A *terD* Domain-Encoding Gene (SCO2368) Is Involved in Calcium Homeostasis and Participates in Calcium Regulation of a DosR-Like Regulon in *Streptomyces coelicolor*

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Although *Streptomyces coelicolor* is not resistant to tellurite, it possesses several *terD* domain-encoding (*tdd*) genes of unknown function. To elucidate the function of *tdd8*, the transcriptomes of *S. coelicolor* strain M145 and of a *tdd8* deletion mutant derivative (the Δ*tdd8* strain) were compared. Several orthologs of *Mycobacterium tuberculosis* genes involved in dormancy survival were upregulated in the deletion mutant at the visual onset of prodiginine production. These genes are organized in a putative redox stress response cluster comprising two large loci. A binding motif similar to the dormancy survival regulator (DosR) binding site of *M. tuberculosis* has been identified in the upstream sequences of most genes in these loci. A predicted role for these genes in the redox stress response is supported by the low NAD+/NADH ratio in the Δ*tdd8* strain. This *S. coelicolor* gene cluster was shown to be induced by hypoxia and NO stress. While the *tdd8* deletion mutant (the Δ*tdd8* strain) was unable to maintain calcium homeostasis in a calcium-depleted medium, the addition of Ca²⁺ in Δ*tdd8* culture medium reduced the expression of several genes of the redox stress response cluster. The results shown in this work are consistent with Tdd8 playing a significant role in calcium homeostasis and redox stress adaptation.

*S. coelicolor* M145 is a soil-dwelling, obligatory aero-bic Gram-positive bacterium with high G+C content (1). This filamentous bacterium is characterized by morphological development starting from vegetative hyphae which grow from their tips and, after a short transition period where programmed cell death takes place, give rise to an aerial mycelium, eventually fragmenting to produce chains of smooth spores (2). The transition period and the aerial mycelium growth period also mark the time when numerous and various genes are modulated in order to allow the production of secondary metabolites, such as the prodigine (Red) antibiotic. Although no or limited sporulation of *S. coelicolor* occurs in liquid media, morphological differentiation evidenced by the formation of a first compartmentalized mycelium which is then replaced by a second multinucleated mycelium has been observed (3, 4).

*S. coelicolor* M145 has a large genome of 7,845 genes whose expression is regulated by complex control systems that allow the growth and the survival of the bacterium under conditions of different environmental stresses. The 21 *terD* domain-encoding genes (*tdd* genes) appear among the genes that are often expressed under stress conditions (5–9). The *TerD* motif, defined as a bacterial stress protein motif (10), was originally associated with Gram-negative bacteria, where it was shown to be involved in conferring resistance to tellurite and toxic xenobiotic compounds. However, *S. coelicolor* does not exhibit resistance to tellurite (11). In addition to *S. coelicolor*, other actinobacteria, *Firmicutes*, and *Proteobacteria*, as well as primitive eukaryotes such as amoebas and diatoms, have genes with *TerD*-like motifs. The presence of *TerD* domain-encoding genes in a wide range of microorganisms suggests that these genes provide a selective advantage in the natural environment, and it has been proposed that tellurite resistance was a consequence of another undetermined biological function and was not the principal role of this class of proteins (10, 12).

While the function of *S. coelicolor* *tdd* genes remains enigmatic, several *tdd* genes appear to be involved in morphological differentiation. For instance, it was demonstrated that deletion or overexpression of *tdd7*, *tdd8*, or *tdd13* affected morphological differentiation or spore production in *S. coelicolor* (11, 13). A strain with a deletion in *tdd8* (SCO2368) produced long chains of short spores with a dense spore wall, while a strain overexpressing *tdd8* produced very few spores of irregular shapes and sizes (11). Several studies also suggest that Tdd8 abundance is modulated in response to different stress conditions such as nitrogen deficiency (8), ethanol shock (5), and growth in the presence of plant extracts (6). Tdd8 is one of the most abundant proteins of the *S. coelicolor* proteome (7) and secretome (6, 14). Tdd8 was overproduced even during the transition phase in cells undergoing programmed cell death (2). As several Tdd proteins, including Tdd8, exhibit calcium binding properties, it has been suggested that these proteins may play an important role in calcium homeostasis (10, 12). Whereas the role of calcium in signal transduction is well established in eukaryotes, it is still elusive in prokaryotes. Nevertheless,

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There is evidence that calcium ions regulate differentiation and adaptation to environmental stresses in bacteria (15).

This study aimed to decipher the function of Tdd8 in *S. coelicolor* by comparing the global gene expression profile, determined by high-density microarrays, of the wild M145 strain with that of the derivative deletion mutant (the Δtdd8 strain). The results from this study suggest that Tdd8 participates in both calcium and redox homeostasis.

**MATERIALS AND METHODS**

**Bacterial strains and culture conditions.** *S. coelicolor* strains M145 (wild strain) and M145Δscc02568 (the Δtdd8 strain) (11) were used in this study. These strains were cultivated at 30°C in liquid culture media in a shaking incubator (250 rpm). Apramycin was added to the Δtdd8 culture media (25 μg/ml) used for the microarray study. For spore production, strains were grown on sporulating mannitol-soya flour-agar medium (MS) (16) for 5 to 7 days. For gene expression studies, *Streptomyces* strains were cultivated by adding 20 μl of a dense spore suspension (5.10^6 CFU/ml) to 50 ml of R5+ or R5− medium. Both media consisted of modified versions of the R5 medium (16). The R5− medium (pH 7.2) was deprived of KH_2PO_4, CaCl_2, and l-proline and supplemented with 6% polyethylene glycol (PEG) 8000 to ensure cell dispersal (3, 16), while the R5+ medium was similar to R5− medium but contained 9 μM CaCl_2. Bacteria were grown in 250-ml flasks containing 4 g of glass beads (3-mm diameter). When appropriate, 2 mM sodium nitroprusside (SNP) was added to the culture medium. *S. coelicolor* cultures could also be grown under conditions of low oxygen concentrations by incubating them within GasPak EZ Campy Container System sachets following the instructions of the manufacturer (BD). A growth curve in the R5− medium was determined for each *Streptomyces* strain by periodically measuring the optical density of the culture at 450 nm. For DNA extraction of *S. coelicolor* M145, the bacterium was grown in 25 ml yeast extract-malt extract (YE) medium (16) for about 40 h.

