Deletion of a regulatory gene within the *cpk* gene cluster reveals novel antibacterial activity in *Streptomyces coelicolor* A3(2)

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Genome sequencing of *Streptomyces coelicolor* A3(2) revealed an uncharacterized type I polyketide synthase gene cluster (*cpk*). Here we describe the discovery of a novel antibacterial activity (abCPK) and a yellow-pigmented secondary metabolite (yCPK) after deleting a presumed pathway-specific regulatory gene (*scbR2*) that encodes a member of the γ -butyrolactone receptor family of proteins and which lies in the *cpk* gene cluster. Overproduction of yCPK and abCPK in a *scbR2* deletion mutant, and the absence of the newly described compounds from *cpk* deletion mutants, suggest that they are products of the previously orphan *cpk* biosynthetic pathway in which abCPK is converted into the yellow pigment. Transcriptional analysis suggests that *scbR2* may act in a negative feedback mechanism to eventually limit yCPK biosynthesis. The results described here represent a novel approach for the discovery of new, biologically active compounds.

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INTRODUCTION

Streptomycetes are ubiquitous soil-dwelling mycelial bacteria characterized by a complex morphology and the ability to produce a vast variety of secondary metabolites, including the majority of antibiotics of natural origin (Challis & Hopwood, 2003). Consequently, the genus *Streptomyces* is of major interest in the search for new biologically active compounds. The genome sequence of the model actinomycete *Streptomyces coelicolor* A3(2) (Bentley *et al.*, 2002) reveals numerous secondary metabolite gene clusters, including 11 for characterized compounds, such as the blue-pigmented polyketide actinorhodin (Bystrykh *et al.*, 1996; Rudd & Hopwood, 1979), the red-pigmented prodiginines (Feitelson *et al.*, 1985) and the calcium-dependent antibiotic (Hopwood & Wright, 1983; Lakey *et al.*, 1983), but also 12 ‘orphan’ gene clusters to which no products have been assigned (Challis & Hopwood, 2003).

Abbreviations: CME, cell methanol extract; PKS, polyketide synthase; qRT-PCR, quantitative RT-PCR.

Two supplementary figures, showing antibiotic production on R2 and R2YE medium, and secondary metabolite production on DNAgar supplemented with *N*-acetylglucosamine and glutamate, and two supplementary tables, showing bacterial strains, plasmids and cosmids used, and primers used, with references, are available with the online version of this paper.

Orphan gene clusters are found not only in *Streptomyces* but also in other actinomycetes, myxobacteria, cyanobacteria and filamentous fungi with the ability to produce biologically active secondary metabolites, and are a promising source of new pharmaceutically useful compounds (Bok *et al.*, 2006; Gross, 2007; Udway *et al.*, 2007). With the increasing number of genome sequences, ‘genome mining’ for new secondary metabolite gene clusters and novel natural products has become a feasible approach to drug discovery. A number of methods have been proposed or adopted to detect the products of orphan gene clusters (Challis, 2008), including *in silico* analysis and prediction of the products of biosynthetic gene clusters, deletion of biosynthetic genes and identification of the corresponding end product by comparative metabolite profiling, heterologous expression of an entire biosynthetic gene cluster (Song *et al.*, 2006), deletion or overexpression of a pleiotropic regulatory gene and overexpression of a pathway-specific activator (Bergmann *et al.*, 2007).

S. coelicolor contains an orphan type I polyketide synthase (PKS) gene cluster, *cpk* (formerly named *kas*), which was initially detected by hybridization to an acetyltransferase (AT) gene (Kuczek *et al.*, 1997). However, apart from the prediction of a constituent aliphatic 12-carbon chain (Pawlik *et al.*, 2007), which was based on the sequence of the PKS subunits *cpkABC*, little could be deduced about the product of the *cpk* gene cluster, which consequently remained ‘orphan’.

ScbR is a γ -butyrolactone receptor protein of *S. coelicolor* that binds to specific DNA sequences in front of its target genes, repressing their transcription (Takano *et al.*, 2001). Binding of γ -butyrolactones synthesized by ScbA at transition phase (Hsiao *et al.*, 2007) prevents ScbR from interacting with DNA. Earlier work had shown that ScbR directly represses expression of *cpkO*, the pathway-specific transcriptional activator gene of the *cpk* cluster, through two binding sites located in the *cpkO* promoter region (Takano, 2006). In agreement with this, transcription of the *cpk* cluster increased in a *scbR* mutant (Takano *et al.*, 2001). There are five *scbR* homologues in *S. coelicolor* (Takano, 2006). Interestingly, one of these, *scbR2*, is part of the *cpk* gene cluster (Pawlik *et al.*, 2007) and thus may be involved in its regulation. ScbR2, together with, e.g. TylQ from *Streptomyces fradiae* and BarB from *Streptomyces virginiae*, appears to constitute a subfamily of γ -butyrolactone receptor homologues that are suggested to act as transcriptional regulators; however, γ -butyrolactone binding has not been experimentally proven (Bibb, 2005; Takano *et al.*, 2001). In this study, *scbR2* was deleted from *S. coelicolor* M145 to assess its role in the regulation of secondary metabolism, resulting in the identification of a novel antibacterial activity.

