RNA-Seq Analysis Reveals a Six-Gene SoxR Regulon in Streptomyces coelicolor

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Abstract

The redox-regulated transcription factor SoxR is conserved in diverse bacteria, but emerging studies suggest that this protein plays distinct physiological roles in different bacteria. SoxR regulates a global oxidative stress response (involving >100 genes) against exogenous redox-cycling drugs in Escherichia coli and related enterics. In the antibiotic producers Streptomyces coelicolor and Pseudomonas aeruginosa, however, SoxR regulates a smaller number of genes that encode membrane transporters and proteins with homology to antibiotic-tailoring enzymes. In both S. coelicolor and P. aeruginosa, SoxR-regulated genes are expressed in stationary phase during the production of endogenously-produced redox-active antibiotics. These observations suggest that SoxR evolved to sense endogenous secondary metabolites and activate machinery to process and transport them in antibiotic-producing bacteria. Previous bioinformatics analysis that searched the genome for SoxR-binding sites in putative promoters defined a five-gene SoxR regulon in S. coelicolor including an ABC transporter, two oxidoreductases, a monoxygenase and an epimerase/dehydratase. Since this in silico screen may have missed potential SoxR-targets, we conducted a whole genome transcriptome comparison of wild type S. coelicolor and a soxR-deficient mutant in stationary phase using RNA-Seq. Our analysis revealed a sixth SoxR-regulated gene in S. coelicolor that encodes a putative quinone oxidoreductase. Knowledge of the full complement of genes regulated by SoxR will facilitate studies to elucidate the function of this regulatory molecule in antibiotic producers.

Introduction

The redox-regulated transcription factor SoxR is present in a diverse range of Proteobacteria and Actinobacteria and homologs are highly similar at the amino acid level [1]. SoxR homologs function as homodimers and have a conserved amino-terminal helix-turn-helix DNA binding domain, suggesting that these proteins bind to and regulate transcription from similar operator sequences. This has been confirmed in organisms where SoxR has been biochemically characterized [2–7]. SoxR homologs also share a conserved sequence (CysX2CysX5Cys) in the carboxy-terminus that has been shown to be necessary for coordinating [2Fe-2S] centers in SoxR proteins from Escherichia coli, Pseudomonas aeruginosa, and Streptomyces coelicolor [5,8,9]. These [2Fe-2S] clusters are central to SoxR’s ability to detect changes in the cellular redox environment and regulate gene expression in response. SoxR was originally discovered in the enterobacterium E. coli where it promotes resistance to redox-cycling drugs like paraquat and menadione [10,11]. Subsequent studies revealed that in this microorganism SoxR mediates its effects in a two-step process. Upon sensing redox stress via its [2Fe-2S] clusters, SoxR activates the expression of a second transcription factor, soxS [12,13]. SoxS, an AraC-type regulator then recruits RNA polymerase to the promoters of >100 genes (the SoxRS regulon), whose protein products cumulatively restore redox homeostasis and repair oxidant-induced cellular damage [14].

The E. coli SoxRS regulon is conserved in other enterobacteria where it functions to confer generalized protection against exogenous redox-cycling compounds. Various lines of research conducted in the past decade indicate that this function may be limited to members of the Enterobacteriaceae, and that the SoxR regulatory network is different in other bacteria. A comprehensive bioinformatic survey of sequenced bacterial genomes revealed that while a soxR homolog is detected in 176 genomes, a soxS homolog is present only in enteric bacteria where it appears to be the solitary gene directly regulated by SoxR [1]. The same study showed that in non-enterics (all of which lack soxS), SoxR is predicted to directly regulate a small number of genes. In further contrast to the apparent function of SoxR in enteric bacteria, none of the putative SoxR targets in non-enterics encode proteins that are typically involved in oxidative stress detoxification and repair. Instead these genes encode membrane transporters and enzymes with homology to proteins that modify small molecules, including antibiotics. The absence of a soxS homolog and the predicted SoxR regulons in non-enterics has given rise to the notion that
SoxR does not regulate a generalized oxidative stress response in the majority of bacteria. This has been corroborated for *Pseudomonas putida*, *P. aeruginosa*, and *S. coelicolor*, where deletion of *soxR* does not result in increased sensitivity to redox-cycling drugs when compared to the parental strain [5,9,15,16]. While the function of SoxR in *P. putida* remains unknown, SoxR regulates gene expression in response to redox-active endogenously produced antibiotics in both *P. aeruginosa* and *S. coelicolor*, indicating that SoxR plays a role that is intimately tied in with the physiology of these organisms [5,6,9,17,18].

