OsdR of *Streptomyces coelicolor* and the Dormancy Regulator DevR of *Mycobacterium tuberculosis* Control Overlapping Regulons

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**ABSTRACT** Two-component regulatory systems allow bacteria to respond adequately to changes in their environment. In response to a given stimulus, a sensory kinase activates its cognate response regulator via reversible phosphorylation. The response regulator DevR activates a state of dormancy under hypoxia in *Mycobacterium tuberculosis*, allowing this pathogen to escape the host defense system. Here, we show that OsdR (SCO0204) of the soil bacterium *Streptomyces coelicolor* is a functional orthologue of DevR. OsdR, when activated by the sensory kinase OsdK (SCO0203), binds upstream of the DevR-controlled dormancy genes devR, hspX, and Rv3134c of *M. tuberculosis*. In silico analysis of the *S. coelicolor* genome combined with in vitro DNA binding studies identified many binding sites in the genomic region around osdR itself and upstream of stress-related genes. This binding correlated well with transcriptomic responses, with deregulation of developmental genes and genes related to stress and hypoxia in the osdR mutant. A peak in osdR transcription in the wild-type strain at the onset of aerial growth correlated with major changes in global gene expression. Taken together, our data reveal the existence of a dormancy-related regulon in streptomycetes which plays an important role in the transcriptional control of stress- and development-related genes.

**IMPORTANCE** Dormancy is a state of growth cessation that allows bacteria to escape the host defense system and antibiotic challenge. Understanding the mechanisms that control dormancy is of key importance for the treatment of latent infections, such as those from *Mycobacterium tuberculosis*. In mycobacteria, dormancy is controlled by the response regulator DevR, which responds to conditions of hypoxia. Here, we show that OsdR of *Streptomyces coelicolor* recognizes the same regulatory element and controls a regulon that consists of genes involved in the control of stress and development. Only the core regulon in the direct vicinity of dosR and osdR is conserved between *M. tuberculosis* and *S. coelicolor*, respectively. Thus, we show how the system has diverged from allowing escape from the host defense system by mycobacteria to the control of sporulation by complex multicellular streptomycetes. This provides novel insights into how bacterial growth and development are coordinated with the environmental conditions.

**KEYWORDS:** Developmental control, *Streptomyces*, dormancy, stress response
Complex natural habitats of bacteria call for rapid response systems to ensure adaption to often-changing environmental conditions. One prevalent mechanism that bacteria such as streptomycetes use to couple environmental stimuli to adaptive responses consists of a sensor kinase (SK) and a cognate response regulator (RR), which act as a two-component signal transduction system (TCS) (Fig. 1) (1, 2). Upon stimulation of the sensory domain of the SK by an external signal, the SK autophosphorylates itself prior to the transfer of the phosphate to a conserved His residue in the RR (1). Typically, the activity of the RR is mediated through DNA binding, although RNA and protein binding activities as well as catalytic activities have also been reported (1, 2).

Soil-borne bacteria, such as streptomycetes, have developed intricate sensory systems to detect nutrient availability and to initiate appropriate response mechanisms. Streptomycetes are industrially important organisms and produce a wide range of natural products, including over 50% of all known antibiotics (3, 4). The bacteria have a complex mycelial lifestyle (Fig. 1) and produce a branching network of vegetative hyphae, which are compartmentalized by cross-walls, making Streptomyces a rare example of a multicellular prokaryote (5). Under environmentally adverse conditions, such as nutrient depletion, streptomycetes initiate a complex developmental program whereby the vegetative mycelium serves as a substrate for a new so-called aerial mycelium. Eventually, the outer part of the aerial hyphae develops into chains of spores (6). In turn, the spores are able to survive periods of unfavorable conditions, such as anaerobiosis (for example, as a result of heavy rainfall). Though the model organism Streptomyces coelicolor is able to survive anaerobic conditions, anaerobic growth has not been reported for this microorganism (7).

The environmental conditions of a streptomycete’s natural habitat are ever-changing, and the complexity of the signals that are received and of the responses that are transmitted is reflected in the large number of TCSs, with the genome of S. coelicolor encoding 85 sensory kinases and 79 response regulators, with 67 known sensor-regulator pairs (8). One such pair is made up of the SK SCO0203 and the RR SCO0204. Unusually, SCO0203 has a second cognate RR in addition to SCO0204, namely, the orphan response regulator SCO3818 (9). The deletion of either RR gene was shown to...
Enhance the production of actinorhodin, the blue-pigmented antibiotic of *Streptomyces coelicolor*. Although no biochemical evidence was provided, it was previously suggested that sensory kinase SCO0203 may be a direct orthologue of DosT, an SK from a well-studied TCS from the pathogenic obligate aerobe *Mycobacterium tuberculosis* (10).

In *M. tuberculosis*, gradual oxygen depletion is sensed by two SKs (DosT and DevS [alternatively known as DosS]) and induces a regulon controlled by the response regulator DevR (alternatively known as DosR), which consists of some 50 genes, including universal stress proteins (USPs), nitroreductases (which allow anaerobic nitrate respiration), redox proteins, and heat shock proteins (11). It is thought that this TCS regulates the escape from the host defense system by promoting dormancy to survive anaerobic conditions, and it is likely that this nonreplicating state plays a major role in the resistance of the bacilli to antibiotics (12, 13). An orthologous oxygen-sensing mechanism in streptomycetes may be essential for the sensing of oxygen levels in soil; under conditions of oxygen depletion, the appropriate response needs to be activated to ensure survival. Alternatively, under nutrient availability (and sufficient oxygen), vegetative hyphae form a very dense mycelium, where oxygen is locally depleted, and this depletion might be regulated via SCO0203/SCO0204.

In this work, we suggest that the TCS pair SCO0203/SCO0204 regulates a dormancy-related response in *S. coelicolor*. Major changes are seen in the global transcription patterns of genes related to stress and development in SCO0204 null mutants. The predicted core regulon of SCO0204, which revolves around the region from SCO0167 to SCO0219 in the *S. coelicolor* genome, contains many dormancy regulon-related genes and is conserved between SCO0204 and the dormancy regulator, DevR, of *M. tuberculosis*. We show binding of SCO0204 upstream of *M. tuberculosis* genes that are part of the DevR regulon as well as binding to the predicted binding site in *S. coelicolor*, including direct binding to developmental genes (which lack a predicted binding site). The locus tags SCO0203 and SCO0204 were named *osdK* and *osdR*, respectively, to highlight their function in response to oxygen availability, stress, and development.