METHODS

Bacterial strains, plasmids and growth conditions. Bacterial strains and plasmids used are listed in Supplementary Table S1. *S. coelicolor* was manipulated as previously described (Kieser *et al.*, 2000). *Escherichia coli* strains were grown and transformed as described by Sambrook & Russell (2001). *Micrococcus luteus* (Tang *et al.*, 2003) and *Bacillus subtilis* (Nakamura *et al.*, 1999) were cultured at 30 °C in LB medium (Sambrook & Russell, 2001). Difco Nutrient Agar (DNAgar), Difco Nutrient Broth (DNB), R2, R2YE, SMMS (Takano *et al.*, 2001) and a minimal medium with sodium glutamate as sole source of nitrogen (Nieselt *et al.*, 2010) were used to detect antibiotic production. When required, 325 mM L-glutamic acid monosodium salt (Sigma) was added to DNB, DNAgar and SMMS. When added to DNAgar, N-acetylglucosamine (GlcNAc) was used at a final concentration of 50 mM. Liquid SMM (Takano *et al.*, 2001) was used to grow cells for the isolation of RNA. Soft nutrient agar (SNA) was used for antibacterial activity tests.

***scbR2* and *scF* mutants.** Mutant alleles of *scbR2* (SCO6286) and *scF* (SCO6272) were constructed in which most of the coding regions were deleted and replaced with an apramycin-resistance gene [*aac(3)IV*] using PCR-targeting (Flett *et al.*, 1997; Gust *et al.*, 2004; MacNeil *et al.*, 1992). Primer pairs mscbR2R/mscbR2F (*scbR2*) and RedirSCO6272Fw/RedirSCO6272Rv (*scF*) were designed to amplify the disruption cassette of pIJ773. The cosmid used for targeting were SCAH10 (*scbR2*) and St1G7 (*scF*) (Redenbach *et al.*, 1996). Deletion of *scbR2* was confirmed by Southern hybridization (data not shown) (Kieser *et al.*, 2000). Construction of the mutants was confirmed by PCR using primers scbRA2/scbRWF3 (*scbR2*) and FwScF/RvScF (*scF*) with genomic DNAs isolated from the parental *S. coelicolor* M145 and potential mutants as templates (data not shown). All primers are listed in Supplementary Table S2. The resulting *scbR2::aac(3)IV* and *scF::aac(3)IV* mutant strains were designated LW3 and LW38, respectively.

For complementation of LW3, *scbR2* together with a 227 bp upstream region was amplified from genomic DNA of *S. coelicolor* M145 using

primers scbR2A_*Xba*I and scbR2FW3_*Xba*I, thereby introducing *Xba*I restriction sites at both ends. The integrity of the *scbR2* coding and promoter region was confirmed by DNA sequencing, and the PCR product was introduced into pGEM-T Easy (Promega) to give plasmid pTE215. A 1.1 kb *Xba*I fragment of pTE215 containing *scbR2* was cloned into pIJ82 (Kieser *et al.*, 2000) to yield plasmid pTE216. The integrative vectors pIJ82 and pTE216 were introduced into the *S. coelicolor* strain LW3 by conjugation. Integration of pIJ82 and pTE216 into the chromosome was confirmed by PCR using primers JGattBI-fwd/JGattPint-rev (Hsiao *et al.*, 2007) and primers scbR2A/scbRWF3, respectively.

Construction of the *S. coelicolor act, red, cda* mutant. Most of the gene cluster for CDA biosynthesis (from SCO3210 to SCO3249) was deleted from *S. coelicolor* M1142 (M145 Δ act Δ red; J. P. Gomez-Escribano, unpublished data) in a two-step process. First, *cdaPSI* and most of *cdaPSII* were replaced with an apramycin-resistance cassette by PCR-targeting followed by homologous recombination using pIJ12145 (Supplementary Table S1), yielding strain M1147. The remaining *cda* genes were then removed by homologous recombination with 2 kb flanking segments using pIJ12146 (Supplementary Table S1). The resulting strain, M1148, was verified by Southern blotting (data not shown).

