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Species-specific residues calibrate SoxR sensitivity to redox-active molecules

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Summary

In enterics, the transcription factor SoxR triggers a global stress response by sensing a broad spectrum of redox-cycling compounds. In the non-enteric bacteria *Pseudomonas aeruginosa* and *Streptomyces coelicolor*, SoxR is activated by endogenous redox-active small molecules and only regulates a small set of genes. We investigated if the more general response in enterics is reflected in the ability of SoxR to sense a wider range of redox-cycling compounds. Indeed, while *Escherichia coli* SoxR is tuned to structurally diverse compounds that span a redox range of –450 to +80 mV, *P. aeruginosa* and *S. coelicolor* SoxR are less sensitive to viologens, which have redox potentials below –350 mV. Using a mutagenic approach, we pinpointed three amino acids that contribute to the reduced sensitivity of *P. aeruginosa* and *S. coelicolor* SoxR. Notably these residues are not conserved in homologues of the Enterobacteriaceae. We further identified a motif within the sensor domain that tunes the activity of SoxR from enterics – inhibiting constitutive activity while allowing sensitivity to drugs with low redox potentials. Our findings highlight how small alterations in structure can lead to the evolution of proteins with distinct specificities for redox-active small molecules.

Introduction

Iron-sulfur clusters (Fe-S) are remarkably diverse in structure and chemistry. Different cluster types span a wide range of redox potentials and the redox potential of a single cluster type can be further tuned by changing its molecular environment (Beinert, 2000). These features enabled the evolution of Fe-S proteins that perform crucial and versatile functions as metabolic enzymes, components of electron transport chains, and redox-sensing regulators of gene expression. The latter act as molecular switches that are either activated or inactivated by specific redox signals (such as oxygen, hydrogen peroxide, superoxide, nitric oxide, or redox-active small molecules) to regulate important aspects of bacterial development and physiology. For example, FNR, a [4Fe-4S] protein, controls the switch between aerobic and anaerobic metabolism in *Escherichia coli* in response to molecular oxygen (Khoroshilova *et al.*, 1997); IscR, a [2Fe-2S] protein, increases the production of Fe-S cluster biogenesis machinery under conditions of oxidative stress (Zheng *et al.*, 2001; Yeo *et al.*, 2006); and SoxR, also a [2Fe-2S] protein, mediates an oxidative stress response to redox-cycling drugs in the enteric bacteria *E. coli* and *Salmonella enterica* (Hidalgo and Demple, 1996). Within this group of redox-sensing transcription factors SoxR is unique in that, unlike the other proteins that are regulated by assembly/disassembly of their Fe-S clusters, the activity of SoxR is modulated by reversible one-electron oxidation–reduction of its [2Fe-2S] clusters (Ding *et al.*, 1996; Gaudu and Weiss, 1996; Ding and Demple, 1997; Gaudu *et al.*, 1997).

In *E. coli* (and related enteric bacteria), SoxR senses redox stress imposed by a broad collection of redox-active compounds including viologens, phenazines and quinones (Table 1; Gu and Imlay, 2011). SoxR transduces these redox signals into a global defence programme via a second transcription factor, SoxS. SoxR is a constitutively expressed regulator bound to the *soxS* promoter poised to detect stress. In the absence of oxidants, SoxR exists in a quiescent state with reduced [2Fe-2S] clusters and *soxS* is not expressed. Exposure to redox-cycling drugs causes oxidation of SoxR's [2Fe-2S] centres, and the oxidized protein activates *soxS* expression by mediating structural changes in the promoter DNA that allow RNA polymerase to initiate transcription (Hidalgo *et al.*, 1995). SoxS in turn recruits RNA polymerase to transcribe > 100 genes, some of which encode proteins that reestablish redox balance and repair oxidant-induced damage (Pomposiello *et al.*, 2001). The SoxRS system in enterics allows for rapid

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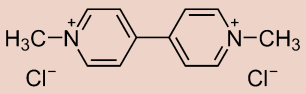
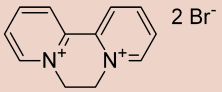
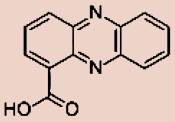
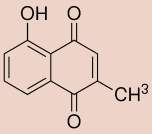
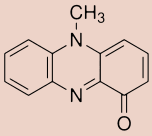
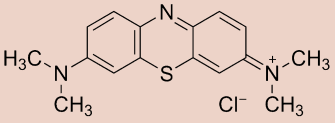
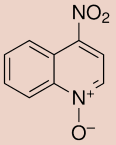
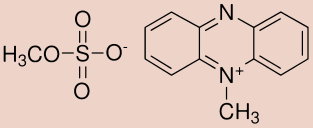
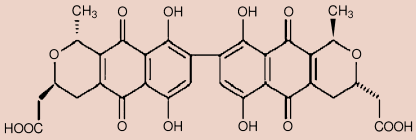
Table 1. Redox drugs used in this work.[a]

Class	Drug	Structure	Redox potential (mV)
Viologen	Paraquat (PQ)		–440 (Steckhan and Kuwana, 1974)
Viologen	Diquat (DQ)		–361 (Steckhan and Kuwana, 1974)
Phenazine	Phenazine-1-carboxylic acid (PCA)		–177 (Price-Whelan *et al.*, 2006)
Napthoquinone	Plumbagin (PB)		–135 (Hakura *et al.*, 1994)
Phenazine	Pyocyanin (Pyo)		–34 (Friedheim and Michaelis, 1931)
Phenothiazine	Methylene blue (MB)		+11 (Kamat *et al.*, 1987)
Quinoline	4-Nitroquinoline-N-oxide (4NQO)		+74 (Biaglow *et al.*, 1978)
Phenazine	Phenazine methosulfate (PMS)		+80 (Moffet *et al.*, 2003)
Anthraquinone	γ-Actinorhodin (Act)		Unknown

a. Drugs are arranged in order of increasing midpoint redox potential. The redox potentials are reported versus the normal hydrogen electrode (NHE).

amplification of the stress signal into a stress response geared towards oxidants.

The SoxRS regulon is unique to enterics. Although *soxR* is widely distributed (and highly similar at the amino acid level) across the Gram-negative Proteobacteria and the Gram-positive Actinobacteria, *soxS* is present exclusively in enterobacteria. An extensive bioinformatic analysis of *soxS*-deficient genomes predicted that in non-enterics SoxR directly regulates a relatively small set of genes that encode putative oxygenases, oxidoreductases, or transporters (Dietrich *et al.*, 2008). This has been verified for the γ-Proteobacterium *Pseudomonas aeruginosa* and the

Actinomycete *Streptomyces coelicolor*, both soil-dwelling organisms notable for producing redox-active secondary metabolites. The SoxR regulon in *P. aeruginosa* consists of a Resistance–Nodulation–Division (RND) efflux pump MexGHI-OmpD (*PA4205–4208*), a major facilitator superfamily (MFS) transporter (*PA3718*), and a monooxygenase (*PA2274*) (Palma *et al*., 2005). In *S. coelicolor*, SoxR regulates a monooxygenase (*SCO1909*) with homology to *PA2274*, two oxidoreductases (*SCO2478*, *SCO4266*), an epimerase/dehydratase (*SCO1178*), and an ABC transporter (*SCO7008*) (Dela Cruz *et al*., 2010; Shin *et al*., 2011). In these bacteria SoxR-regulated genes are induced in stationary phase during the production and secretion of redox-active metabolites – phenazines in the case of *P. aeruginosa* and the benzochromanequinone polyketide actinorhodin (Act) in the case of *S. coelicolor* (Dietrich *et al*., 2006; Dela Cruz *et al*., 2010; Shin *et al*., 2011). This is not a mere correlation as expression of each SoxR regulon is dependent on production of the redox-active compounds by the microbe (Dietrich *et al*., 2006; Dela Cruz *et al*., 2010; Shin *et al*., 2011). These observations support the view that SoxR evolved to regulate the machinery that processes/transports endogenous redox-active metabolites in producer organisms. The enterobacteria (which do not produce redox-active secondary metabolites) are unique in that SoxR regulates only one gene, *soxS*. They may have acquired *soxR* via lateral gene transfer, taking advantage of its redox-sensing abilities to regulate a generalized stress response (SoxS regulon) against toxic redox-cycling compounds.

Given that SoxR performs distinct functions in different bacteria, we asked if the differences in SoxR functionality are manifested only by its regulons, or if SoxR from different species also sense different inputs. We hypothesized that SoxR from *P. aeruginosa* and *S. coelicolor* sense redox molecules that resemble their endogenous activators (phenazines and anthraquinones respectively), while *E. coli* SoxR, given its involvement in a general stress response, senses a broader spectrum of redox-active compounds. Here we report that the non-enteric SoxR proteins are indeed more restricted in the range of molecules they sense compared with their *E. coli* counterpart, and we have identified key features that contribute to the differential sensitivities. This study provides insight into the evolutionary fine-tuning of this redox-sensing transcription factor that adapted it to serve the needs of organisms with different physiologies.