**RESULTS**

**Analysis of the two-component regulatory system OsdKR.** SCO0203 (OsdK) and SCO0204 (OsdR) form a two-component regulatory system (9) and are encoded by the *osdR-osdK* operon. OsdK has 41% and 42% amino acid identity (57% amino acid similarity) with DevS and DosT, respectively (see Fig. S1 in the supplemental material), and it was postulated as a possible ortholog of the dormancy sensory kinases of *M. tuberculosis* (14). Indeed, of the 18 amino acid residues required for oxygen sensing (15, 16), 15/18 residues of DosT and 12/18 residues of DevS are conserved in OsdK (Fig. S1). The interaction between the RR DevR and its target site is known in structural detail (17). OsdR and DevR share 61% amino acid identity (79% amino acid similarity) (Fig. S1 and S2), and comparison of the residues in the DevR and OsdR proteins revealed that 11 of the 13 residues implicated in DNA binding are conserved between DevR and OsdR (Fig. S2).

To test whether *S. coelicolor* OsdR could bind to the recognition site of *M. tuberculosis* DevR, electrophoretic mobility shift assays (EMSAs) were performed. His$_6$-tagged OsdK and OsdR were purified, and the sensory kinase OsdK was autophosphorylated using $^{32}$P-radiolabeled ATP and then used to transphosphorylate OsdR (Fig. S3). OsdR transphosphorylation could be achieved with autophosphorylated OsdK. However, OsdR readily lost its phosphosignal in the presence of OsdK, as previously observed for DevRS/DosT. Therefore, acetyl phosphate (AcP) was used as phosphor donor (18). As probes for EMSAs we used three mycobacterial promoters that are known targets of DevR (18), namely, the promoters for devR, Rv3134c (which is located upstream of devR and encodes a universal stress domain protein), and *hspX*, which encodes a latency-related heat shock protein. As negative controls, DNA fragments of the upstream region of *dasR* of *S. coelicolor* and AT-rich DNA from *Escherichia coli* were used. OsdR bound with low affinity to the DNA fragment encompassing the *dasR* promoter region, while no binding was seen when AT-rich *E. coli* control DNA was used (Fig. 2A). Interestingly,
FIG 2 Binding site of OsdR (SCO0204) and comparison to that of M. tuberculosis DevR. (A) EMSAs using purified His₆-tagged OsdR on known targets of DevR in M. tuberculosis. Both phosphorylated OsdR (OsdR-P) and nonphosphorylated OsdR (OsdR) were used in the assays. OsdR-P was obtained after AcP phosphorylation in vitro. Numbers on the horizontal axis refer to micromolar concentrations. F, free DNA fragment; C, complexes of DNA and protein. (B) Weblogo representation of cis-regulatory elements identified upstream of osdR, identified from the upstream regions of osdR orthologues in 12 Streptomyces species (see Materials and Methods). For comparison, the upstream regulatory element recognized by DevR (97) is presented. (C) Gene synteny between the loci around osdR in S. coelicolor (top) and devR in M. tuberculosis (bottom). Functional categories are given in the figure, and black dots indicate predicted OsdR binding sites, with that of upsA (SCO0200) surrounded by a red ring. Orthologues are presented in the same colors, and when multiple genes with similar functions are present, they appear in patterns.
OsDR bound well to all probes for the mycobacterial target genes (Fig. 2A). Furthermore, similar differential affinities for the three fragments were observed as described previously for DevR in *M. tuberculosis* (18), with stronger binding upstream of Rv3134c and hspX than to the autoregulatory site of devR. Nonphosphorylated OsDR bound significantly less efficiently to the probes. Taken together, these data strongly suggest that OsDR and the dormancy regulator DevR recognize the same upstream regulatory elements, with phosphorylation by OsDK required to enhance DNA binding.

**In silico prediction of the OsDR regulon of *S. coelicolor*.** The OsDR consensus sequence was deduced by searching the upstream regions of *osdR* orthologues from 12 *Streptomyces* species for possible similar *cis*-acting regulatory elements using MEME (19). This identified a 16-nucleotide palindromic consensus sequence, 5′-AGGGCCGATCGGCCCT, which conforms well to the consensus sequence found in *M. tuberculosis* (Fig. 2B). The *S. coelicolor* genome was then scanned by PREDetector (20), using a position weight matrix (see Table S3 in the supplemental material) based on 12 predicted upstream elements as inputs. With a cutoff score of 8.0 for medium reliability (21), PREDetector identified putative binding sites for OsDR upstream of 27 transcription units. There was a total of 43 binding sites that may affect the transcription of 85 genes, which therefore may qualify as the direct OsDR response regulon (Table 1).

Eight binding sites were identified upstream of genes/operons in the vicinity of *osdR*, including *osdR* itself, controlling 20 of the 22 genes in the region between SCO0198 and SCO0219 (Fig. 2C). Comparison with the genomic region around *M. tuberculosis devR* revealed significant gene synteny (Fig. 2C). Of the 11 *S. coelicolor* genes for USP domain proteins, 8 are found in the genomic region between SCO0167 and SCO0021, and in *M. tuberculosis*, *usp* genes are part of the DevR regulon. SCO0213 to SCO0219 encode a nitrate transporter and nitrate reductase, which also prominently feature in the DevR regulon. When a lower cutoff score of 6.0 was used, PREDetector predicted a possible 27 elements in the regions SCO0167 to SCO0181 and SCO0198 to SCO0219.

**Specificity analysis of OsDR binding to the predicted regulatory element of uspA.** To investigate whether OsDR binds specifically to the predicted nucleotide sequence, a 50-mer probe of the upstream region of *uspA* (SCO0200), centered on the predicted binding site, was used as a probe (see Table S2 in the supplemental material). Indeed, AcP-phosphorylated OsDR (OsDR~P) bound well to the DNA fragment (Fig. 3A). Some retarded DNA remained in the wells of the gel, likely due to bridging, whereas each of the monomers of the OsDR dimer bound to a different probe rather than to the same site, which can result in long concatemers, as was observed for, e.g., NagR in *Bacillus subtilis* (22) and DasR in *S. coelicolor* (23).

