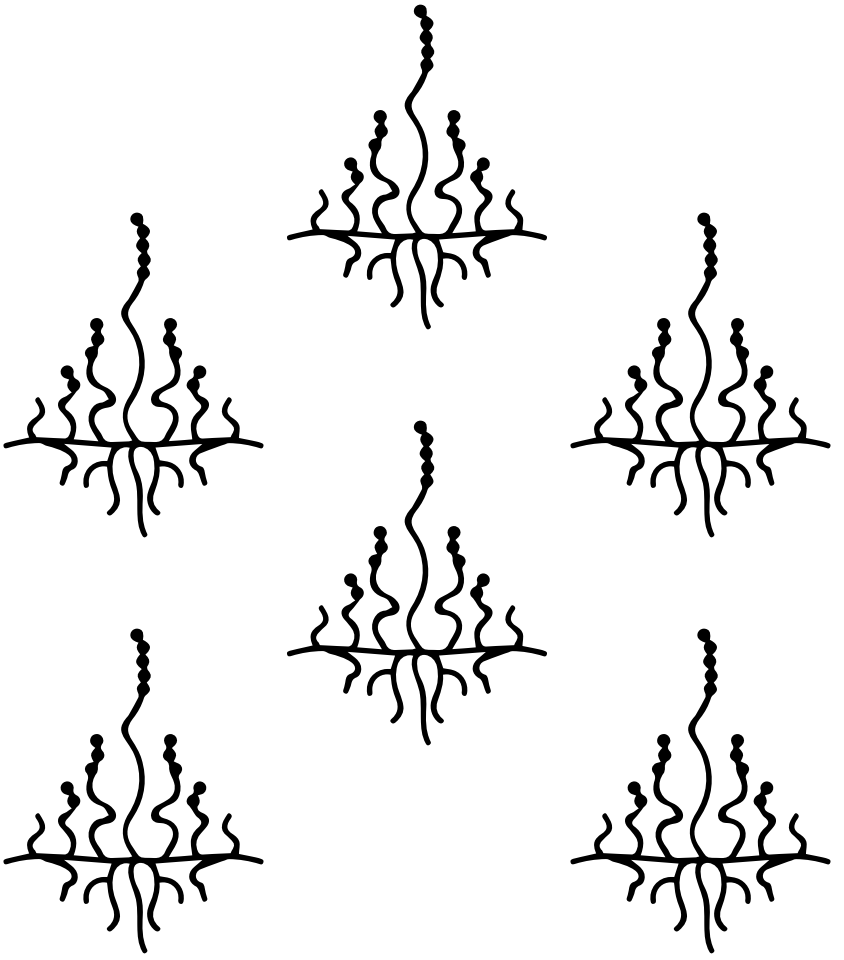
The ecology and evolution of microbial warfare in streptomyces

Westhoff, S.



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Chapter 6

Transcriptomic profiling of *Streptomyces coelicolor* colonies grown in close association with *Kitasatospora* sp. MBT66

Sanne Westhoff, Kay Nieselt, Robin Harmening,
Daniel E. Rozen, Gilles P. van Wezel

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MANUSCRIPT IN PREPARATION

ABSTRACT

Microbes respond to microbial competition using offensive strategies such as the production of bioactive compounds or defensive strategies such as the production of biofilms or spores. For the filamentous *Streptomyces* bacteria, renowned for their production of antibiotics, co-culturing approaches that simulate competition have mainly focused on increased production of secondary metabolites. Here, we show that major transcriptional changes occur during interaction of a colony of the model organism *Streptomyces coelicolor* with *Kitasatospora* sp. MBT66, which produces molecules that inhibit the growth of *S. coelicolor*. During competition, 13% of the ORFs in the *S. coelicolor* genome were differentially expressed, with genes involved in development, secondary metabolism and transport significantly enriched. Interestingly, genes for the volatile organic compounds (VOCs) geosmin and 2-methylisoborneol, compounds that were recently shown to facilitate spore dispersal by attracting arthropods, were highly upregulated in response to competition. The results presented here suggests the possibility that *Streptomyces* make fight or flight decisions during microbial competition, deciding between the production of antibiotics and accelerated sporulation and dispersal.

INTRODUCTION

Bacterial competition can be mediated through competition for resources or through the production of toxic compounds, known as interference competition (Granato *et al.*, 2019). Both forms of competition can generate unfavourable conditions, via resource or toxin-mediated stress, which bacteria act to escape or overcome, for example through biofilm formation or sporulation (Caro-Astorga *et al.*, 2020). These behaviours are tightly regulated and require cells to respond to environmental information, including sensing nutrient gradients, cell damage or other cues such as quorum sensing molecules (Leroux *et al.*, 2015; Cornforth and Foster, 2013). While there is abundant evidence that environmental cues provided by competing microorganisms can change the behavior of bacteria, how this occurs at a transcriptional level remains poorly understood.

Streptomycetes are filamentous bacteria that belong to the order of Actinomycetales and live in soil or aquatic ecosystems. Streptomycetes exhibit a remarkably complex multicellular life cycle: germination of spores leads to the outgrowth of vegetative hyphae, which grow by tip extension and branching to form a dense mycelial network. In the reproductive phase, aerial hyphae are formed that grow into the air and differentiate into chains of unigenomic spores, a process that is governed by a complex pathway with many molecular checkpoints (Barka *et al.*, 2016; Flärdh and Buttner, 2009). Streptomycetes are renowned for the capacity to make specialized metabolites, many of which are used as antibiotics, antifungals, anti-cancer drugs and immunosuppressants (Barka *et al.*, 2016; Bérdy, 2012; Hopwood, 2007). The genes for these secondary metabolites are organized in biosynthetic gene clusters (BGCs) that are tightly controlled (Bibb, 2005; van der Heul *et al.*, 2018). Genome mining revealed that streptomycetes harbour a much larger capacity to produce specialised metabolites than originally believed (Bentley *et al.*, 2002; Ikeda *et al.*, 2003; Ohnishi *et al.*, 2008). These are referred to as cryptic BGCs and are not expressed under standard lab conditions. Conditions that elicit the production of these secondary metabolites are expected to correspond to cues that streptomycetes encounter in their natural environment (Rutledge and Challis, 2015; Bergeijk *et al.*, 2020). These include environmental stresses such as nutrient starvation, changes in pH and cellular damage through the presence of toxic compounds (Zhu *et al.*, 2014).

