

1 **The *SCO1731* methyltransferase modulates actinorhodin production and morphological**
2 **differentiation of *Streptomyces coelicolor* A3(2)**

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17

1 **Abstract**

2 *Streptomyces coelicolor* is a Gram-positive microorganism often used as a model of physiological
3 and morphological differentiation in streptomycetes, prolific producers of secondary metabolites
4 with important biological activities. In the present study, we analysed *Streptomyces coelicolor*
5 growth and differentiation in the presence of the hypo-methylating agent 5'-aza-2'-deoxycytidine
6 (5-aza-dC) in order to investigate whether cytosine methylation has a role in differentiation. We
7 found that cytosine demethylation caused a delay in spore germination, aerial mycelium
8 development, sporulation, as well as a massive impairment of actinorhodin production. Thus, we
9 searched for putative DNA methyltransferase genes in the genome and constructed a mutant of the
10 *SCO1731* gene. The analysis of the *SCO1731::Tn5062* mutant strain demonstrated that inactivation
11 of *SCO1731* leads to a strong decrease of cytosine methylation and almost to the same phenotype
12 obtained after 5-aza-dC treatment. Altogether, our data demonstrate that cytosine methylation
13 influences morphological differentiation and actinorhodin production in *S. coelicolor* and expand
14 our knowledge on this model bacterial system.

15

16

1 **Introduction**

2 Base methylation is a DNA modification present in all kingdoms of life, including bacteria. The
3 methylation of cytosines is an important epigenetic mark, well known in higher eukaryotes to
4 control transcriptional regulation that can cause repression or activation of gene expression. The
5 correct inheritance of epigenetic patterns is crucial to cell processes while atypical DNA
6 methylation is linked to numerous diseases, disorders and abnormalities^{1,2}. DNA methyltransferase
7 (Dnmt1) and UHRF1 (ubiquitin-like, containing PHD and RING finger domains) are recognized as
8 the main players in the preservation of DNA methylation in mammals.

9 In bacteria the majority of DNA methyltransferases described are part of restriction-modification
10 (RM) systems. A RM system consists of a restriction endonuclease and a DNA (adenine or
11 cytosine) methyltransferase. Usually, base methylation protects host DNA from DNA cleavage by
12 the associated endonuclease. ‘Orphan’ DNA methyltransferase genes can be found in many
13 bacterial genomes and probably derive from ancestral RM systems that lost the cognate restriction
14 enzyme. Additional roles in regulating several important cellular processes, such as initiation of
15 DNA replication, DNA repair and gene regulation, were proposed for bacterial adenine
16 methyltransferases³⁻⁸. The most famous examples are the adenine DNA methyltransferases Dam
17 and CcrM. In *Escherichia coli* Dam is important for gene expression as well as other cellular
18 processes, like DNA replication initiation and DNA repair⁹⁻¹¹. In *Caulobacter crescentus* and other
19 *Alphaproteobacteria* CcrM is essential to regulate gene expression and controls more than 10% of
20 the genes necessary for its cell cycle progression¹². Recently, roles in regulating gene expression
21 were also given to orphan cytosine methyltransferases of *Helicobacter pylori* and *Escherichia coli*.
22 In *H. pylori* an orphan cytosine methyltransferase influences the expression of genes involved in
23 motility, adhesion, and virulence¹³. In *E. coli*, the Dcm cytosine methyltransferase controls the
24 expression of two ribosomal protein genes, the drug resistance transporter gene *sugE* at early
25 stationary phase^{10,14,15} and the expression of genes associated with stationary phase¹⁶.

26 5-azacytidine (5-azaC) and 5-aza-2'-deoxycytidine (5-aza-dC) are cytosine DNA methylation
27 inhibitors routinely used to demethylate DNA in a variety of eukaryotes to assess the consequences
28 of cytosine DNA methylation loss^{17,18}. They are nucleoside analogs that are converted
29 intracellularly to the corresponding 5'-triphosphates upon cell entry; 5-azaC is incorporated into
30 both RNA and DNA, whereas 5-aza-dC only into DNA¹⁸⁻²². When these analogues are
31 incorporated, cytosine-5 DNA-dependent cytosine methyltransferases are locked on the DNA and
32 inhibited with the consequence of decreased 5-methylcytosines in newly replicated DNA^{20,21}.
33 Recently, 5-azaC use was applied to *E. coli* where it was found to modulate transcriptome²³.