We then designed four mutant 50-mer probes containing single mutations (designated 50a and 50b), a double mutation (50ab), or a quintuple mutation (50x) of the most conserved nucleotides of the binding site. In line with the predicted importance of the conserved nucleotide positions in the consensus sequence (Fig. 2B), nucleotide permutations significantly decreased the binding of OsDR to the probes, such that the single G→T substitution at position 3 (50a) and the G→T substitution at position 11 (50b) lowered binding efficiency by around 50%, which was further reduced by mutating both positions (Fig. 3A). Binding was abolished when five of the conserved nucleotides were mutated (50x). We also performed a competition assay with unlabelled DNA on the radiolabeled wild-type 50-mer *uspA* probe. Increasing the amount of the unlabeled wild-type *uspA* probe strongly inhibited binding by OsDR, while addition of unlabeled competitor DNA with 5 permutations in the binding site (50x) had no effect on OsDR binding (Fig. 3B). Taken together, these experiments provide conclusive evidence that OsDR specifically recognizes the predicted regulatory element.

**Verification of the regulon predictions by EMSAs.** Next we tested DNA binding by OsDR to predicted targets using EMSAs of PCR-amplified DNA probes (Table S2). These were *uspA*, *osdR*, SCO2637 (for a serine protease), and SCO2967 (for a carboxypeptidase), and the intergenic regions between the divergent genes SCO0027 and
SCO0208 (for another USP domain protein and pyruvate phosphate dikinase), SCO5978 and SCO5979 (for a hypothetical protein and an enoyl coenzyme A [enoyl-CoA] hydratase), and SCO6040 and SCO6041 (for a lipoprotein and a protoporphyrinogen oxidase). All the predicted binding sites were bound by OsdR, with most probes fully bound by OsdR-P (at 1 μM), except SCO2637, which was bound with 2-fold-lower affinity (Fig. 4A). This suggests that phosphorylation (by OsdK) leads to enhanced

### Table 1: Predicted binding sites for SCO0204 (OsdR) in Streptomyces coelicolor

<table>
<thead>
<tr>
<th>Locus tag</th>
<th>Function</th>
<th>Sequence</th>
<th>Position</th>
<th>Score</th>
<th>Cotranscribed gene(s)</th>
<th>Function(s)</th>
</tr>
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<tr>
<td>SCO0204c</td>
<td>OsdR, two-component response regulator</td>
<td>AGGGCCGCGTCGGCCCC</td>
<td>−81</td>
<td>13.74</td>
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<td>SCO0200c</td>
<td>Universal stress protein</td>
<td>GGGGGCCGACGTCGGCTT</td>
<td>−100</td>
<td>12.49</td>
<td>SCO0199c/SCO0198c</td>
<td>Zinc-deprived alcohol dehydrogenase, universal stress protein</td>
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<tr>
<td>SCO0215c</td>
<td>Nitroreductase family protein</td>
<td>AGGGCCGTCCAAGGCCC</td>
<td>−99</td>
<td>12.24</td>
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<td></td>
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<tr>
<td>SCO0208c</td>
<td>Pyruvate phosphate dikinase</td>
<td>CAGGGCCGACGTCGGCTT</td>
<td>−102</td>
<td>12.19</td>
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<td></td>
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<tr>
<td>SCO0207c</td>
<td>Universal stress protein</td>
<td>GGGGGCCGACGTCGGCTT</td>
<td>−144</td>
<td>10.08</td>
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<tr>
<td>SCO5979c</td>
<td>Enoyl-CoA hydratase</td>
<td>CAGGGACCTTCCGGCCCC</td>
<td>−88</td>
<td>11.62</td>
<td>SCO5980</td>
<td>Bifunctional hydroxylase, oxidoreductase</td>
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<td>SCO2637c</td>
<td>Secreted serine protease</td>
<td>AGGGCCGCGTCGGCCTT</td>
<td>−53</td>
<td>11.27</td>
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<tr>
<td>SCO5978c</td>
<td>Hypothetical protein</td>
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<td>−53</td>
<td>11.27</td>
<td>SCO5980</td>
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<tr>
<td>SCO0038c</td>
<td>Sigma factor</td>
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<td>−53</td>
<td>11.27</td>
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<td>SCO0168c</td>
<td>Crp-like regulatory protein</td>
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<td>−284</td>
<td>10.75</td>
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<tr>
<td>SCO0167c</td>
<td>Universal stress protein</td>
<td>GGGGGCGACGTCGGCTT</td>
<td>−53</td>
<td>10.75</td>
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<td></td>
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<tr>
<td>SCO0216c</td>
<td>Nitrate reductase alpha chain NarG2</td>
<td>GGGGGCCGACGTCGGCTT</td>
<td>−53</td>
<td>10.75</td>
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<td></td>
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<tr>
<td>SCO5410c</td>
<td>Hypothetical protein</td>
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<td>−53</td>
<td>10.75</td>
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<tr>
<td>SCO6040c</td>
<td>Lipoprotein</td>
<td>GGGGGCGACGTCGGCTT</td>
<td>−53</td>
<td>10.75</td>
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<tr>
<td>SCO3431c</td>
<td>EmrB/QacA subfamily transporter</td>
<td>GGGGGCGACGTCGGCTT</td>
<td>−53</td>
<td>10.75</td>
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<tr>
<td>SCO6041c</td>
<td>Protoporphyrinogen oxidase</td>
<td>GGGGGCGACGTCGGCTT</td>
<td>−53</td>
<td>10.75</td>
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<tr>
<td>SCO0214c</td>
<td>Nitrate-nitrite transporter protein</td>
<td>GGGGGCGACGTCGGCTT</td>
<td>−53</td>
<td>10.75</td>
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<td>SCO0355c</td>
<td>Conserved hypothetical protein</td>
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<td>−53</td>
<td>10.75</td>
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</tr>
<tr>
<td>SCO0179c</td>
<td>Possible Crp-like regulatory protein</td>
<td>GGGGGCGACGTCGGCTT</td>
<td>−53</td>
<td>10.75</td>
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<tr>
<td>SCO0164c</td>
<td>Hypothetical protein with DksA/TraR family C4 zinc finger domain</td>
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<td>−53</td>
<td>10.75</td>
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<tr>
<td>SCO0163c</td>
<td>Sensor kinase</td>
<td>GGGGGCGACGTCGGCTT</td>
<td>−53</td>
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<tr>
<td>SCO0356c</td>
<td>Peptidyl-prolyl cis-trans isomerase</td>
<td>GGGGGCGACGTCGGCTT</td>
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<tr>
<td>SCO0355c</td>
<td>Conserved hypothetical protein</td>
<td>GGGGGCGACGTCGGCTT</td>
<td>−53</td>
<td>10.75</td>
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</tbody>
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**a**SCO numbers in boldface were tested by EMSA.