The SoxR regulon in *P. aeruginosa* is induced during the production of phenazine antibiotics in stationary phase, and consists of two membrane transporters (encoded by *PA4205-PA4208* and *PA3718*) and a monoxygenase enzyme (*PA2274*) [15,17]. The *S. coelicolor* SoxR regulon is also induced in stationary phase during the production of the benzochromane-quinone blue-pigmented antibiotic actinorhodin (Act), and is similar to the *P. aeruginosa* regulon in encoding an ABC-type membrane transporter (*SCO7008*) and four redox enzymes (*SCO1178*, *SCO1909*, *SCO2478*, *SCO4266*) [5,6]. It is noteworthy that two of these genes encode products that are similar to enzymes that catalyze tailoring steps in the Act biosynthetic pathway. Specifically, *SCO4266* is similar to the oxidoreductases ActVI-ORF2 (37% identity; 49% similarity) and ActVI-ORF4 (34% identity; 44% similarity), and ActVI-ORF5 resembles the monoxygenase ActVA-ORF6 (38% identity; 55% similarity) [19]. *SCO1909* is also similar to *PA2274* (37% identity; 45% similarity), the SoxR-regulated monoxygenase in *P. aeruginosa* [19]. An independent study had also described four of the SoxR targets in *S. coelicolor* as genes whose expression is temporally coordinated with Act (*eca*), and whose levels are reduced in an Acton producing strain: *SCO7008* (ecaA), *SCO1909* (ecaB), *SCO1178* (ecaC), *SCO4266* (ecaD) [20]. The composition of the *P. aeruginosa* and *S. coelicolor* SoxR regulons, and the fact that they are induced by endogenously produced redox-active antibiotics, suggests that SoxR evolved to sense endogenous metabolites and activate machinery to process and transport them in these two phylogenetically divergent bacteria. This notion is further supported by the observation that both *P. aeruginosa* and *S. coelicolor* soxR mutants display de-regulated antibiotic production/secretion [1,6].

Both the SoxR regulon in *P. aeruginosa*, and the SoxRS regulon in *E. coli* were characterized using microarray-based expression profiling [14,15]. The five-gene SoxR regulon in *S. coelicolor*, on the other hand, was identified by conducting an *in silico* search of the genome for SoxR-binding sites located upstream of predicted open reading frames (ORFs) [1,5,6]. Given the morphological complexity and large genome size of *S. coelicolor* (3.7 megabases), it is possible that the bioinformatic screen for SoxR-regulated genes may have missed potential targets. This analysis would also have failed to identify genes that are indirectly regulated by SoxR (i.e. via an intermediate transcription factor as in *E. coli*). In this study we conducted a whole genome transcriptome comparison of the wild type *S. coelicolor* strain M145 and a soxR null mutant using RNA-Seq with the goal of gaining a comprehensive picture of the SoxR regulatory network in this medically important antibiotic-producer. This analysis revealed a sixth SoxR-regulated gene in *S. coelicolor*, in addition to the five confirmed targets.

### Materials and Methods

**Bacterial strains, culture conditions and RNA isolation**

The *S. coelicolor* strains used in this study are listed in [Table 1](#). For RNA-Seq and qRT-PCR experiments, about 10⁸ spores were cultured on R2YE plates [21] that were overlaid with cellophane and grown at 30°C for either 24 h (prior to the production of Act) or 72 h (when Act-proficient cells produce Act), with biological replicates obtained for the 72 h cultures. To harvest, cells were incubated with RNAProtect bacterial reagent (Qiagen) for 5 min at room temperature, scraped off the cellophane, pelleted by centrifugation for 10 min at 5,000×g, and frozen at −80°C. Cells were lysed by incubation for 15 min at 30°C in TE buffer containing 15 mg/mL lysozyme, followed by 30 s of sonication on ice. Total RNA was extracted with an RNeasy plant mini kit (Qiagen) according to manufacturer’s instructions. Contaminating DNA was removed by treating for 1 h at 37°C with 5 units of RNase-free DNase I (Qiagen). The RNA preparation was subject to one extraction with acidified phenol-chloroform, followed by an extraction with chloroform-isoamyl alcohol. RNA was ethanol precipitated overnight at −80°C, washed with 80% ethanol and resuspended in nuclease-free water. RNA purity and concentration were determined using a Nanodrop or Qubit spectrophotometer. RNA quality was assessed by agarose gel electrophoresis or a Nano Bioanalyzer. The absence of contaminating DNA was confirmed by the absence of product following a 30-cycle PCR reaction using RNA as template and hnhB primers [Table S1].

**Library construction and RNA sequencing**

Library construction and sequencing of RNA transcripts was performed by Fasteris SA (Switzerland). Briefly, RNA samples were treated to reduce ribosomal RNA levels using the Ambion MICROBExpress kit. RNA transcripts were fragmented using a buffered zinc solution. The first cDNA strand was synthesized by reverse transcription using random primers in the presence of dUTPs. After second strand synthesis and adapter ligation, the first cDNA strand was digested with uracil-DNA glycosylase. The remaining fragments were PCR amplified and 150–200 bp amplicons were selected by polyacrylamide gel electrophoresis. The resulting cDNAs underwent high-throughput sequencing in

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**Table 1. Streptomyces coelicolor strains used in this study.**

| Strain | Genotype or description | Reference |
|--------|-------------------------|-----------|
| M145   | SCP1SCP2 derivative of A3(D) | [21]       |
| M511   | ∆actIV-ORF4 derivative of M145 | [32]       |
| M145-1A | Markerless ∆soxR derivative of M145 | [5]         |
| 145/pSET152 | M145 transformed with pSET152 | [5]         |
| ∆soxR/pSET152 | M145-1A transformed with pSET152 | [5]         |
| ∆soxR/psoxR | M145-1A transformed with pSET152:soxR | [5]         |