**RNA extraction and cDNA labeling for the microarray study.** To examine the changes in transcriptome profiles associated with the presence or absence of the *tdd8* gene, total RNA was extracted from the wild strain and the Δtdd8 strain at the visual onset of Red production.

Prior to RNA extraction, culture samples were treated with RNA Protect Bacterium Reagent (Qiagen) and the bacterial cells were pelleted by centrifugation (3,450 × g for 10 min) and resuspended in 1 ml TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Cells were centrifuged and pelleted again and stored at −80°C. For RNA extraction, a hybrid protocol between the procedures proposed by the manufacturers of the RNeasy Plus mini kit (Qiagen) and mirVana miRNA isolation kit (Ambion) was developed. Bacteria were resuspended in 0.2 ml lysozyme buffer (15 mg/ml in TE) and incubated for 15 min at room temperature. RLT buffer (0.6 ml) from a Qiagen RNeasy Plus mini kit was added, and samples were transferred to 2-ml tubes containing a stainless steel bead and agitated on a TissueLyser (Qiagen) twice for 2 min. The mixture was centrifuged at 10,000 × g for 5 min, and the supernatant was recovered. Two phenol-chloroform extractions and a final chloroform extraction were performed. The upper aqueous layer was transferred to a genomic DNA (gDNA) elimination filter (Qiagen RNeasy Plus minikit) and centrifuged at 10,000 × g for 30 s. The extraction continued following the manufacturer’s protocol for a mirVana mRNA isolation kit (Ambion). The RNA was eluted with 100 μl of purified water and quantified using a NanoDrop ND-1000 spectrophotometer. The RNA quality was checked using an Agilent Bioanalyzer RNA chip. Fluorescently labeled cDNA was produced for each RNA sample by reverse transcription (RT); 10 to 15 μg of RNA was used for each cDNA synthesis/labelling procedure in a total volume of 30 μl with 5.1 μl of random primers (Invitrogen). The RNA-primer mix was denatured at 70°C for 10 min. After snap cooling on ice, 6 μl of First Strand Buffer, 3 μl of 100 mM dithiothreitol (DTT), 0.6 μl of a deoxy-nucleoside triphosphate (dNTP) solution (25 mM dATP, dGTP, and dTTP and 10 mM dCTP), 400 U of SuperScript III (Invitrogen), and 1.5 μl Cy3-CTP were added. The reaction mixtures were incubated in the dark at 25°C for 10 min, to allow primers to anneal, and then at 42°C for 4 h.

**Genomic DNA labeling.** DNA of *S. coelicolor* M145 was extracted according to the procedure described by Kieser et al. (16). Fluorescently labeled genomic DNA (gDNA) was generated using 2 to 3 μg DNA that was added to 3 μg random primers in a total volume of 41.5 μl and was denatured at 95°C for 5 min. A 1-μl volume of a dNTP mixture (5 mM dATP, dGTP, and dTTP and 2 mM dCTP), 1.5 μl Cy5-dCTP, 5 μl Klenow DNA polymerase buffer, and 1 μl of Klenow polymerase (5 U/μl) were added to the DNA solution, and the mixture was incubated overnight at 37°C in the dark. The labeled samples were purified through the use of a MiniElute PCR purification kit (Qiagen).

**Microarray hybridization and data processing.** *S. coelicolor* M145 genome arrays (format 2 × 105K OGT v3 microarray; Agilent Technologies), designed by Oxford Gene Technology in collaboration with Colin P. Smith’s team at the University of Surrey (United Kingdom), were used in this study (17). The cDNA synthesis and labeling and genomic DNA labeling were done as previously described (http://www.surrey.ac.uk/lhms/microarrays/Downloads/Protocols/Strep_hyb_protocol_1005.pdf). For hybridization in a microarray, 40 pmol of Cy3 and 30 pmol of Cy5-labeled cDNA and gDNA (respectively) in a total volume of 104 μl were mixed with 26 μl of Agilent blocking buffer and 130 μl of 2× hybridization buffer (oligonucleotide aCGH hybridization kit; Agilent Technologies) and denatured at 95°C for 3 min. After loading of the labeled samples on the OGT v3 microarray, the slide was placed in a hybridization chamber (Agilent Technologies) and rotated at 20 rpm for 40 h at 65°C. Each slide was washed into Wash 1 oligonucleotide cGH buffer (Agilent Technologies) for 5 min and then successively transferred into warm (37°C) Wash 2 oligonucleotide cGH buffer (Agilent Technologies) for 1 min, an acetonitrile-filled container for 1 min, and a drying and stabilization solution (Agilent Technologies) for 30 s.