**b**Position relative to the start of the gene.

**c**The cutoff score calculated using the PREDetector algorithm and based on the position weight matrix in Table S3 in the supplemental material.

**d**Genes known or predicted to be cotranscribed with the gene and therefore likely influenced by the regulatory element.

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SC00208 (for another USP domain protein and pyruvate phosphate dikinase), SCO5978 and SCO5979 (for a hypothetical protein and an enoyl coenzyme A [enoyl-CoA] hydratase), and SCO6040 and SCO6041 (for a lipoprotein and a protoporphyrinogen oxidase). All the predicted binding sites were bound by OsdR, with most probes fully bound by OsdR-P (at 1 μM), except SCO2637, which was bound with 2-fold-lower affinity (Fig. 4A). This suggests that phosphorylation (by OsdK) leads to enhanced...
binding of OsdR to its binding sites. The combined predictions and EMSA data reveal some 50 likely OsdR target genes or gene clusters, of which at least 13 have orthologues that are controlled by DevR in *M. tuberculosis* (*osdR*, SCO0167, SCO0198, *uspA* [SCO0200], and SCO0207 and genes for nitrate reductase subunits).

**Transcriptional analysis of OsdR targets.** To analyze the transcriptional control by OsdR, promoter probing was performed using the Red promoter probing system (see Materials and Methods) in the nonpigmented *S. coelicolor* strain M512 and the M512 *osdR* mutant derivative GSTC6. Promoter-probe vectors harboring the upstream regions of *uspA* (SCO0200), *osdR*, and SCO0207 were introduced into *S. coelicolor* M512 and the mutant GSTC6, and the promoter activity was analyzed, with as a control the empty vector or the vector with the *whiG* promoter, which is transcribed constitutively (the developmental control of the gene product *WhiG* is governed primarily at the posttranslational level). While the empty vector did not show activity and *whiG* transcription was not affected by the deletion of *osdR*, *P* _SCO0200_, *P* _SCO0204_, and *P* _SCO0207_ were

**FIG 3 EMSAs with OsdR on a predicted *S. coelicolor* binding site.** (A) Analysis of the OsdR binding site by mutation of highly conserved nucleotides in the *upsA* (SCO0200) binding site. Twenty femtomoles of a 50-mer DNA was incubated with increasing micromolar concentrations of OsdR–P. Replacements of the *upsA* binding site are indicated with red letters in the sequences; the 50-mer *dasR* fragment was used as a control. wt, wild type. (B) Competition assays using 10 μM protein and 20 fmol of labeled 50-mers centered on the *upsA* binding site. Increasing concentrations of the unlabeled competitor 50-mer were added, using either the wild-type *upsA* 50-mer or a mutated *upsA* 50-mer with 5 substitutions of highly conserved nucleotides (50x). − and + refer to the presence of phosphorylated OsdR (OsdR–P); competitor DNA is given in micromolar units. F, free DNA fragment; C, complexes of DNA and protein.
EMSAs with OsdR on selected *S. coelicolor* targets. (A) EMSAs were performed on DNA fragments harboring predicted binding sites upstream of the indicated genes. (B) EMSAs of *ssgB* (SCO1541) and *whiE* (SCO5319 and SCO5320-SCO5321). Concentrations of nonphosphorylated OsdR (OsdR) and phosphorylated OsdR (OsdR~P) are given in micromolar amounts. F, free DNA fragment; C, complexes of DNA and protein.
all active in M512 but poorly or not expressed in the mutant (Fig. 5), strongly suggesting that the genes are transcriptionally activated by OsdR.

Global transcription profiling of the osdR null mutant by DNA microarray analysis. Phenotypic analysis of the M145 osdR null mutants GSTC2 and GSTC3 as well as M145 ΔosdK (GSTC1) and M145 ΔosdRK (GSTC4) on MS medium indicated earlier formation of mycelial hyphae in the osdR mutants and accelerated sporulation and enhanced production of the gray spore pigment (Fig. 6A). In the absence of both Osdr and Osdk, this phenotype was not observed. High-resolution imaging by cryo-scanning electron microscopy revealed that the spores had a normal morphology (Fig. 6B). Observation of the spores with laser confocal microscopy indicated a strong delay in the germination of spores of the osdR null mutant compared to that of spores of the parental strain (Fig. 6C). Staining of dead and viable spores showed that this delay in germination in the GSTC2 mutant was not due to extensive accumulation of dead spores, as the proportions of viable/dying spores were comparable between S. coelicolor M145 and its osdR mutant derivative GSTC2.

To obtain a global overview of the effect of the deletion of osdR on transcription, microarray analysis was performed using RNA extracted from S. coelicolor M145 and its osdR null mutant GSTC2 grown on minimal medium (MM) agar plates overlaid with cellophane discs. Biomass was harvested at time points corresponding to vegetative growth (24 h), the onset of aerial growth (30 h), aerial growth (36 h), early sporulation (42 h), and sporulation (54 h) in the parental S. coelicolor M145. RNA from two independent biological replicate experiments was subsequently used as a template for cDNA synthesis/Cy3-dCTP labeling and subsequently hybridized onto oligonucleotide-based S. coelicolor whole-genome DNA microarrays (see Materials and Methods). By rank product analysis, a list of genes whose levels of expression were statistically significantly different was obtained at a percentage of false positives (PFP) of <0.01. With the additional cutoff of a minimum 2-fold change in the levels of transcription between the wild type and mutant, a list of over 800 genes whose transcription was significantly altered in the osdR null mutant was obtained (see Table S4 in the supplemental material). Classes of genes that were overrepresented were related to stress, anaerobic growth, and development. Notably, and as detailed further below, many of the genes that were differentially expressed between the wild type and osdR mutant had particularly strongly altered mRNA levels at 36 h. Suggestively, transcription of Osdr itself peaks at 36 h in wild-type cells, as shown in the present study and as established previously (see, e.g., reference 24).

Stress-related genes and the chromosomal region around osdRK. The majority of the genes encoding universal stress proteins are located in the vicinity of osdk and osdR, and several are predicted or proven members of the direct Osdr regulon (see above). Of these, SCO0167, SCO0172, SCO0181, and SCO0200 (uspA) were all down-regulated at one or more time points in the mutant (Fig. 7A). The same was observed
for the genes for the nitrate reductase system Nar2 (SCO0216 to SCO0219) at 36 h (Fig. 7C). *S. coelicolor* has three different nitrate reductases (Nar1 to -3) for anaerobic respiration, each active at different stages of development (25, 26). Genes for the two other nitrate reductase systems were not affected (see Table S4 in the supplemental material).