1 Streptomycetes are Gram positive soil bacteria with CG rich genomes (70%). They are industrially
2 very important because they produce two thirds of all clinically relevant secondary metabolites²⁴.
3 *Streptomyces coelicolor* A(3)2 strain M145 is the best-known species of the *Streptomyces* genus at
4 both genetic and molecular level²⁵⁻²⁷ and it has long been considered as the model streptomycete for
5 studying physiological (antibiotic production) and morphological differentiation. *Streptomyces*
6 *coelicolor* A(3)2 M145 produces three well characterised antibiotics (actinorhodin, blue pigment,
7 Act; undecylprodigiosin, red pigment, Red; calcium-dependent lipopeptide antibiotic, CDA), and
8 has been described to encode for up to 30 additional secondary metabolites²⁸. *S. coelicolor* M145
9 exhibits a complex developmental cycle that includes sporulation and developmentally associated
10 programmed cell death^{29,30}. In a solid culture (i.e. GYM) five different cell types are sequentially
11 produced: the unigenomic spores, the first mycelium (MI), the second mycelium (MII), aerial cells
12 and sporulating cells. After spore germination, a viable vegetative mycelium grows on the surface
13 and within the agar matrix forming the first compartmentalized mycelium that undergoes a highly
14 ordered PCD. The remaining viable segments of these hyphae enlarge and form the second
15 multinucleated mycelium MII that comprises (i) the MII substrate that grows within the agar matrix,
16 (ii) the aerial MII characterized by hydrophobic layers and (iii) the sporulating MII, which
17 undergoes a second round of PCD followed by spore formation. *S. coelicolor* life cycle is regulated
18 at different levels by extracellular signals and quorum sensing-related factors, multiple master
19 regulators, and biochemical pathways, such as *bald*, *white* and *sky*^{27,31,32}, but little is known
20 regarding the effect of DNA methylation controlling differentiation.

21 *S. coelicolor* M145 has a stringent type IV restriction-modification system that cleaves exogenous
22 methylated DNA, and for its successful transformation it is first necessary to demethylate DNA
23 constructs in a *dam⁻ dcm⁻* mutant strain of *E. coli*³³. Recently, an endonuclease capable to bind to 5-
24 methyl-cytosine containing DNA in all sequence contexts was characterized³⁴. Years ago, the role
25 of DNA methyltransferases in *Streptomyces antibioticus* and *S. coelicolor* was investigated by
26 treating the cultures with demethylating agents and it was found that methylation could influence
27 development and differentiation³⁵⁻³⁷.

28 In this study, we investigated whether *S. coelicolor* M145 genome undergoes differential DNA
29 cytosine methylation during the growth cycle and whether treatment with a demethylating agent (5-
30 aza-dC) could affect growth and differentiation. We found that DNA cytosine methylation is
31 modulated during development and that demethylation impairs morphological differentiation and
32 actinorhodin production. Thus, we searched for DNA methyltransferase genes in the genome and
33 constructed a mutant in a putative DNA methyltransferase gene. Our data showed that in the

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between text and references 29,30

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1 *SCO1731::Tn5062* strain, methylation levels decreased and growth and differentiation were
2 delayed, similarly to the effects caused by the treatment of *S. coelicolor* M145 with the
3 demethylating agent. To the best of our knowledge, this is the first study that demonstrates the
4 involvement of cytosine methylation in the control of morphological and physiological
5 differentiation in a microorganism.

6

7 **RESULTS**

8 **DNA cytosine methylation varies during development of Streptomyces**

9 Genomic DNA was extracted from different developmental stages of *S. coelicolor* M145, *S.*
10 *avermitilis* ATCC 31267, *S. griseus* NBRC 102592 and *S. lividans* 1326 and analyzed by dot blot
11 assay using the antibody against 5-MeC (Fig. 1). To our surprise, this analysis showed that cytosine
12 methylation is higher at the MI stage than at the MII stages in all the conditions (solid GYM
13 cultures and sucrose-free R5A liquid medium) and species analysed (Fig. 1). Aerial hyphae (MII_{48h})
14 showed to have the lowest levels of methylated DNA in *S. coelicolor* development in solid GYM
15 cultures (Fig. 1a), while MII hyphae (MII_{55h}) showed the lowest methylation levels in liquid
16 sucrose-free R5A cultures (Fig. 1b).

17 *S. coelicolor* M145, *S. griseus* and *S. avermitilis* degrade exogenous methylated DNA, while *S.*
18 *lividans* does not^{38,39}. *S. lividans* 1326 and *S. coelicolor* M145 are different in accepting methylated
19 DNA, but they have a very similar genome and a similar development⁴⁰. Despite of that, DNA
20 cytosine methylation during development is comparable in all the *Streptomyces* species analyzed
21 (higher at the MI stage), indicating that it does not depend on RM systems.