Microarrays were scanned at 532 nm for the Cy3 channel and 635 nm for the Cy5 channel with a Agilent DNA microarray dual-laser scanner. The resulting images were processed using Agilent Feature Extraction software (v. 9.1, Agilent Technologies) and the default protocols except that “no within-array normalization” and background correction were used. The feature extraction output files were imported into R (software programming language) (version 2.5.0; http://www.R-project.org) and normalized using the LIMMA package (18); global median within-array normalization followed by “scale” across-array normalization was applied to the log([cDNA/gDNA]) ratios of the expression arrays. Flagging of poor-quality spots was performed (19), and probes were filtered out of the data set if they did not yield a good-quality spot across gene expression data sets. Probes targeting the coding regions of a gene were averaged such that each annotated protein-encoding gene of *S. coelicolor* was represented by a single value. The averaged data sets from two independent experiments were then analyzed for differential levels of expression in strain M145 and the Δtdd8 strain using rank product analysis at P < 0.01.

**Bioinformaticss.** Potential regulatory motifs were predicted with a two-step MEME search. The MEME program (20) is a tool for motif discovery resulting in an output of a position-specific scoring matrix (PSSM) representing the predicted motif. The input for the MEME search was built using 300-bp upstream sequence segments of a subset of genes identified by transcriptomic analysis. The PSSM for the predicted binding box was then used to search all the upstream sequences of genes in the entire *S. coelicolor* genome using the MAST algorithm (20), and only motifs with an E value of <10 were considered.

**RT-qPCR.** The expression of several genes of interest was investigated under various growth conditions. RNA was extracted from strain M145 and Δtdd8 strain cultures as described above. cDNA synthesis was carried out on 2 μg of RNA using a SuperScript III first-strand synthesis system for RT-PCR (Invitrogen) according to the manufacturer’s specifications. Primers used for reverse transcription real-time quantitative PCR (RT-qPCR) are listed in Table 1. Real-time PCR was run on a Stratagene Mx3000P system (Agilent Technologies Inc.). Reaction mixtures con-
green Supermix (Bio-Rad) and 1

TABLE 1

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Ca2+ or the developmental stage on gene expression in strain M145 and the Δtdd8 strain were calculated relative to gyrA expression using the following threshold cycle (Ct) formula: 2–(ΔΔCt) = (ΔCt target gene – CT gyrA) (ΔCt method), Statistix 9 software was used to determine significantly statistically different groups by analysis of variance (ANOVA) followed by a least significant difference (LSD) test. The effect of stress on gene expression in the wild strain was determined using the ΔΔCt method (21). Fold changes were calculated and analyzed with REST 2009 software (22).

**NAD/NADH ratio quantification.** The ratios of NAD and its reduced form (NADH) were determined on cell extracts with a NAD/NADH quantitation kit (Sigma-Aldrich). S. coelicolor cultures were sampled at the onset of Red production and after 66 h of growth, and the cells were then pelleted (10,000 × g). Cell extracts were obtained according to the manufacturer’s instructions. Two samples of each culture were analyzed, and the experiment was repeated three times.

**Intracellular free calcium concentration measurements.** The concentration of free intracellular Ca2+ ([Ca2+]i) was monitored using the calcium-sensitive fluorescent indicator Fura-2 acetoxyethyl (AM) (ATT Bioquest). Strain M145 and the Δtdd8 strain were grown in R5 medium supplemented or not supplemented with CaCl2 or SNP (see the description of the growth conditions for details). Culture samples (3 ml) were taken at the visual onset of Red antibiotic production (Red onset) and washed twice by centrifugation in 100 mM KCl–30 mM MOPS (morpholinepropanesulfonic acid) solution buffer, pH 7.2. Fura-2 AM (5 μM final concentration) was added to the cell suspension and incubated at 30°C for 60 min. For Fura-2 excitation, cells were illuminated at two alternating wavelengths, 340 and 380 nm, and emitted light was monitored at 530 nm (23–25) using a PTI spectrofluorometer equipped with Felix software. The [Ca2+]i was calculated based on a standard curve for Fura-2 made using a calcium calibration buffer kit (Biotium) following the manufacturer’s instructions. The experiments were performed with four biological replicates.

**RESULTS**

Deletion of the tdd8 gene modifies the transcriptome profile of *S. coelicolor*. As growth kinetics differed within strains (Fig. 1), the transcriptomes of strain M145 and the Δtdd8 strain were compared at the visual onset of Red production, which corresponds to the transition period between the compartmentalized-mycelium (first mycelium) and multinucleated-mycelium (second mycelium) states in a liquid culture (3,4). To ensure that the differences observed in the microarray experiments were not simply due to differences in the growth stages, microarray results were validated for several genes by performing RT-qPCR on cDNA from bacteria of the wild and deletion strains sampled at the visual onset of Red production (Table 2) and at different time points (Fig. 2). Microarray analyses revealed that the transcriptome profile of the deletion strain was determined using the gyrA reference gene (SCO3873) was used in all RT-qPCR studies as an internal control since its expression levels remained constant under all tested conditions (data not shown). The effects of the presence of

FIG 1 Growth curve of *Streptomyces coelicolor* strains in R5 medium represented as the optical density at 450 nm (O.D.450 nm) of subsamples diluted 10 times. The arrows indicate the visual onset of Red production.
strain was significantly altered at this growth stage. Compared to the wild strain, a total of 267 genes showed differential (either up- or downregulated) levels of expression at the onset of Red biosynthesis in the Δtdd8 strain (see Table S1 in the supplemental material). Upregulated genes included the gene cluster responsible for calcium-dependent antibiotic production (SCO3210 to SCO3222 and SCO3227 to SCO3249) and two conserved operons (cvn7 and cvn9) of unknown function.