Deletion of *osdR* had a major effect on the transcription of many of the genes that were previously shown to be involved in stress management (27–31), such as the response to redox and (thiol) oxidative, osmotic, and temperature stress (Fig. 7B; see also Table S4 in the supplemental material). The σ factor gene sigL, which is involved in osmoprotection and oxidative stress (32), was upregulated, as was *catB*, but most of the stress-related genes were significantly downregulated. This included genes that in
Heat maps of stress- and development-related genes differentially expressed between the osdR mutant and its parent, S. coelicolor M145. Transcription patterns (expressed as fold changes between the osdR mutant and the wild type) are presented for genes close to osdRK (A), stress-related genes (B), anaerobic-growth-related genes (C), and developmental genes (D). RNA was isolated from MM agar during vegetative growth (24 h), vegetative/aerial growth (30 h), aerial growth (36 h), aerial growth/early sporulation (42 h), and sporulation (54 h). Blue indicates downregulation (<0.5) and red indicates upregulation (>2.0) in the mutant; intermediate fold changes are represented in white. See Table S4 in the supplemental material. db #, database locus tag.

**Colour key**

- 0.1
- 0.5
- 1.0
- 2.0
- 10

**FIG 7** Heat maps of stress- and development-related genes differentially expressed between the osdR mutant and its parent, S. coelicolor M145. Transcription patterns (expressed as fold changes between the osdR mutant and the wild type) are presented for genes close to osdRK (A), stress-related genes (B), anaerobic-growth-related genes (C), and developmental genes (D). RNA was isolated from MM agar during vegetative growth (24 h), vegetative/aerial growth (30 h), aerial growth (36 h), aerial growth/early sporulation (42 h), and sporulation (54 h). Blue indicates downregulation (<0.5) and red indicates upregulation (>2.0) in the mutant; intermediate fold changes are represented in white. See Table S4 in the supplemental material. db #, database locus tag.
B. subtilis are part of the oxidative-stress response regulon (33), namely, katA, trxA, trxB, msrA, a catR/perR-like gene, and the genes for the oxidative-stress-related σ factor/anti-σ factor pair SigR/RsrA (34–36), as well as genes involved in protein degradation and folding, such as clpP1 to clpP2 (SCO2618 to SCO2619), dnaK, hspR, groEL1, groEL2, groES, genes encoding the proteasome (SCO1643 to SCO1644), and several cold shock genes. Zinc-related genes like those of the gene cluster for the zincophore coelbactin, were downregulated at all time points except 36 h, at which time levels of transcription were comparable between wild-type and osdR mutant cells (Fig. S4). Sufficient zinc is necessary for processes related to protein folding, redox balance, and oxygen stress (37–39). Similar changes in expression were observed for genes related to sulfur, cysteine synthesis, and thiol homeostasis (Fig. S4), which are involved in the management of (thiol) oxidative, redox, or osmotic stress (40, 41).

Developmental control. Major changes were observed in the global transcription profile of developmental genes, with a very distinctive pattern of upregulation of many sporulation genes in the absence of osdR at most time points, while early-developmental (bld) genes were downregulated at the same time points (Fig. 7D). Transcription of other genes, namely, ssgB, ssgG, smeA-ssfA, chpADFG, rdlAB, and sapB, all followed the same pattern, with a sharp peak at the onset of sporulation in wild-type cells and, instead, a steady increase in the mutant (see Table S5 in the supplemental material). SsgB and SsgG are members of the actinomycete-specific family of SsgA-like proteins (SALPs) (42) and determine the positions of septum sites during sporulation-specific cell division (43, 44). SmeA and SsfA are also involved in the control of septation as well as DNA segregation (45), and the rdl and chp genes encode the rodlin and chaplin proteins, respectively, which form amyloid-like structures to create a water-repellent hydrophobic sheath around aerial hyphae and spores (46–48). SapB is a lanthipeptide that acts as a signaling molecule for the onset of development (49, 50). The same transcriptional upregulation was observed for the whiE gene cluster for the spore pigment WhiE (51) and for the whiE-like gene cluster from SCO7449 to SCO7453, which also produces a spore pigment (52) (Fig. 7D; Table S4). The upregulation of sporulation genes correlates well to the accelerated development and enhanced pigmentation of osdR mutants (Fig. 6A).

Conversely, the early-developmental genes were downregulated in the osdR null mutant, including bldC, bldG, bldM, bldN, and crp, as well as chpCEH. The crp gene encodes the cAMP receptor protein that controls spore germination and early development (53, 54). The reduced expression of crp correlates with the observed strong delay in the germination of spores of the osdR null mutant (Fig. 6C). bldG encodes a developmental anti-σ factor antagonist that controls the activity of the stress σ factor σH, bldM and whi encode orphan response regulators that control complex developmental pathways (55), and bldN encodes a σ factor that is required for the transcription of, among other genes, the chp and rdl genes (56, 57). The downregulation of chpCEH contrasts with the upregulation of the other chp genes, which is the first time that such differential regulation has been observed. Interestingly, the chpCEH genes have been shown to belong to the early chp genes and are sufficient to support aerial development, while the other chp genes as well as rdlAB are produced significantly later during development (58). This is again consistent with the concept that OsdR represses sporulation and activates early-development processes.

Differential expression at 36 h. Interestingly, some 200 genes showed deregulated expression at the 36-h time point. These genes include 22 genes in the genomic region between SCO160 and SCO0220, as well as other members of the direct or indirect OsdR regulon that are involved in nitrogen metabolism and anaerobic respiration genes (e.g., nar2, ureAB, nirB, glnD, glnII, glnK, and draK), development (whiE and whiE-like genes, ssgB, chp, and rdl), stress management, etc. (see Table S5 in the supplemental material). These genes all showed a sharp rise or drop of transcription at 36 h in wild-type cells, with transcription recovering at 42 h, while such a sharp change in transcript levels was not seen in the osdR null mutant. The deregulated transcription
of these genes in wild-type cells corresponds to a peak in osdR transcription at 36 h. A sharp peak in the expression of osdR toward the end of exponential growth in liquid cultures was observed by others, both in shake flasks (59) and in a fermentor (60). The transition from exponential to stationary phase roughly corresponds to the onset of aerial growth in surface-grown cultures. Interestingly, another peak in transcription was observed around 5 h after spore germination (61), which may correspond to OsdR’s control of early events.