22

23 **Effect of cytosine demethylation using 5-aza-dC on *S. coelicolor* M145 differentiation**

24 Thus, to assess whether the modulation of cytosine methylation could have a role on morphological
25 and physiological differentiation, a treatment with the hypomethylating agent 5'-aza-2'-
26 deoxycytidine (5-aza-dC) was performed. Experiments for the set-up of the cytosine DNA
27 demethylation treatment were carried out, as described in [MM](#). *Streptomyces coelicolor* M145
28 cultures were treated with 5 µM of the hypomethylating agent 5-aza-dC every 12h.

29 Analysis under confocal laser scanning microscopy (CLSM) after SYTO 9 and PI staining
30 demonstrated that 5-aza-dC reduced spore germination up to 65% in respect to the 95% of the
31 untreated culture after 9h of growth. **After 12h, the spores of the treated culture were germinated as**

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1 those of the untreated culture (Fig. 2 a-c). Growth curves on solid medium of the untreated and the
2 5-aza-dC treated *S. coelicolor* M145 cultures revealed that there was a little effect of 5-aza-dC on
3 bacterial growth for the first 63h; after 63h, the treated culture grew very slowly and remained in
4 the stationary phase (Fig. 2d). At 72h and 96h the 5-aza-dC treated samples showed the
5 multinucleated secondary mycelium (MII) characterized by non-septate branching non-sporulating
6 hyphae (Fig. 2f), while the untreated culture presented spore chains and single spores (Fig. 2e). In
7 the 5-aza-dC treated culture, the undecylprodigiosin (red pigment) and actinorhodin (blue pigment)
8 productions were decreased compared to the untreated culture (Fig. 2g-h).

9 In liquid sucrose-free R5A cultures, the addition of 5-aza-dC caused a decrease of growth rate (Fig.
10 3a) and a delay in germination; indeed, ungerminated spores were still present at 20h in the treated
11 culture, as visualized by CLSM (Supplementary Fig. S1). In the 5-aza-dC treated liquid culture,
12 undecylprodigiosin production started later in the treated culture, but after 100h, the yields were
13 similar (Fig. 3b), while actinorhodin production was 4-fold decreased and started later in respect to
14 the untreated culture (Fig. 3c).

15 So far, these experiments revealed that 5-aza-dC induces a delay in morphological differentiation
16 both in liquid and solid medium, influencing spore germination, mycelium development and
17 sporulation; in addition, actinorhodin yield was impaired.

18

19 Construction of a mutant in the putative DNA methyltransferase *SCO1731* gene

20 A bioinformatics search revealed that in *S. coelicolor* M145 genome there are annotated 38 genes
21 coding for putative DNA methyltransferases (Table 1). The expression profile of these putative
22 DNA methyltransferases was compared to the transcriptomic data, previously obtained using the
23 same growth media²⁹. This search revealed that *SCO1731* displayed the highest transcription level
24 among the putative methyltransferase genes in MI and has orthologs in *S. lividans* 1326 genome
25 (100% identity), *S. avermitilis* ATCC 31267 (79%) and *S. griseus* strain NBRC 102592 (64%).

26 Thus, since methylation levels were found higher in MI we evaluated if this gene is important for
27 cytosine methylation by generating a mutant using a cosmid containing the gene interrupted by the
28 transposon Tn5062⁴¹. In liquid sucrose-free R5A cultures, disruption of *SCO1731* did not
29 significantly alter the growth kinetics (Fig. 4a) and spore germination (Supplementary Fig. S1) of
30 the *SCO1731::Tn5062* mutant strain, indicating that this gene is not critical for bacterial growth
31 under the used conditions.

32 Dot blot analysis demonstrated that the cytosine methylation levels were strongly reduced in the
33 *SCO1731::Tn5062* mutant strain at 20h (MI) (from $xx \pm SD$, to $yy \pm SD$) (Fig.4b). Differently, the

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Aza treated cultures show slower growth in R5A (Fig. 3A). So there is something more than SCO1731 in the effect observed in aza-treated cultures.