Interactions with other microorganisms are known to inhibit or promote germination, growth and sporulation (Vetsigian *et al.*, 2011; Xu and Vetsigian, 2017; Yamanaka

et al., 2005). Biotic interactions can also change antibiotic production in streptomycetes (Traxler *et al.*, 2012, 2013; Abrudan *et al.*, 2015; Lee *et al.*, 2020) (and Chapter 5 of this thesis). Several diffusible molecules have been identified that induce this effect, including siderophores, that chelate iron from the environment, antibiotics and quorum sensing molecules (Traxler *et al.*, 2012; Lee *et al.*, 2020; Xu *et al.*, 2010; Zou *et al.*, 2014; Wang *et al.*, 2014). While these studies prove that strains respond to competitor induced resource stress or compounds, it is still largely unknown what other responses, besides changing antibiotic and siderophore production, are elicited and how this is realized at a molecular level.

Recently, the role of volatile compounds in the chemical ecology and in warfare of Actinobacteria was also highlighted (Avalos *et al.*, 2018, 2020; Schulz-Bohm *et al.*, 2017). Specifically, the volatile compounds geosmin and 2-MIB that are commonly produced by *Streptomyces* spp., have been shown to attract arthropods that can disperse spores and were also regulated by sporulation specific transcription factors (Becher *et al.*, 2020).

To begin to unravel the molecular mechanisms of microbial competition in *Streptomyces*, here we study transcriptional responses of the model species, *Streptomyces coelicolor*, during growth and development with and without an antagonistic competitor. The production of coloured secondary metabolites, including the blue actinorhodin, and the fully sequenced and annotated genome make this strain an ideal candidate for phenotypic screening and trancscriptomic analysis.

RESULTS

Phenotypic responses to interaction

During a previous study, colonies of *Streptomyces coelicolor* were co-cultured with different Actinobacteria, without physical contact, and tested for changes in phenotype and secondary metabolite production (Chapter 5 of this thesis). When grown in close proximity to *Kitasatospora* sp. MBT66 (from now on called MBT66) on a defined minimal medium, colonies of *S. coelicolor* underwent a clear change in phenotype. After 4 days of co-culture, a blue ring was seen around the colony edge, while no blue colouration was seen during growth in isolation, suggesting that MBT66 induced the production of the blue-pigmented antibiotic actinorhodin (Act) (FIG. 1). In addition, morphological changes were observed, including the formation of wrinkles and bald patches in the mycelium during the interaction. No difference

in pH of the media around the colonies was detected when *S. coelicolor* was either grown alone or next to MBT66.

We next set out to establish the nature of the cue that lead to this change in phenotype. To establish if volatile organic compounds (VOCs) may play a role, a strip of agar was removed between the colonies, inhibiting all communication via soluble materials, but enabling interactions via volatile compounds. No change in phenotype was observed (FIG. 1),

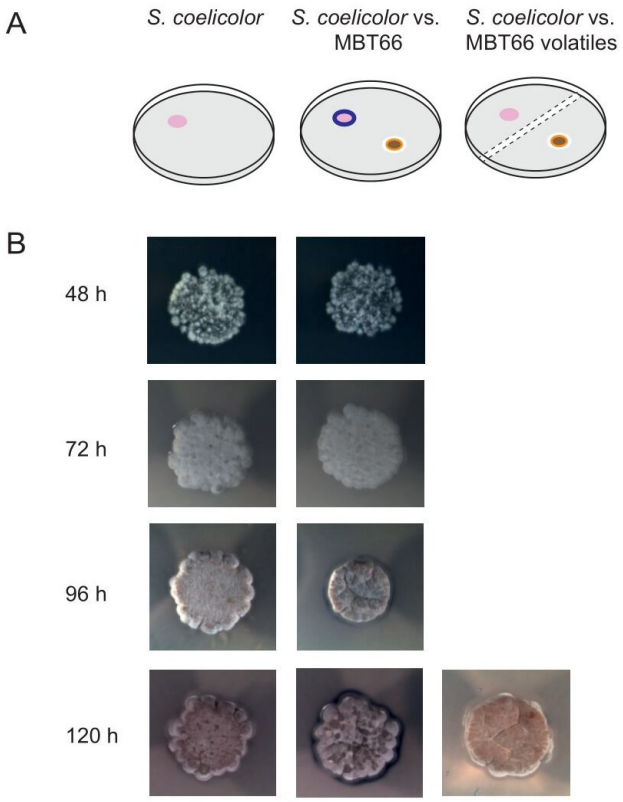


Fig. 1. Phenotypic analysis of *S. coelicolor* grown alone and in interaction with *Kitasatospora* sp. MBT66. (A) Schematic depicting *S. coelicolor* growth alone or in interaction. Small minimal medium agar plates were inoculated with 1 ul spots containing 10^3 spores to form colonies. *S. coelicolor* was either grown alone (left) or in co-culture with MBT66 (middle). The effect of volatiles on the interaction was studied by removing a slice of agar in between the colonies to prevent diffusion between the two strains (right). (B) Representative images of the *S. coelicolor* colonies from the experiments described in (A) at different timepoints.

indicating that the phenotypic changes in *S. coelicolor* resulted from molecules diffusing through or nutrients disappearing from the agar. We next examined if MBT66 was capable of producing compounds that inhibit the growth of *S. coelicolor*, by inoculating *S. coelicolor* in a soft agar overlay on a 4 day old *Kitasatospora* colony. This revealed that the MBT66 colony indeed inhibited *S. coelicolor* growth. We then 'pre-conditioned' the media by growing a colony of MBT66 for 1-4 days, before removing the colony and inoculating a *S. coelicolor* adjacent to removed colony. We found that also then *S. coelicolor* growth was inhibited, whereby a high quantity of Act was produced, suggesting that MBT66 produced one or more bioactive compound(s) regardless of the presence or absence of *S. coelicolor*. These effects were visible on media pre-conditioned with a colony of MBT66 grown for 2 days, but not if MBT66 was removed earlier. As nutrient stress is a well-known inducer for secondary metabolite production, we tested whether the same response was observed when we supplemented the conditioned medium with additional resources. Replenishing the media with the original resource levels (MM with 0.5% glycerol) did not mitigate the observed effects, indicating that these are not caused by resource competition, but by interference competition.