**Verification by RT-qPCR and EMSAs.** To corroborate the microarray data, reverse transcription-quantitative PCR (qPCR) analysis was performed on independent RNA samples isolated from the mycelia of *S. coelicolor* M145 and its osdR null mutant GSTC2 grown under the same conditions as those used to prepare RNA samples for microarray analysis. The results were normalized using rpsI (SCO4735) as the internal standard, and RNA obtained from mycelia of M145 grown for 24 h was used to normalize the results between the different qPCR runs. Similar trends in expression profiles were observed in both sets of transcript analyses (Fig. 8; see also Fig. S5 in the supplemental material). Expectedly, no osdR transcripts were detected in the osdR null mutant. The peak in the transcription of osdR after 36 h in wild-type cells, both in the microarray and in the qPCR data, again suggests that osdR plays an important regulatory role at this stage of the life cycle (Fig. 8). Downregulation of upsA (SCO0200) in the osdR mutant together with the binding of OsdR to the upstream regulatory element strongly suggests that upsA transcription is transactivated by OsdR. ssgB (SCO1541) transcription was higher in the mutant, which corresponds well with the accelerated development and enhanced spore pigmentation of GSTC2 (Fig. 6). The transcription of SCO5320 and SCO5321, which are part of the whiE gene cluster for the gray spore pigment, was increased at several time points (though whiE transcription also characteristically peaked at 36 h in the wild-type strain).

While no regulatory elements were predicted upstream of ssgB or within the whiE cluster, EMSAs showed specific binding by phosphorylated OsdR to ssgB and to the intergenic region between genes SCO5320 and SCO5321 (Fig. 4B), while the promoters of SCO5319 and SCO5316 (the latter is not shown) were only weakly bound by OsdR in vitro. Considering the lack of binding of nonphosphorylated OsdR to the upstream regions of SCO5316 and SCO5319 and the weak binding of OsdR–P, it is unclear whether these two genes are directly controlled by OsdR in vivo.

**DISCUSSION**

The two-component regulatory system (TCS) formed by OsdK (SCO0203) and OsdR (SCO0204) shows significant sequence similarity to the dormancy TCS in *Mycobacterium tuberculosis* (16). In this work, we show not only that the OsdR binding site conforms very well to the binding site for DevR in *M. tuberculosis* but also that OsdR recognizes the regulatory elements upstream of key genes of the *M. tuberculosis* dormancy regulon and with affinities similar to those of DevR. EMSAs established OsdR binding to short, 50-bp DNA sequences containing the predicted recognition site, and the specificity was validated by the decrease in binding upon changing of one or more nucleotides of the consensus sequence. Thus, the TCS formed by OsdK and OsdR is most likely orthologous to the dormancy control system DosT/DevS/DevR in *M. tuberculosis*. This is further supported by gene synteny, as many genes for USP domain proteins are in the vicinity of the TCSs in the respective organisms. Despite hundreds of millions of years of evolution, some 15 targets are conserved between the DevR-controlled dormancy regulon of *M. tuberculosis* and the regulon predicted to be controlled by OsdR in *S. coelicolor*. Most of these lie in the region around osdR, namely, SCO0167, uspA, osdR, SCO0207, SCO0215, and SCO0216 to SCO0219 (narG2-narJ2).

The sensory kinase OsdK activates its cognate response regulator, OsdR, by phosphorylation and enhances its DNA binding capability, as shown by the enhanced binding of OsdR–P in the EMSAs. Combined, the *in silico* predictions and *in vitro* validation by EMSAs indicate that around 50 genes or gene clusters are controlled directly by OsdR. Analysis of the transcriptional changes in the osdR null mutant by
FIG 8  Microarray and RT-qPCR expression profiles of genes deregulated in the osdR mutant. RNAs for microarray analysis (left) and RT-qPCR (right) profiling were prepared from independent cultures. For time points, see Fig. 7. The expression profiles of the wild type (black circles) and the osdR mutant (gray triangles) from the microarray (left) and RT-qPCR (right) were compared. Genes of interest tested were SCO0200 (uspA), SCO0204 (osdR), SCO1541 (ssgB), and SCO5320 (whiE). See also Fig. S4 in the supplemental material. Note that the graphs are not at the same scale.
global transcription profiling revealed the deregulation of numerous stress-related
genes, including numerous stress-related genes in the region around osdR. A distinctive
pattern of deregulation of developmental genes was evident, with upregulation of
sporulation genes (including whiE, whiI, smeA-ssfA, rdiAB, ssgBG, ramS, and the late chp
genes) and downregulation of genes involved in early development (bldC, bldG, bldM,
bldN, crp, and the early chp genes), which corresponds well to the observed accelerated
development of osdR mutants. The transcriptional data suggest that OsdR controls a
hinge point in development. This is perhaps best illustrated by the divergent transcription
of the chp genes in the osdR mutant. It has previously been shown that the chpCEH
genesis are expressed earlier than the other chp genes and also that the ChpCEH proteins
are sufficient to form the characteristic chaplin layer on the outside the aerial hyphae
and spores and to support aerial growth. BldN was previously shown to control all of
the chp genes (48, 56), which does not explain the difference in chp gene expression
profiles. Our data show that in the osdR null mutant, transcription of bldN and chpCEH
is reduced, while the other chp genes as well as rdiAB are upregulated. Therefore, we
propose that fine-tuning of chp and rdi gene expression is maintained by OsdR.

Some of the differentially expressed genes that lack an obvious consensus se-
quence, in particular the ssgB and genes of the whiE gene cluster, were bound by OsdR
in vitro. This indicates that the OsdR regulon may be larger than anticipated, and some
members of the regulon may be controlled by so-called class II binding sites, in other
words, sites that do not conform to the predicted consensus sequence site. Similar
duality has been shown for many other functionally diverse global regulatory networks
in bacteria, including those controlled by LexA (62) and Crp (63) in E. coli, SpoOA in
B. subtilis (64), CtrA in Caulobacter crescentus (65), and Crp (66), GlnR (67), PhoP (68),
and DasR (69) in Streptomyces. For B. subtilis SpoOA, some 15% of the total binding sites
were not bound in vitro (64).