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Table 2. Genes identified by RNA-Seq as SoxR-dependent in stationary phase.a.

| SCO number | Decrease in ΔsoxR versus WT on Day 3b | Increase in WT on Day 3 vs. Day 1c | Predicted functiond |
|------------|--------------------------------------|-----------------------------------|---------------------|
|            | Fold change  | q-valued | Fold Change | q-valued | |
| 0319       | 84          | 2e-28    | >100        | 9e-12    | Hypothetical protein |
| 0320       | 79          | 3e-21    | 52          | 5e-10    | Quinone oxidoreductase |
| 1178 (ecaC)| 52          | 2e-21    | 49          | 1e-9     | NAD-dependent epimerase/dehydratase |
| 4266 (ecaD)| 30          | 2e-28    | 14          | 4e-7     | Oxidoreductase |
| 7688       | 28          | 8e-2     | 1           | 1e0      | Hypothetical protein |
| 1177       | 23          | 7e-18    | 32          | 2e-7     | GntR-family transcriptional regulator |
| 0321       | 18          | 7e-18    | 26          | 9e-8     | Carboxylestrase |
| 1909 (ecaB)| 18          | 2e-22    | 11          | 7e-6     | Monooxygenase |
| 1734       | 15          | 4e-11    | 24          | 2e-5     | Secreted cellulose binding protein |
| 4021       | 11          | 6e-2     | 2           | 8e-1     | Two-component histidine kinase |
| 2478       | 7           | 3e-1     | >100        | 1e3      | Flavoprotein reductase |
| 7682       | 6           | 1e-4     | <1          | 8e-1     | Non-ribosomal peptide synthase |
| 4157       | 6           | 6e-1     | 4           | 4e-1     | Protease |
| 4020       | 6           | 3e-3     | 2           | 8e-1     | Two-component response regulator |
| 6165       | 5           | 3e-1     | >100        | 8e-4     | Hypothetical protein |
| 1659       | 5           | 8e-3     | 1           | 1e0      | SoxR |
| 2878 (ecaD)| 5           | 4e-1     | <1          | 5e-1     | Hypothetical protein |
| 7008 (ecaA)| 5           | 3e-9     | 37          | 2e-9     | ABC transporter |

aGenes are organized in decreasing order of SoxR-dependence as determined by RNA-Seq.
bRNA for this comparison was obtained from 3-day old WT or ΔsoxR cultures, both of which were blue-pigmented.
cRNA for this comparison was obtained from 3-day old WT (blue-pigmented) or 1-day old WT (unpigmented) cultures.
dFalse discovery rate adjusted q-values were calculated according to the Benjamini-Hochberg procedure [27].
eConfirmed SoxR-targets that are directly regulated by SoxR in response to Act production [5,6].
fThese genes are unlikely to be Act-dependent since they were not differentially expressed between 3-day old versus 1-day old WT samples, and were not considered for further analysis.
gThese genes are unlikely to be Act-dependent since they were not differentially expressed between 3-day old versus 1-day old WT samples, and were not considered for further analysis.
hSCO1697 is soxR which is constitutively expressed over the course of development, and is not autoregulated [5].

Read mapping and differential expression

The sequenced reads were mapped to the S. coelicolor genome [22], using BWA v0.5.9 [23] and read counts for each annotated transcript were compiled using BEDtools without regard to strand [24], on the web-based platform Galaxy [25–27]. For both the wild type and ΔsoxR libraries, ~97% of total reads were mapped to the S. coelicolor genome. Differential gene expression was assessed using DESeq with library sizes normalized by the median count ratios across transcripts and false discovery rate adjusted q-values were calculated according to the Benjamini-Hochberg procedure [28]. The data supporting the results of this work are available in NCBI’s Gene Expression Omnibus and accessible through GEO series accession number GSE57268 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE57268).

Re-annotation of SCO0319, SCO0320, SCO0321 region

To identify alternative potential transcripts in the region surrounding SCO0319–SCO0321 the S. coelicolor genome region spanning SCO0318 to SCO0321 (310329–322255) was scanned for open reading frames (ORFs) using Geneious (v.7.1.4, Biomatters). Large ORFs (>500 bp) that spanned the region of SCO0319 to SCO0320 were selected and a BLAST search [29] was performed to identify related sequences and elucidate potential gene function.

Quantitative RT-PCR

The cDNA templates for qRT-PCR were generated from total RNA with iScript (Bio-Rad). The primers used for qRT-PCR (Integrated DNA Technologies) were designed using Primer3 software [30], with a melting temperature of 60°C, length of ~20nt, and amplicon length of ~100 bp (Table S1). Each qRT-PCR reaction (20 μL) contained 25 ng cDNA, 250 nM each of forward and reverse primer, and 10 μL Power Sybr green PCR master mix (Applied Biosystems). qRT-PCR reactions were carried out in a StepOne PCR machine (Applied Biosystems) with the following reaction parameters: 10 min at 95°C; 40 two-step amplification cycles with 15 s denaturation at 95°C and 1 min annealing and extension at 60°C; final dissociation stage for 15 min to generate a melting curve and verify specificity of amplification products. Samples were assayed in duplicate and the target signal standardized to the level of the housekeeping sigma factor hrdB.

