservation at specific regions (Appendix Fig S10A). These regions encompass the loops of the first and the middle hairpins and the downstream single-stranded RNA stretch (Appendix Fig S10B). In this study, we show that the middle hairpin B and the single-stranded region of OxyS interact with the nusG mRNA target of E. coli. As for nusG, conservation analysis of the interaction between OxyS and nusG predicts two possible base-pairing sites within nusG mRNAs: the first overlaps the nusG ribosome-binding site, and the second is located about 20–30 nt upstream of the Shine–Dalgarno sequence of nusG. Of 35 examples examined, 32 were predicted to harbor nusG/OxyS interaction; of which 14 were predicted to interact with both sites, and 17 were predicted to interact with the site overlapping the Shine–Dalgarno sequence and/or AUG of nusG or with the site located upstream (Appendix Fig S11). The OxyS sequence and structural conservation and the conservation of the responsive site in nusG indicate that regulation of nusG by OxyS is an important part in the bacterial response to oxidative stress. Coexistence of nusG with kilR or with \( \lambda \) kil gene is predicted in 16 of the respective examples, of which in 14 instances nusG is also predicted to base-pair with OxyS (Appendix Fig S12). Eighteen strains predicted to carry nusG/OxyS interaction lack kilR or \( \lambda \) kil. The effect of OxyS on nusG, a highly conserved and essential transcription factor in strains lacking Kil proteins, and the physiological impact this interaction may have remains to be addressed in future studies.

Small RNAs deploy a variety of mechanisms to cope with stress conditions such as, for example, reducing expression of outer membrane proteins in response to membrane stress, decreasing expression of iron-containing proteins in response to limiting iron or down-regulating the expression of sugar transporters, while up-regulating sugar de-phosphorylation in response to glucose–phosphate stress (Wagner & Romby, 2015). In all of these cases, the sRNAs cope with stress conditions by targeting genes directly linked to the specific stress. OxyS introduces a new regulatory concept in which a transient growth inhibition caused by this sRNA facilitates damage repair, thus enabling a higher number of cells to resume growth following stress. Whether additional sRNAs exploit the toxic coexistence of genes like nusG and kilR to coordinate cell growth with damage control remains to be addressed in further studies.

Materials and Methods

Bacterial growth conditions

*Escherichia coli* (MG1655) was grown at 37°C (200 rpm) in LB medium (pH 6.8). Ampicillin (100 μg/ml), tetracycline (10 μg/ml), chloramphenicol (20 μg/ml), and kanamycin (40 μg/ml) were added where appropriate. *Plac* and *Ptac* promoters were induced with isopropyl β-D-thiogalactoside (IPTG; 1 mM) as indicated. (List of strains, plasmids, and DNA primers used in this study appears in Appendix Tables S1–S3.)

Strain construction

Gene deletion mutants were generated using the gene disruption method as described (Yu et al., 2000). For construction of deletion mutants, tetracycline, chloramphenicol, or kanamycin, cassettes were amplified using mini-Tn10 chromosomal cassette and plasmids pKD3 or pKD4, respectively (Datsenko & Wanner, 2000). The PCR product (5–10 μg) purified using the Wizard SV PCR cleanup system (Promega, Madison, WI) was introduced into DY378 cells grown at 30°C to OD_{600} of 0.4–0.6 and then transferred to 42°C for 15 min. The mutations were transferred into a fresh genetic background of MG1655 *mat::lacZ* by P1 transduction. The resistance cassettes were eliminated using pCP20 (Datsenko & Wanner, 2000). To construct ΔlacZ::Tn10, the chromosomal region flanked by genome coordinates 363264 and 366272 (GenBank entry NC_000913.3) was replaced by the tetR and tetA genes using primers 2081 and 2082. *lacZ* gene disruption was examined by PCR using flanking primers (2083 and 2084). To construct ΔoxyS::kan, the chromosomal region flanked by genome coordinates 4158322 and 4158375 (GenBank entry NC_000913.3) was replaced by the *kan* gene using primers 2169 and 2170. *oxyS* gene disruption was examined by PCR using flanking primers, 2026 and 2027. To construct ΔΔlacZ::cat, the chromosomal region flanked by genome coordinates 1417950 and 1418235 (GenBank entry NC_000913.3) was replaced by the *cat* gene using primers 2265 and 2295. *kil* gene disruption was examined by PCR using flanking primers 2267 and 2296. To construct NusG-SPA fusion in the chromosome, primes were designed to amplify the SPA tag together with the kanamycin resistance cassette from plasmid pJL148 (Zeghouf et al., 2004). At least 42 nt of homologous sequences was allowed. MDS42 nusG-SPA-kan was constructed using primers 2216 and 2217. The PCR products were gel-purified and then transform into MDS42 cells carrying pKD46 plasmid (Datsenko & Wanner, 2000). Insertions were confirmed by PCR using primers 2182 and 2219. The products were sequenced using primer 2227. The fusions were transferred into strains by P1 transduction.