Analysis of differentially expressed genes during interaction

In order to better understand the changes occurring at the cellular level in *S. coelicolor* during this competitive interaction, we compared the transcriptional profiles of *S. coelicolor* colonies grown alone versus growth in proximity to colonies of MBT66. We chose four timepoints, starting at 48 h, when MBT66 had produced the inducing compound, up to 120 h when we observed Act production by *S. coelicolor*. Among the 7824 proteins encoded by the *S. coelicolor* genome, 1041 (approximately 13%) were differentially expressed genes (DEG) between growth alone and during co-culture at the different timepoints ($P < 0.05$) (FIG. 2). Most DEGs were found when comparing samples obtained after 48 and 72 h (FIG. 2A). Only 11 genes were differentially expressed at all four timepoints (FIG. 2B and D). Of these DEGs one was upregulated at all timepoints (SCO3436, encoding a possible fatty acid CoA ligase) and two were downregulated (SCO2408, for a possible aminotransferase and SCO7189, for a hypothetical protein). SCO1418, encoding a possible integral membrane protein, was upregulated at 48 h, but downregulated at all following timepoints. The remaining 7 DEGs were all downregulated up to 96 h, but upregulated at 120 h. These include SCO2210–2212, encoding a glutamine synthetase (glnII) and two hypothetical proteins; SCO5583–5585,

encoding an ammonium transporter (amt), nitrogen regulator protein P-II (glnB) and probable protein pII uridylyltransferase (glnD); and SCO5840, for a probable transcriptional regulator.

We screened all DEGs for enriched functional categories using GO and Panther terms ($P < 0.05$), which revealed that transporter proteins were significantly enriched at 72 h (FIG. 2C). Specifically, anion transport and inorganic anion transport were detected as significantly enriched processes at 72 and 96 h, respectively. Other transporters that were

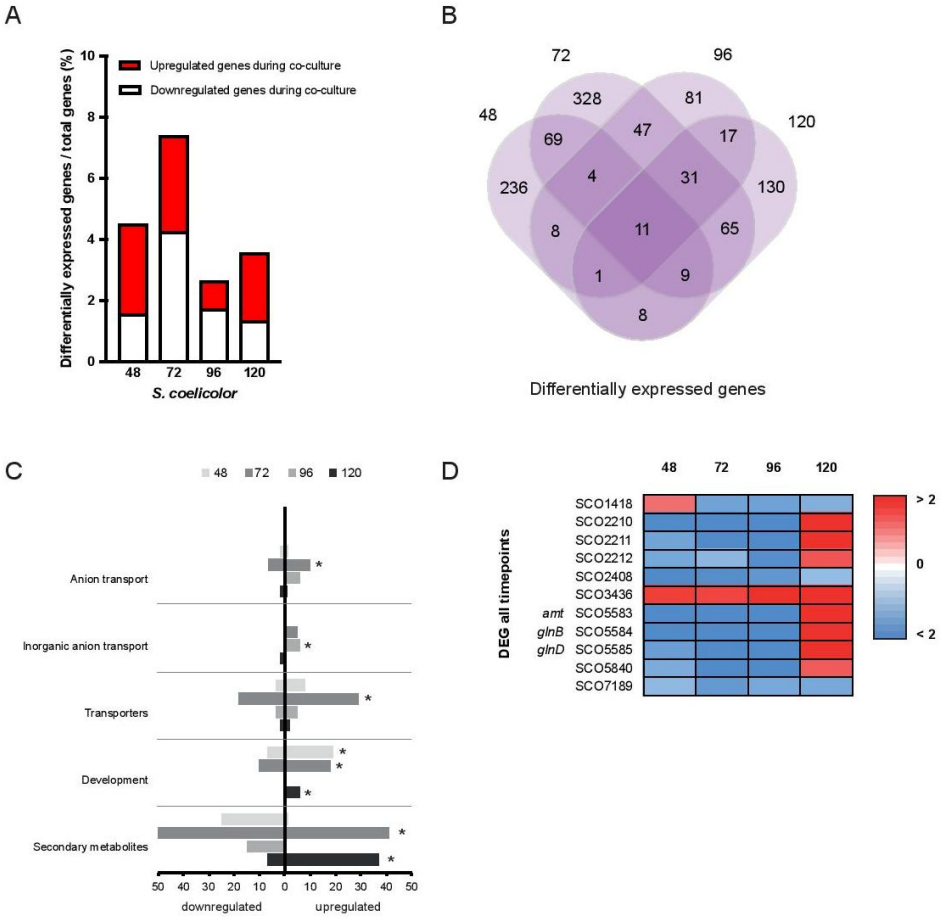


Fig. 2. Differentially expressed genes (DEG) during co-culture. (A) Number of up- and down-regulated genes at the different timepoints during co-culture. (B) Venn diagram showing all DEG. (C) DEG in enriched functional categories, stars indicate significant enrichment at $p < 0.05$. (D) Heatmap depicting the genes that are differentially expressed at all four timepoints.

differentially expressed included, amongst others, those involved in cation transport and a multidrug efflux protein (FIG. 3A). In addition, we analyzed processes specific for Streptomyces, including development and secondary metabolite production, which revealed that DEG involved in these processes were significantly enriched (FIG. 2C).

Differential expression of developmental genes during interaction

Many developmental genes were differentially expressed as result of the interaction, which is in accordance with the observed changes in morphogenesis (FIG. 3B). Developmental genes were significantly enriched in the interaction at 48 h, 72 h and 120 h (FIG. 2C). All of the differentially expressed developmental genes were upregulated at these timepoints, apart from *bldKa* and *whiG,* which were downregulated. The *bldK* gene cluster encodes an oligopeptide transporter that is required for development (Nodwell *et al.*, 1996), and *whiG* encodes the sigma factor WhiG that is required for the early stages of aerial hyphae formation (Mendez and Chater, 1987).

Upregulated developmental genes included the *whiE* gene cluster and the *whiE*-like genes (gene cluster SCO7449–7453) that both produce spore pigments (Salerno *et al.*, 2013) (Kelemen *et al.*, 1998), *whiD,* which is required for the differentiation of aerial hyphae into mature spore chains, and the sporulation genes *ssgA* and *ssgB*, which are required for the activation of sporulation-specific cell division and therefore also for sporulation (Keijser *et al.*, 2003; Van Wezel *et al.*, 2000). The chaplin and rodlin proteins, encoded by respectively the *chp* and *rdl* genes, form amyloid-like fibrils that lower the water surface tension to allow aerial growth and cover aerial structures, making them hydrophobic (Claessen *et al.*, 2003, 2002; Elliot *et al.*, 2003). The *chp* and *rdl* genes were significantly upregulated during the interaction at 72 h or 120 h, as was the *ramCSAB* operon involved in the production of SapB, a lanthibiotic-like peptide that acts as a signaling molecule for the onset of development (Kodani *et al.*, 2004; Willey *et al.*, 1991).