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1 *SCO1731::Tn5062* mutant had a slight effect on cytosine methylation after 55h of growth (MII)
2 (Supplementary Fig. S2). Actinorhodin (blue pigment) was not observed in the *SCO1731* mutant
3 strain in liquid sucrose-free R5A even after 96h of growth (Fig. 5), revealing that the mutant is
4 impaired in actinorhodin production. *SCO1731::Tn5062* mutant cultures produced
5 undecylprodigiosin (red color) (Fig. 5).
6 Likewise, the inactivation of *SCO1731* caused a marked delay in morphological and physiological
7 differentiation on solid GYM (Fig. 6): aerial mycelium formation started at 96h in the
8 *SCO1731::Tn5062* mutant strain compared to the 48h in the wild-type strain; spore chains were not
9 formed up to 96h in the mutant strain compared to 72h in the wild-type strain; actinorhodin (blue
10 color) and undecylprodigiosin (red color) were strongly reduced in the mutant strain.
11 These results indicate that *SCO1731* is responsible for methylation of cytosine in MI and it is
12 involved in the regulation of actinorhodin production and morphological differentiation.
13 A complemented strain harboring a copy of the *SCO1731* ORF and its upstream region large
14 enough to include the promoter region, was generated. In the *SCO1731_compl* strain, the
15 methylation levels were restored to 70% of the wild-type methylation level (Fig. 4b), the
16 morphological development (aerial mycelium and sporulation) was fully restored (Fig. 6), and
17 actinorhodin production was reestablished at 96h, but not at 72h (Fig. 5). The control strain,
18 containing a copy of the empty integrative vector used for complementation (*S. coelicolor*+pNG3),
19 showed a normal antibiotic production profile (Fig. 5) and development (Fig. 6), excluding an effect
20 of pNG3 integration on the phenotypes observed in the *SCO1731_compl* strain.

21

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1 Discussion

2 Cytosine and adenine methylation are epigenetic mechanisms to control gene expression in
3 eukaryotic and prokaryotic organisms, respectively. While adenine methylation has been largely
4 studied in many bacterial systems and it was shown to influence crucial vital processes, such as
5 bacterial cell cycle, only a few studies have so far been published on cytosine methylation in
6 bacteria, mostly in *Escherichia coli*^{4,5,16,23}. Years ago, some attempts to find a role for DNA
7 methyltransferases in *S. antibioticus* and *S. coelicolor* were reported. These studies applied different
8 compounds known to block cytosine methylation (e.g. 5-azacytidine and sinefungin), but no clear
9 role for cytosine methylation was established³⁵⁻³⁷. Here, we demonstrate that methylation levels are
10 modulated throughout the growth cycle in both, solid and liquid media. This result is of particular
11 interest, since *S. coelicolor* transformation efficiency depends on the methylation status of
12 exogenous DNA³⁸⁻⁴⁰. We also demonstrate that the hypo-methylating agent 5'-aza-2'-deoxycytidine
13 (5-aza-dC) causes a delay in spore germination, aerial mycelium differentiation and sporulation in
14 solid medium and **affects growth and spore germination in liquid medium**; in addition, actinorhodin
15 production is massively impaired in both solid and liquid media; differently, **undecylprodigiosin**
16 **production is retarded, but the yields in the treated cultures are similar to the untreated ones.**
17 Unfortunately, our results cannot be compared to previous reports using hypo-methylating agents in
18 *Streptomyces*³⁵⁻³⁷, since 5-azacytidine is incorporated into both DNA and RNA, and sinefungin is an
19 inhibitor of SAM-dependent cytosine and adenine methyltransferase, while in our experiments we
20 used 5-aza-2'-deoxycytidine that is only incorporated in DNA. Thus, the effect we noticed is
21 essentially due to cytosine methylation in the genome.

22 We demonstrate that *SCO1731* codes for a cytosine methyltransferase involved in the cytosine
23 methylation accompanying *Streptomyces* differentiation. In the *SCO1731::Tn5062* mutant strain,
24 cytosine methylation was reduced to 22% compared to the parental strain during the MI stage. Our
25 results cannot rule out that other methyltransferases may be responsible for residual methylation nor
26 that other methyltransferases may be expressed following *SCO1731* activation in a cascade manner.
27 In fact, 38 genes coding for putative methyltransferases are present in *S. coelicolor* genome, making
28 this a reasonable hypothesis. Among them, *SCO1731* was transcribed in MI at higher levels, when
29 cytosine methylation is higher than in other growth stages both in liquid and solid medium.
30 Moreover, the *SCO1731::Tn5062* mutant shows the same phenotype observed in the 5-aza-2'-
31 deoxycytidine treated cultures regarding the effect on actinorhodin production, aerial mycelium
32 differentiation and sporulation, that resulted impaired in the *SCO1731::Tn5062* mutant and the 5-
33 aza-2'-deoxycytidine treated cultures; differently, the delay observed in undecylprodigiosin