Electrophoretic mobility shift assay (EMSA)

A gel mobility shift assay was used to assess the binding of purified SoxR to promoter DNA. The method of Dela Cruz et al. [5] was used to purify histidine-tagged SoxR and generate DIG-labeled DNA probes. Primers used to PCR amplify the promoter DNA fragments are listed in Table S1. For the
Table 3. Validation of RNA-Seq results by quantitative RT-PCR.

| SCO Number | Fold decrease in ΔsoxR versus WTb | Fold decrease in Δact versus WTc | Predicted function          |
|------------|----------------------------------|---------------------------------|----------------------------|
| Potential SoxR-targets\(^d\) |                                  |                                 |                            |
| 0319       | 4                                | 24                              | Hypothetical protein       |
| 0320       | 86                               | 34                              | Quinone oxidoreductase     |
| 0321       | 59                               | 61                              | Carboxylesterase           |
| 1177       | 9                                | 11                              | GntR-family transcriptional regulator |
| 1734       | 8                                | 3                               | Secreted cellulase binding protein |
| 4020\(^e\) | 1                                | 1                               | Two-component response regulator |
| 4021       | 10                               | 2                               | Two-component histidine kinase |
| 4157\(^e\) | 1                                | 2                               | Protease                   |
| 6165\(^e\) | 1                                | <1                              | Hypothetical protein       |

isoR and confirmed SoxR-targets

|                      |     | 60 | 29 | NAD-dependent epimerase/dehydratase |
|----------------------|-----|----|----|--------------------------------------|
| 1697                 | 47  |    |    | SoxR                                 |
| 1178 (ecaC)          | 30  |    |    | Monoxygenase                          |
| 1909 (ecaB)          | 30  |    |    | Flavoprotein reductase                |
| 2478                 | 15  |    |    | Oxidoreductase                        |
| 4266 (ecaD)          | 29  |    |    | ABC transporter                       |
| 7008 (ecaA)          | 15  |    |    |                                      |

*RNA for qRT-PCR validation was obtained from independent biological samples.

\(^d\)Differential gene expression in WT and ΔsoxR in 3-day old cultures assessed by qRT-PCR. Gene expression was standardized to the housekeeping sigma factor, hrdR, and normalized to WT.

\(^b\)Differential gene expression in WT and the Δact strain (M511) in 3-day old cultures assessed by qRT-PCR. Gene expression was standardized to the housekeeping sigma factor, hrdR, and normalized to WT.

\(^e\)Genes are arranged in order of increasing SCO number.

\(^c\)These genes did not demonstrate SoxR- or Act-dependent expression in the qRT-PCR validation assay and were eliminated from further analysis.

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binding reaction, 6 fmol of DNA probe was incubated with SoxR (0 to 20 nM) for 15 min at 25°C in binding buffer (10 mM Tris-HCl, pH 8, 75 mM KCl, 0.1 mM dithiothreitol, 10% glycerol, 2 mM MgCl\(_2\), 0.1 μM poly(dI)-poly(dC), 2 mM dAMP) in a total volume of 30 μL. For the competition assay, 3,000 fmol of unlabeled probe (specific competitor) was added to the reaction mixtures. Protein-bound and uncomplexed DNA products were separated on a 5% polyacrylamide gel (Tris-HCl, pH 8, 75 mM KCl, 0.1 mM dithiothreitol, 10% glycerol, 2 mM MgCl\(_2\)) and detected using a DIG gel shift 2nd-generation kit (Roche) according to manufacturer's instructions.

Reverse Transcription PCR (RT-PCR)

RT-PCR reactions were carried out on total RNA using the OneStep RT-PCR kit (Qiagen) according to manufacturer’s instructions. All RT-PCR reactions consisted of 30 cycles using primers listed in Table S1. Reactions were analyzed on TAE: agarose gels and nucleic acids were visualized using ethidium bromide.

Bioinformatic analysis of Streptomyces genomes for potential SoxR-regulated genes

Whole genome sequences of Streptomyces species downloaded from the NCBI genomes database [http://ftp.ncbi.nih.gov/genomes/] were searched for potential homologs of the genes of the Streptomyces coelicolor SoxR regulon using blastn [29], retaining only the best matching genomic region for each gene. If two of the regulon genes matched to the same region of the genome, only the best matching gene was reported. For each retained matching sequence, the region 1 kb upstream was searched for the soxbox binding motif [1] using a position-specific scoring matrix applied via the BioPython toolkit [31]. The nucleotide frequencies for each target genome were used to calculate expected background match rates, and matches with a log-odds score for the soxbox motif greater than 10 are reported.