Several genes that are known to be involved in morphological differentiation and sporulation were differentially expressed in the Δtdd8 strain (Table 3). For example, expression of most chaplin genes, coding for proteins forming a filamentous sheath on aerial mycelium and spores, was increased in the Δtdd8 strain at the onset of Red production. Furthermore, wblA and sapA, encoding spore-associated proteins, as well as a whiB-like gene, were overexpressed in the absence of Tdd8 (Table 3).

Redox homeostasis is impaired in the Δtdd8 strain. The genome sequence of S. coelicolor reveals a number of genes related to those of facultative anaerobes that are involved in adaptation to anaerobic conditions and growth under O2-limiting conditions (26). Several of these genes were upregulated in the Δtdd8 strain at the onset of Red biosynthesis. Table 4 shows the list of these genes, their probable functions, and their characteristics.

Two gene loci located in an arm region of the chromosome were clearly upregulated in the Δtdd8 strain at the visual onset of Red biosynthesis. The first region is located between SCO0161 and SCO0181 and the second between SCO0197 and SCO0220. Of the 21 genes in the first locus (SCO0161 to SCO0181), 17 were more expressed in the deletion mutant (Table 4). This first locus includes a gene encoding the Crp/Fnr-like transcriptional regulator (SCO0168), a putative alcohol dehydrogenase (SCO0179), five universal stress protein (USP) genes (SCO0167, SCO0172, SCO0178, SCO0180, and SCO0181), a nitrreductase gene (SCO0162), cystathionine β-synthase (CBS)-domain encoding genes (SCO0169 and SCO0170), and a gene coding for a probable pyridoxamine 5'-phosphate oxidase (SCO0174).

In the second locus, 20 of 24 genes were upregulated in the Δtdd8 strain (Table 4). This locus includes orthologs of the respiratory nitrreductase genes (SCO0216 to SCO0219) of Bacillus subtilis along with SCO0213, a gene sharing similarity with narK2 of M. tuberculosis that encodes a nitrreductase transporter. It also includes genes sharing sequence similarity with dosS, dosR, and dosT of M. tuberculosis (SCO0203, SCO0204 and SCO0211), a two-component regulatory system responding to a reduced respiratory chain. Several genes of this second locus were related to those found in the first one: a putative alcohol dehydrogenase gene (SCO0199), a putative nitrreductase gene (SCO0215), genes encoding proteins with USP motifs (SCO0198 and SCO0200), a CBS motif (SCO0210), or a pyridoxamine 5'-phosphate oxidase motif (SCO0197 and SCO0214). Several genes of these two loci have orthologs found in M. tuberculosis, where they have been shown to be regulated by DosR (DevR) in response to hypoxia and NO (28).

To ensure that the differential levels of expression of the gene loci were not due to the differences in the growth rates between the strains, the levels of expression of two genes of each locus (SCO0171, SCO0174, SCO0213, and SCO0214) and of ureA (SCO0136), a gene involved in nitrogen metabolism, were determined by RT-qPCR at different time points during the growth of both strain M145 and the Δtdd8 strain. While the expression of genes SCO0171, SCO0174, and SCO0215 peaked at the visual onset of Red production in both the wild strain and the mutant, these genes were substantially overexpressed in the Δtdd8 strain (Fig. 2). SCO0212 was also clearly overexpressed in the Δtdd8 strain not only at the onset of Red production but also in other developmental stages (Fig. 2). These data indicate that the overexpression of the two gene clusters in the deletion mutant is attributable to the loss of Tdd8. The slight differences in ureA expression levels observed between strains might, however, be attributable to their respective growth rates (Fig. 2).

Other genes located outside the two loci, but also possibly involved in “redox stress responses,” were upregulated in the Δtdd8 strain. This is the case for SCO7310, a gene encoding a member of the Crp/Fnr transcriptional regulators; the cydABC gene operon; the succinate dehydrogenase/fumarate reductase gene; and orthologs of the B. subtilis narR and hmpA11 regulation system that are implicated in NO detoxification (30). The latter genes and the two loci described above may represent a cluster of genes that respond to redox stress. Consequently, the effect of redox stress on the

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**TABLE 2**: RT-qPCR validation of microarray data for 10 genesa

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<th>Log2-transformed fold changeb</th>
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a Fold change data of the Δtdd8 strain compared to the wild strain were log2 transformed.
b Regression analysis generated slope y = 0.797x − 0.063 and R² = 0.7444.

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**FIG 2**: Expression levels (± standard deviations [SD]) of five genes determined by RT-qPCR and calculated relative to gyrA expression (ΔCt method), in the wild (squares) and Δtdd8 (circles) strains, as a function of the developmental stage.
expression of 11 selected genes of the putative redox stress response cluster (8 genes of the redox stress response loci, \(tdd8\), and 2 genes located outside the loci) was analyzed in \(S. \) coelicolor M145 cultures.

The differential levels of expression of these 11 genes between M145 cultures grown under stress or control conditions are shown in Table 5. Hypoxia and SNP, a NO donor, induced expression of all the genes tested. In contrast, hypoxia and the nitrosative stress had a significant negative effect on \(tdd8\) expression (Table 5).

Upregulation of genes associated with the putative redox stress response cluster in the \(\Delta tdd8\) strain suggests that the deletion of \(tdd8\) causes a redox imbalance in \(S. \) coelicolor. The values determined for the NAD/NADH ratio, an indicator of redox status in the bacterial cells, differed at the onset of Red production within the \(\Delta tdd8\) strain and M145, suggesting that there is indeed a redox imbalance. The ratio was significantly lower in the \(\Delta tdd8\) strain (1.01 ± 0.18) than in the wild strain (5.23 ± 0.79). After 66 h of growth, the NAD/NADH ratios were low for both strains (1.55 ± 0.19 and 1.30 ± 0.05 for strains M145 and the \(\Delta tdd8\) strain, respectively).