Results

SoxR protects E. coli*, but not* P. aeruginosa *or* S. coelicolor*, against redox-cycling drugs*

In *E. coli* and related enterobacteria, SoxR mediates a general stress response against redox-cycling compounds by activating the SoxS regulon. By contrast, non-enterics lack a SoxS regulon. Instead, SoxR directly regulates a small set of genes, making a general stress response unlikely. To test this, we exposed *soxR* deletion mutants of *E. coli* (which contains *soxS*), the Gram-negative *P. aeruginosa* PA14, and the Gram-positive *S. coelicolor* M145 (both of which lack *soxS*) to a diverse array of redox-cycling compounds using a filter disk assay. In agreement with previous reports, an *E. coli* Δ*soxR* mutant was more sensitive to most tested redox-cycling agents such as pyocyanin (Pyo), plumbagin (PB), and 4-nitroquinoline-N-oxide (4NQO), compared with wild type (Greenberg *et al*., 1990; Tsaneva and Weiss, 1990; Fig. 1A). The *E. coli* Δ*soxR* mutant was no more sensitive to the viologen diquat (DQ) than wild type, and a previous study had shown this to also be true for paraquat (PQ), another viologen (Greenberg *et al*., 1990). This appears to be a strain-specific phenomenon, since a different *E. coli* Δ*soxR* mutant strain was more sensitive to PQ than wild type (Tsaneva and Weiss, 1990). In contrast to *E. coli*, the *S. coelicolor* Δ*soxR* mutant and wild type were equally sensitive to all tested compounds (Fig. 1B). *P. aeruginosa* was generally more resistant to all drugs tested in this study. The wild type and Δ*soxR* mutant were resistant to Pyo, PB, and 4NQO, and were equally sensitive to DQ (Fig. 1C). While SoxR does not contribute to *S. coelicolor* or *P. aeruginosa*'s resistance to redox-cycling drugs under the conditions tested here, it is worth noting that it might do so under other experimental conditions. It is also worth noting that the *P. aeruginosa* Δ*soxR* mutant has a colony morphology phenotype (Dietrich *et al*., 2008), which can be reverted by complementation with *E. coli* or *P. aeruginosa* SoxR (Fig. S1).

P. aeruginosa *and* S. coelicolor *SoxRs sense a narrower spectrum of redox drugs than* E. coli *SoxR*

Our finding that SoxR did not contribute to resistance against redox-cycling agents in *P. aeruginosa* and *S. coelicolor* is consistent with the notion that the enteric-specific SoxS regulon governs a general stress response. To explore the hypothesis that the SoxR regulons in *P. aeruginosa* and *S. coelicolor* may be specific to phenazines and Act, respectively, we posited that SoxR itself might be optimized to sense specific redox inputs. Given the role of SoxR in *E. coli* as a general stress-response regulator, we predicted this protein would respond to a broad spectrum of redox-cycling molecules, while *P. aeruginosa* and *S. coelicolor* SoxRs would only respond to molecules that resemble their endogenous activators, i.e. phenazines and anthraquinones respectively.

To quantify the SoxR response to a wide spectrum of redox-cycling compounds, we employed a β-galactosidase assay using a *soxS* promoter-*lacZ* reporter in *E. coli*. SoxR

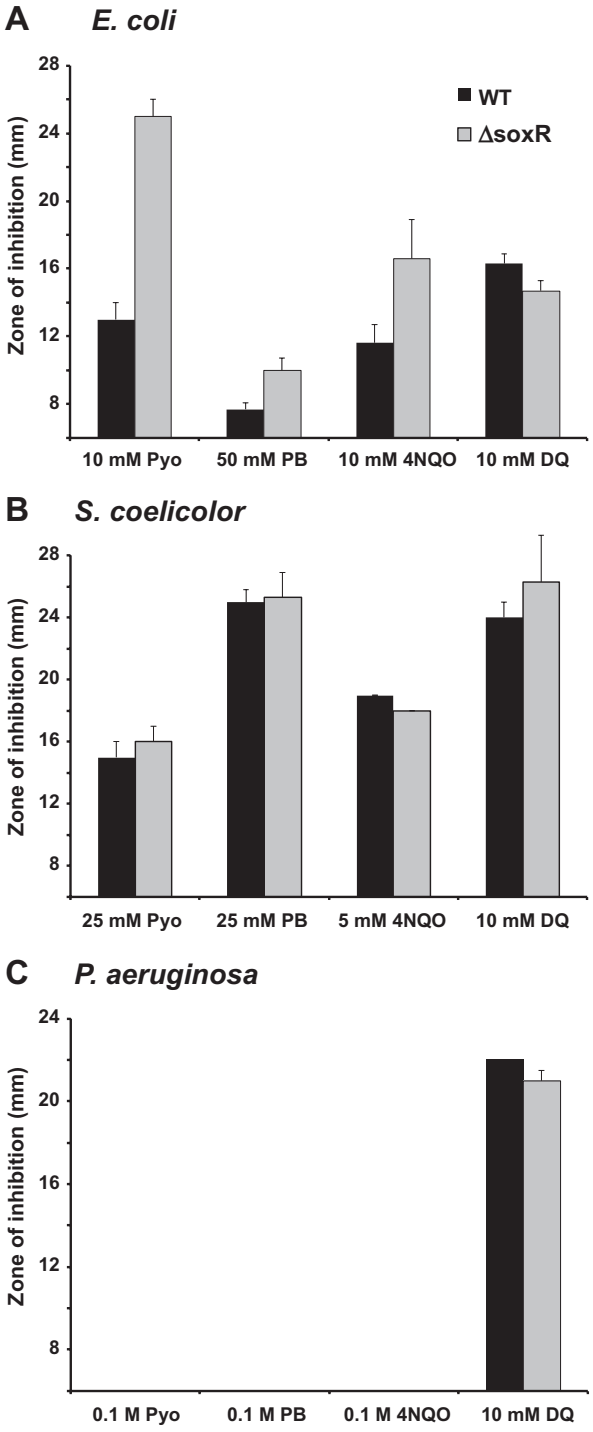


Fig. 1. *S. coelicolor* and *P. aeruginosa* Δ*soxR* mutants are not hypersensitive to superoxide-generating agents. Paper disks soaked with solutions of the indicated compounds were placed on bacterial lawns of wild type (black columns) or Δ*soxR* mutant (grey columns) growing on nutrient agar plates. Zones of growth inhibition around the disks were recorded after 24 h at 37°C for *E. coli* (A) or 48 h at 30°C for *S. coelicolor* (B) and *P. aeruginosa* (C). A zone of inhibition of 6 mm corresponds to the diameter of the disk and indicates that there was no detectable inhibition zone. The data represent the means of three to five replicates ± standard deviations (bars; some not visible).

orthologues from *E. coli*, *P. aeruginosa*, and *S. coelicolor* have very similar DNA binding domains, and all three proteins bind to the *soxS* promoter with high affinity *in vitro* (Fig. S2C). Therefore, using the *soxS* promoter-*lacZ* reporter seemed a reasonable approach. The three *soxR* genes (each with an N-terminal histidine-tag) were transformed into an *E. coli* Δ*soxRS* mutant lysogenized with a λ[*soxS* promoter-*lacZ* reporter] (Table 2). Transformed cells were grown to exponential phase and then treated with representative drugs that span a wide range of redox potentials and belong to different structural classes (Table 1). The phenazines Pyo and phenazine-1-carboxylic acid (PCA) are produced by *P. aeruginosa*, while phenazine methosulfate (PMS) is synthetic.

Escherichia coli SoxR was strongly activated (albeit to different extents) by all nine drugs tested (Fig. 2A). *P. aeruginosa* SoxR was activated at levels comparable to *E. coli* SoxR by PCA, PB, Pyo, methylene blue (MB), 4NQO, PMS, and Act, but at significantly lower levels by the viologens PQ and DQ (Fig. 2A). PQ elicited 15-fold lower β-galactosidase activity in cells expressing *P. aeruginosa* SoxR compared with *E. coli* SoxR. Ethyl viologen, which has a similar midpoint redox potential as PQ (–480 mV), was also a weak inducer of *P. aeruginosa* SoxR activity (data not shown). The response to DQ, which has a higher redox potential than PQ (–360 mV) was more robust (2200 Miller units), but still only about half that of *E. coli* SoxR (4600 Miller units). Thus *P. aeruginosa* SoxR has low sensitivity to the viologens that have redox potentials more negative than –350 mV (Fig. 2B).