Extensive studies of the DosT and DevS signaling systems have indicated that,
during hypoxia, the dissociation of oxygen from the SKs results in the transition from
the inactive to the active states of these proteins. With the initial DevR hypoxic
response mediated by DosT, which has a higher dissociation constant than DevS, the
response is then maintained through DevS. Differences in the local structures surround-
ing a heme in either SK result in different oxygen affinities (15, 16). Additionally,
ascorbic acid, nitric oxide, and carbon monoxide also induce the DevR regulon (70). NO
has been shown to activate DosT under aerobic conditions by displacement of oxygen
(71), while DevS acts as a redox sensor of the electron transport system and a decrease
activates the SK under aerobic conditions (72). The similarity of the amino acid residues
involved in signal recognition by DosT/DevS and OsdK suggests that oxygen is the
major candidate as a sensory signal. Indeed, Daigle and colleagues showed that osdR,
as well as many genes in the genomic region around osdR, were strongly upregulated
in wild-type cells under both low-oxygen conditions and when cells were grown with
sodium nitroprusside, an NO donor (10). Additional evidence for the oxygen stress-
related function of OsdR was provided by a study of the proteomes of large versus
small pellets (73), in which oxygen depletion within large pellets—which created local
anaerobic conditions—resulted in the upregulation of various proteins expressed from
the OsdR-controlled SCO0168-SCO0208 genomic region (26).

In liquid-grown cultures, where S. coelicolor forms large mycelial pellets (causing
oxygen transfer problems toward the center of the clump [74]), and on solid-grown
cultures (7), local oxygen depletion occurs. OsdKR-mediated oxygen sensing may well
be responsible for the response to microaerobic conditions, during which the bacte-
rium switches metabolism to meet the challenge of low oxygen. Still, streptomyces
cannot grow anaerobically, despite the presence of an arsenal of genes for enzymes
associated with anaerobic metabolism (75). This has previously been referred to as the
“anaerobic paradox.” This is exemplified by the surprising presence of three nitrate
reductases in S. coelicolor, and our work shows that one of these is directly controlled
by OsdR. Alternatively, S. coelicolor may undergo a state of dormancy as a means of
survival. Indeed, while S. coelicolor cannot grow in oxygen-deprived soil, it is able to
survive periods of anaerobiosis in which it remains dormant (7). Sporulation is a state of dormancy, and the fact that spore germination is significantly delayed in osdR null mutants without affecting spore viability (Fig. 6C) supports the notion that osdR controls this dormancy state. This delay was corroborated independently by imaging the germination of 500 spores of the wild type and the osdR mutant using light microscopy (not shown).

The transcriptional changes at 36 h of growth in the osdR null mutant are noteworthy, and while the results need to be worked out further, they may have major implications for the control of the switch from early- to late-developmental growth. Interestingly, such a clear transition in the global transcriptional profile of S. coelicolor has been reported previously, during growth in a fermentor. Distinctive sharp increases and decreases in the transcription of many genes were observed at this time point, and importantly, this includes several genes of the OsdR regulon, namely, genes in the nitrate reductase cluster adjacent to osdR (SCO0212-SCO0220), bldN, the bldN-controlled chp genes, and several other developmental genes (60). We observed a similar distinctive change in gene expression at 36 h in surface-grown cultures of wild-type cells, with many of the genes of the OsdR regulon, as well as osdR itself, showing expression in the wild-type strain different from that in the osdR null mutant. To some extent, the data from surface- and liquid-grown cultures can be compared, with many developmental genes upregulated in liquid-grown cultures at the time corresponding to the transition from exponential to stationary growth, suggesting that the phase of growth cessation in submerged culture is comparable to the onset of development (59). Our data provide a first indication that OsdR may play a major role in mediating a switch in gene expression during the transition from normal to developmental growth. The transcription of osdR also shows a peak almost immediately after germination (61), which suggests that OsdR may play a similar role during the transition from dormancy to early growth. Such a role of OsdR in mediating a rapid and global change in gene expression requires further investigation.

In summary, the TCS OsdKR of S. coelicolor is orthologous to the dormancy TCS system of M. tuberculosis, with OsdR regulating development and stress management in S. coelicolor. The signal activating this response system is likely related to stress, such as nutrient deprivation or hypoxic stress; however, this remains to be confirmed. OsdK also partners with SCO3818 (9), which adds an extra level of complexity. This also means that deletion of osdR may not completely inactivate the OsdK-based sensory system in S. coelicolor. The system may be even more complicated, as sensory kinase SCO3948 has a higher amino acid identity to OsdK than any other SK encoded by the S. coelicolor genome. Mutational and functional analysis followed by a system-wide analysis of the effects of all possible members of the control system on global gene expression should establish the level of cross talk between the two sensory systems and how they control the stress response of the complex soil bacterium Streptomyces.

MATERIALS AND METHODS

Bacterial strains and media. The bacterial strains described in this work are listed in Table S1 in the supplemental material. E. coli strains JM109 and ET12567 were grown and transformed by standard procedures (76). S. coelicolor A3(2) M145 was the parent for the osdK (GSTC1), osdR (GSTC2 and GSTC3), and osdK (GSTC4) null mutants. S. coelicolor M512 (M145 ΔredD ΔactII-ORF1) (77) was the parent strain for the osdR null mutant GSTC6, and M512 and GSTC6 were the hosts for promoter probing experiments (78). Preparation of protoplasts, transformations, and conjugations were performed according to routine procedures (79). R5 medium was used for regeneration of protoplasts and MS medium (79) for the selection of mutants, for the preparation of spores, and for phenotypic characterization of mutants. To obtain mycelia for transcript analysis, strains were grown on minimal medium (agar plates with mannitol [1%, wt/vol] [79]).

Preparation of gene knockout constructs. Details for all plasmids and mutants are presented in Table S1 in the supplemental material. The gene replacement strategy was as described previously (80) and used the highly unstable vector pWHM3 (81), harboring around 1,500 bp of flanking region on either side of the gene targeted for deletion, and the genes of interest were replaced by the apramycin resistance cassette aacC4 (82). PCRs were performed as previously described (83) with the oligonucleotides listed in Table S2. Plasmids pGW3738 and pGW3737 allowed gene replacement of osdK and osdR, respectively. To create an in-frame osdR deletion mutant (designated GSTC3), construct pGW3737, which carries only the flanking regions, was used for homologous recombination. Construct pGW3738 was
designed for the construction of an in-frame osdRK double mutant (called GSTC4) by combining the upstream region of osdR (obtained from pGWS377) and the downstream region of osdK (obtained from pGWS378). GSTC6 (MS12 ΔosdR) was created for promoter probing purposes using the same approach as for the S. coelicolor M145 osdR mutant.

**Protein isolation, phosphorylation of OsdR, and electrophoretic mobility shift assays.** His6-tagged OsdR and OsdK were overexpressed from plasmids pET0203 and pET0204 in E. coli BL21(DE3) (9). The plasmids were a kind gift from Weihong Jiang (Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China). Proteins were isolated using Ni-nitrotriacetic acid (NTA) chromatography as described previously (84).