Table 5: Differential expression of genes known to be involved in \(S. \) coelicolor morphological differentiation at the onset of Red in the \(\Delta tdd8\) strain, revealed by microarray analyses

<table>
<thead>
<tr>
<th>SCO no.</th>
<th>Assigned gene</th>
<th>Protein function</th>
<th>Relative expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCO0409</td>
<td>sapA</td>
<td>Spore-associated protein precursor</td>
<td>3.31 ± 1.00</td>
</tr>
<tr>
<td>SCO1674</td>
<td>chpC</td>
<td>Chaplin C</td>
<td>2.33 ± 0.81</td>
</tr>
<tr>
<td>SCO1675</td>
<td>chpH</td>
<td>Chaplin H</td>
<td>6.20 ± 1.60</td>
</tr>
<tr>
<td>SCO1800</td>
<td>chpE</td>
<td>Chaplin E</td>
<td>10.09 ± 1.03</td>
</tr>
<tr>
<td>SCO2699</td>
<td>chpG</td>
<td>Chaplin G</td>
<td>2.90 ± 1.02</td>
</tr>
<tr>
<td>SCO2717</td>
<td>chpD</td>
<td>Chaplin D</td>
<td>4.40 ± 1.33</td>
</tr>
<tr>
<td>SCO3323</td>
<td>bldN</td>
<td>Sigma factor bldN</td>
<td>22.53 ± 1.47</td>
</tr>
<tr>
<td>SCO3324</td>
<td>rsbN</td>
<td>Anti-sigma factor</td>
<td>2.97 ± 0.94</td>
</tr>
<tr>
<td>SCO3579</td>
<td>wblA</td>
<td>Regulatory protein</td>
<td>4.34 ± 1.24</td>
</tr>
<tr>
<td>SCO4768</td>
<td>bldM</td>
<td>Two-component regulator</td>
<td>4.64 ± 1.30</td>
</tr>
<tr>
<td>SCO5112</td>
<td>bldKA</td>
<td>ABC transport system integral membrane protein</td>
<td>0.78 ± 0.23</td>
</tr>
<tr>
<td>SCO5113</td>
<td>bldKB</td>
<td>ABC transport system lipoprotein</td>
<td>0.49 ± 0.03</td>
</tr>
<tr>
<td>SCO5114</td>
<td>bldKC</td>
<td>ABC transport system integral membrane protein</td>
<td>0.41 ± 0.16</td>
</tr>
<tr>
<td>SCO5116</td>
<td>bldKE</td>
<td>Peptide transport system ATP-binding subunit</td>
<td>0.37 ± 0.08</td>
</tr>
<tr>
<td>SCO5147</td>
<td>sigE</td>
<td>Sigma factor E</td>
<td>2.39 ± 0.74</td>
</tr>
<tr>
<td>SCO7647</td>
<td>cabC</td>
<td>Calcium-binding protein</td>
<td>2.24 ± 0.69</td>
</tr>
</tbody>
</table>

Identification of a DosR-like binding motif in several genes of the \(S. \) coelicolor redox stress response gene cluster. The presence of two gene loci where most genes were simultaneously upregulated suggested the presence of common binding sites in the promoter regions for these genes. Several of these predicted gene products also share sequence similarity with those of \(M. \) tuberculosis that belong to the DosR regulon. A putative binding motif search using the upstream sequences of genes upregulated within the two genetic loci whose data are presented in Table 5 (see Table S1 in the supplemental material) revealed a regulatory motif with the exception of the two developmental genes \(nsrR\) and \(SigH\) (Table 2) was determined in both the \(\Delta tdd8\) strain and strain M145 at the onset of Red biosynthesis. The level of transcription was significantly higher in the \(\Delta tdd8\) strain than in the wild strain for all genes tested (Fig. 5) in the R5− medium. In the \(\Delta tdd8\) strain, addition of calcium significantly reduced the expression of all genes tested, with the exception of the two developmental genes \(chpC\) and \(sigE\) (SCO1674 and SCO5147) and genes \(nsrR\) and \(hmpA1\) (SCO7427 and SCO7428), where no DosR-like binding motif is observed. The level of transcription of genes CO0162, CO0168, CO0171, CO0174, CO0204, CO0212, CO0215, and CO0216 in the \(\Delta tdd8\) strain grown in R5+ medium was similar to that observed in wild M145 grown in R5− medium. Growth of M145 in the presence of a higher calcium concentration did not affect gene expression or affected it only slightly.
### TABLE 4 Characteristics of a gene cluster possibly involved in redox stress response overexpressed in the ΔtddΔ8 strain compared to the wild-type strain at the onset of Red