In stark contrast to the high levels of β-galactosidase activity produced by *E. coli* and *P. aeruginosa* SoxR, *S. coelicolor* SoxR produced relatively low signals in response to all tested drugs (Fig. 2A). As such we were unable to draw any meaningful conclusions about *S. coelicolor* SoxR activation using the heterologous *E. coli* system. We therefore investigated this transcription factor's activity in its native background. Because this protein is activated by the endogenous metabolite Act, it was necessary to monitor its response to exogenous drugs in *S. coelicolor* M511, a strain that does not synthesize Act (Table 2). Cells were grown for 20 h in liquid culture before a 30 min exposure to the redox-cycling drugs listed in Table 1. SoxR activity was assessed by monitoring the expression levels of two of its target genes, *SCO2478* and *SCO4266*, by quantitative real-time PCR (qRT-PCR). In addition to Act, PCA, PB, Pyo, MB, 4NQO and PMS induced SoxR-target gene expression to high levels over background (Fig. 2C). Drug-induced expression of *SCO2478* and *SCO4266* was SoxR-dependent since these mRNAs were not detectable in M511Δ*soxR* cells that were similarly treated (data not shown). Similar to *P. aeruginosa* SoxR, only the viologens PQ and DQ failed to activate SoxR to any appreciable level (Fig. 2C). Thus,

Table 2. Bacterial strains and plasmids used in this study.

Strain/plasmid	Genotype/description	Source/reference
E. coli		
GC4468	K12 *rpsL thi soxR*$^{+}$ *soxS*$^{+}$	Greenberg *et al.* (1990)
DJ901	Δ(*soxRS*) derivative of GC4468	Greenberg *et al.* (1990)
EH46	DJ901 lysogenized with λ(*soxS* promoter-*lacZ*)	Hidalgo and Demple (1997)
EH86	DJ901 lysogenized with λ(16 bp spacer mutant *soxS* promoter-*lacZ*)	Hidalgo and Demple (1997)
P. aeruginosa		
PA14	Clinical isolate UCBPP-PA14	Rahme *et al.* (1995)
PA14Δ*soxR*	PA14 with a deletion in *soxR*	Dietrich *et al.* (2006)
WTp*mexgfp*	PA14 with insert of *mexG* promoter fused to *gfp* reporter gene	This study
Δ*soxR*p*mexgfp*	PA14Δ*soxR* with insert of *mexG* promoter fused to *gfp* reporter gene	This study
S. coelicolor		
M145	SCP1^{-}, SCP2^{-}	Kieser *et al.* (2000)
M145-1A	M145 with a deletion in *soxR*	Dela Cruz *et al.* (2010)
M511	Δ*act*II-ORF4 derivative of M145	Floriano and Bibb (1996)
M511Δ*soxR*	Δ*soxR* derivative of M511	Dela Cruz *et al.* (2010)
Plasmids		
pSE380	*trc* promoter-containing plasmid with *lacI*^{q} gene (Ampicillin^{r})	Invitrogen
pSE380:H-ECO	N-terminally histidine-tagged *E. coli soxR* gene in pSE380	This study
pSE380:H-PA	N-terminally histidine-tagged *P. aeruginosa soxR* gene in pSE380	This study
pSE380:H-SCO	N-terminally histidine-tagged *S. coelicolor soxR* gene in pSE380	This study
pSET152	Apramycin^{r} *lacZα* MCS *rep*^{pUC}	Bierman *et al.* (1992)
pSET152:H-SCO	N-terminally histidine-tagged *S. coelicolor soxR* gene in pSET152	This study
pSET152:H-ΔC	N-terminally histidine-tagged truncated *S. coelicolor soxR* gene in pET152	This study
pUCp18	Carbenicillin^{r}, pUCP18 vector containing a *P. aeruginosa* origin of replication	Schweizer (1991)
pUC:*ECsoxR*	N-terminally histidine-tagged *E. coli soxR* gene in pUCp18	This study
pUC:*PAsoxR*	N-terminally histidine-tagged *P. aeruginosa soxR* gene in pUCp18	This study
pYL122	Ampicillin^{r}, *rhlA-gfp* transcription fusion in mini-CTX-*lacZ*	Lequette and Greenberg (2005)

S. coelicolor and *P. aeruginosa* SoxR sense redox-active molecules in the same range of redox potential.

The inability of *S. coelicolor* SoxR to complement an *E. coli* Δ*soxR* mutant could result from inefficient protein expression or failure to effect *soxS* transcription. We confirmed that this protein is stably expressed in *E. coli* by immunoblot analysis (Fig. S2A). Furthermore, *S. coelicolor* SoxR binds efficiently to the *E. coli soxS* promoter *in vitro* (Fig. S2C) and *in vivo* (Fig. S2B), and stimulates transcription of the *soxS* gene *in vitro* (Fig. S2D). Interestingly, the C-terminus of *S. coelicolor* SoxR has an additional 22-residues not present in homologues from members of the Enterobacteriaceae or pseudomonads (Fig. 3A). In fact, an extended C-terminal region is found in SoxR proteins from several other *Streptomyces* species (data not shown) and is peculiar to this genus. Given that this is the most obvious structural difference between *S. coelicolor* SoxR and its *E. coli* and *P. aeruginosa* counterparts, we asked if this region could be involved in the regulation of *S. coelicolor* SoxR. To test this, we constructed a mutant that lacks the extreme C-terminal 22-residues by engineering a stop codon at position 154 (see Fig. 3A). We confirmed that the mutant protein is expressed in *E. coli* and interacts with the *soxS* promoter (Fig. S2B). The transcriptional activity of this protein in response to PMS was measured in the *E. coli* Δ*soxRS* mutant lysogenized with λ[*soxS* promoter-*lacZ* reporter] (Table 2). Figure 4A shows that PMS induced similar (low) β-galactosidase levels in cells expressing wild-type or truncated *S. coelicolor* SoxR proteins. Deletion of the C-terminus also did not affect the activity of this protein when expressed in a *S. coelicolor* Δ*soxR* strain. We introduced the truncated gene into the Δ*soxR* strain via the pSET152 vector and monitored the transcription of two SoxR target genes, *SCO4266* and *SCO1178*, by qRT-PCR. The results indicated in Fig. 4B show that, in response to the endogenous activator Act, both wild-type SoxR and the truncated mutant protein activated *SCO4266* and *SCO1178* expression to levels similar to those observed in wild-type cells (WT/pSET152). Thus, the extreme C-terminal region is dispensible for *S. coelicolor* SoxR function, and at this point we have no ready explanation for why *S. coelicolor* SoxR failed to complement the *E. coli* Δ*soxR* mutant.

Mutations in specific residues of P. aeruginosa *SoxR alters its specificity for redox-active molecules*

The transcriptional assays described in Fig. 2 demonstrated that *P. aeruginosa* and *S. coelicolor* SoxR were more selective than *E. coli* SoxR, with reduced sensitivity to compounds with low redox potentials (viologens). The activation profiles for *P. aeruginosa* and *S. coelicolor* SoxR (responsive to PMS but not PQ) were reminiscent of *E. coli*