Secondary metabolite production, antibacterial activity assays and preparation of methanol extracts. For agar-grown cultures of *S. coelicolor*, 4×10^7 spores were streaked out in a 2.5×2.5 cm square prior to incubation at 30 °C in darkness. When testing antibacterial activity, plates were overlaid after 27 h of growth with 2 ml SNA containing 0.4% (v/v) of *E. coli*, *B. subtilis* or *M. luteus* overnight cultures. After incubation overnight at 30 °C, zones of bacterial growth inhibition were recorded by scanning the bottom of the plates.

For liquid-grown cultures, 2×10^8 *S. coelicolor* spores were inoculated into 50 ml DNB supplemented with glutamic acid (Glu-DNB) in a 250 ml unbaffled Erlenmeyer flask (containing a 26 cm stainless steel coiled spring) and incubated with shaking at 220 r.p.m. and 30 °C for 27 h to an OD₄₅₀ between 1.20 and 1.26. Cells from 1 ml of culture were collected by centrifugation. A 200 μ l volume of the culture supernatant was transferred to a 96-well plate and the rest of the liquid discarded. To quantify yellow pigment production, the A_{400–700} of the culture supernatant was determined on a plate spectrophotometer (SpectraMax Plus384, Molecular Devices). The cell pellet was resuspended in 1 ml Tris/EDTA (Kieser *et al.*, 2000) and 200 μ l was transferred to a 96-well plate, which was scanned from the bottom. The remaining 800 μ l of cell suspension was diluted with buffer to an OD₄₅₀ below 1 to determine the growth of the culture.

To prepare cell methanol extracts (CMEs), mycelium from 5 ml samples of 27 h Glu-DNB cultures and from 24 h glutamate-based minimal medium cultures was collected by centrifugation at 4 °C and the cell pellet resuspended in 500 μ l methanol. After incubation on ice for 10 min with occasional mixing, cells were removed by centrifugation and the supernatant (CME) collected. To compare secondary metabolite production, 200 μ l of the Glu-DNB-derived CME was transferred to a 96-well plate, which was scanned from the bottom. To assay the antibiotic activity of the CME and the Glu-DNB culture supernatant, 50 μ l aliquots were concentrated to 10 μ l in a SpeedVac concentrator (New Brunswick Scientific) in approximately 10 min and dropped directly onto the surface of an LB agar plate containing 0.4% (v/v) of an overnight culture of *B. subtilis*. The plates were allowed to dry for a few minutes and incubated overnight at 30 °C. Pictures were taken from the bottom to determine inhibition of *B. subtilis* growth.

S1 nuclease protection assay. M145, M751 and M752 were grown in SMM, and RNA was isolated as previously described (Takano, 2006). The S1 nuclease protection assay was conducted as described

by Takano *et al.* (2001). The probe for *scbR2* was made using primers scbR2-REV/SCBR-FW to generate a 540 bp fragment. The probe for *cpkJ* was made using primers ksER/ksEF (Takano *et al.*, 2005a) to generate a 502 bp fragment. pBR322 digested with *MspI* was used as a marker. The results shown for *hrdB* have been previously published (Takano *et al.*, 2005a).

Reverse transcription and quantitative RT-PCR (qRT-PCR).