<table>
<thead>
<tr>
<th>SCO no.</th>
<th>Putative function(s) of the corresponding protein</th>
<th>Fold change(s)</th>
<th>Comments (reference)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>First redox stress response locus</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SCO0161</td>
<td>Hypothetical protein</td>
<td>3.27 ± 0.26</td>
<td>32.6% identity to Agc nitroreductase of <em>M. tuberculosis</em> where the corresponding gene is induced by hypoxia and regulated by DosR (27)</td>
</tr>
<tr>
<td>SCO0162</td>
<td>Nitroreductase</td>
<td>5.92 ± 0.86</td>
<td>56.7% and 47.3% identity to SCO0169 and SCO0210, respectively</td>
</tr>
<tr>
<td>SCO0165</td>
<td>Hypothetical protein</td>
<td>3.45 ± 0.25</td>
<td>27.6% identity to PncB (nicotinate phosphoribosyltransferase) from <em>Escherichia coli</em> involved in NADH/NAD homeostasis (29)</td>
</tr>
<tr>
<td>SCO0166</td>
<td>Regulator</td>
<td>2.32 ± 0.25</td>
<td></td>
</tr>
<tr>
<td>SCO0167</td>
<td>Universal stress protein</td>
<td>19.71 ± 0.14</td>
<td></td>
</tr>
<tr>
<td>SCO0168</td>
<td>Transcriptional regulator</td>
<td>27.13 ± 0.94</td>
<td>Member of CRP/FNR family of transcriptional regulators</td>
</tr>
<tr>
<td>SCO0169</td>
<td>Cystathionine β-synthase</td>
<td>18.07 ± 0.16</td>
<td>31.5% identity to Hrp1, a hypoxic response protein regulated by DosR in <em>M. tuberculosis</em> (28)</td>
</tr>
<tr>
<td>SCO0170</td>
<td>Cystathionine β-synthase</td>
<td>8.40 ± 0.37</td>
<td>26.2% identity to PncB (nicotinate phosphoribosyltransferase) from <em>Escherichia coli</em> involved in NADH/NAD homeostasis (29)</td>
</tr>
<tr>
<td>SCO0171</td>
<td>Nicotinate phosphoribosyltransferase</td>
<td>4.93 ± 0.34</td>
<td></td>
</tr>
<tr>
<td>SCO0172</td>
<td>Universal stress protein</td>
<td>3.47 ± 0.43</td>
<td></td>
</tr>
<tr>
<td>SCO0173</td>
<td>OsmC</td>
<td>6.94 ± 2.62</td>
<td></td>
</tr>
<tr>
<td>SCO0174</td>
<td>Pyridoxamine 5'-phosphate oxidase</td>
<td>16.85 ± 2.56</td>
<td>38% identity to Rv0569 from <em>M. tuberculosis</em> where the corresponding gene is regulated by DosR (28)</td>
</tr>
<tr>
<td>SCO0177</td>
<td>Membrane protein</td>
<td>12.01 ± 1.86</td>
<td></td>
</tr>
<tr>
<td>SCO0178</td>
<td>Universal stress protein</td>
<td>2.15 ± 0.11</td>
<td></td>
</tr>
<tr>
<td>SCO0179</td>
<td>Alcohol dehydrogenase</td>
<td>14.00 ± 0.31</td>
<td></td>
</tr>
<tr>
<td>SCO0180</td>
<td>Universal stress protein</td>
<td>4.80 ± 1.31</td>
<td></td>
</tr>
<tr>
<td>SCO0181</td>
<td>Universal stress protein</td>
<td>9.48 ± 1.56</td>
<td></td>
</tr>
<tr>
<td><strong>Second redox stress response locus</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SCO0197</td>
<td>Pyridoxamine 5'-phosphate oxidase</td>
<td>7.10 ± 1.16</td>
<td>42.1% identity to Rv0080 of <em>M. tuberculosis</em> where the corresponding gene is regulated by DosR (28); 62.2% and 31.1% identity to SCO0214 and SCO0174, respectively</td>
</tr>
<tr>
<td>SCO0198</td>
<td>Universal stress protein</td>
<td>6.07 ± 1.00</td>
<td></td>
</tr>
<tr>
<td>SCO0199</td>
<td>Alcohol dehydrogenase</td>
<td>10.90 ± 2.32</td>
<td></td>
</tr>
<tr>
<td>SCO0200</td>
<td>Universal stress protein</td>
<td>13.49 ± 4.25</td>
<td></td>
</tr>
<tr>
<td>SCO0201</td>
<td>Thiosulfate dehydrogenase (quinone)</td>
<td>10.24 ± 1.41</td>
<td></td>
</tr>
<tr>
<td>SCO0203</td>
<td>DosS-like</td>
<td>2.38 ± 0.18</td>
<td>43.5% identity to redox sensor histidine kinase DosS of <em>M. tuberculosis</em> where the corresponding gene is regulated by DosR (28)</td>
</tr>
<tr>
<td>SCO0204</td>
<td>DosR-like</td>
<td>12.63 ± 0.71</td>
<td></td>
</tr>
<tr>
<td>SCO0208</td>
<td>Pyruvate phosphate dikinase</td>
<td>3.48 ± 0.68</td>
<td></td>
</tr>
<tr>
<td>SCO0209</td>
<td>Hypothetical protein</td>
<td>9.32 ± 0.19</td>
<td></td>
</tr>
<tr>
<td>SCO0210</td>
<td>Cystathionine β-synthase</td>
<td>4.00 ± 0.25</td>
<td></td>
</tr>
<tr>
<td>SCO0211</td>
<td>DosT-like</td>
<td>2.93 ± 0.25</td>
<td></td>
</tr>
<tr>
<td>SCO0212</td>
<td>Hemerythrin domain-containing protein</td>
<td>21.77 ± 1.15</td>
<td></td>
</tr>
<tr>
<td>SCO0213</td>
<td>Nitrate/nitrite transporter</td>
<td>15.82 ± 5.56</td>
<td>49.4% identity to NarK2 of <em>M. tuberculosis</em> where the corresponding gene is regulated by DosR (28)</td>
</tr>
<tr>
<td>SCO0214</td>
<td>Pyridoxamine 5'-phosphate oxidase</td>
<td>8.03 ± 0.36</td>
<td>46.5% identity to Rv0080 of <em>M. tuberculosis</em> where the corresponding gene is regulated by DosR (28); 62.2% and 36.6% identity to SCO0197 and SCO0174, respectively</td>
</tr>
</tbody>
</table>

(Continued on following page)
The effect of various calcium concentrations (0 μM, 1 μM, 5 μM, and 9 μM) on the expression of the \textit{tdd8} strain was also determined in M145 at the onset of Red biosynthesis. No significant expression differences between treatments were observed (data not shown).