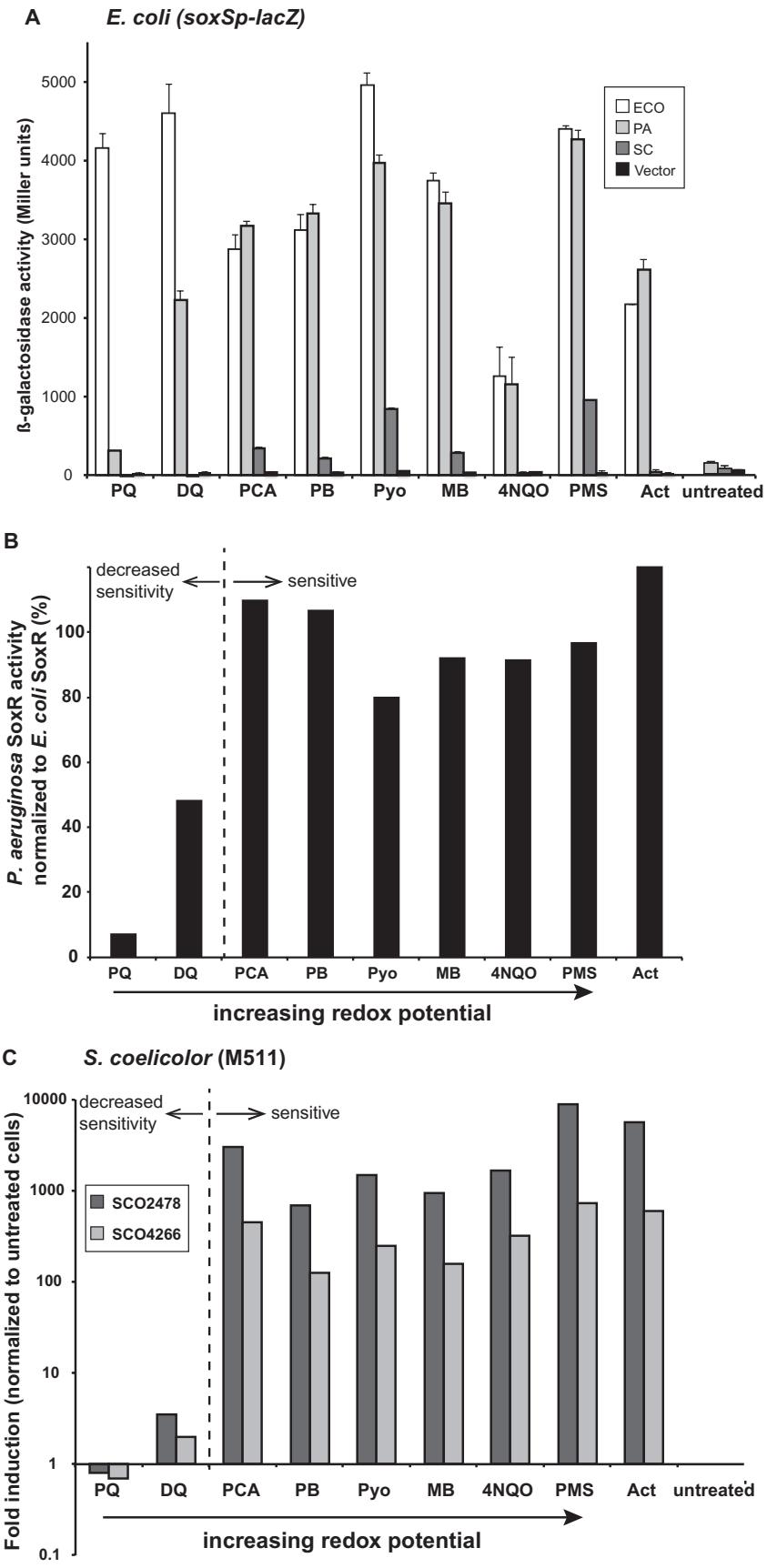


Fig. 2. *P. aeruginosa* and *S. coelicolor* SoxRs sense a narrower spectrum of redox-active compounds than *E. coli* SoxR.

A. *E. coli* strain EH46 (Δ*soxRS* lysogenized with λ[*soxS* promoter-*lacZ* reporter) expressing histidine-tagged *E. coli* SoxR (white columns), *P. aeruginosa* SoxR (light grey columns), *S. coelicolor* SoxR (dark grey columns) or empty vector (black columns) were untreated or treated with 200 μM PQ, 200 μM DQ, 500 μM PCA, 25 μM PB, 20 μM Pyo, 25 μM MB, 50 μM 4NQO, 20 μM PMS or 25 μM Act for 1 h before the assay for β-galactosidase activity. The results shown represent the means and standard errors (bars; some not visible at this scale) of three independent experiments. Dose–response curves for most of these drugs are shown in Fig. S3.

B. *P. aeruginosa* SoxR activity normalized to that of *E. coli* SoxR indicates that the former displays reduced sensitivity to drugs with redox potentials below –300 mV. The data in this figure are the same as the *P. aeruginosa* SoxR data in Fig. 2A.

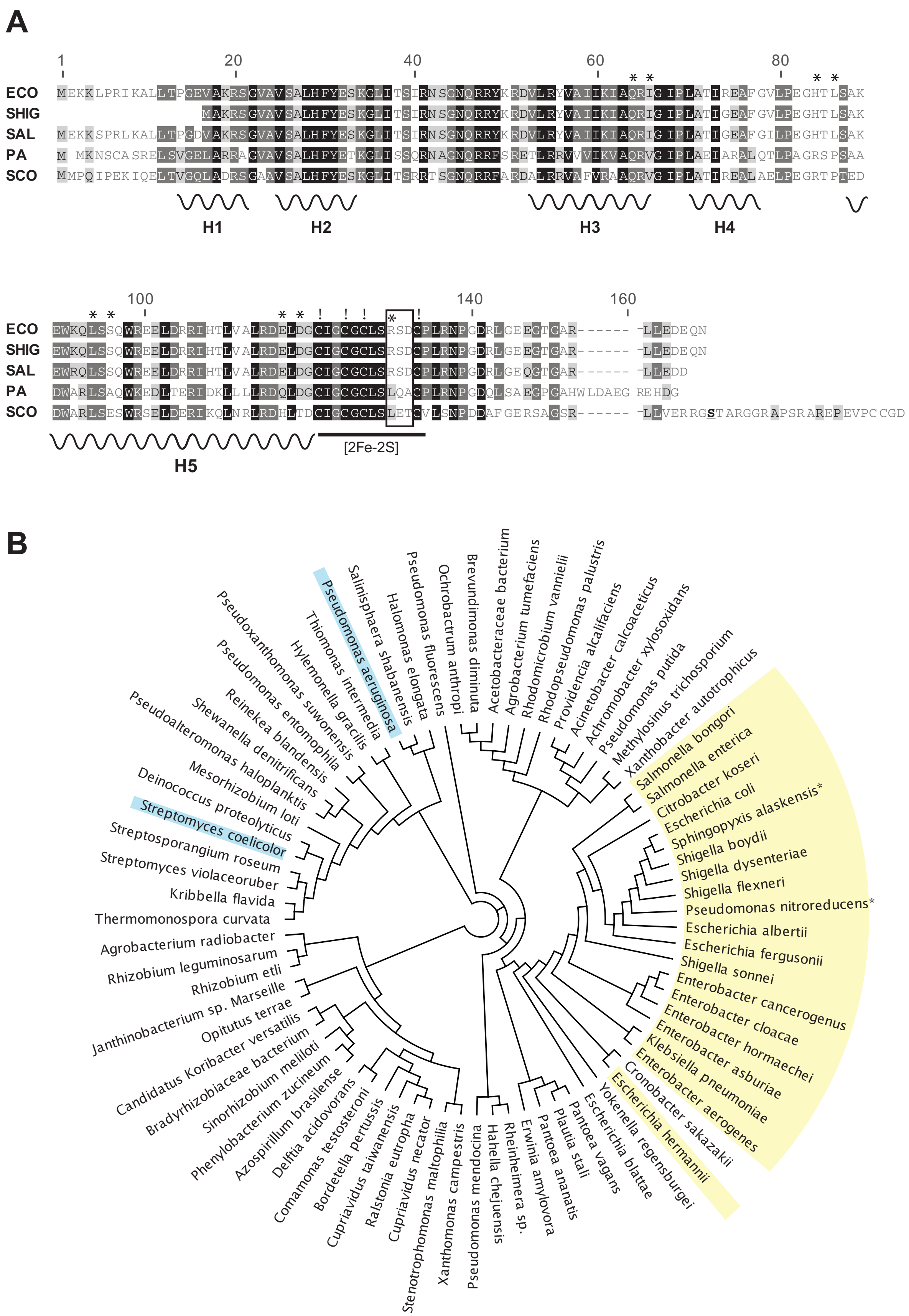
C. The Act-deficient *S. coelicolor* strain M511 was grown for 20 h in R5⁻ medium, then exposed for 30 min to 1 mM PQ, 1 mM DQ, 500 μM PCA, 100 μM PB, 10 μM Pyo, 25 μM MB, 1 mM 4NQO, 10 μM PMS or 10 μM Act, or left untreated. qRT-PCR was performed on RNA extracted from these cells to detect induction of SoxR-target genes *SCO2478* (dark grey columns) and *SCO4266* (light grey columns). Signals were standardized to the level of the housekeeping sigma factor, *hrdB*, and fold-induction was normalized to untreated M511 cells. Note that the *y*-axis is shown as a logarithmic scale.

Fig. 3. Sequence comparison of SoxR homologues.
A. A BLAST analysis was performed for *E. coli* SoxR against all available bacterial genomes. The 250 closest homologues were aligned using ClustalW, and shown is an alignment of five of these SoxR proteins from the enterics *E. coli* (ECO), *Shigella flexneri* (SHIG), *Salmonella enterica* (SAL), and the non-enterics *P. aeruginosa* (PA), and *S. coelicolor* (SCO). Black, dark grey or light grey boxes surrounding residues indicate 100%, 80–100% or 60–80% similarity between all 250 SoxRs (Blosum62 score matrix with threshold of 1). Secondary structural motifs are based on the crystal structure of *E. coli* SoxR (Watanabe *et al*., 2008): H1 and H2 indicate the helix–turn–helix motif that makes specific contacts with the *soxS* promoter; H3 and H4 form a second helix–turn–helix motif within the DNA binding domain; H5 indicates the dimerization helix. The four conserved cysteine residues that anchor the [2Fe-2S] cluster are indicated by exclamation marks (!). A three-residue, hypervariable motif in the [2Fe-2S] region is indicated by a box. In most species of the Enterobacteriaceae this motif is RSD. Other residues that influence the redox-sensing properties of *E. coli* SoxR are marked by asterisks (*) (Chander and Demple, 2004). The serine residue that was changed to a stop codon to construct the C-terminal truncated *S. coelicolor* SoxR mutant is underlined.
B. Based on the alignment of 250 SoxR proteins a tree was generated with Geneious Pro 5.6 using the neighbour-joining method (Saitou and Nei, 1987; Drummond *et al*., 2011). For clarity only one member of each represented species is shown. Species that contain the RSD motif in SoxR are highlighted in yellow; these all belong to the Enterobacteriaceae excepting *Pseudomonas nitroreducens* and *Sphingopyxis alaskensis*. *P. aeruginosa* and *S. coelicolor* SoxR are highlighted in blue.