RNA was isolated as previously described (Takano *et al.*, 2005a) from M145, LW3 and LW6 [*cpkO::aac(3)IV*] grown in SMM to OD₄₅₀ values of: M145, (1) 0.61 (13.3 h), (2) 1.36 (16.3 h), (3) 1.6 (21.3 h); LW3, (1) 0.46 (17.5 h), (2) 0.89 (21 h), (3) 1.48 (25 h); LW6, (1) 0.54 (19 h), (2) 0.72 (21.5 h), (3) 0.90 (25 h). The LW6 samples are the same as those described in Takano *et al.* (2005a). DNase I treatment (10 U DNase I, Roche) was performed on 10 µg of each RNA sample used for the qRT-PCR assays, and the absence of chromosomal DNA was confirmed by PCR using primers hrdBrt1 and hrdBrt2 (Hsiao *et al.*, 2007). To synthesize cDNA, 2 µg RNA, 1.8 µg random hexamers (Invitrogen) and dNTPs to a final concentration of 1 mM each were incubated at 72 °C for 10 min; to this, 5 × First-Strand Buffer (Invitrogen), 10 mM DTT, 100 U SuperScript II (Invitrogen) and RNase-free water were added to give a final volume of 30 µl, and the mixture was incubated at 50 °C for 100 min and 75 °C for 15 min. qRT-PCR experiments were performed using the ABI Prism 7500 FAST system (Applied Biosystems). Each 25 µl reaction contained 12.5 µl SYBR Green PCR Master Mix (Applied Biosystems), 6.6 ng cDNA, 10 pmol each primer and 9.5 µl water. The reaction parameters were: 95 °C for 10 min, followed by 40 two-step amplification cycles consisting of 30 s denaturation at 95 °C and 1 min of annealing and extension at 60 °C. A final dissociation step was used to generate a melting curve and consequently verify the specificity of the amplification procedure. Real-time PCR was monitored and analysed by the Sequence Detection System version 1.3 software (Applied Biosystems), and relative expression levels were normalized to mRNA derived from the major vegetative sigma factor gene (*hrdB*). Levels of gene expression for *actII-4*, *cpkC*, *cpkE*, *cpkO* and *redD* are shown as fold-change relative to the M145 time point 1 exponential growth phase sample (Fig. 6a), and for *scbR2* relative to the M145 time point 2 sample (Fig. 6b), since no *scbR2* transcripts were detected at M145 time point 1. All samples were run in triplicate.

RESULTS

Deletion of *scbR2* reveals a diffusible yellow pigment

A deletion mutant of *scbR2*, which encodes a protein with 32 % amino acid sequence identity to ScbR and which is located in the *cpk* gene cluster, was made to assess the role of this putative regulatory gene in secondary metabolism in *S. coelicolor*. The resulting strain LW3 [M145 *scbR2::aac(3)IV*] was assessed for secondary metabolite production during growth on R2YE agar medium (Supplementary Fig. S1). Deletion of *scbR2* resulted in a marked reduction in actinorhodin production, and the appearance of a previously unseen yellow diffusible pigment on DNagar. To confirm that production of the yellow compound and the lower level of actinorhodin synthesis resulted solely from *scbR2* deletion, *scbR2* was reintroduced into LW3. Secondary metabolite production was restored to the levels observed in the parental strain M145 (Fig. 1). M145 and LW3 were subsequently compared on a range of agar

media, including DNagar and R2, with essentially the same results. Production of the yellow compound was now also evident in M145, but only when grown on DNagar, peaking earlier in growth and at a lower level than in LW3 (Fig. 1). Production of the yellow compound was not observed on minimal medium (SMMS; Fig. 2a). Growth on MS agar, on which *S. coelicolor* sporulates well, revealed no detectable effect of the deletion of *scbR2* on morphological development or on antibiotic production (data not shown). Since production of the yellow compound was maximal on DNagar, where actinorhodin production was apparently abolished in LW3, this medium was used for further analysis.

The yellow compound is produced in a carotenoid deletion mutant

S. coelicolor produces intracellular yellow-pigmented carotenoids when grown under light (Takano *et al.*, 2005b). To rule out the possibility that the observed yellow compound could reflect carotenoid production, an *S. coelicolor* mutant blocked in carotenoid synthesis (M145Δ*crtEIVB*; Takano *et al.*, 2005b) was grown on DNagar (Fig. 1). The mutant closely resembled M145 in yellow compound production. Consequently, the newly described yellow pigment is not related to carotenoid production. Moreover, all of the cultures shown were incubated in darkness, where carotenoid synthesis is not induced (Takano *et al.*, 2005b).

Yellow compound (yCPK) biosynthesis requires a functional *cpk* gene cluster

To exclude the possibility that the yellow compound was related to any of the characterized secondary metabolites produced by *S. coelicolor*, strain M1148 (M145 Δ*act* Δ*red* Δ*cda*) was grown on DNagar and production of the pigment confirmed (Fig. 1). Strain M1146 (M145 Δ*act* Δ*red* Δ*cda* Δ*cpk*), which lacks the entire *cpk* gene cluster, P100 [M145 *cpkC::aac(3)IV*] (Pawlik *et al.*, 2007) and LW6 [M145 *cpkO::aac(3)IV*] (Takano *et al.*, 2005a), which are defective in the biosynthetic PKS subunit CpkC and in the pathway-specific activator of the *cpk* cluster CpkO, respectively, were also tested for yellow compound production. They failed to produce the yellow pigment (Fig. 1), suggesting that it is indeed the product of the orphan type I PKS gene cluster *cpk*. The yellow compound was therefore named yCPK (yellow *coelicolor* polyketide).