1 production, spore germination and growth in the 5-aza-2'-deoxycytidine treated cultures was not
2 observed in the *SCO1731::Tn5062* mutant. This further indicates that *SCO1731* is not the only
3 methyltransferase participating in the regulation of *Streptomyces* development. Interestingly, the
4 complemented mutant strain restored partially the methylation levels to 70% and if on the one hand
5 this was sufficient to restore the correct morphological development, on the other one said
6 methylation level was not sufficient to re-establish the onset of the actinorhodin production, further
7 supporting that some other cellular events do occur. This kind of multilevel regulation would be not
8 far from other bacterial systems, better investigated for influence of DNA methylation on gene
9 expression. For instance, in *C. crescentus* many genes are controlled by the CcrM methyltransferase
10 and are also co-regulated by other global cell cycle regulators, demonstrating an extensive cross talk
11 between DNA adenine methylation and the complex regulatory network governing cell cycle
12 progression¹².

13 Given that *S. coelicolor* undergoes a complex life cycle with two programmed cell death events, we
14 hypothesized that the hypermethylation of genomic DNA in MI could be a signal that activates
15 DNA cleavage in some cells leading to cell death and allowing a controlled life cycle. In *E. coli*, it
16 was demonstrated that cell death occurs upon an induced cytosine hypermethylation of genome⁴²⁻⁴⁴.
17 Even if this were the case, it would still be difficult to explain how adjacent cells perceive different
18 stimuli and follow different fates²⁹. If this hypothesis were correct, PCD and differentiation of *S.*
19 *coelicolor* would have to be blocked after 5-aza-dC treatment and in the *SCO1731::Tn5062* mutant
20 strain, and this is not the case. Notwithstanding it is possible that 5-aza-dC effect is temporary and
21 that other methyltransferases or pathways are activated in the *SCO1731::Tn5062* mutant strain. An
22 alternative hypothesis about the biological role of cytosine methylation, is that it influences gene
23 expression, as it occurs in other systems^{14-16,23,45}. Methylation of cytosines in eukaryotic promoters
24 leads to repression of transcription and to an activation when it affects gene bodies⁴⁵. In *E. coli* the
25 absence of the *dcm* gene leads to a differential expression of 510 genes, i.e. two ribosomal protein
26 genes and the drug resistance transporter gene *sugE*, at stationary phase^{14,15,16}. Future work will be
27 addressed to identifying *SCO1731* target genes and to comparing the methylome of *S. coelicolor*
28 parental and the *SCO1731::Tn5062* mutant strains.

29 Overall, this is the first report that correlates DNA cytosine methylation with differentiation in *S.*
30 *coelicolor* and attributes a DNA methyltransferase function to the *SCO1731* gene. Our results show
31 that both, the treatment with 5-aza-2'-deoxycytidine as well as the inactivation of the *SCO1731*
32 gene, result in a strong impairment in morphological differentiation (delay in aerial mycelium and
33 sporulation) and an impediment in actinorhodin production. Our results reveal, for the first time,

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- 1 that epigenetics, through methylation of cytosines, control morpho-physiological differentiation in
- 2 *S. coelicolor* unveiling new levels of complexity of gene expression and regulation in this
- 3 microorganism.

1 **Methods**

2
3 **Bacterial strains and media.** Bacterial strains, plasmids and cosmids are listed in Table 2 The
4 strains were grown in sucrose-free R5A, GYM and SFM, and maintained by following procedures,
5 reported in Manteca *et al.*⁴⁶. *Escherichia coli* strains were grown at 37°C in solid or liquid 2xYT⁴⁷
6 medium supplemented with the appropriate antibiotics.

7
8 **Dot Blot assay.** Genomic DNAs were extracted by salting out procedures, as described in Lo
9 Grasso *et al.*⁴⁸. Dot blot assay was performed following the protocol described in Caracappa *et al.*
10⁴⁹. Briefly, genomic DNA was denatured at 95 °C for 10 min, spotted on nitrocellulose filter
11 (Hybond ECL, GE Healthcare Life Sciences) and fixed by UV (2 cycles at 700 J). The spotted
12 DNA was detected by staining the filter with 0.02% (w/v) methylene blue in 0.3 mol/L sodium
13 acetate (pH 5.2). After removal of the staining solution, the methylated cytosines were detected
14 using the anti-5-methylcytosine mouse antibody (Calbiochem) and the secondary goat anti-mouse
15 IgG-H&L chain specific peroxidase conjugate (Calbiochem). The images were taken using
16 Chemidoc (Chemi Hi sensitivity) and SuperSignal®West Femto maximum sensitivity substrate
17 (Life Technologies). Spots of the same area were manually labelled and quantified by Molecular
18 Imager ChemiDoc XRS System Biorad. Percentage of methylation level was reported as arbitrary
19 units per mm² (AU/mm²). The experiments were performed at least twice and in triplicate.
20 Genomic DNAs, extracted from *Escherichia coli* DH10B and *Escherichia coli* ET12567/pUZ8002
21 strains, were used as positive and negative control, respectively. The experiments were performed at
22 least twice and in triplicate.