SoxR mutant proteins that were reported several years ago (Chander *et al*., 2003; Chander and Demple, 2004). In those studies, nine residues were identified that, when individually changed, rendered *E. coli* SoxR insensitive to PQ, but fully responsive to PMS (Fig. 5A). It was suggested that changes in these residues alter the redox-reactivity of SoxR, rendering the protein hyposensitive to certain redox signals. Thus, while these mutant proteins are still activated by the strongly oxidizing drug PMS (redox potential of +80 mV), they are unresponsive to the less oxidizing drug PQ (redox potential of −440 mV). Of these nine residues, only two are conserved in *S. coelicolor* SoxR (Q63, L93) and three are conserved in *P. aeruginosa* SoxR (Q62, L92, D115) (Fig. 3A). We hypothesized that changing the non-conserved residues in *P. aeruginosa* or *S. coelicolor* SoxR to those found in *E. coli* SoxR might decrease their drug-selectivity, i.e. that the mutant proteins would respond to PQ. To exclude any SoxR-independent differences between the species, such as drug uptake, we expressed the mutant proteins in *E. coli*. Because *S. coelicolor* SoxR is only weakly active in *E. coli*, we focused our comparison on *P. aeruginosa* and *E. coli* SoxR.

We individually mutated six of the aforementioned residues in *P. aeruginosa* SoxR (V64I, R82H, P84L, A94S, Q113E, L125R) and analysed the resulting variants using the β-galactosidase assay described previously. As shown before, while *E. coli* SoxR was activated with similar efficiency by both PQ and PMS, wild-type *P. aeruginosa* SoxR was strongly activated by PMS but only weakly by PQ (Fig. 5B). Mutant proteins R82H, A94S and Q113E resembled wild-type *P. aeruginosa* SoxR (Fig. 5B). Two amino acid substitutions, V64I and P84L, conferred PQ-sensitivity to *P. aeruginosa* SoxR, essentially converting this protein into its *E. coli* counterpart (Fig. 5B). The L125R substitution, which alters a residue in the [2Fe-2S] cluster region, rendered *P. aeruginosa* SoxR constitutively active; in untreated cells, this variant displayed ~ 80% of the activity obtained in the presence of PQ or PMS (Fig. 5B). To determine if the constitutive activity of the L125R variant depends on the [2Fe-2S] clusters, we introduced a C118→A mutation into this background (C118AL125R). The equivalent cysteine→alanine mutation in *E. coli* and *S. coelicolor* SoxRs results in cluster-deficient proteins (Bradley *et al*., 1997; Dela Cruz *et al*., 2010). The C118AL125R variant showed a ~ threefold reduction in constitutive activity compared with the L125R single variant, indicating the importance of the [2Fe-2S] clusters (Fig. 5C). The relatively high basal level activity of the C118AL125R protein was also produced by the C118A single mutant protein (Fig. 5C) and had been previously observed with cluster-deficient *E. coli* SoxR (Bradley *et al*., 1997).

The constitutive activity displayed by the L125R *P. aeruginosa* SoxR variant was unexpected, given that *E. coli* SoxR (which has an arginine in this position) is not constitutive. It is interesting that SoxR homologues from enteric species contain an arginine in this position, which is replaced by a hydrophobic residue (most often leucine) in SoxR from almost all non-enteric species analysed (Figs 3A and S4A). Our finding that the presence of arginine within the [2Fe-2S] domain makes SoxR constitutively active, as was observed with the L125R *P. aeruginosa* SoxR variant, conflicted with the fact that *E. coli* SoxR is not constitutive. We hypothesized that this difference might be attributed to other amino acids in the vicinity of the [2Fe-2S] cluster that modulate *E. coli* SoxR activity, tuning it so that it is only active in the presence of redox-active drugs. A closer examination of the SoxR sequences from enteric and non-enteric bacteria revealed that while the [2Fe-2S] cluster domain is highly conserved, it contains a three-residue hypervariable sequence (Fig. S4A). In most species of the Enterobacteriaceae it is comprised of a hydrophilic 'RSD' motif. However, it is not conserved in SoxRs from other bacteria, including *P. aeruginosa*, which instead contains the sequence LQA (Figs 3A and S4A). In fact, we found only two species not belonging to the Enterobacteriaceae, *Pseudomonas nitroreducens* and *Sphingopyxis alaskensis*, that contain the RSD motif (Fig. 3B). We speculated that the RSD motif is crucial for the *E. coli*-specific activation of SoxR. First, we asked if it is the presence of the arginine residue that renders the L125R *P. aeruginosa*


A
1 20 40 60 80
ECO MEKKLPRIKALLTPGEVAKRSGVAVSALHFYESKGLITSIRNSGNQRRYKRDVLRYVAIIKIAQRIGIPLATIREAFGVLPEGHTLSAK
SHIG MAKRSGVAVSALHFYESKGLITSIRNSGNQRRYKRDVLRYVAIIKIAQRIGIPLATIGEAFGVLPEGHTLSAK
SAL MEKKSPRLKALLTPGDVAKRSGVAVSALHFYESKGLITSIRNSGNQRRYKRDVLRYVAIIKIAQRIGIPLATIGEAFGILPEGHTLSAK
PA M MKNSCASRELSVGELARRAGVAVSALHFYETKGLISSQRNAGNQRRFSRETLRRVVIKVAQRVGIPLAEIARALQTLPAGRSPSAA
SCO MMPQIPEKIQELTVGQLADRSGAAVSALHFYESKGLITSRRTSGNQRRFARDALRRVAFVRAAQRVGIPLATIREALAELPEGRTPTED
H1 H2 H3 H4
100 140 160
ECO EWKQLSSQWREELDRRIHTLVALRDELDGCIGCGCLSRSDCPLRNPGDRLGEEGTGAR------ -LLEDEQN
SHIG EWKQLSSQWREELDRRIHTLVALRDELDGCIGCGCLSRSDCPLRNPGDRLGEEGTGAR------ -LLEDEQN
SAL EWRQLSSQWREELDRRIHTLVALRDELDGCIGCGCLSRSDCPLRNPGDRLGEQGTGAR------ -LLEDD
PA DWARLSAQWKEDLTERIDKLLLRRDQLDGCIGCGCLSLQACPLRNPGDQLSAEGPGAHWLDAEG REHDG
SCO DWARLSESWRSELDERIKQLNRLRDHLTDCIGCGCLSLETCVLSNPDDAFGERSAGSR------ -LLVERRGSTARGGRAPSRAREPEVPCCGD
H5
[2Fe-2S]
B
Pseudomonas aeruginosa
Salinisphaera shabanensis
Halomonas elongata
Pseudomonas fluorescens
Ochrobactrum anthropi
Brevundimonas diminuta
Acetobacteraceae bacterium
Agrobacterium tumefaciens
Rhodomicrobium vannielii
Rhodopseudomonas palustris
Providencia alcalifaciens
Acinetobacter calcoaceticus
Achromobacter xylosoxidans
Pseudomonas putida
Methylosinus trichosporium
Xanthobacter autotrophicus
Salmonella bongori
Salmonella enterica
Citrobacter koseri
Escherichia coli
Sphingopyxis alaskensis*
Shigella boydii
Shigella dysenteriae
Shigella flexneri
Pseudomonas nitroreducens*
Escherichia albertii
Escherichia fergusonii
Shigella sonnei
Enterobacter cancerogenus
Enterobacter cloacae
Enterobacter hormaechei
Enterobacter asburiae
Klebsiella pneumoniae
Enterobacter aerogenes
Cronobacter sakazakii
Escherichia hermannii
Yokenella regensburgei
Escherichia blattae
Pantoea vagans
Plautia stali
Pantoea ananatis
Erwinia amylovora
Rheinheimera sp.
Hahella chejuensis
Pseudomonas mendocina
Xanthomonas campestris
Stenotrophomonas maltophilia
Cupriavidus necator
Ralstonia eutropha
Cupriavidus taiwanensis
Bordetella pertussis
Comamonas testosteroni
Delftia acidovorans
Azospirillum brasilense
Phenylobacterium zucineum
Sinorhizobium meliloti
Bradyrhizobiaceae bacterium
Candidatus Koribacter versatilis
Opitutus terrae
Janthinobacterium sp. Marseille
Rhizobium etli
Rhizobium leguminosarum
Agrobacterium radiobacter
Thermomonospora curvata
Kribbella flavida
Streptomyces violaceoruber
Streptosporangium roseum
Streptomyces coelicolor
Deinococcus proteolyticus
Mesorhizobium loti
Pseudoalteromonas haloplanktis
Shewanella denitrificans
Reinekea blandensis
Pseudomonas entomophila
Pseudoxanthomonas suwonensis
Hylemonella gracilis
Thiomonas intermedia