yCPK production in *S. coelicolor* is enhanced in glutamate-supplemented medium

Since yCPK production in M145 was difficult to detect, an attempt was made to define the medium conditions for improved yCPK synthesis. One of the accessory proteins of the *cpk* gene cluster, CpkG, shows high homology to pyridoxalphosphate-dependent class III aminotransferases that directly metabolize the amino group from amino

Fig. 1. Secondary metabolite production on DNagar. Strains were patched in a square and incubated on DNagar at 30 °C, and the production of pigmented secondary metabolites was recorded at the times indicated.

acids (Pawlik *et al.*, 2007) and belongs to the AAT-I protein superfamily. Another member of this family, FdtB from *Aneurinibacillus thermoaerophilus*, has been shown to use glutamate, but not glutamine, alanine and aspartate as amino group donors (Pfoestl *et al.*, 2003). Therefore, glutamate (Glu) was added to DNagar and SMMS in addition to or replacing the Casamino acids (CA) present in SMMS. On SMMS (with CA), yCPK production was not observed (Fig. 2a) with the parental strain M145 nor with LW3. On SMMS with glutamate in addition to CA, the yellow pigment was detected only after 40 h of growth (Fig. 2a). Optimal production was observed when CA were replaced by glutamate and on DNagar medium supplemented with glutamate (Fig. 2a, b). While yellow pigment production in M145 appeared to peak after approximately 2 days of growth, accumulation in LW3 persisted, yielding apparently large amounts on both Glu-SMMS and Glu-DNagar (Fig. 2a, b). yCPK was still visible in apparently large amounts even after 6 days of growth on Glu-DNagar (Fig. 2b). yCPK production was seen only with the *cpk*⁺ *S. coelicolor* strains and not with the *cpk* mutant strains (Fig. 2b), indicating that yCPK production requires a functional *cpk* gene cluster even when using media yielding apparently copious amounts of yellow pigment.

yCPK is found in liquid culture supernatants and not inside the cells

S. coelicolor strains M145, LW3, M1146 and M1147 [M145 $\Delta act \Delta red cdaPS::aac(3)IV$] were grown to equivalent biomasses for 27 h in the liquid version of the optimized yCPK production medium (Glu-DNB). As observed on solid media, yCPK production was seen only with the *cpk*⁺ *S. coelicolor* strains and not with the *cpk* mutant (Fig. 3). The absorption spectra (400–700 nm) of the culture supernatants were determined using Glu-DNB as blank. Absorption peaked at 460 nm in the presence of the yellow pigment (Fig. 3). Therefore, A_{460} values were used to compare the amounts of yCPK produced. In agreement with yCPK production on solid Glu-DNagar (Fig. 2b), yCPK was detected in M145 and M1147, and over-produced in LW3, and was not produced in the *cpk* mutant M1146.

Cells of M1147 separated from the culture medium by centrifugation were non-pigmented, despite the production of yCPK, whereas the yellow compound was clearly visible and detected in the culture supernatant. It was also readily observed in culture supernatants of M145 and LW3, while putative yellow pigment production in the corresponding mycelia may have been masked by the production

Fig. 2. yCPK production on (a) (modified) SMMS and (b) DNAgar supplemented with glutamate. Strains were incubated at 30 °C on (a) SMMS, SMMS containing glutamate (Glu-SMMS) and SMMS supplemented with glutamate and without Casamino acids (Glu-SMMS without CA), and (b) on glutamate-supplemented DNAgar. Production of pigmented secondary metabolites was recorded at the indicated time points.

of Red (Fig. 3). Thus, in liquid-grown cultures, yCPK occurs extracellularly, consistent with the observed secretion of the yellow pigment from yCPK-producing strains grown on agar (Figs 1 and 2).

***S. coelicolor* displays *cpk*-dependent antibacterial activity**

Several strains were grown on Glu-DNAgar plates for 27 h and then overlaid with SNA containing *B. subtilis*, *M. luteus* or *E. coli*. After incubation overnight, growth of *B. subtilis* was inhibited by the yCPK-producing strains M145, LW3 and M1147, but not by the *cpk* mutant strains M1146 and P100 (Fig. 4). *cpk*-dependent antibacterial activity was also observed against *M. luteus* and *E. coli* (Fig. 4). In addition to yCPK production, antibacterial activity was also enhanced in the *scbR2* mutant strain LW3 when compared with the parental strain M145 (Fig. 4). These results suggest that the observed antibiotic activity coincides with the production of yCPK and that it also requires the *cpk* gene cluster. In the absence of glutamate in the medium, antibacterial activity was markedly lower or even fully absent (data not shown).