23
24 **5-aza-dC treatment.** Preliminary experiments were performed to set up the demethylation. The
25 amount of 5-aza-dC (Sigma) to add to the medium was chosen after checking the effect of
26 increasing concentrations of the drug on the cells on solid medium GYM (Supplementary Fig. S3).
27 5-azadC is reported to have a half-life of 20h-24h under conditions of physiological temperature
28 and neutral pH⁵⁰, so the treatment was repeated every 24h, from 0 to 96h. A control experiment was
29 done in parallel using DMSO (the solvent of 5-aza-dC). 5 µM 5-aza-dC was the highest
30 concentration in which the cells were still viable, while 10 and 15 were lethal for the cells, indeed a
31 halo of growth inhibition was present. This is in accordance with results reported for *E. coli*²³.
32 In addition, a treatment was carried out to liquid cultures every 12 and 24h, from 0 to 36h. A control
33 experiment was done in parallel using DMSO. The efficiency of demethylation was evaluated after
34 48h of growth of *S. coelicolor* in the presence of 5 µM of 5-azadC added every 24h and 12h, by dot

1 blot analysis (Supplementary Fig. S4). The results revealed that the efficiency of the treatment
2 carried out every 24h was 72%, while every 12h was 99,5%. Thus, for the demethylation
3 experiments treatment was carried out with 5 µM 5-azadC added every 12h.

4
5 **Confocal laser scanning microscopy analysis (CLSM).** Culture samples were processed for
6 microscopy at different incubation time points following the protocol reported in Manteca *et al.* ⁴⁶.
7 Cells were stained with the LIVE/DEAD Bac-Light bacterial viability kit (Invitrogen), that contains
8 the SYTO 9 green fluorescent stain for labelling all the cells and the non-cell-permeating nucleic
9 acid stain (propidium iodide, PI) for detecting the dead cells. The samples were observed under a
10 Leica TCS-SP2-AOBS confocal laser-scanning microscope at a wavelength of 488 nm and 568 nm
11 excitation and 530 nm (green) or 640 nm (red) emissions. A significant number of images was
12 analyzed in a minimum of three independent culture analyses.

13
14 **Antibiotic quantification.** To measure actinorhodin (intracellular and extracellular), cells were
15 broken in their culture medium by adding KOH 0.1 N. Cellular debris was discarded by
16 centrifugation, and actinorhodin was quantified spectrophotometrically with UV/visible
17 spectrophotometer, applying the linear Beer-Lambert relationship to estimate concentration
18 ($\epsilon_{640}=25,320$). Undecylprodigiosin was measured after vacuum drying of the mycelium, followed
19 by extraction with methanol, acidification with HCl (to 0.5 N), and spectrophotometric assay at 530
20 nm, again using the Beer-Lambert relationship to estimate concentration ($\epsilon_{530}=100,500$).
21 Reproducibility has been corroborated by at least three independent cultures at various
22 developmental time points.

23
24 **Disruption of the *SCO1731*.** To generate the *SCO1731::Tn5062* mutant, the cosmid I11.2.G06
25 containing a copy of the gene interrupted by the transposon Tn5062⁴¹ was used. It contains the
26 apramycin and kanamycin resistance cassettes in the transposon and in the cosmid, respectively.
27 After transformation by interspecific conjugation with *E. coli* ET12567/pUZ8002 as a donor strain,
28 8 apramycin resistant colonies were obtained. Genomic DNA was extracted from 4 mutants and
29 analyzed by PCR for the presence of apramycin (~1.3 kbp) and the absence of kanamycin (0.9 kbp)
30 resistance cassette. The following primers were used Kana_F 5'-GATGGCTTTCTTGCCGCC3-'
31 and Kana_R 5'-TCGGTCATTTCTGAACCCC-3', Apra_F 5'-
32 CGGGGTACCCTCACGGTAACTGATGCC-3' and Apra_R 5'-ATTTTAATGCGGATGTTGCG-
33 3' to amplify apramycin or kanamycin resistance cassette, respectively. Two samples (*SCO1731-3*

1 and -4) had the expected profile (Supplementary Fig. S5) and they were analyzed by Southern Blot
2 using genomic DNA digested with *Sall* and Tn5062 as a probe.