In vitro autophosphorylation of 30 pmol of OsdK was performed with [γ-32P]radiolabeled ATP as described previously (9). For transphosphorylation of OsdR, 30 pmol of OsdR was autophosphorylated in 10 μl and incubated for 20 min at 30°C. Following a chill on ice, 80 pmol of OsdR was added. In vitro phosphorylation of OsdR for electrophoretic mobility shift assays (EMSAs) was achieved using the phosphor donor acetyl phosphate (AcP) as described previously (18). EMSAs with [γ-32P]radiolabeled probes were performed as previously described (85).

The OsdR binding site was predicted and used to scan the S. coelicolor genome by PREDetector (20). This binding sequence was investigated by binding assay experiments with wild-type and mutated 50-mers of the predicted binding site upstream of SCO0200. The most-conserved nucleotides in the predicted binding sites (Table 1 and Fig. 2B) were identified, and single (50a, 50b), double (50ab), and quintuple (50x) substitutions were introduced (for 50-mer oligomers, see Table S2 in the supplemental material).

**Promoter probing.** Promoter probing experiments were performed using the redD system as described previously (78). The nonpigmented mutant S. coelicolor M512 lacks the pathway-specific activator genes actII-ORF4 and redD (77). When redD is transcribed from a promoter element cloned into the promoter-probe vector pl2587 (78), the RED biosynthetic pathway is activated, which can be monitored by a nondiffusible red pigment. Constructs for the redD promoter-probe system were created for the promoters of SCO0200, osdR, and SCO0207, using the whiG promoter as the control (Table S1). The promoter fragments were amplified by PCR, and EcoRI/BamHI-digested fragments were cloned into pl2587, resulting in the constructs pGWS345, pGWS1058, pGWS1059, pGWS1060 (for probing of whiG), SCO0200, osdR, and SCO0207.

**Transcript analysis.** RNA was isolated from S. coelicolor M145 (wild-type strain) and its osdR mutant GSTC2 by harvesting biomass from cellophane disks on MM with 1% mannitol after 24, 30, 36, 42, and 54 h of growth. Total RNA was isolated as described previously (85).

**Microarray analysis.** The quality and integrity of the RNA was tested with the Agilent 2100 Bioanalyzer (Agilent Technologies). The RNA was reverse transcribed into cDNA using Cy3-dCTP (http://www.surrey.ac.uk/fhms/microarrays/Downloads/Protocols/index.html). Together with Cy5-dCTP-labeled S. coelicolor M145 genomic DNA as the common reference, the samples were hybridized onto 44,000 60-mer oligonucleotide microarray slides (86). The fluorescent signals on the slides were captured by an Agilent microarray scanner with Feature Extraction software (Agilent Technologies). Within-array normalization (global median) followed by cross-array normalization was performed in R (http://www.r-project.org) using Limma (version 2.5.0) (87, 88). Rank product analysis by means of the R packages RankProd (89) and RankProdIt (90) was applied to identify significantly differentially expressed genes (for which the probability of false prediction [PFP] value was <0.01) between the wild type and mutant at each time point.

**RT-qPCR analysis.** For RT-qPCR analysis, cDNA was generated using the iScript Advanced cDNA synthesis kit (Bio-Rad Laboratories). RT-qPCRs were performed on 200 ng RNA with the iTaq universal SYBR green supermix (Bio-Rad Laboratories), using rpsI (SCO4735) as an internal control. Each reaction mixture was tested in triplicate and for normalization between different plates, with the 24-h wild-type sample as the reference. An average of all three measurements was used to calculate normalized expression.

**Microscopy.** Cryo-scanning electron microscopy was performed as described previously (91) with a JEOL JSM6700F microscope. Stereomicroscopy was done using a Zeiss Lumar.V12 stereomicroscope. Confocal laser-scanning microscopy was performed with a Leica TCS-SP2 microscope and Leica confocal software. Staining of dead and viable Streptomyces filaments and spores was performed as described previously (92) using the cell-impermeable nucleic acid stain SYTO 9 (for live cells). Samples were examined at wavelengths of 488 and 566 nm for excitation and 530 nm (green) and 630 nm (red) for emission.

**Bioinformatics analysis.** Motif searching was performed with InterProScan (93) and Pfam 24.0 (94). Protein homology searches were performed using BLASTp (95). The comparative analysis of the upstream regions of OsdR orthologues was performed with MEME (19), using orthologues from S. coelicolor, S. clavuligerus, S. scabies, S. ghanensis, S. bingchengensis, S. cattleya, S. sviceus, S. vinidochromogenes, S. griseoaurantiacus, Streptococcus sp. E14, Streptococcus sp. TR54, and S. hygroscopicus. The S. coelicolor genome was scanned for possible similar cis-acting regulatory elements using PREDetector (20). The consensus sequence for the predicted binding site of OsdR was visualized using WebLogo (96). The M. tuberculosis DevR binding site logo was created based on the primary DevR binding sites identified in reference 97.

**Accession numbers.** The microarray expression data have been deposited in ArrayExpress (with the accession number E-MTAB-4597). The GenBank nucleotide sequence accession number of M. tuberculosis DosR is P9WGK0, and that of DevR is NP_217648.
SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://dx.doi.org/10.1128/mSystems.00014-16.

Table S1, PDF file, 0.4 MB.
Table S2, PDF file, 0.5 MB.
Table S3, PDF file, 0.3 MB.
Table S4, PDF file, 0.1 MB.
Table S5, PDF file, 1 MB.
Figure S1, TIF file, 1 MB.
Figure S2, TIF file, 2.4 MB.
Figure S3, TIF file, 0.1 MB.
Figure S4, TIF file, 0.3 MB.
Figure S5, TIF file, 0.3 MB.

ACKNOWLEDGMENTS

We are grateful to Weihong Jiang for providing plasmids pET0203 and pET0204 and to Tom Ottenhoff for providing genomic DNA of M. tuberculosis Rv37.

FUNDING INFORMATION

This work, including the efforts of Elodie Tenconi and Sébastien Rigali, was funded by Federal office for Scientific Affairs Belgium (PAI no. P7/44). This work, including the efforts of Giselda Bucca, Emma Laing, and Colin P. Smith, was funded by Biotechnology and Biological Sciences Research Council (BBSRC) (BB/D011582). This work was funded by NWO | Stichting voor de Technische Wetenschappen (STW) (10379).

REFERENCES


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