The downregulated developmental genes are involved in the commitment to development, while the upregulated genes are involved in later steps of the sporulation, which could indicate a shift in the timing of development, with development occurring earlier during interaction with *Kitasatospora* colonies.

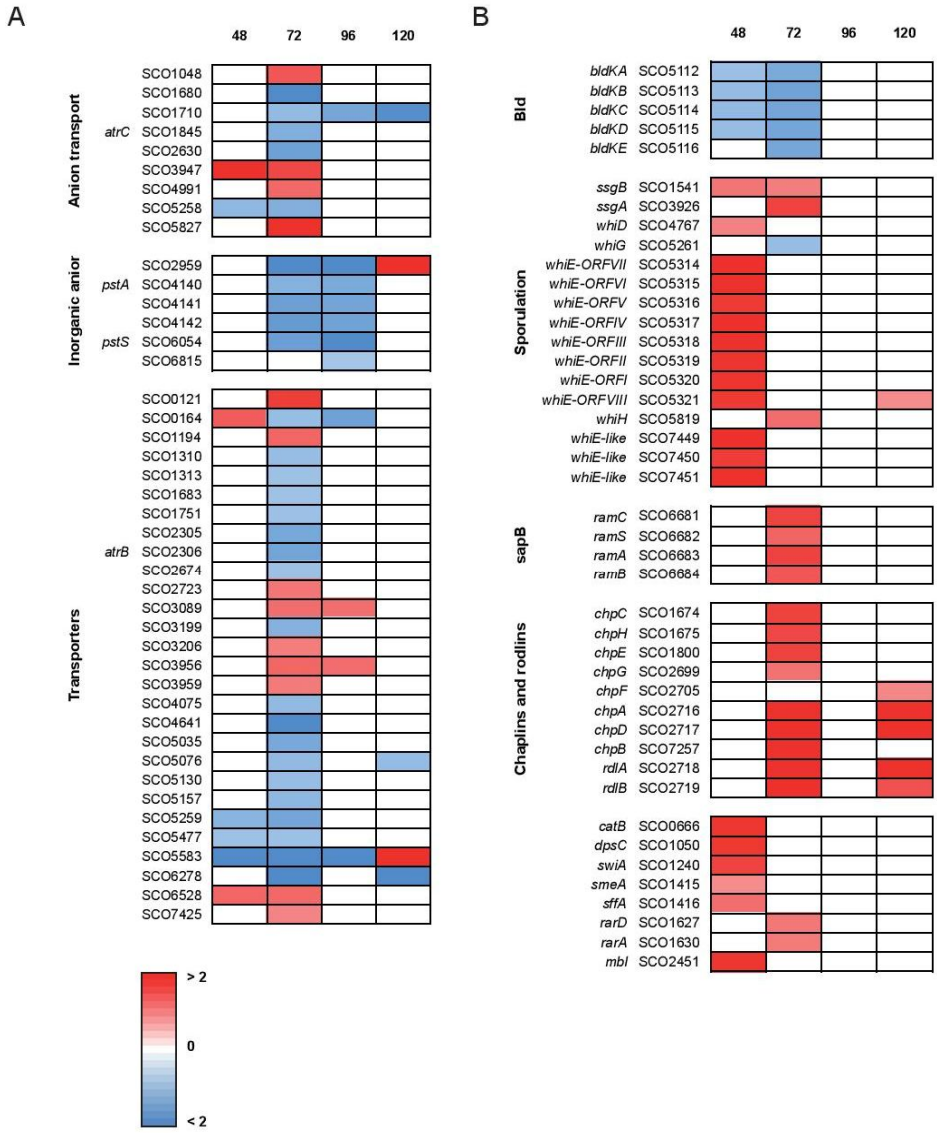
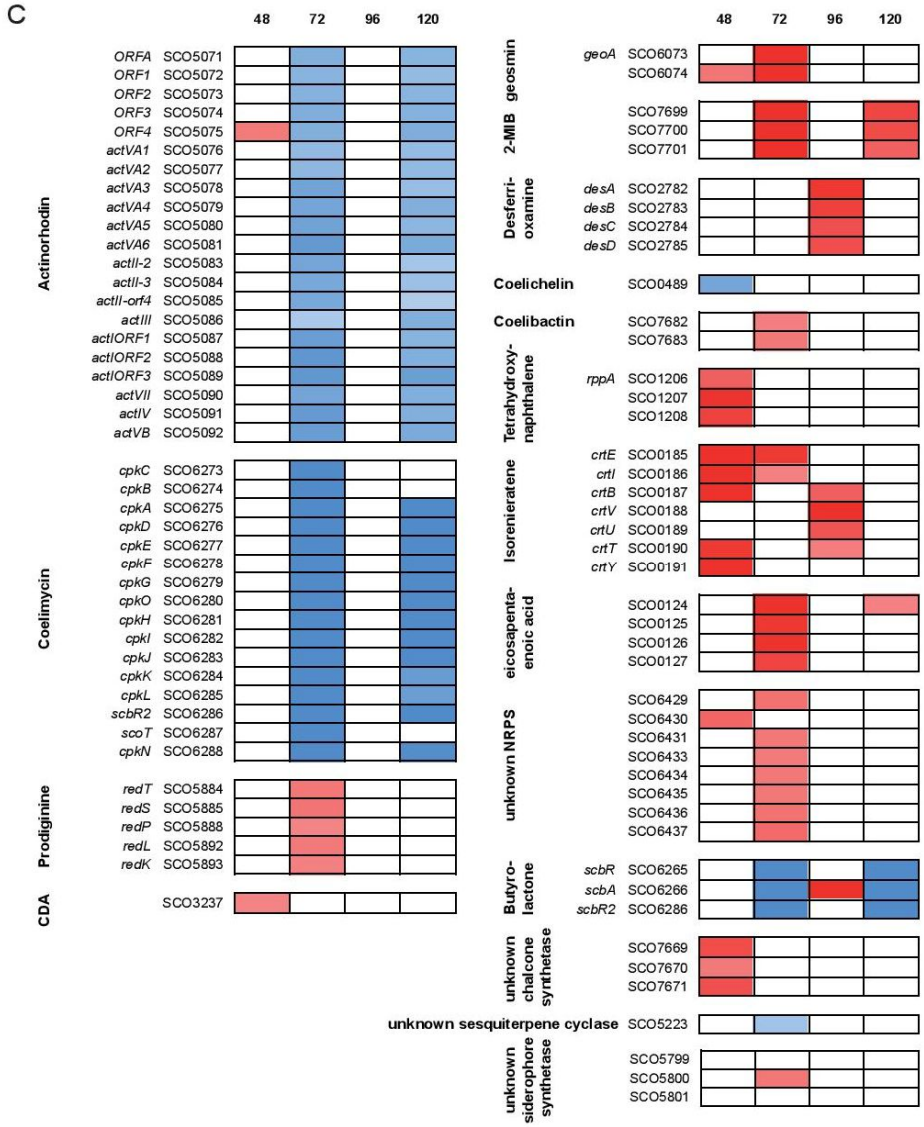


Fig. 3. Heatmaps showing DEG involved in (A) transport, (B) development and (C) secondary metabolism.