To demonstrate further that the observed antibiotic activity was derived from the *cpk* gene cluster and that production of the antibacterial compound occurred in liquid medium, *S. coelicolor* strains M145, LW3, M1146 and M1147 were grown in liquid Glu-DNB and a glutamate-based minimal medium, and CMEs were prepared. Antibacterial activity was analysed in the culture supernatant and in the CMEs. Only the CMEs from M145, LW3 and M1147 gave antibacterial activity, but not extracts from M1146 (Fig. 3). The antibacterial activity in LW3 was observed at 22–44 h of growth in liquid media (data not shown). These results are consistent with the bioactivity assays obtained with agar-grown cultures (Fig. 4), which indicates that the observed antibiotic activity requires a functional *cpk* gene cluster.

Antibacterial activity does not require the yellow pigment

ScF and CpkH are homologues that show 53 and 49% amino acid identity, respectively, to AclO, a putative oxidoreductase involved in aclacinomycin production in *Streptomyces galilaeus* (Pawlik *et al.*, 2007). ScF and CpkH

Fig. 3. Production of yCPK and abCPK in liquid cultures. Strains were grown in glutamate-supplemented DNBroth for 27 h to equivalent biomasses. Cells were separated from the culture supernatant (s/n) and resuspended in colourless buffer. Also, CMEs were prepared and both CMEs and s/n were tested for antibacterial activity against *B. subtilis* ('Bioactivity CME' and 'Bioactivity s/n', respectively). To quantify yCPK production, the absorption spectra ($A_{400-700}$) of the s/n were determined. Values for the maximal absorption at A_{460} , reflecting the amount of yCPK produced, are given on the right.

are associated to the cell wall and have been shown to be substrates of the *S. coelicolor* Tat protein export system (Widdick *et al.*, 2006). In addition, CpkD also possesses a Tat system signal peptide (Pawlik *et al.*, 2007). A *tatC* deletion mutant, impaired in the Tat protein secretion

system (strain TP4; Widdick *et al.*, 2006), neither produced yCPK nor showed antibiotic activity (Fig. 4). Thus, late steps of the *cpk* biosynthetic pathway may take place outside the mycelium. To test this further, LW38 [M145 *scF::aac(3)IV*] was created. As expected, LW38 did not

Fig. 4. *cpk* cluster-related bioactivity against *B. subtilis*, *M. luteus* and *E. coli*. Strains were grown on glutamate-supplemented DNAgar for 27 h and overlaid with SNA containing *B. subtilis*, *M. luteus* or *E. coli*. To determine inhibition of bacterial growth, the plates were further incubated at 30 °C overnight and photographed from the bottom.

produce yCPK, whereas Red and Act were still produced (Fig. 2b). In contrast to the other *cpk* cluster mutants tested, despite the lack of yCPK production, LW38 still inhibited growth of *B. subtilis*, *M. luteus* and *E. coli* (Fig. 4). This suggests the existence of a colourless compound, distinct from yCPK but also derived from the *cpk* cluster, that possesses antibiotic activity (abCPK; antibiotic *coelicolor* polyketide). The results also indicate a possible role for ScF in the extracellular conversion of antibacterial abCPK into yCPK. The proposed relationship between the two CPK metabolites, however, will require confirmation by structure elucidation of both compounds, and by purification of the ScF protein and *in vitro* conversion of abCPK into yCPK.

Another indication that the *cpk*-related antibiotic activity does not derive from yCPK came from the analysis of CMEs. The colourless abCPK can be detected in the CME obtained from unpigmented cells of M1147 (Fig. 3). On the other hand, so far it has not been possible to detect antibiotic activity in a culture supernatant where the yellow-pigmented yCPK is found (Fig. 3).

Expression of *scbR2* is growth phase-dependent in M145, repressed in a *scbA* mutant and constitutive in a *scbR* mutant

To understand the relationship between ScbA, ScbR and ScbR2, transcription of *scbR2* was analysed in M145 and in congenic *scbA* and *scbR* mutants. The transcriptional start site of *scbR2* was first determined by high-resolution S1 nuclease protection analysis and found to lie 50 nt upstream of the ScbR2 translational start site [an adenine at nucleotide 6946379 in the NCBI genome sequence NC_003888 (GenBank accession no. AL645882) (Fig. 5a, b)]. While short direct and inverted repeats were detected in the intergenic region between *scbR2* and divergent *scoT*, DNA sequences resembling previously reported binding sites for ScbR and its homologues ArpA, BarA and FarA (Takano *et al.*, 2005a) were not observed.