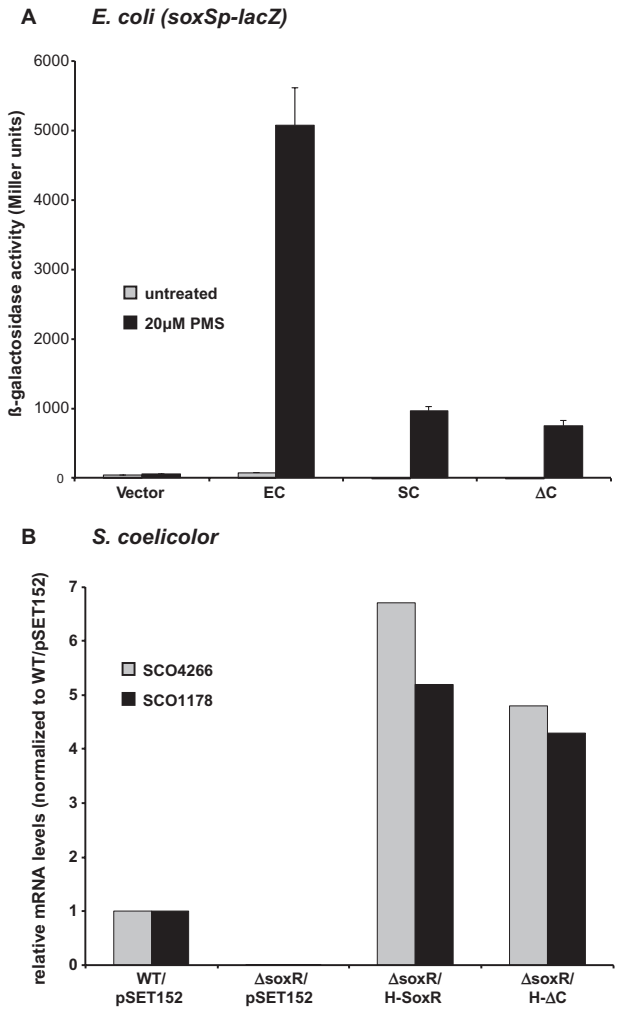


Fig. 4. The extended C-terminal region of *S. coelicolor* SoxR is not important for function.
A. *E. coli* cells (strain EH46) expressing histidine-tagged wild-type *E. coli* or *S. coelicolor soxR* alleles, or the *S. coelicolor* C-terminal truncated mutant from pSE380-based plasmids were either untreated (grey columns) or treated with 20 μM PMS (black columns) for 1 h before the assay for β-galactosidase activity. The results represent the means and standard errors (bars; some not visible on this scale) of three independent experiments.
B. qRT-PCR was performed on RNA isolated from the following *S. coelicolor* strains: WT/pSET152, Δ*soxR*/pSET152, and a Δ*soxR* strain complemented with wild-type *soxR* (H-SoxR) or the C-terminal truncated mutant (H-ΔC), grown in R5$^-$ liquid medium for 3 days at which point Act was robustly produced. The expression levels of SoxR target genes, *SCO4266* (grey columns) and *SCO1178* (black columns) were standardized to the level of *hrdB* and normalized to expression in WT/pSET152.

SoxR mutant constitutively active, or the lack of its native leucine. For this we generated an L125A mutant, which proved to be constitutively active (Fig. 5C). This suggested that the absence of L125 was sufficient to make *P. aeruginosa* SoxR constitutively active. Next we tested if the serine and aspartate residues within this motif are responsible for preventing constitutive activity. Introduction of mutations Q126S or A127D alone had no effect on *P. aeruginosa* SoxR activity (Fig. 5C). In contrast, introduction of both mutations into the L125R background (LQA→RSD), such that the [2Fe-2S] cluster domain was now an exact replica of *E. coli* SoxR, dampened the constitutive phenotype significantly, while still allowing sensitivity to PQ (Fig. 5C). Interestingly, changing QA→SD in the L125A background (LQA→ASD) had no attenuating effect on the constitutive activity of the single mutant protein (Fig. 5C). Similarly, the LQA→AAA triple mutant protein also demonstrated high constitutive activity (Fig. 5C). This showed that, while rendering *P. aeruginosa* SoxR constitutively active could be achieved by a variety of substitutions of L125, the 'dampening effect' required a specific combination of residues in the hypervariable region (Fig. 5C).

Having demonstrated that a three-residue substitution (LQA→RSD) in the [2Fe-2S] region is sufficient to transform the activity of *P. aeruginosa* SoxR to mimic that of *E. coli* SoxR, we tested if the same would hold true in reverse. We changed RSD→LQA in *E. coli* SoxR, such that its [2Fe-2S] region replicated that of *P. aeruginosa* SoxR, and found that this change did not affect its activity; this mutant protein retained sensitivity to PQ (Fig. S4B). Interestingly, a mere substitution of R127→L rendered *E. coli* SoxR insensitive to PQ, highlighting the importance of this position in the regulation of SoxR activity (Fig. S4B, Chander and Demple, 2004).

Discussion

SoxR regulates a global stress response against redox-cycling drugs in *E. coli*. As such this protein is engineered to sense and respond to a wide spectrum of redox-active molecules that vary in structure and redox potentials. This is not the case for *P. aeruginosa* and *S. coelicolor* in which SoxR performs a more specific role as suggested by the small number of genes it regulates in response to endogenous redox-active signals. Given the functional differences of SoxR homologues across species, we asked if the sensitivities of *P. aeruginosa* and *S. coelicolor* SoxR were tuned towards compounds that resemble phenazines and Act respectively. We found this to be partially true. While *E. coli* SoxR was activated by structurally distinct drugs that span a range in redox potentials from ~ –450 to ~ +80 mV, *P. aeruginosa* and *S. coelicolor* SoxR appeared to be less sensitive to the viologens that have redox potentials below ~ –300 mV. Although SoxR appears to be tuned to sense drugs based on their redox potentials, we cannot rule out other contributing factors, such as structural features, charge and hydrophobicity.

What is the mechanism underlying SoxR's differential selectivity for drugs? An earlier study on *E. coli* SoxR had identified residues that, when mutated, reduce the pro-