C
48
72
96
120
Actinorhodin
ORFA SCO5071
ORF1 SCO5072
ORF2 SCO5073
ORF3 SCO5074
ORF4 SCO5075
actVA1 SCO5076
actVA2 SCO5077
actVA3 SCO5078
actVA4 SCO5079
actVA5 SCO5080
actVA6 SCO5081
actII-2 SCO5083
actII-3 SCO5084
actII-orf4 SCO5085
actIII SCO5086
actIORF1 SCO5087
actIORF2 SCO5088
actIORF3 SCO5089
actVII SCO5090
actIV SCO5091
actVB SCO5092
Coelimycin
cpkC SCO6273
cpkB SCO6274
cpkA SCO6275
cpkD SCO6276
cpkE SCO6277
cpkF SCO6278
cpkG SCO6279
cpkO SCO6280
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scoT SCO6287
cpkN SCO6288
Prodiginine
redT SCO5884
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redP SCO5888
redL SCO5892
redK SCO5893
CDA
SCO3237
geosmin
geoA SCO6073
SCO6074
2-MIB
SCO7699
SCO7700
SCO7701
Desferri-oxamine
desA SCO2782
desB SCO2783
desC SCO2784
desD SCO2785
Coelichelin
SCO0489
Coelibactin
SCO7682
SCO7683
Tetrahydroxy-naphthalene
rppA SCO1206
SCO1207
SCO1208
Isorenieratene
crtE SCO0185
crtI SCO0186
crtB SCO0187
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crtT SCO0190
crtY SCO0191
eicosapenta-enoic acid
SCO0124
SCO0125
SCO0126
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unknown NRPS
SCO6429
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SCO6436
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Butyro-lactone
scbR SCO6265
scbA SCO6266
scbR2 SCO6286
unknown chalcone synthetase
SCO7669
SCO7670
SCO7671
unknown sesquiterpene cyclase SCO5223
unknown siderophore synthetase
SCO5799
SCO5800
SCO5801

Differential expression of secondary metabolite genes during interaction

The production of secondary metabolites is often affected by microbial interactions, as exemplified by antibiotics or siderophores (Traxler *et al.*, 2013; Lee *et al.*, 2020; Abrudan *et al.*, 2015) (and Chapter 5 of this thesis). We noticed significant changes to genes within 17 of the 22 BGCs of *S. coelicolor,* although in six cases only a minority of the genes in the BGC was differentially expressed (Bentley *et al.*, 2002) (FIG. 3C). Two of the five antibiotic BGCs showed a change during interaction, namely those for Act and coelimycin biosynthesis, which were downregulated in a similar pattern. The downregulation of the *act* gene cluster at 72 and 120 h during the interaction is surprising, because the blue-pigmented Act was visually increased.

The *S. coelicolor* gamma-butyrolactone quorum sensing molecule SCB1 plays a role in the regulation of these antibiotics by stimulating their production (Takano, 2006; Takano *et al.*, 2005). The gamma-butyrolactone receptor gene *scbR* (SCO6265) and the SCB1 biosynthetic gene *scbA* (SCO6266) were both downregulated at 72 and 120h, consistent with the downregulation of the *act* and *cpk* clusters. A similar downregulation was seen for the gene encoding pseudo gamma-butyrolactone receptor ScbR2, which does not bind the endogenous signaling molecule SCB1, but instead binds Act and undecylprodigiosin (Xu *et al.*, 2010).

Iron competition through the use of iron-chelating siderophores influences microbial interactions, because iron availability impacts the timing of development and of secondary metabolite production (Lambert *et al.*, 2014; Traxler *et al.*, 2012). *S. coelicolor* possesses at least three BGCs for the production of siderophores, namely desferioxamine (SCO2782-2785), coelichelin (SCO0489–0499)and coelibactin (SCO7681–7691) and in addition contains a BGC for an unknown siderophore (SCO5799–5801) (Bentley *et al.*, 2002). During interaction with MBT66, the desferioxamine BGC of *S. coelicolor* was upregulated at 96h, perhaps reflecting iron stress due to the presence of a competing strain. In addition, single genes in the BGCs for coelibactin, coelichelin and the putative siderophore synthase were differentially expressed.

Interestingly, genes involved in the production of the volatile organic compounds (VOCs) geosmin and 2-methylisoborneol (2-MIB) were highly upregulated during the interaction (FIG. 3C). Together, the sesquiterpene geosmin and the monoterpene 2-MIB lend the earthy aroma to soil. These two VOCs are widspread in streptomycetes, with geosmin nearly completely conserved (Martín-Sánchez *et al.*, 2019). A recent study links the production of these two compounds to sporulation through two sporulation-specific transcription factors, BldM for 2-MIB and WhiH for geosmin (Becher *et al.*, 2020). In accordance with this study we

also see a concomitant upregulation of *whiH* and *geoA* at 72 h, but we do not see an upregulation of *bldM* expression during co-culture.

Quantifying actinorhodin production

Although we visually observed an increase in Act production by the appearance of a blue ring around the colony during interaction with MBT66, we observed downregulation of the Act BGC at two of the four timepoints (72 and 120 h). To quantify the difference in Act production, the compound was extracted from agar-grown colonies. Surprisingly, there was no difference in Act production between colonies grown in isolation or next to MBT66, as measured by absorption at 640 nm (Fig. 4A). To further explore this result, we used a reporter strain that has the gene for eGFP under the control of the promoter of *actII*-ORF4, which encodes the pathway-specific activator of the *act* cluster. Imaging of the colonies showed that after 72h of growth, when downregulation of the *act* cluster was observed in the transcriptome experiments, there was no significant difference in the total GFP expression across the colony (Fig. 4B-C). However, for colonies in co-culture GFP expression was not uniform across the colony, and was significantly higher in the outer ring compared to a colony grown alone (two-sample T-test, $p = 0.041$) (Fig. 4D). This suggests variation in the localization of Act production in these two conditions, which can explain the observed phenotype.