Expression of the *cpk* gene cluster was previously detected by microarray analysis using *S. coelicolor* grown in SMM (Takano *et al.*, 2005a). Therefore, to determine the role of ScbR and ScbA in regulating *scbR2* expression, S1 nuclease protection analysis was carried out on RNA isolated at different phases of growth from cultures of M145, M751 ($\Delta scbA$) and M752 ($\Delta scbR$) grown in SMM. Consistent with the results presented for SMMS agar-grown cultures (Fig. 2a), none of the three strains produced the yellow compound (data not shown). However, transcription of *scbR2* in M145 increased dramatically in transition phase before decreasing slightly once the culture entered stationary phase (Fig. 5c). In the *scbA* mutant, expression of *scbR2* was abolished, while it was constitutively expressed throughout growth in the *scbR* mutant (Fig. 5c).

Expression of the putative yCPK biosynthetic gene *cpkJ* was also examined. In M145, expression was detected during

transition and early stationary phase, while little or no expression was observed in M751 (Fig. 5c). In M752, expression of *cpkJ* was detected at high levels during exponential growth and at a lower intensity in stationary phase. As expected, the *scbA* mutant did not produce yCPK on Glu-DNAgar (Fig. 2b), presumably because production of γ -butyrolactones is abolished and ScbR constitutively represses *cpkO* expression and consequently the *cpk* cluster. This is consistent with the lack of expression of *scbR2* in M751 (Fig. 5c). In agreement with repression of *cpkO* expression by ScbR (Fig. 5c), the *scbR* mutant did produce yCPK (Fig. 2b).

qRT-PCR reveals that ScbR2 represses transcription of the *cpk* cluster

To obtain further insight into the regulation of the *cpk* gene cluster, expression of the putative regulatory genes *cpkO* and *scbR2*, and the predicted biosynthetic genes *cpkC* and *cpkE*, was assessed by qRT-PCR. RNA was isolated at three different time points from strains M145, LW6 and LW3 grown in SMM liquid media. In agreement with the S1 nuclease protection results (Fig. 5c), qRT-PCR analysis revealed transcription of *scbR2* and all three *cpk* genes during transition phase in M145 (Fig. 6a, b). Expression of *scbR2*, *cpkE* and *cpkC* was not observed in the *cpkO* mutant LW6 (Fig. 6a, b), consistent with previous results (Takano *et al.*, 2005a) and with the role of CpkO as a transcriptional activator of the *cpk* cluster. As expected, transcription of *cpkO* was not detected in the $\Delta cpkO$ deletion mutant LW6 (Fig. 6a), since the primers used correspond to the deleted region. Expression of *cpkC*, *cpkE* and *cpkO* occurred in M145 during late transition phase, while in LW3, all three genes were transcribed earlier and eventually at much higher levels (12.9-, 6.8- and 6.6-fold, respectively; Fig. 6a). This indicates a negative role for ScbR2 in regulating the expression of all three *cpk* genes tested.

Since the *S. coelicolor* butanolide system was originally identified through its influence on Act and Red production (Takano *et al.*, 2001), the expression of the pathway-specific activator genes of the *act* and *red* gene clusters, *actII-4* and *redD*, respectively, was also examined. Expression of both *actII-4* and *redD* was detected during exponential phase in LW6, much earlier than in M145 and LW3. Expression of *redD* was enhanced 2.4-fold in LW3 during early transition phase when compared with M145 and LW6 (Fig. 6a).

DISCUSSION

Discovery of a yellow pigment and an antibiotic compound in *S. coelicolor* by deletion of a regulatory gene

Deletion of the gene encoding the γ -butyrolactone receptor homologue ScbR2 led to the discovery of an as yet unseen yellow metabolite, yCPK, and a previously undetected

Fig. 6. qRT-PCR analysis of *actII-4*, *redD*, *cpkC*, *cpkE*, *cpkO* and *scbR2*. cDNA was synthesized from RNA isolated from liquid SMM cultures of *S. coelicolor* M145, LW6 and LW3. E and T indicate the exponential and transition phases of growth, respectively. Gene expression is shown (a) as fold-change relative to the M145 exponential growth phase sample (13.3 h) or (b) relative to the M145 early transition phase sample (16.3 h), since no *scbR2* transcripts were detected with M145 after 13.3 h.