Results

RNA-Seq analysis reveals several novel genes as potential SoxR targets

The previously described five-gene SoxR regulon in S. coelicolor was identified bioinformatically [5,6]. In order to identify other potential SoxR-regulated genes that might have been missed by this bioinformatic approach, we conducted RNA-Seq analysis to detect genes differentially expressed between wild type and a ΔsoxR mutant strain. RNA substrates were extracted from hyphae grown on R2YE medium. Because Act (or a precursor) is a known physiological activator of SoxR in S. coelicolor [5,6], samples were collected three days post-inoculation when cells were actively producing this blue-pigmented antibiotic. As a control, samples were also collected 24 h post-inoculation, when no pigmented antibiotics were visible (and SoxR is quiescent). To identify genes that were potentially both SoxR- and Act-dependent, we focused on those that met two criteria: (i) were differentially expressed between wild type and a ΔsoxR mutant strain. RNA substrates were extracted from hyphae grown on R2YE medium. Because Act (or a precursor) is a known physiological activator of SoxR in S. coelicolor [5,6], samples were collected three days post-inoculation when cells were actively producing this blue-pigmented antibiotic. As a control, samples were also collected 24 h post-inoculation, when no pigmented antibiotics were visible (and SoxR is quiescent). To identify genes that were potentially both SoxR- and Act-dependent, we focused on those that met two criteria: (i) were differentially expressed between wild type and ΔsoxR in 3-day old cultures (Act produced); (ii) were also differentially expressed between 3-day old (blue) and 1-day old (unpigmented) wild type cultures. As predicted, the five established members of the SoxR regulon (ecaA-ecaD, SCO2478) were all significantly overexpressed in wild type compared to the
ΔsoxR mutant (Table 2). Each showed a ≥4-fold differential expression in wild type versus ΔsoxR, and a q-value of ≤0.5. Using these parameters (≥4-fold differential expression and a q-value of ≤0.5), twelve novel genes were discovered to be potentially up-regulated by SoxR in stationary phase (Table 2). Of these twelve genes, three (SCO7688, SCO7682, SCO2878) did not display differential expression between 1-day and 3-day old wild type cultures (Table 2). These genes are thus unlikely to be Act-dependent and were eliminated from further analysis.

Quantitative real-time PCR confirms SoxR- and Act-dependence in a subset of genes identified by RNA-Seq

To validate the RNA-Seq results, the SoxR-dependence of the nine newly identified genes was analyzed by quantitative real time PCR (qRT-PCR). RNA was obtained from independent biological samples (wild type and ΔsoxR) following growth for three days on complex agar (R2YE) medium. Six of the nine newly identified genes were significantly overexpressed in wild type as compared to ΔsoxR (≥4-fold difference) by qRT-PCR analysis: SCO0319, SCO0320, SCO0321, SCO1177, SCO1734, SCO4021 (Table 3). The other three genes that were tagged as SoxR-dependent by RNA-Seq (SCO4020, SCO4157, SCO6165) did not demonstrate differential expression between wild type and ΔsoxR in the qRT-PCR assay and were eliminated from further analysis (Table 3).

Since SoxR is transcriptionally active only in Act-producing cells, SoxR-dependent genes should also demonstrate Act-dependence. This is true for the five previously confirmed SoxR targets (Table 3). To determine if the six new candidate SoxR-targets demonstrate Act-dependence, their expression levels were compared in 3-day old wild type and the Act-deficient strain M511 (a strain with an in-frame deletion of the pathway-specific regulator of Act biosynthesis, actII–ORF4) [32]. Four of the six new candidate SoxR targets (SCO0319, SCO0320, SCO0321, and SCO1177) were significantly under-expressed (≥11-fold difference) in M511 compared with wild type, while SCO1734 and SCO4021 showed a more modest (2–3 fold) difference in expression levels between the two backgrounds (Table 3).

To further confirm that reduced expression of the six new potential SoxR-targets in the ΔsoxR background was due to SoxR-deficiency, we conducted a complementation experiment. Using qRT-PCR, the expression levels of the relevant genes (along with

Figure 1. Complementation analysis to confirm SoxR-dependence of genes identified by RNA-Seq. qRT-PCR was performed on RNA isolated from WT/pSET152, ΔsoxR/pSET152, and a ΔsoxR strain complemented with wild-type soxR (pSoxR), that were grown on R2YE plates for 3 days. The expression levels of all genes were standardized to the level of the constitutively expressed housekeeping sigma factor, hrdB, and normalized to expression in WT/pSET152. The results represent the means and standard deviation (bars; some are not visible on this scale) of four independent experiments.

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known controls) were analyzed in the complemented ΔsoxR strain that expresses soxR from a chromosomally integrated plasmid pSET152 (Table 1). Figure 1 shows that expression of SCO1697 (soxR), and its five confirmed targets (ecaA-ecaD, SCO2478) was restored in the soxR null strain complemented with a wild type copy of soxR (soxR/pSoxR). Furthermore, expression of three genes that showed significant Act-dependence (Table 3), SCO0320, SCO0321 and SCO1177, was also rescued in the soxR-complemented strain (Figure 1). By contrast, SCO0319, SCO1734 and SCO4021 failed the complementation test. In this experiment, SCO0319 showed similar expression levels in the ΔsoxR and the complemented ΔsoxR backgrounds, while SCO1734 and SCO4021 were similarly expressed in wild type, ΔsoxR and the complemented ΔsoxR backgrounds (Figure 1). Given these results SCO0319, SCO1734 and SCO4021 were eliminated as SoxR-targets.