Plasmid construction

To construct Plac-oxyS, the oxyS sequence from its transcription start site and 27 nt downstream of its transcription terminator was PCR-amplified from MG1655 chromosomal DNA using primers 2026 and 2027 and cloned into the Aattl and HindIII restriction sites of pBR-plac (Guillier & Gottesman, 2006). To construct Ptac-nusG-lacZ translation fusion in the single-copy plasmid pBOG552 (Hershko-Shalev et al., 2016), the nusG fragment was PCR-amplified from MG1655 using primers 2179 and 2181 and then cloned into the EcoRI and SmaI sites of pKK177-3-lacZ. The Ptac-nusG fragment was sub-cloned into pBOG552 using the BamHII site. To construct Plac-kilR, kilR sequence was amplified from MG1655 chromosomal DNA using primers 2700 and 2701 and cloned into the Aattl and HindIII restriction sites of pBR-plac. To construct P_{secE-nusG}, the sequence of secE-nusG was PCR-amplified using primers 2377 and 2378 and then cloned into HindIII and BamHI restriction sites of pACYC184. To construct P_{secE-nusG}, we deleted the secE gene from the plasmid P_{secE-nusG} using primers 2725 and 2727. To construct P_{secE-nusG}, the sequence of secE was PCR-amplified using primers 2377 and 2727 and then cloned into HindIII and BamHI restriction sites of pACYC184. All pACYC (P15A) plasmids carry the OxyS complementary sequence. The cloning in pACYC plasmids destroyed the tet resistance cassette.
Random mutagenesis

Random mutagenesis of OxyS wild type and OxyS<sub>A69C</sub> was carried out using hydroxylamine as described before (Hershko-Shalev et al., 2016).

Site-directed mutagenesis

Mutations A69C, A69C,C70U, C56U,C58U, C76U,C77U, and C76G, C77G were generated by PCR using P<sub>lac-<i>oxyS</i></sub> (pSA86) and two tail-to-tail divergent primers of which one carried the desired mutation. The PCR product was gel-purified and subjected to blunt end ligation. Likewise, the mutations in <i>nusG</i> were generated in <i>Ptac-nusG</i> (pKK177-3). The mutated <i>Ptac-nusG</i> fragment was sub-cloned into pBGO552 using the BamHI site.

Survival assays

Overnight cultures of MG1655 strains as indicated in the legends, carrying control or OxyS plasmids, were grown from fresh transformation plates. Starters were diluted 1/100 in 20 ml LB (125-ml Erlenmeyer flasks) and grown at 37°C (200 rpm). IPTG (1 mM) was added at the time of dilution, where indicated H<sub>2</sub>O<sub>2</sub> (1 mM) was added at OD<sub>600</sub> ~ 0.1 for 30 min. Thereupon, the cells were washed and suspended in fresh warm LB medium. Samples were taken at 30, 60, and 90 min after wash, diluted in 1× PBS, and plated. Each sample was plated twice. Colonies were counted, and the percentage of survival rate was calculated. Cultures with no OxyS plasmids were treated and assayed as above except for IPTG induction.