**DISCUSSION**

Little information is available on the role and function of \textit{tdd} genes in \textit{S. coelicolor}, but Tdd8 shows calcium binding properties.

**TABLE 4 (Continued)**

<table>
<thead>
<tr>
<th>SCO no.</th>
<th>Putative function(s) of the corresponding protein</th>
<th>Fold change(s)(^a)</th>
<th>Comments (reference)(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCO0215</td>
<td>Nitroreductase</td>
<td>2.93 ± 0.71</td>
<td>35.7% identity to \textit{Rv3131}, a nitroreductase from \textit{M. tuberculosis} where the corresponding gene is induced by hypoxia and regulated by DosR ((28)); 43.0% identity to SCO0162</td>
</tr>
<tr>
<td>SCO0216-SCO0219</td>
<td>Respiratory nitrate reductases G2, H2, I2, and J2</td>
<td>NarG2, 12.94 ± 0.63; narH2, 11.34 ± 1.00; narJ2, 8.14 ± 2.31; narJ2, 16.89 ± 1.90</td>
<td>63.3%, 60.6%, 43.1%, and 47.7% identity to \textit{M. tuberculosis} nitrate reductases NarG, NarH, NarJ, and NarX, respectively, where narX is regulated by DosR ((27))</td>
</tr>
<tr>
<td>SCO0220</td>
<td>Hypothetical protein</td>
<td>10.76 ± 0.75</td>
<td>SnoA, domain-containing protein</td>
</tr>
</tbody>
</table>

\(^a\) Determined by the microarray study.

\(^b\) CRP, cyclic AMP receptor protein; FNR, fumarate and nitrate reduction regulator.

**TABLE 5 Differential levels of gene expression between \textit{S. coelicolor} M145 (wild strain) cultures exposed to a low concentration of O\(_2\) or to SNP and control cultures**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Putative function of the corresponding protein or description</th>
<th>Fold change determined by RT-qPCR(^a)</th>
<th>Low (5%–15%) SNP O(_2) concn (2 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCO0162</td>
<td>Nitroreductase</td>
<td>26.8</td>
<td>22.8</td>
</tr>
<tr>
<td>SCO0168</td>
<td>Crp/Fnr-like protein</td>
<td>11.2</td>
<td>2.0</td>
</tr>
<tr>
<td>SCO0171</td>
<td>Nicotinate phosphoribosyltransferase</td>
<td>4.6</td>
<td>3.2</td>
</tr>
<tr>
<td>SCO0174</td>
<td>Pyridoxamine 5(^{\prime})-phosphate oxidase</td>
<td>13.4</td>
<td>10.1</td>
</tr>
<tr>
<td>SCO0204</td>
<td>DosR-like</td>
<td>7.2</td>
<td>3.9</td>
</tr>
<tr>
<td>SCO0212</td>
<td>Hemerythrin motif-containing protein</td>
<td>2.4</td>
<td>2.8</td>
</tr>
<tr>
<td>SCO0215</td>
<td>Nitroreductase</td>
<td>8.1</td>
<td>3.0</td>
</tr>
<tr>
<td>SCO0216</td>
<td>NarG2</td>
<td>1.6</td>
<td>1.9</td>
</tr>
<tr>
<td>SCO2368</td>
<td>Tdd8</td>
<td>0.2</td>
<td>0.5</td>
</tr>
<tr>
<td>SCO7427</td>
<td>NsrR</td>
<td>14.9</td>
<td>8.6</td>
</tr>
<tr>
<td>SCO7428</td>
<td>HmpA1</td>
<td>41.4</td>
<td>15.0</td>
</tr>
</tbody>
</table>

\(^a\) All values were significantly different from those of the control treatment (nonsupplemented R5– medium) at \(P < 0.05\).
A mutation mutant might therefore represent a compensatory response to the absence of Tdd8.

The most striking observation at the onset of Red production was, however, the fact that several genes involved in the redox stress response were overexpressed in the Δtdd8 strain. Most of these genes were located in two distinct loci, and their respective promoter regions possess a DosR-like binding motif. These include the DosS-like (SCO0203), DosR-like (SCO0204), and DosT-like (SCO0211) genes which represent orthologs of *M. tuberculosis*, where DosS and DosT act as sensors of redox stress (induced by respiration-impairing gases such as NO) and hypoxia for the DosR transcriptional factor (37). Interestingly, proteome comparisons in *S. coelicolor* populations of small and large pellets revealed that proteins encoded by genes flanking the dosR-like gene (SCO0204) were overrepresented in large pellets, where a higher level of oxygen stress is assumed to occur (38). In *Mycobacterium* spp., DosR controls the expression of genes necessary for dormancy survival (32, 37). Nitroreductase genes, CBS motif-containing genes, and pyridoxamine 5'-phosphate oxidase genes belong to the DosR regulon in *M. tuberculosis* (28), and similar genes were found in multiple copies in the two loci identified in this study. A remarkable redundancy of USP genes was detected in these loci. Such genes are also present in several bacterial genomes where they appear to be involved in survival under anaerobic conditions (39).