In summary, RNA-Seq analysis followed by qRT-PCR validation identified eight genes that are both SoxR- and Act-dependent. Five of these are previously confirmed members of the S. coelicolor SoxR regulon [5,6]. Of the newly identified SoxR-induced genes, it appeared that SCO0320 (homologous to the carboxy-terminal half of SLI_0274, a quinone oxidoreductase in Streptomyces lividans, Figure S2) and SCO0321 (carboxylesterase) may be transcriptionally coupled. As redox-associated enzymes, SCO0320 and SCO0321 functionally cluster with the known SoxR-targets, ecaB, ecaC, ecaD and SCO2478. SCO1177 encodes a putative GntR-family transcriptional regulator. The GntR family members (so named for the Bacillus subtilis repressor of the gluconate operon) normally act as transcriptional repressors, and regulate gene expression in response to nutritional and/or other environmental signals [33].

SoxR binds to the promoter of only one of its new putative targets (with a twist)

As mentioned before, SoxR homologs from different bacteria have highly conserved DNA binding domains, and thus bind to similar operator sequences (soxbox) in the promoters of their target genes. We previously demonstrated that SoxR directly binds to the promoters of its five known targets in S. coelicolor (all of which share a similar promoter architecture) to directly activate their transcription [5]. However, visual inspection of the DNA region upstream of the newly identified potential SoxR targets, SCO0320, SCO0321, and SCO1177 failed to reveal potential SoxR docking sites. Nevertheless, we decided to empirically determine if SoxR binds to the promoters of its five known targets in S. coelicolor (all of which share a similar promoter architecture) to directly activate their transcription [5]. However, visual inspection of the DNA region upstream of the newly identified potential SoxR targets, SCO0320, SCO0321, and SCO1177 failed to reveal potential SoxR docking sites. Nevertheless, we decided to empirically determine if SoxR binds to the promoters of its three new putative target genes by electrophoretic mobility shift assays (EMSA). Towards this end we incubated purified SoxR with DIG-end-labeled DNA fragments that span ~170-200 bp upstream of each predicted ORF. While these assays showed that SoxR bound at the expected dissociation constant of 5 nM to the promoter of

Figure 2. RNA-Seq reads visualized on Integrative Genomics Viewer. Reads were obtained from RNA isolated from 3-day old wild type and ΔsoxR samples grown on R2YE medium. Red indicates the positive strand; blue indicates the negative strand; scale bar indicates the chromosomal position in the S. coelicolor M145 genome. A) SCO0319-SCO0321; B) SCO1177-SCO1179; C) SCO1908-SCO1910; D) SCO2477-SCO2479; E) SCO4265-SCO4267; F) SCO7007-SCO7009.

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ecaD (a known target), we were unable to detect binding to any of the DNA probes for the new potential targets, even at the highest concentration tested (20 nM; data not shown).

Given these puzzling findings, we examined the RNA-Seq reads for all SoxR-regulon members more closely on Integrative Genomics Viewer (Figure 2) [34,35]. The five known SoxR targets (ecaA-ecaD, SCO2478) appeared as expected, displaying strong expression on the annotated DNA strand in the wild type background, and very low (to negligible) reads in the soxR background (Figure 2B–F). An examination of the RNA-Seq reads for the SCO0319-SCO0321 region in S. coelicolor, however, revealed a few unexpected features. According to the annotation in StrepDB (http://strepdb.Streptomyces.org.uk), SCO0320 and SCO0321 are transcribed from the same strand, while SCO0319 is divergently transcribed from the complementary strand (Figure 2A and 3A). However, the RNA-Seq reads for all three genes mapped on the same strand, and furthermore, large numbers of reads mapped to the intergenic regions between the three genes (Figure 2B). To investigate the possibility that these reads derived from a previously unannotated transcript, we reannotated and manually curated all possible ORFs within this region. An alternate ORF that spans the region of what is currently annotated as SCO0319 and SCO0320 was identified, which we have named SCO0320ext (corresponding to chromosomal positions 319614-320569; Figure 3B). A BLAST search [29] of the translated amino acid sequence from SCO0320ext revealed that its N-terminal region is homologous to SLI_0274, a quinone oxidoreductase in S. lividans, the C-terminus of which had previously been identified as homologous to SCO0320 (Figure S2). Intriguingly, the DNA region upstream of SCO0320ext revealed a potential SoxR binding site and a promoter architecture that resembles the promoters of the five confirmed SoxR target genes in S. coelicolor. SoxR binding sites are indicated in bold type, and the inverted arrows depict the sequence of dyad symmetry. The asterisks indicate conserved nucleotides within the SoxR binding site. The transcriptional start site of E. coli soxS is labeled +1, and the −10 and −35 sequences are indicated. The number of nucleotides to the predicted start codons of the different genes is shown.

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Figure 3. Reannotation of the SCO0319-SCO0321 region in S. coelicolor. (A) SCO0319 is annotated in StrepDB as transcribed divergently from SCO0320 and SCO321. (B) Reannotation of the same region using Geneious (v.7.1.4 Biomatters) predicts that SCO0320 initiates further upstream within the SCO0319 ORF; the reannotated ORF is renamed SCO0320ext. A conserved SoxR-binding site (soxbox) is positioned upstream of SCO0320ext. (C) The putative promoter of SCO0320ext is aligned with the E. coli soxS promoter and the promoters of five confirmed SoxR target genes in S. coelicolor. SoxR binding sites are indicated in bold type, and the inverted arrows depict the sequence of dyad symmetry. The asterisks indicate conserved nucleotides within the SoxR binding site. The transcriptional start site of E. coli soxS is labeled +1, and the −10 and −35 sequences are indicated. The number of nucleotides to the predicted start codons of the different genes is shown.