β-galactosidase assays

Overnight cultures of MG1655 relA::cat, <i>ΔoxyS{l}i::frt</i>, lacZ::<i>Tn</i>10, lac<sup>fl</sup> carrying <i>Ptac-nusG-lacZ</i> translational fusion (pSCh101<sup>+</sup> <i>kan</i>) as well as <i>Plac</i> control and <i>Plac-oxyS</i> plasmids as indicated, were diluted 1/100 in 10 ml LB medium supplemented with ampicillin and kanamycin. The cultures grown to OD<sub>600</sub> ~ 0.1 were treated with IPTG (1 mM) to induce transcription of both <i>nusG-lacZ</i> and OxyS. β-Galactosidase activity was measured at 120 min after IPTG induction. To measure OxyS effects on <i>λfhlA-lacZ</i> fusion, MG1655 <i>λfhlA-lacZ::kan</i>, relA::cat, lac<sup>fl</sup>, lacZ::<i>Tn</i>10 carrying <i>Ptac-oxyS</i> wild type and mutants were diluted 1/100 in 5 ml LB medium supplemented with ampicillin. The cultures grown to OD<sub>600</sub> ~ 1.0 were treated with IPTG (1 mM) to induce OxyS expression. β-Galactosidase activity was measured at 60 min after IPTG induction.

RNA extraction

To isolate total RNA, cultures grown as indicated in the specific legends were pelleted and resuspended in 50 μl of 10 mM Tris–HCl (pH 8) containing 1 mM EDTA. Lysozyme was added to 0.9 mg/ml, and the samples were subjected to three freeze–thaw cycles. Total RNA was purified using TRI reagent (Sigma) according to the manufacturer’s protocol. For real-time analysis, the purified RNA was ethanol-precipitated in the presence of 0.3 M sodium acetate. To estimate the half-life of <i>secE-nusG</i> mRNA, cultures of MG1655 relA::cat, lac<sup>fl</sup>, and MG1655 relA::cat, lac<sup>fl</sup>, <i>ΔoxyS{l}i::kan</i> grown to early-exponential phase in LB medium were treated with 1 mM hydrogen peroxide. After 2 min, rifampin (0.2 mg/ml) was added. Samples were taken at 2, 4, 6 min after the rifampin addition.

Northern analysis

RNA samples (10–20 μg) were denatured for 10 min at 70°C in 98% formamide loading buffer, separated on 8 M urea–6% polyacrylamide gels, and transferred to Zeta-Probe GT membranes (Bio-Rad Laboratories) by electroblotting. To detect OxyS RNA, the membranes were hybridized with <sup>32</sup>P-end-labeled <i>oxyS</i> primer (492) in modified CHURCH buffer (Church & Gilbert, 1984). To detect <i>secE-nusG</i> full-length RNA, samples (15 μg) were denatured for 10 min at 70°C in MOPS loading buffer, separated on 1.4% agarose gels, and transferred to Zeta-Probe GT membranes by capillary transfer. <i>secE-nusG</i> mRNA levels were detected using anti-<i>nusG</i>-labeled riboprobe synthesized using PCR template (2620 and 2554) as previously described (Hershko-Shalev et al., 2016). Riboprobe hybridization buffer contained 50% formamide, 3.5% SDS, 250 mM NaCl, 82 mM Na<sub>2</sub>HPO<sub>4</sub>, and 40 mM NaH<sub>2</sub>P<sub>4</sub> at pH 7.2. After 2 h at 50°C, the membranes were washed for 20 min at 50°C in 2× SSC, 1% SDS, then for 20 min at 55°C in 1× SSC, 0.5% SDS, and last for 20 min at 60°C in 0.5× SSC, 0.1% SDS. tmRNA (105a) was used as a loading control (primer 1912).