Although *S. coelicolor* cannot grow in the total absence of oxygen, the bacterium is capable of microaerobic growth and can

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**FIG 3** Representation of the two putative *Streptomyces coelicolor* redox stress response loci. Genes upregulated in the Δtdd8 strain at the onset of Red production are presented as colored arrows. The red dots indicate the presence of a DosR-like binding motif in intergenic regions. The consensus sequence motif is shown with the size of the base representative of its degree of conservation in the 19 input sequences analyzed. PPK2, polyphosphate kinase 2; CRP, cyclic AMP receptor protein; FNR, fumarate and nitrate reduction regulator; PPDK, pyruvate phosphate dikinase.

**FIG 4** Intracellular free Ca²⁺ concentration (+SD) in *Streptomyces coelicolor* strains determined under various growth conditions. Bacteria were cultivated in R5 medium supplemented or not supplemented with CaCl₂ (a) and R5 medium supplemented or not supplemented with the NO donor sodium nitroprusside (SNP) (b). Data with the same letter are not statistically significantly different (LSD test, *P* < 0.05).
even survive several weeks under conditions of strict anaerobiosis (26). The expression of S. coelicolor genes containing a DosR-like binding motif responded to the same external stimuli (hypoxia and NO) as the M. tuberculosis DosR regulon (28,31), indicating that the DosR-like regulon of S. coelicolor is also probably involved in adaptation to redox stress. Interestingly, gene SCO0212 located in the second locus and lacking the DosR-like binding site showed an expression profile that differed from the profiles of the other genes of the loci (Fig. 2). No difference was observed between the wild strain and the Δtdd8 strain in terms of resistance to oxygen stress, whereas the Δtdd8 strain appeared more sensitive than strain M145 to a stress induced by SNP in the absence of calcium (see Fig. S1 and S2 in the supplemental material). This last observation was unexpected, since both low-oxygen- and SNP-induced stresses resulted in reduced transcription of tdd8.

Although genes with a DosR binding motif have been found in a variety of actinobacteria, including Rhodococcus spp., Nocardia farcinica, and Sarccharopolyspora spp. (32), this is the first report of a DosR-like regulon in a Streptomyces species. The DosR-like regulon of S. coelicolor appeared to belong to a larger redox stress response gene cluster since expression of some genes located outside the loci and lacking a DosR-like binding site, such as nsrR and hmp, which are responsible for sensing and detoxification of NO, concomitantly responded to the same stimuli (hypoxia and NO).

Expression of a redox stress response gene cluster in the Δtdd8 strain suggests a role for tdd8 in redox homeostasis. Sensing changes in the redox state can be directly detected in bacteria via interaction of O2, NO, or CO with heme-containing proteins or Fe-S proteins (40). Tdd8 does not belong to these classes of proteins, and it is unlikely that it acts as a direct sensor for O2, NO, or CO. Changes in the redox state could, however, be indirectly sensed by pools of molecules within the cell that include metal ions, such as Ca2+. It has been proposed that Ca2+ acts as a second messenger by controlling membrane permeativity to proteins (42,43). Ca2+ signaling may also be involved in adaptation to various stress responses, as tdd8 expression or Tdd8 production is modulated under various stress conditions, including hypoxia (references 6, 7, and 8 and this work). The reduced expression of tdd8 in M145, when the strain was grown in R5 medium under conditions of nitrosative stress, correlated with the reduced free intracellular calcium concentration, providing further evidence for the role of Tdd8 in maintaining the intracellular calcium concentration when S. coelicolor cells are grown in poor-calcium medium. Interestingly, an extracellular calcium supply restored to the Δtdd8 mutant a level of intercellular free calcium similar to that associated with M145 in the R5 medium and concomitantly reduced the expression of the genes belonging to the DosR-like regulon. Addition of calcium in a Δtdd8 culture had, however, no significant effect on the expression of the nsrR and hmp genes, which belong to the redox stress response cluster but not to the DosR-like regulon.

The high level of NADH present in Δtdd8 cells at the visual onset of Red biosynthesis indicates the need for the mutant to balance its redox status, but the wild strain also appeared to suffer (though at a

FIG 5 Relative levels of expression (ΔCt + SD, determined by RT-qPCR) of genes of the redox stress response cluster and developmental genes in Streptomyces coelicolor M145 and Δtdd8 strains in R5− (black bars) and R5+ (white bars) culture media. Values accompanied by the same letter do not statistically significantly differ (LSD test, P < 0.05).
lesser extent) from redox stress at the visual onset of Red biosynthesis because at least some genes with a DosR-like binding motif were specifically expressed at this developmental stage. This stage is characterized by growth arrest and programmed cell death prior to the appearance of the second mycelium (4). It has been proposed that the growth arrest could be triggered by local oxygen depletion in the developing mycelium (2). Expression of the DosR-like regulon at the onset of Red production supports this hypothesis. Furthermore, overproduction of TdD8 in cells undergoing the programmed cell death (2) as well as the overexpression of the putative redox stress response cluster in the ΔtdD8 strain at the onset of Red synthesis suggests not only a role for TdD8 during growth under conditions of specific environmental stresses but also a role in the proper morphological development of S. coelicolor. The role of the DosR-like regulon in the morphological differentiation process of S. coelicolor is under investigation.

This work brings evidence that TdD8 plays a significant role in calcium homeostasis even if the mechanism of action remains elusive. Despite the fact that very little is known about calcium signaling in prokaryotes, this report suggests the presence of calcium regulation by TdD8 for redox stress adaptation in S. coelicolor.

ACKNOWLEDGMENTS

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REFERENCES