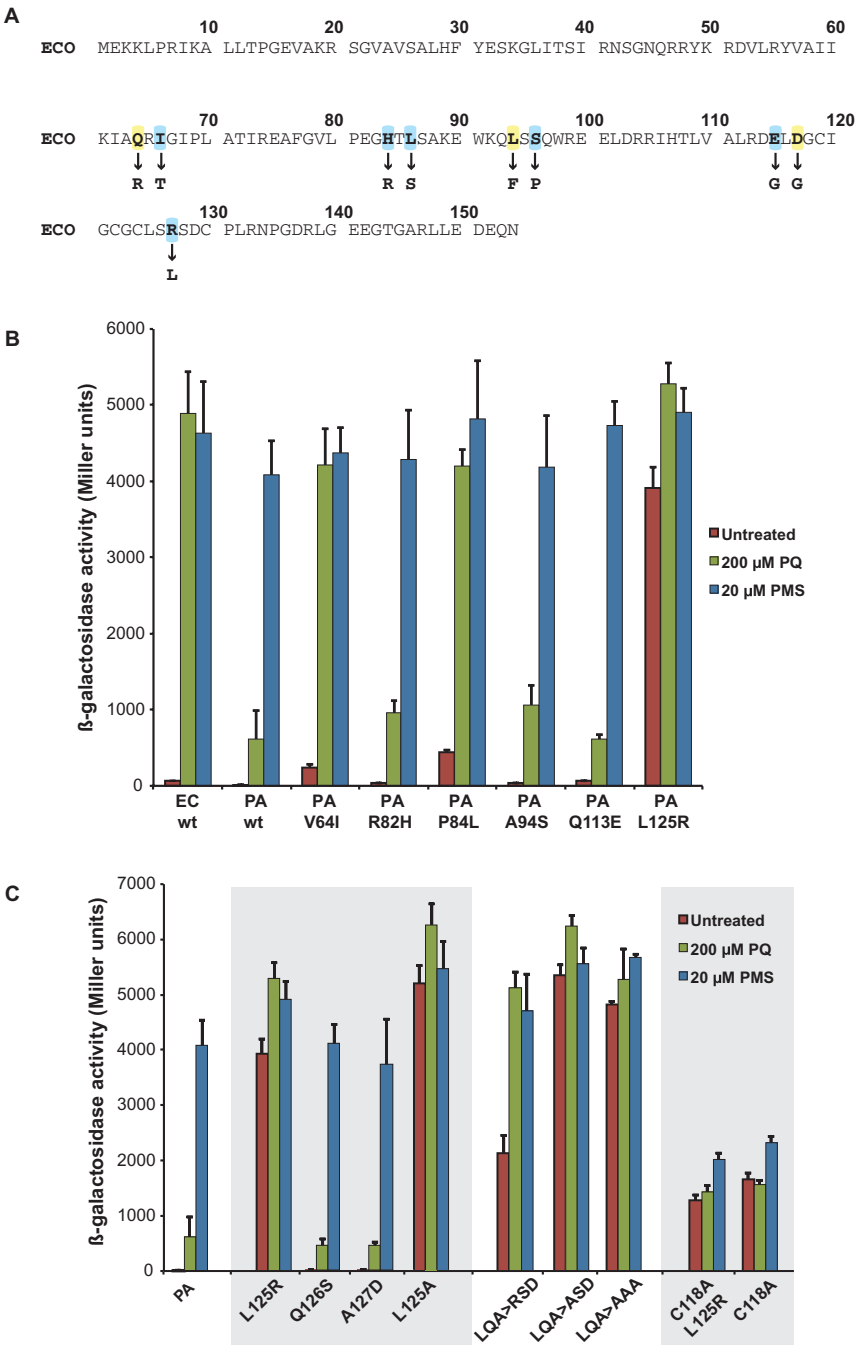


Fig. 5. Mutations that alter drug selectivity of *P. aeruginosa* and *E. coli* SoxR.
A. The amino acid sequence of *E. coli* SoxR is shown. The nine residues that had previously been shown to influence the redox-sensing properties of SoxR are in bold type; the substitutions that caused reduced sensitivity to PQ are indicated below the wild-type sequence (Chander and Demple, 2004). Three of these residues (Q64, L94 and D117) are conserved in *P. aeruginosa* SoxR; the six residues highlighted in blue are not conserved between *P. aeruginosa* and *E. coli* SoxR, and were targeted for mutagenesis in the former.
B. EH46 cells expressing wild-type *E. coli* or *P. aeruginosa soxR* alleles, or *P. aeruginosa soxR* mutant alleles from pSE380-based plasmids were untreated (red columns), or treated with 200 µM PQ (green columns) or 20 µM PMS (blue columns) for 1 h before the assay for β-galactosidase activity. The results represent the means and standard errors of three independent experiments.
C. EH46 cells expressing wild-type or mutant *P. aeruginosa soxR* alleles from pSE380 plasmids were treated as in (B). The mutations in these alleles are all within the [2Fe-2S] cluster domain. The SoxR variants L125R, C118A, and C118AL125R are expressed at similar levels in EH46 cells as assessed by immunoblot analysis (data not shown).

tein's reactivity to drugs with low redox potentials (such as PQ). Six of these residues (Ile66, His84, Leu86, Ser96, Glu115, Arg127 in *E. coli*) are conserved in SoxR homologues from enterics (which display broad drug selectivity), but not in those from non-enterics (which show narrower drug selectivity). Changing the corresponding residues in *P. aeruginosa* SoxR to those found in *E. coli* SoxR revealed that three of the six residues individually affected drug sensitivity. Substitutions V64→Ile and Pro84→Leu both increased the sensitivity of *P. aeruginosa* SoxR to PQ, and the variants were indistinguishable from *E. coli* SoxR (Fig. 5B). The Leu125→Arg change resulted in constitutive activity (Fig. 5B). These amino acids are conserved in *S. coelicolor* SoxR (V65, P85, L126; Fig. 3A), which like *P. aeruginosa* SoxR showed reduced sensitivity to viologens (Fig. 2C). These findings emphasize the importance of these amino acids in SoxR sensitivity to redox-active small molecules, and suggest structural changes that *E. coli* SoxR may have evolved if its *soxR* gene was acquired by horizontal gene transfer.

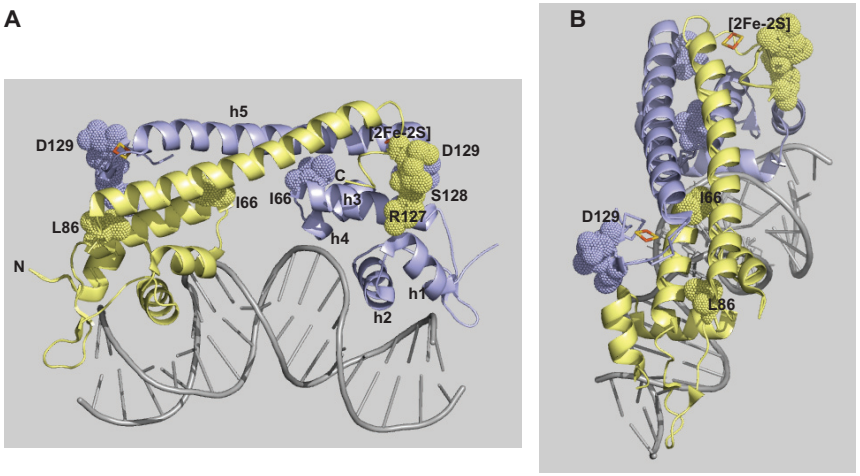


Fig. 6. Location of key residues in *E. coli* SoxR. The structure of *E. coli* SoxR protein complexed with the *soxS* promoter is depicted side-on (A) or from the top (B) (Watanabe *et al*., 2008; pdb 2ZHG). The amino- and carboxy-termini are indicated by N and C respectively, on one of the monomers. Helices labelled h1–h4 comprise the DNA binding domain; h5 is the dimerization domain; the [2Fe-2S] cluster in one monomer is labelled. Residues (I66, L86, R127, S128, D129) identified as playing an important role in tuning the redox-reactivity of SoxR are shown. The images were created using MacPyMOL Molecular Graphics System, Version 1.5, Schrodinger, LLC.

SoxR forms a homodimer. Each subunit contains three distinct domains: a DNA binding domain composed of four helices (h1–h4), a coiled-coil dimerization helix (h5) and the C-terminal sensor domain that contains the [2Fe-2S] clusters (Fig. 6). The crystal structure for the oxidized SoxR dimer bound to DNA shows that helices 3 and 4 within the DNA binding domain make hydrophobic contacts with helix 5 within the same subunit (Watanabe *et al*., 2008). Furthermore, the metal binding domain of one subunit is stabilized by interactions with helices 3, 4 and 5 of the other monomer. The structure of reduced SoxR is unknown, but using Raman spectroscopy, Kobayashi *et al*. (2011) showed that the relative orientations of helices 3 and 4 (in the DNA binding domain) and helix 5 (dimerization domain) depend on the redox state of SoxR. It is tempting to speculate that transmission of oxidative signals from the [2Fe-2S] clusters to the DNA involves an orchestrated rearrangement of the metal binding, dimerization and DNA binding domains, thereby explaining how the redox signal may be propagated from the [2Fe-2S] clusters to the DNA. Conversely, potential structural changes that result from DNA binding of SoxR have dramatic effects on the redox potential of its [2Fe-2S] cluster, highlighting the fine-tuned feedback between the DNA binding and sensory domains (Gorodetsky *et al*., 2008). It would therefore not be surprising that even small changes in the protein structure impact sensing and activation. It is peculiar that previous measurements of SoxR redox potentials gave almost identical results for *E. coli* and *P. aeruginosa* SoxR in solution (–280 mV; Ding *et al*., 1996; Gaudu and Weiss, 1996; Kobayashi and Tagawa, 2004), and in the DNA-bound forms (+200 mV; Gorodetsky *et al*., 2008). A potential caveat of these analyses is that the redox potential of SoxR was not measured *in vivo* (i.e. bound to DNA within a cell). A previous study by Koo *et al*. (2003) suggests that SoxR may be part of a transient protein complex. We speculate that these interactions may differ for SoxRs from different species and could affect their redox potentials differentially *in vivo*. A recent paper (Fujikawa *et al*., 2012) also demonstrated that *E. coli* SoxR was more readily oxidized by superoxide than was *P. aeruginosa* SoxR, hinting at differences in their redox potentials. At present, we can only speculate that the tuning of SoxR's specificity towards redox-active compounds is based on its redox potential. However, other factors (such as differences in structure and charge) may also contribute to the differential recognition of small molecules. Independent of the precise mechanism, we identified key residues that regulate SoxR specificity: Ile66, His84, Leu86, Ser96, Glu115, Arg127 in *E. coli* SoxR. Ile66, His84 and Leu86 are located at the interface between helix 3 in the DNA binding domain and the dimerization helix 5 (Fig. 6), which makes them good candidates for mediating the functional interaction between DNA binding and sensing. Leu86, for example, which is located just upstream of helix 5, forms hydrophobic interactions with Tyr56 and Ile59 in helix 3 (Watanabe *et al*., 2008). Interestingly, helix 3 also interacts with the sensor domain of the second dimer subunit (Fig. 6). Our findings demonstrate that even point mutations can change the sensitivity towards specific compounds.