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Figure 4. SoxR binds to the promoter of SCO0320ext in vitro. A DIG-end-labeled DNA fragment spanning the putative promoter region of SCO0320ext was incubated with increasing amounts of purified histidine-tagged SoxR protein. Protein-bound complexes (C) and free DNA (F) were separated on a 5% native polyacrylamide gel. The specificity of SoxR binding was demonstrated by the addition of a 500-fold molar excess of unlabeled competitor probe.

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To test if SoxR binds to the putative promoter of SCO0320ext, we conducted EMSA with purified SoxR and a DNA fragment that spans the region upstream of SCO0320ext. Figure 4 shows that SoxR binds to this region with high affinity; the amount of
protein needed to bind 50% of the DNA was between 5 and 10 nM (Figure 4), which is comparable to the affinity of SoxR for its other target genes in *S. coelicolor* [5]. Specificity of binding was demonstrated by addition of 500-fold excess of unlabelled competitive DNA that resulted in displacement of SoxR from the labeled probe (Figure 4). This result, combined with the qRT-PCR assays described above, strongly suggests that SCO0320ext is a direct SoxR target, and the sixth member of the SoxR regulon in *S. coelicolor*.

SCO0321 and SCO1177 are not SoxR targets, but artifacts of transcriptional read-through

These results help explain how SoxR regulates transcription of SCO0320ext. However, we were left with two other potential SoxR targets (SCO0321 and SCO1177) that do not appear to harbor a SoxR-binding site in their putative promoters, leaving the mechanism of their regulation unclear. Given that the RNA-seq reads for SCO0321 are concentrated within the 5' end of the gene (Figures 2A and 5A), we propose that SCO0321 is not a real SoxR-target, but likely an artifact of transcriptional read-through from SCO0320ext. The primers that were used in the previously described qRT-PCR validation assays for SCO0321 (0321N-F and 0321N-R; Figure 5B) bind within the 5' region of the gene that demonstrated high RNA-Seq reads (Figure 5A). However, when we used a different set of primers (0321C-F and 0321C-R; Figure 5B) that bind within the 3' region of the gene, we did not observe an RT product (Figure 5C). This result is consistent with the lack of RNA-seq reads within the 3' region of SCO0321 and indicates that the complete sequence of SCO0321 is not independently transcribed under our experimental conditions.

An examination of the RNA-Seq reads for SCO1177 similarly suggested that this gene is not a SoxR-target but an artifact of transcriptional read-through from the adjacent gene, SCO1178 (ecaC), a confirmed SoxR regulated gene. As annotated in StrepDB, SCO1177 and ecaC are convergently transcribed from opposite strands, however all RNA-Seq reads for SCO1177 map to the sense strand for ecaC (Figure 2B). Moreover, the reads for SCO1177 cluster towards the 3' end of the gene that is closest to ecaC, and could readily be explained by transcriptional read-through of ecaC. Here, SCO1177 was detected as a SoxR-target in our initial RNA-Seq analysis because read counts were compiled without regard to strand specificity. Furthermore, the primers used in the qRT-PCR validation assays for SCO1177 happen to prime within the 3' region of this gene that demonstrated high RNA-Seq reads (Figure 2B). It should be noted that we did not observe significant reads in the region corresponding to the sense strand of SCO1177 in either wild type or ΔsoxR strains at any of the time points sampled (day 3 reads shown in Figure 2B; day 1 data not shown). Thus, SCO1177 also does not appear to be expressed under the growth conditions used in this work.

Discussion

In this work we expanded the SoxR regulon in *S. coelicolor* by comparing the transcriptionomes of wild type and ΔsoxR null mutant strains in stationary phase using RNA-Seq. This regulon is composed of six genes induced by SoxR in response to the redox-active antibiotic Act that is produced in stationary phase. Five of these genes (ecaA-ecaD, SCO2478) were previously identified as SoxR-dependent using a bioinformatics approach designed to identify genes based on the presence of a SoxR-binding site in putative promoter regions [5,6]. The Act-dependent expression of four of the SoxR-targets (ecaA-ecaD) was independently described by Huang and co-workers [20]. The sixth *S. coelicolor* SoxR-regulon member identified in this work, SCO0320ext, was missed by the bioinformatic search for SoxR-targets, and was also not previously identified as an “eca” gene.