3 The expected restriction profile of the mutant is shown in the Supplementary Fig. S6. Southern blot
4 analysis revealed that both putative mutants *SCO1731::Tn5062* had the expected restriction profile,
5 two bands of approximately 2.8 and 2.7 kb.

6
7 **Complementation of *SCO1731::Tn5062* mutation.** A copy of *SCO1731*, placed under the control
8 of its promoter was amplified via PCR using Phusion High-Fidelity DNA Polymerase (Thermo),
9 using the primers 1731_SpeI F 5'-GGACTAGTTGGCTGCCTCCTTACGGAT-3' and 1731_compl
10 R 5'-AAGATATCGTCTGGACGAGGACGAGTTC-3' and was then cloned into pCR™-Blunt II-
11 TOPO®. The sequences were checked via DNA sequencing using the M13 universal primers prior
12 to subcloning them into pNG3⁴⁸ constructing the plasmid pNG3-1731compl (Table 2). The plasmid
13 was used to transform the mutant *SCO1731* strain by interspecific conjugation generating the
14 *SCO1731_compl* strain. Thus, 32 colonies of putative *SCO1731_compl* strain were obtained after
15 the growth in SFM with hygromycin. After 3 passages of these colonies on GYM with hygromycin,
16 genomic DNA was extracted by 10 putative complemented strains and analyzed by PCR using the
17 primers *SCO4848_F* 5'-CGTCGATCCCCTCGGTTG-3' and *SCO4848_R*. 5'-
18 GAGCCGGGAAAGCTCATTCA-3'. These primers amplified a fragment of 617 bp only if pNG3 is
19 integrated at the *attB* site of *SCO4848*. Six of eight samples had the expected profile
20 (Supplementary Fig. S7) and only the *SCO1731_compl*-8 was used for further experiment.

21

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18

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26 **Author Contributions** A.P. performed the experiments. A.P., A.M. and R.A. analyzed the data.
27 A.M. and R.A. conceived, designed the work and wrote the manuscript. All authors contributed to
28 the critical discussion of the manuscript, read and approved the final manuscript.

29 **Competing Interests:** The authors declare that they have no competing interests.

30

1 **Figure legend**

2 **Fig. 1: Methylation levels along the different growth phases of *S. coelicolor*, *S. lividans*, *S.***
3 ***griseus* and *S. avermitilis*.**

4 Genomic DNA was extracted from bacterial cultures grown both in liquid and on solid medium and
5 analyzed by dot blot assay with antibody against 5MeC. Bars represent methylation levels in
6 arbitrary units (AU) quantifying dot blot signal intensities. Error bars were obtained from three
7 independent experiments. Dot blots are shown below the bars. MI and MII stages are indicated. a)
8 solid cultures. b) liquid cultures. MII_{48h} and MII_{72h} correspond to aerial and sporulating aerial
9 hyphae, respectively.

Comentario [AM11]: Revise that this is correct. It might be (a)

Comentario [VA12]: Done

10

11 **Fig. 2 Effect of 5-aza-dC treatment on *S. coelicolor* morphological and physiological**
12 **differentiation on solid GYM.**

13 a-b) CLSM analysis (LIVE/DEAD Bac-Light bacterial viability kit staining) of the untreated and
14 treated cultures after 5, 7 and 8h from seeding of the same spore stock on GYM plates with or
15 without 5-aza-dC. c) Percentage of spore germination after 5h, 7h, 8h and 9h of growth of untreated
16 and treated cultures. d) Growth curves of untreated and treated cultures. e-f) CLSM analysis of
17 untreated and treated cultures at 72 and 96h. g-h) Macroscopic view of undecylprodigiosin (red
18 color) and actinorhodin (blue color) production of untreated and treated cultures at 50 and 72h on
19 GYM plates.

20

21 **Fig. 3: Effect of 5-aza-dC treatment in sucrose-free liquid R5A cultures.**

22 a) Growth. b) Undecylprodigiosin production. c) Actinorhodin production. Continuous and dashed
23 lines indicate the untreated and the treated culture, respectively.

Comentario [AM13]: Y axis in graph c starts on 0, but graphs a and b starts in a negative value. Please, unify it. It is not important, but is unusual.

Comentario [VA14]: done

24

25 **Fig. 4: Growth and cytosine methylation of the *S. coelicolor* wild-type strain, the**
26 ***SCO1731::Tn5062* mutant and the complemented *SCO1731::Tn5062* mutant, in sucrose-free**
27 **R5A cultures.**

28 a) Growth curves. b) cytosine methylation levels at 20-hours (MI). AU indicate arbitrary units of
29 methylation levels.