Western of SPA-tagged <i>nusG</i>

Overnight cultures of SPA-tagged strains were diluted 1/100 and grown shaking (200 rpm) at 37°C in 100 ml LB (250-ml Erlenmeyer flasks). H<sub>2</sub>O<sub>2</sub> (1 mM) was added at OD<sub>600</sub> ~ 0.1 for 2, 5, and 7 min. Samples were pelleted and then fluidized in 1× Laemmli sample buffer (Bio-Rad), heated at 95°C for 5 min, and centrifuged for 5 min. 10<sup>7</sup> cells of each sample were analyzed on SDS–PAGE (12%). The proteins were transferred to a nitrocellulose membrane (Sartorius); the blots were blocked with BSA and skim milk and probed with FLAG M2 monoclonal antibody (Sigma-Alrich) according to the manufacturer’s protocol. The membranes were also probed with Hfq-specific antibodies as a loading control. The proteins were visualized using secondary antibody Anti-Mouse IgG-Alkaline Phosphatase (FLAG) or Anti-Rabbit IgG-Alkaline Phosphatase (Hfq) (Sigma-Alrich) according to the Alkaline Phosphatase development protocol (Promega).

Real-time PCR

RNA concentrations were determined using a NanoDrop machine (NanoDrop Technologies). DNA was removed by DNase treatment according to the manufacturer’s instructions (RQ1 RNase-free DNase, Promega). 2 μg of DNA-free total RNA was used for cDNA synthesis using MMLV reverse transcriptase and random primers (Promega). <i>kil</i> cDNA levels were analyzed by real-time PCR using specific primers (2531–2532) and SYBR green mix (Absolute SYBR GREEN ROX MIX, ABgene) with Rotor-gene 3000A (Corbett) according to the manufacturer’s instructions. The level of 16S rRNA (<i>rrsB</i>; primers 1309–1310) (Park et al, 2007) was used to normalize <i>kil</i> levels. The relative amount of cDNA was calculated using the
standard curve method. A standard curve was obtained from PCR on serially diluted genomic DNA as templates and was analyzed using Rotor-gene analysis software 6.0.

**In vitro RNA synthesis**

Purified PCR fragments of *nusG* (207 bp) and *oxyS* wild type and mutants (173 bp) generated using primers 2220–2221 and 2238–2027 were used as templates to produce *nusG* and *OxyS* RNAs of 176 nt and 109 nt, respectively. The RNAs were synthesized in 50 µl reactions containing T7 RNA polymerase (25 units: New England Biolabs), 40 mM Tris–HCl (pH 7.9), 6 mM MgCl₂, 10 mM dithiothreitol (DTT), 20 units RNase inhibitor (CHIMERx), 500 µM of each NTP, and 200 ng of purified PCR templates carrying the sequence of the T7 RNA polymerase promoter. To synthesized fully labeled RNA, 500 µM of each, UTP, GTP, CTP, and 40 µM ATP was used with 10 µCi [³²P]-ATP (specific activity 800 Ci/mmol). Synthesis was allowed to proceed for 2 h at 37°C and then terminated by heating (70°C, 10 min). To remove DNA template, 4 U of turbo DNase I (Ambion) was added (37°C, 30 min), followed by phenol/chloroform extraction and ethanol precipitation in the presence of 0.3 M ammonium acetate.

**Primer extension**

Annealing mixtures containing in DEPC-treated water, 0.05 pmol of *in vitro*-synthesized *nusG* RNA, 0.6 pmol of 5’ end-labeled *nusG*-specific primer (2221) without or with 12 pmol of *in vitro*-synthesized OxyS RNAs (wild type and mutants) were heated for 10 min at 70°C and then chilled on ice for 20 min. Thereafter, the reactions were incubated at 37°C in 20 mM Tris–HCl, 10 mM magnesium acetate, 0.1 M NH₄Cl, 0.5 mM EDTA, 2.5 mM β-mercaptoethanol, and 0.5 mM each dNTP for 15 min. The extension products were separated on 6% sequencing gels, alongside with sequencing reactions.

**RNase protection assay**

0.06 pmol of labeled *nusG* RNA and 15 and 30 of *OxyS* RNAs were precipitated and annealed as described before (Rio et al., 2011).

After overnight annealing at 45°C, the mixture was subjected to ribonuclease (A and T1) degradation at room temperature for 10 min. The products were separated on 8% sequencing gels, alongside with labeled RNA Marker (Decade Thermo Fisher Scientific).