DISCUSSION

Streptomyces genomes encode the biosynthetic machinery for a large variety of secondary metabolites that play a role in intermicrobial competition, including antibiotics and siderophores. Moreover, they have the means to escape unfavourable circumstances through a complex developmental program resulting in sporulation. Secondary metabolite production and development are tightly regulated and intertwined and an important question is how environmental cues during microbial interactions affect these processes. Here, we analyzed transcriptional changes through time in a *S. coelicolor* colony when grown in close proximity to colonies of the antagonistic *Kitasatospora* sp. MBT66. Our data show that transcription in *S. coelicolor* is altered significantly by the presence of the competitor, whereby the transcription of 13% of its genes was changed, with most changes happening at the 72h timepoint. Specifically, DEGs were enriched for the functional categories related to transport, secondary metabolite production and, most interestingly, development.

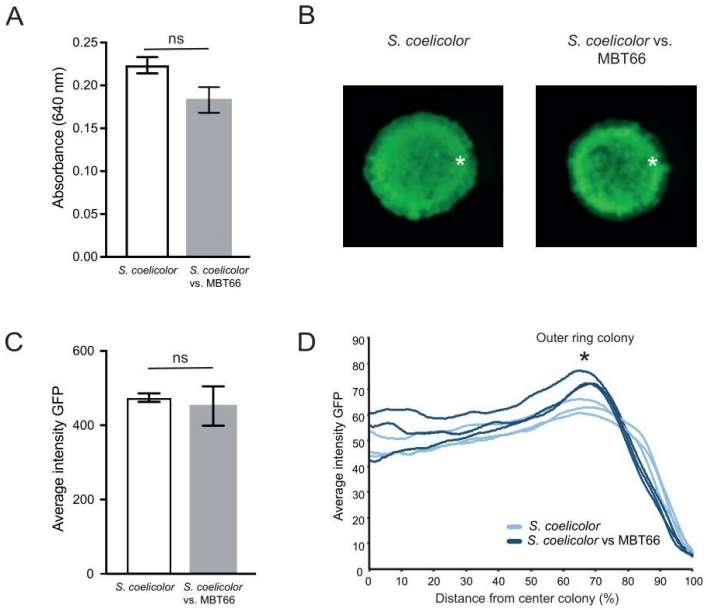

Fig. 4. Actinorhodin production during co-culture. (A) Actinorhodin production of *S. coelicolor* alone or in interaction measured by absorption at 640nm. (B) Colonies of a *S. coelicolor* ActII-ORF4-GFP promoter fusion strain at 72h alone or in co-culture with MBT66. (C) Average intensity of GFP in the two conditions. (D) ActII-ORF4-GFP expression patterns, measured as average GFP intensity from the center of the colony, during growth alone and in interaction with MBT66. Star corresponds to the stars in (B) to indicate the outer ring of the colony.

A commonly reported response to competition, possibly biased by researchers' focus on antibiotics, is the induction of secondary metabolite production in streptomycetes (Lee *et al.*, 2020; Traxler *et al.*, 2013; Ueda *et al.*, 2000; Bertrand *et al.*, 2014; Schäberle *et al.*, 2014). In contrast to these earlier results, we found that the Act and Cpk BGCs were downregulated at the transcriptional level. We previously reported the suppression of antibiotic production as a common response to competition (Abrudan *et al.*, 2015) (Chapter 5 of this thesis) and the results presented here corroborate this finding at the transcriptomic level. However, the downregulation of *act* gene expression apparently contrasts the observed competition-mediated accumulation of Act around *S. coelicolor* colonies (FIG 1). While quantification assays showed no relevant changes in Act content of colonies grown alone or in interaction, a significant, though small, increase in *actII-ORF4* promoter activity was observed when GFP was used as a

reporter. Since a small increase in the expression of a pathway-specific activator may have a large impact on expression of the entire BGC (van der Heul *et al.*, 2018), this could explain the strong Act accumulation around the colonies in competition. Targeted production at the site of interaction could benefit the producer by increasing local concentrations and possibly mitigate some of the costs. Bacteriocin producing cells that are under immediate attack have been shown to self-lyse to release toxins, decreasing the cost of production by sacrificing already dying cells (Granato and Foster, 2020). Moreover, *S. coelicolor* colonies are partitioned via a division of labour into cells that vary in their antibiotic production (Zhang *et al.*, 2020); if similar partitions occur spatially, this could generate the patterns we observe.

The timing of secondary metabolite production in *Streptomyces* is linked to the developmental cycle (van der Heul *et al.*, 2018). While the molecular mechanisms underlying this association are known, the ecological implications of sporulation or changes in developmental timing are less well studied. *Streptomyces* likely defend their resources by secreting antibiotics (Westhoff *et al.*, 2020). An alternative strategy that may entail different costs or benefits is to escape or survive unfavorable environments by producing resistant spores. *Streptomyces* colonies produce huge numbers of small hydrophobic spores with a relatively thick coat, containing protective small molecules including sugars such as trehalose and heat shock proteins (Bobek *et al.*, 2017). In this dormant state, cells become widely resistant as they arrest their growth, discontinue replication and become metabolically inactive (Bobek *et al.*, 2017). Spores not only protect genetic information during unfavorable conditions, but are also adapted to wind and water dispersal (Gebbink *et al.*, 2005). In this way, sporulation serves either dispersal to more favourable environments or survival in the hostile environment to germinate when circumstances have improved. The results of our transcriptomic study imply that the developmental programme is modified by microbial interactions, possibly leading to an earlier commitment to sporulation. Studying not only the timing of sporulation during microbial competition, but also spore quantity and viability, will further our understanding as to whether sporulation can be seen as a strategic choice to mediate competition.