SCO0320ext encodes a putative quinone oxidoreductase with homology to SLI_0274 in *S. lividans* and SAV_4018 in *Streptomyces avermitilis* (Table 4). Both the *S. lividans* and *S. avermitilis*
homologs of SCO0320ext contain putative SoxR-binding sites in their promoters suggesting that they may be under SoxR regulation in these organisms as well. It is peculiar that only the amino-terminal half of SCO0320ext is homologous to the other two proteins. As illustrated in Figure S2, there is almost complete sequence identity between the amino-terminal halves of SCO0320ext and SLI_0274, but clear divergence in the carboxy-terminal halves. An alignment of the nucleotide sequences of SLI_0274 and SCO0320ext shows a single base difference (deletion of cytosine 494) in SCO0320ext that causes a shift in the open reading frame (Figure S2A). Interestingly, SCO0320 (as annotated in StrepDB) is almost identical to the carboxy-terminal half of SLI_0274 (Figure S2). Thus it appears that S. coelicolor M145 acquired a mutation making what was originally one gene (that corresponding to SCO0320ext), appear to be two separate genes (SCO0319 and SCO0320). Analysis of the nucleotide sequence of this region in two other S. coelicolor A3(2) derivatives (M600 and J1501) showed the same base deletion found in M145 (data not shown). At this point it is unclear if SCO0320ext is functional in S. coelicolor, or if the mutation has any physiological consequences for the organism.

SoxR is typically considered a transcriptional activator. Nevertheless, we analyzed our RNA-Seq data for genes whose expression may be inhibited by SoxR. Several candidate genes were found to be overexpressed in the ΔsoxR mutant compared to wild type (≥4-fold difference; Table S2). From this list, thirteen genes showed statistically significant differential expression (q-value ≤ 0.1), and were selected for validation by qRT-PCR performed on RNA isolated from independent biological samples. Of these, only six showed significantly different expression levels (≥4-fold) in wild type and the ΔsoxR mutant by qRT-PCR (Table S3). However, none of these six genes passed the complementation test, where we expected lower expression in the wild type and soxR-complemented backgrounds compared to the ΔsoxR-deficient background. Instead, most were overexpressed in the soxR-complemented strain contrary to the predicted pattern (Figure S1). It can thus be concluded that SoxR does not function as a transcriptional inhibitor in S. coelicolor.

As described earlier, SoxR stimulates the production of a similar group of genes (transporters and redox enzymes) in S. coelicolor and P. aeruginosa in response to endogenously produced antibiotics; Act in the former and phenazines in the latter. This homology of SCO0320ext contain putative SoxR-binding sites in their promoters suggesting that they may be under SoxR regulation in these organisms as well. It is peculiar that only the amino-terminal half of SCO0320ext is homologous to the other two proteins. As illustrated in Figure S2, there is almost complete sequence identity between the amino-terminal halves of SCO0320ext and SLI_0274, but clear divergence in the carboxy-terminal halves. An alignment of the nucleotide sequences of SLI_0274 and SCO0320ext shows a single base difference (deletion of cytosine 494) in SCO0320ext that causes a shift in the open reading frame (Figure S2A). Interestingly, SCO0320 (as annotated in StrepDB) is almost identical to the carboxy-terminal half of SLI_0274 (Figure S2). Thus it appears that S. coelicolor M145 acquired a mutation making what was originally one gene (that corresponding to SCO0320ext), appear to be two separate genes (SCO0319 and SCO0320). Analysis of the nucleotide sequence of this region in two other S. coelicolor A3(2) derivatives (M600 and J1501) showed the same base deletion found in M145 (data not shown). At this point it is unclear if SCO0320ext is functional in S. coelicolor, or if the mutation has any physiological consequences for the organism.

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Streptomyces S. coelicolor shows that all six SoxR-regulated genes in the Supporting Information cetes. An analysis of seven species surveyed showed a potential soxbox upstream suggesting about 13 percent of species that are annotated Streptomyces to 33 percent for SoxR homologs in other Streptomyces species, showing that these homologs may be autoregulated. The suite of SoxR-regulated genes in S. coelicolor is fairly well conserved in the other streptomycetes analyzed, ranging from 100 percent conservation of ecaA to 33 percent for ecaC (Table S4; Figure 6). When found, these genes commonly contain a potential soxbox within one kilobase pairs upstream (Table S4; Figure 6). S. coelicolor produces Act per se, members of this genus are known to produce other biologically active secondary metabolites. These molecules could serve as signals that trigger SoxR activity in other streptomycetes. Knowledge of the individual genes regulated by SoxR will facilitate further studies to elucidate the function of this regulatory protein and its regulon in antibiotic producers.

Supporting Information

Figure S1. Complementation analysis to further assess the SoxR-dependence of genes identified by RNA-Seq.

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Figure S2. Sequence alignment of SCO0320ext and SCO0320 with the quinone oxidoreductase SLI_0274 from S. lividans.

Table S1. Primers used in this study.

Table S2. Genes identified by RNA-Seq as upregulated in AsoxR compared to WT in stationary phase.

Table S3. Validation of RNA-Seq data reported in Table S2 by quantitative RT-PCR.

Table S4. tblastn matches of genes of the SoxR regulon to Streptomyces whole genome sequences regions. Soxbox positions are listed relative to the 5’ end of the blast hit.

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Author Contributions

Conceived and designed the experiments: MC NN JAS. Performed the experiments: MC NN JAS. Analyzed the data: MC NN JAS. Contributed reagents/materials/analysis tools: MC. JAS. Contributed to the writing of the manuscript: MC NN JAS.
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