**EMSA**

Fully labeled *in vitro*-synthesized wt and quadruple mutant *nusG* RNAs (176 nt, 0.01 pmol, and 0.004 pmol, respectively) without and with increasing concentrations of *OxyS* (5, 10, and 15 pmol left panel, or 2, 4 right panel) were incubated for 15 min at 85°C. Thereafter, the mixtures were incubated at 37°C or 42°C for 60 min in binding buffer (6.7 mM Tris-acetate (pH 7.4), 3.3 mM Na-acetate, 10 mM DTT, and 10 mM MgCl₂). The RNA samples were separated on 4.5% nondenaturing polyacrylamide gels (19:1) in 20 mM Tris–HCl (pH 7.5), 60 mM KCl, and 10 mM MgCl₂ (50 volts for 5–6 h at 4°C).

**Mutagenesis assays**

Overnight cultures carrying OxyS plasmids wild type and mutant with or without a plasmid expressing *ftsQAZ* (*pFtsZ*) were diluted 1/100 in 7 ml LB medium supplemented with ampicillin (for *oxyS* plasmids) and tetracycline (for *pFtsZ*). The cultures were grown to OD₆₀₀ ~ 0.1 (37°C, 200 rpm) prior to the addition of IPTG (1 mM). At 15 min of IPTG induction, the cultures were exposed to 5 mM hydrogen peroxide at room temperature (22°C), without shaking for 15 min. Thereupon, 0.5 ml of cells was mixed with 2 ml fresh LB and grown overnight (23 h). To determine frequencies of mutagenesis, aliquots were taken after 23 h and plated on LB plates containing 100 µg/ml of rifampicin. The numbers of Rif² mutants were normalized to the numbers of viable cells at the 23-h time point. To estimate the number of mutations due to KilR expressed from Plac-*kilR* plasmid, cells were treated as above, except that expression of *kilR* was induced using 20 µM of IPTG instead of 1 mM. To estimate the number of mutations in wild type and *oxyS* mutant, cultures at OD₆₀₀ ~ 0.1 were exposed to 1 mM hydrogen peroxide for 30 min (37°C, 200 rpm). The numbers of Rif² were calculated following the protocol described above.

**Fluorescence microscopy**

Overnight cultures were sub-cultured in fresh LB medium supplemented with 1 mM IPTG and Amp. Tetracycline was included only in cultures that expressed *pFtsQAZ*. Cultures were grown at 37°C for 3 h before imaging. All the samples were spotted on PBS agar pad for imaging. DNA was stained with DAPI (Sigma-Aldrich) at a final concentration of 2 µg/ml. Cells were visualized and photographed using Nikon Eclipse Ti-E inverted microscope equipped with Perfect Focus System (PFS) and ORCA Flash 4 camera (Hamamatsu photonic). Images were processed using NIS-Elements-AR software. To determine cell length distribution, > 250 cells from a 4 × 4 matrix field (16 individual fields) were randomly selected, and their length was measured using NIS-Elements-AR software. The values given are data obtained from three independent experiments, a total of > 750 cells.

**Computational prediction of OxyS homologs and targets**

Iterative blasting and structural filtering implemented in the GLASSgo web server (http://rna.informatik.uni-freiburg.de/GLASSgo/Input.jsp) was used to detect potential OxyS homologs. The maximum allowed *E*-value was set to 1, the minimum required identity was 58%, and the structure-based filtering value was set to 2. This search yielded 603 candidates belonging to 10 different genera and some uncharacterized enterobacterial sequences, representing at least 27 different species and more than 500 different strains. The computational OxyS target prediction was conducted using IntaRNA and CopraRNA (Wright et al., 2014) on the web server version with standard parameters.

**Expanded View** for this article is available online.

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Author contributions
SB, ME-W, JE, JC, SG, MH, and PRW performed the experiments. SB, ME-W, JE, JC, SG, MH, PRW, SA, and WRH conceived the experiments and analyzed the data. SA wrote the manuscript. SA and WRH managed the project.

Conflict of interest
The authors declare that they have no conflict of interest.

References


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