We also observed upregulation of genes for the VOCs geosmin and 2-methylisoborneol, although we were unable to confirm the levels of geosmin and 2-MIB produced during co-culture with MBT66 in this study, as both strains produced these volatile compounds (unpublished observations). Geosmin production has been linked to sporulation, and mutants unable to produce aerial mycelia do not produce it (Bentley and Meganathan, 1981; Schöller *et al.*, 2002). Recently, the sporulation specific transcription factors BldM and

WhiH were found to regulate the production of 2-MIB and geosmin respectively (Becher *et al.*, 2020). Whether increased expression of the genes for these VOCs during co-culture is a result of co-regulation with development through these transcription factors or instead represents a specific response to competition remains to be tested. An increase in production of these odorous VOCs in response to competition will attract agents for dispersal, such as arthropods and larger animals, thereby facilitating the dispersal of spores away from harmful competition. Transcriptomic analysis of *S. coelicolor* grown in co-culture with the predatory bacterium *Myxococcus xanthus* revealed a similar upregulation of genes for VOCs, indicating that this could be a general response to microbial competition (Lee *et al.*, 2020). Analyzing the expression of these genes or directly measuring the production of the VOCs during microbial competition, could further our knowledge on the biological role and regulation of these common secondary metabolites.

Taken together, our results illustrate the changes that may occur in a developing *Streptomyces* colony during microbial competition. The data suggest that *Streptomyces* colonies do not only respond to competition by counterattacking through the increase of secondary metabolite production. Instead, they indicate that *Streptomyces* can modify their behavior to escape unfavorable environments by accelerating development so as to generate spores, the dispersal of which may be facilitated by the VOCs geosmin and 2-MIB. While the production of antibiotics implies aggression, sporulation ensures long-term survival or dispersion to new environments, suggesting *Streptomyces* bacteria balance the fine line between fight or flight during competitive interactions.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Streptomyces coelicolor A3(2) M145 and *Kitasatospora* sp. MBT66 (Zhu *et al.*, 2014) were used in this study. All experiments were performed on minimal medium agar (MM) containing 500 mg L-Asparagine (Duchefa Biochemie, The Netherlands), 500 mg KH2PO4 (Duchefa Biochemie, The Netherlands), 200 mg MgSO4.7H2O (Duchefa Biochemie, The Netherlands), 10 mg Fe2SO4.7H2O (Sigma Aldrich, MO, USA) and 20 g agar (T.M.Duche & Sons Ltd, Wilmslow, UK) per litre, adjusted to pH 7.2, and supplemented with 0.5% glycerol. Small petri dishes (35 mm) containing 4 ml of MM were inoculated with 1 ul spots containing 10^3 spores and were

incubated at 30°C. *S. coelicolor* was inoculated alone or one cm away from a colony of MBT66 or on MBT66 conditioned media as indicated.

RNA isolation

To isolate RNA from agar grown colonies, we inoculated *S. coelicolor* and MBT66 colonies as described above in three biological replicates and sampled destructively at four timepoints (48, 72, 96 and 120 h). At each timepoint an image was taken of a representative colony for each condition using a Leica-M80 stereo microscope. Colonies were excised from the agar using the backside of a P200 or P1000 pipet tip depending on colony size. Excess agar was removed from the bottom of the agar plug, resulting in a 2 mm thick agar disc containing the colony that was immediately frozen in liquid nitrogen and stored at -80°C. Two metal bullets (4 mm in diameter) were added to each sample and subsequently, samples were ground using a TissueLyser II (Qiagen, Hilden, Germany) in pre-chilled adapters at 30 Hz for 1 min. We adapted the Triton X-100/EDTA RNA isolation protocol described by Van Dessel et al. (2004). Briefly, the frozen material was suspended in 1 ml pre-warmed lysis buffer (5% (v/v) Triton X-100 and 10 mM EDTA pH 8.0) by vortexing. Supernatant was transferred to a clean tube before equal volume of phenol/chloroform (50:50, pH 4.9 – 5.5, VWR) was added. The sample was mixed by vortexing and centrifuged at 12,000 x g for 15 minutes and the aqueous phase was used for RNA isolation. The phenol/chloroform step was repeated once to ensure the removal of cell debris and proteins. Total nucleic acids were precipitated by addition of 125 l 1M Tris-HCl (pH 8.0), 50 l 5 M NaCl and 750 l chilled ethanol absolute and incubation at -20°C for at least 20 min. After centrifugation (5 min, 7,500 x g) the pellet was washed with 1 ml 80% EtOH. After centrifugation (5 min, 7500 x g) EtOH was removed and the pellets were air dried and resuspended in RNAse-free water. Subsequently, RNA was treated with 5 units RNase-free DNaseI (New England BioLabs) for 1 hour to remove DNA. Phenol/chloroform step was repeated to remove debris and protein and total nucleic acids were precipitated by addition of 25 ul 1M Tris-HCl pH 8.0, 10 ul 5M NaCl and 300 ul chilled EtOH absolute and incubation at -20°C for at least 20 min. After centrifugation (5 min, 7,500 x g) the pellet was washed with 1 ml 80% EtOH. After centrifugation (5 min, 7500 x g) EtOH was removed and the pellets were air dried and resuspended in RNAse-free water.

The RNA concentration was measured using Qubit RNA BR Assay Kit (ThermoFisher Scientific), RNA purity was assessed using A260/A280 and A260/A230 ratio using the Nano

Drop ND-1000 Spectrophotometer (PEQLAB). RNA Integrity Number was estimated using RNA 6000 Nano Kit (Agilent) and the Bioanalyzer 2100 (Agilent).

Library preparation and sequencing

A total of 1 μg of total RNA was subjected to rRNA depletion using Ribo-Zero rRNA Removal Kit Bacteria (Illumina). The cDNA libraries were constructed using the resulting tRNA and the NEBNext Ultra II Directional RNA Library Prep Kit (NEB). Libraries were sequenced as single-read (75 bp read length) on an Illumina NextSeq500 platform at a depth of 8–10 million reads each.

RNA-Seq data assessment and analysis

Sequencing statistics including the quality per base and adapter content assessment of resulting transcriptome sequencing data were conducted with FastQC v0.11.5 (Andrews). All reads mappings were performed against the reference strain of *Streptomyces coelicolor* A3(2) (RefSeq ID NC_003888.3). The mappings of all samples were conducted with HISAT2 v2.1.0 (Kim *et al.*, 2015). As parameters spliced alignment of reads was disabled and strand-specific information was set to reverse complemented (HISAT2 parameter --no-spliced-alignment and --rna-strandness "R"). The resulting mapping files in SAM format were converted to BAM format using SAMtools v1.6 (Li *et al.*, 2009). Mapping statistics, including SSP estimation, percentage of mapped reads and fraction exonic region coverage, were conducted with the RNA-Seq module of QualiMap2 v2.2.2-dev (Okonechnikov *et al.*, 2016). Gene counts for all samples were computed with featureCounts v1.6.0 (Liao *et al.*, 2014) based on the annotation of the respective reference genome, where the selected feature type was set to transcript records (featureCounts parameter -t transcript). To assess variability of the replicates of each time series, a PCA was conducted with the DESeq2 package v1.20.0 (Love *et al.*, 2014).