(*scbR2*) located in an orphan secondary metabolite gene cluster resulting in the production of a related antibiotic substance (abCPK) that had not been previously recognized in the parental strain. The emergence of multiple antibiotic-resistant bacterial pathogens has created an urgent need for new antibiotics. The results reported here may underpin an important route to the discovery of novel antibiotics that are currently 'hidden' within the genomes of secondary metabolite-producing organisms. The number of secondary metabolite gene clusters in sequenced actinomycetes genomes far exceeds the number of secondary metabolites known to be made by these strains (Omura *et al.*, 2001), and these 'cryptic' gene clusters potentially represent an immense source of novel, pharmaceutically useful compounds.

We hypothesize that after synthesis of the polyketide backbone by the type I PKS subunits CpkABC and modification by tailoring enzymes, a precursor polyketide is transported across the membrane. Transport may be performed by CpkF, a putative transmembrane efflux protein encoded within the *cpk* cluster (Pawlik *et al.*, 2007). This intermediate would then be further modified outside the cell by the secreted oxidoreductases CpkH and Scf, and maybe also by the putative secreted monooxygenase CpkD. Interestingly, our results suggest that the antibacterial intermediate abCPK is converted into the yellow pigment yCPK by Scf.

ScbR2 is under the control of the *S. coelicolor* butanolide system and represses yellow compound production

The absence of *scbR2* expression in a *scbA* mutant and the constitutive expression of *scbR2* in a *scbR* mutant (Fig. 5c) suggest that *scbR2* expression is controlled by the ScbAR butanolide system. Previously, the pathway-specific activator gene of the *cpk* cluster, *cpkO*, has been shown to be negatively regulated by ScbR, which binds to the *cpkO* promoter region and represses transcription. The expression profile of *scbR2* resembles that of *cpkO* (Takano *et al.*, 2005a). However, direct regulation of *scbR2* by ScbR seems unlikely, since no ScbR binding sites could be detected in the *scbR2* promoter region (Takano *et al.*, 2005a). The absence of a *scbR2* transcript in the Δ *cpkO* mutant suggests that *scbR2* is part of the CpkO regulon.

The onset of *scbR2* transcription in M145 occurs approximately 2 h later than that of *cpkO* and *cpkJ* during early transition phase (Takano *et al.*, 2005a). Interestingly, the yCPK pigment decreased markedly after 27.5 h, while in the *scbR2* deletion mutant it was sustained for a much longer period of time (Fig. 1). Consequently, it is possible that ScbR2 serves to ultimately suppress expression of the *cpk* gene cluster. This role is also implied by the overexpression of *cpkO*, *cpkC* and *cpkE* in the *scbR2* mutant (Fig. 6a), in which *cpkC* and *cpkE* may be indirectly regulated by ScbR2 via the derepression of the positive regulator CpkO. Since CpkO activates both *scbR2* and the

biosynthetic *cpk* genes, this would constitute negative feedback regulation of γ CPK biosynthesis.

Actinorhodin production was highly impaired in the *scbR2* mutant on all media tested and not visible on DNagar even after 10 days of growth (Fig. 1). The expression of *actII-4* was not impaired in the *scbR2* mutant; however, the induction in late transition phase observed in the parent was not detected. Therefore, it is unlikely that ScbR2 regulates the transcription of the actinorhodin gene cluster via the activator ActII-4. The decreased actinorhodin production may reflect competition for precursor supply, as actinorhodin and γ CPK are known and predicted (Pawlik *et al.*, 2007), respectively, to be derived from acyl-CoA compounds.

There are several other *Streptomyces* species in which pairs of putative γ -butyrolactone binding proteins are involved in the regulation of secondary metabolite production (reviewed by Takano, 2006). For example, in *Streptomyces ambofaciens*, AlpZ and AlpW are involved in the regulation of alpomycin and orange pigment synthesis (Bunet *et al.*, 2008). In *Streptomyces virginiae*, BarA is the primary negative transcriptional regulator of virginiamycin biosynthesis (Kinoshita *et al.*, 1997; Nakano *et al.*, 1998, 2000). Binding of the virginiae butanolide autoregulator VB to BarA derepresses antibiotic production and also a corresponding resistance mechanism. However, in a strain mutated in the second γ -butyrolactone homologue, BarB, virginiamycin is produced earlier. This suggests that BarB may also have a negative role in virginiamycin production, retarding synthesis until the cells acquire resistance against the antibiotic (Matsuno *et al.*, 2004). Interestingly, in many of these examples, only one of the pair of homologous proteins (for example, ScbR and BarA) has been shown to be a true γ -butyrolactone binding protein, and it is conceivable that the other partner has lost this ability and instead acquired an alternative function to fine-tune secondary metabolite production.

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