A particularly intriguing example is the [2Fe-2S] binding site itself. Although it is remarkably conserved among SoxR homologues, it contains a hypervariable stretch of three residues (Figs 3A and S3A). Strikingly, within almost all enterics we found it to be conserved as the charged RSD motif (Figs 3B and S4A). In contrast, in *P. aeruginosa* it is replaced by LQA (Fig. 3A). When *P. aeruginosa* SoxR was mutated to replace the LQA motif with RQA, the resulting variant displayed strong constitutive activity (Fig. 5B). Thus having an arginine residue within the [2Fe-2S] domain makes SoxR constitutively active. However, when the original LQA motif was mutated to RSD (so that the [2Fe-2S] cluster was now identical to

that in *E. coli* SoxR), the level of constitutive activity significantly decreased, but the protein still retained the ability to respond to PQ (Fig. 5C). Thus, the RSD motif in SoxRs from Enterobacteriaceae is essential for fine-tuning the protein's specificity – preventing constitutive activity while retaining low selectivity for drugs.

Considering that the RSD motif is almost exclusively found in the Enterobacteriaceae, it is tempting to speculate that it is an evolutionary adaptation specific to this bacterial family (Fig. S4A). In this context, it is interesting that an LQA→RSD substitution in *P. aeruginosa* SoxR mimicked *E. coli* SoxR but the analogous RSD→LQA *E. coli* mutant remained sensitive to PQ (Fig. S4B). The idea that protein evolution from one functional state to another may not be reversible due to the occurrence of secondary mutations has recently been demonstrated (Bridgham *et al*., 2009). The fact that the LQA motif has different effects on PQ sensitivity in *E. coli* and *P. aeruginosa* SoxR indicates that specific residues outside of the [2Fe-2S] region contribute to the regulatory function of the hypervariable region.

Our findings give insight into the diversity of SoxR proteins with respect to their ability to sense redox-active compounds. They demonstrate how minor changes in the primary sequence can lead to the evolution of SoxR proteins with narrow- or broad-range sensing capacities.

Experimental procedures

Bacterial strains and plasmids

Bacterial strains and plasmids that were utilized or constructed in this study are listed in Table 2.

Redox-cycling drugs

The redox-cycling drugs used in this study are listed in Table 1 along with their chemical structures and midpoint redox potentials. All chemicals were purchased from Sigma, with the exception of PCA which was purchased from Princeton Biomolecular Research, and γ-Act which was extracted from *S. coelicolor* cells as described by Bystrykh *et al.* (1996). PQ, DQ, MB, and PMS were dissolved in water; PB, Pyo, PCA and Act in dimethylsulphoxide; 4NQO in acetone.

Drug susceptibility tests

The effects of various redox active drugs on the growth of wild type and Δ*soxR E. coli*, *P. aeruginosa* and *S. coelicolor* cells were determined using a disk diffusion assay (strains are listed in Table 2). *E. coli* and *P. aeruginosa* cells were grown for 16 h at 37°C in Luria–Bertani (LB) medium, 100 µl added to 4 ml of melted soft nutrient agar (Difco), then plated on nutrient agar plates (Difco). *S. coelicolor* spores (~ 10^8) were similarly plated. Six-millimetre Whatman paper disks impregnated with 15 µl of drug were placed onto the agar. *E. coli* plates were incubated at 37°C for 24 h, and *P. aeruginosa* and *S. coelicolor* plates at 30°C for 48 h, after which the zone of growth inhibition around each disk was recorded.

Cloning of his-tagged soxR genes for complementation analysis in E. coli *and* P. aeruginosa

For complementation analysis in *E. coli*, the *soxR* alleles from *E. coli*, *P. aeruginosa*, and *S. coelicolor* were expressed as N-terminally histidine-tagged proteins from the plasmid pSE380 under the control of the *trc* promoter (Table 2). The coding region of the *soxR* alleles (including the histidine-tag) was PCR-amplified from pET16b-based vectors (Chander and Demple, 2004; Gorodetsky *et al*., 2008; Dela Cruz *et al*., 2010) using primers pET-F and pET-R (Table S1) and Pfu Polymerase (Stratagene). The PCR fragments were digested with BamHI and SalI and ligated into pSE380. The resulting plasmids containing *soxR* alleles with a 10-histidine tag attached to the N-terminus were sequenced on both strands and transformed into *E. coli* strain EH46 or EH86 for β-galactosidase assays (Table 2).

For complementation analysis in *P. aeruginosa*, the histidine-tagged *soxR* proteins were subcloned from pSE380 into the BamHI/SalI site of the vector, pUCp18 (Table 2), and expressed under the control of the *lac* promoter. The resulting clones were sequenced on both strands and transformed into *P. aeruginosa* strain PA14Δ*soxR* (Table 2).

Construction of soxR mutant alleles

Mutations in the *P. aeruginosa* or *E. coli soxR* genes were generated using the GENEART site-directed mutagenesis kit from Invitrogen according to the manufacturer's instructions. Mutations in the *S. coelicolor soxR* gene were generated using the QuikChange site-directed mutagenesis kit from Stratagene following manufacturer's recommendations. Plasmid pSE380, containing the histidine-tagged *soxR* genes from *P. aeruginosa*, *E. coli*, or *S. coelicolor* were used as templates for mutagenesis along with the mutagenic primers listed in Table S1. All mutations were verified by DNA sequence analysis.

For expression of the histidine-tagged C-terminal truncated *soxR* gene in *S. coelicolor*, the coding region (along with the 10-histidine tag) was PCR-amplified from pSE380 using Pfu polymerase and primers 380F-Bam and 380R-Bam (Table S1), and subcloned into the BamHI site of the integrating vector pSET152, to yield H-ΔC. The histidine-tagged WT *S. coelicolor soxR* gene was similarly constructed to yield H-SoxR. The pSET152-based plasmids were introduced into the *S. coelicolor* Δ*soxR* strain M145-1A by intergenic conjugation from *E. coli* ET12567/pUZ8002.

β-Galactosidase assay to measure complementation in an E. coli Δ*soxR mutant*

The ability of the various SoxR homologues (and mutant derivatives) to complement an *E. coli* Δ*soxR* strain was assessed by measuring β-galactosidase activity in EH46 cells (Table 2) expressing the various histidine-tagged SoxR

proteins from pSE380-based plasmids as previously described (Chander *et al.*, 2003). Cells were treated with various redox-active drugs for 1 h with shaking at 220 r.p.m.

β-Galactosidase assays were also used to analyse the stable production and *soxS* promoter binding ability of the various SoxR proteins *in vivo*. Strain EH86 (Table 2) was transformed with the aforementioned plasmids and grown for 2.5 h in the absence of oxidative stress before the lysates were assayed for β-galactosidase activity.

qRT-PCR assay in S. coelicolor

Liquid R5$^-$ medium (Huang *et al.*, 2001) was inoculated with 10^7 *S. coelicolor* spores ml^{-1} and grown at 30°C with shaking at 220 r.p.m. for the indicated times. Cells were harvested by incubating with RNAprotect bacterial reagent (Qiagen) for 5 min at room temperature, centrifuging for 10 min at 5000 *g*, and frozen at –80°C. Total RNA was extracted and qRT-PCR assays conducted as previously described (Dela Cruz *et al.*, 2010). The primers used for qRT-PCR are listed in Table S1.

Construction of P. aeruginosa *mexG-gfp reporter strains and Gfp fluorescence quantification*

The *mexG* promoter region was PCR-amplified from PA14 genomic DNA using primers pmexG-F and pmexG-R (Table S1), and cloned into the HindIII/EcoRI site of the vector pYL122 (Table 2). The p*mexG-gfp* reporter fusion was integrated into the *attB* site of *P. aeruginosa* PA14 or PA14Δ*soxR* using a previously described protocol (Lequette and Greenberg, 2005).

To quantify Gfp fluorescence, the p*mexG-gfp* reporter strains expressing histidine-tagged *E. coli* or *P. aeruginosa soxR* from pUCp18, were grown in LB medium supplemented with carbenicillin (300 μg ml^{-1}) for 16 h at 37°C. Cultures were then diluted 100-fold and grown for an additional 3 h (to logarithmic phase), before finally diluting to an optical density of 0.05 at 500 nm into a 96-well plate (Costar). The optical density and fluorescence was monitored for 19 h using a Synergy four-plate reader (BioTek). The excitation wavelength was 488 nm; emission wavelength was 520 nm. Data were acquired using the Gen5 program.

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Supporting information

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