Differentially expressed genes were identified using DESeq2 of the bioconductor R library version '1.24.0' (Love *et al.*, 2014). For each timepoint, genes were classified as differentially expressed between the state "grown alone" (GA) and state "HM125" if the sum of reads per gene was higher than 10, the absolute log2 fold change was larger than 1, and the adjusted p value was larger than 0.05.

GO enrichment analysis

Gene ontology (GO) biological process, protein class and pathway enrichment analyses were performed on differentially expressed genes using the Panther web service (Thomas *et al.*, 2003). An enrichment test was performed for the set of developmental genes of *Streptomyces coelicolor* (see Table S1). This was done using the two-sided Fisher's exact test using the SciPy version 1.4.1 package in python 3.7.3 (Fisher, 1992; Virtanen *et al.*, 2020).

Quantification of actinorhodin production

Actinorhodin was extracted from agar grown colonies. Colonies were excised from agar using a pipet tip and an equal volume of methanol was added. After overnight incubation at room temperature, KOH (1N final concentration) was added and the concentration of actinorhodin was measured by UV absorption at 640 nm with a Amersham Biosciences Ultrospec 2100 pro. To study the localization of actinorhodin production, an actII-ORF4-GFP promoter fusion strain (provided by Huib Verheul/Dennis Claessen) was grown alone or in co-culture with MBT66 for 72h and was imaged using a Leica MZ16FA fluorescence stereo microscope. The green fluorescent images were created using a 450-490 nm excitation and 500-550 nm bandpass emission. Intensity of fluorescence was quantified using ImageJ.

SUPPLEMENTAL INFORMATION

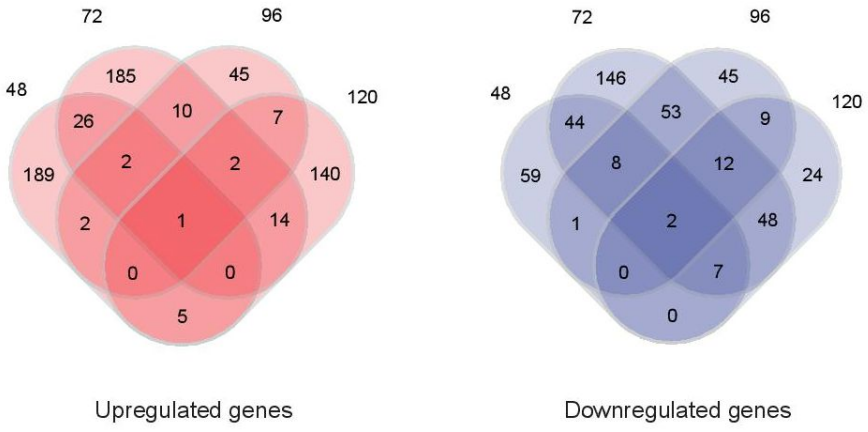

Fig. S1. Venn diagrams showing all differentially expressed upregulated and downregulated genes.

Table S1. List of *S. coelicolor* developmental genes

SCO number	Name	SCO number	Name	SCO number	Name
SCO0409	sapA	SCO2610	mreC	SCO5115	bldKD
SCO0596	dpsA	SCO2611	mreB	SCO5116	bldKE
SCO0666	catB	SCO2619	clpP1	SCO5141	samR
SCO0762	sti1	SCO2699	chpG	SCO5231	dasR
SCO1050	dpsC	SCO2705	chpF	SCO5261	whiG
SCO1240	swiA	SCO2716	chpA	SCO5314	whiE
SCO1385	lcm	SCO2717	chpD	SCO5315	whiE
SCO1386	lcm	SCO2718	rdlA	SCO5316	whiE
SCO1387	lcm	SCO2719	rdlB	SCO5317	whiE
SCO1415	smeA	SCO2736	citA	SCO5318	whiE
SCO1416	sffA	SCO2792	bldH	SCO5319	whiE
SCO1480	sIHF	SCO2924	ssgG	SCO5320	whiE
SCO1489	bldD	SCO2950	hupA	SCO5321	whiE
SCO1513	relA	SCO2968	ftsX	SCO5556	HupS
SCO1541	ssgB	SCO2969	ftsE	SCO5577	smc
SCO1626	rarE	SCO3034	whiB	SCO5723	bldB
SCO1627	rarD	SCO3095	divIC	SCO5750	ftsK
SCO1628	rarC	SCO3158	ssgE	SCO5751	rodZ
SCO1629	rarB	SCO3323	bldN	SCO5756	dpsB
SCO1630	rarA	SCO3549	bldG	SCO5819	whiH
SCO1662	ParJ	SCO3571	crp	SCO5967	sflB
SCO1674	chpC	SCO3886	ParA	SCO5999	acoA
SCO1675	chpH	SCO3887	ParB	SCO6029	whiI
SCO1749	sflA	SCO3925	ssgR	SCO6071	cprB
SCO1800	chpE	SCO3926	ssgA	SCO6312	cprA
SCO1950	whiA	SCO4034	sigN	SCO6681	ramC
SCO2075	ddbA	SCO4035	sigF	SCO6682	ramS
SCO2077	divIVA	SCO4091	bldC	SCO6683	ramA
SCO2078	sepG	SCO4126	cmdA	SCO6684	ramB
SCO2079	sepF	SCO4127	cmdB	SCO6685	ramR
SCO2080	ylmE	SCO4128	cmdC	SCO6722	ssgD
SCO2081	ylmD	SCO4129	cmdD	SCO7175	ssgF
SCO2082	ftsZ	SCO4130	cmdE	SCO7257	chpB
SCO2083	ftsQ	SCO4131	cmdF	SCO7289	ssgC
SCO2085	ftsW	SCO4543	whiJ	SCO7449	whiE-like
SCO2090	ftsI	SCO4767	whiD	SCO7450	whiE-like
SCO2091	ftsL	SCO4768	bldM	SCO7451	whiE-like
SCO2451	mbl	SCO4928	cya	SCO7452	whiE-like
SCO2607	sfr	SCO5112	bldKA	SCO7453	whiE-like
SCO2608	pbp2	SCO5113	bldKB	tRNA	bldA
SCO2609	mreD	SCO5114	bldKC		