Comentario [AM15]: Why not include the dot blot under the bars like in Fig. 1?

Comentario [VA16]: done

30

31

1 **Fig. 5: Actinorhodin production in sucrose-free R5A cultures of the *S. coelicolor* wild-type**
2 **strain, the *SCO1731::Tn5062* mutant and the *SCO1731::Tn5062* complemented strain.**

3 Macroscopic view of laboratory flasks is shown at different developmental time points (48h, 72h
4 and 96h). Blue color corresponds to actinorhodin; red colour corresponds to undecylprodigiosin.

5

6 **Fig. 6: Morphological differentiation of the *S. coelicolor* wild-type strain, the**
7 ***SCO1731::Tn5062* mutant and the *SCO1731::Tn5062* complemented strain on GYM plates.**

8 Macroscopic view (left panels); CLSM images (right panels) taken after staining the cells with the
9 LIVE/DEAD Bac-Light bacterial viability kit. Arrows indicate spore chains.

10

Comentario [AM17]: Why the plate of the complemented strain at 48-hours is green?

Comentario [VA18]: No idea!
It was the picture

1 **Table 1 List of putative methyltransferases annotated in *S. coelicolor* genome (StrepDB - The**
 2 **Streptomyces Annotation Server).**

3 Ratios of log₂ of gene expression between MII24h/MI are reported. A negative ratio indicates that
 4 the gene is more transcribed in MI, a positive one that is more transcribed in MII 24h. The ratios
 5 were taken from Yagiie *et al.*²⁹.

Putative methyltransferase	Log 2 Ratio MII _{24h} /MI	Expression phase
SCO1731	-1.8623	MI
SCO0190	-1.3723	MI
SCO4504	-1.298	MI
SCO1969	-1.1342	MI
SCO7445	-0.8099	MI
SCO5972	-0.6938	MI
SCO0408	-0.5595	MI
SCO2098	-0.3133	MI
SCO5895	-0.3038	MI
SCO3545	-0.2817	MI
SCO2317	-0.2732	MI
SCO2814	-0.2385	MI
SCO3215	-0.2133	MI
SCO7055	-0.1974	MI
SCO2170	-0.1867	MI
SCO2670	-0.1226	MI
SCO5589	-0.1108	MI
SCO1555	-0.0979	MI
SCO5094	-0.0884	MI
SCO6844	-0.0768	MI
SCO0594	-0.0681	MI
SCO0760	-0.0136	MI
SCO0648	0.0093	MII
SCO3744	0.1059	MII
SCO2338	0.1692	MII
SCO6541	0.2415	MII
SCO1162	0.3867	MII
SCO5146	0.4161	MII
SCO0929	0.5308	MII
SCO6549	0.6532	MII
SCO6928	0.7393	MII
SCO3452	0.7782	MII
SCO0835	0.7845	MII
SCO0826	0.8163	MII
SCO7452	0.8841	MII
SCO5257	1.3063	MII

SCO0392	2.0070	MII
SCO0995	2.4922	MII

1
2 **Table 2. Bacterial strains, plasmids and cosmids used in this study.**

	Description	Origin or reference
Bacterial strains		
<i>S. coelicolor</i> M145	SCP1 SCP2	³⁹
<i>S.coelicolor</i> SCO1731::Tn5062	SCO1731::Tn5062, Am ^R	This study
<i>Streptomyces lividans</i> 1326	SCP1 SCP2	³⁹
<i>Streptomyces griseus</i> NBRC 102592		NBRC
<i>Streptomyces avermitilis</i> ATCC 31267		ATCC
<i>Escherichia coli</i> DH10B	F- <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>) φ80 <i>lacZ</i> ΔM15 Δ <i>lacX74</i> <i>recA1 endA1 araD139</i> Δ(<i>ara, leu</i>)7697 <i>ga/U ga/K λ- rpsL nupG</i>	Invitrogen
<i>Escherichia coli</i> ET12567/pUZ8002	F- <i>dam</i> -13::Tn9 <i>dcm6 hsdM hsdR recF143 zjj201::Tn10 galK2 galT22 ara14 lacY1 xyl-5 leuB6</i>	³⁹
Plasmid/Cosmid		
pCR™-Blunt II-TOPO®	Zero Blunt® TOPO® PCR Cloning Kit, Km ^R	Invitrogen
pNG3	<i>bla</i> cloned into pNG1/ <i>HindIII</i> / <i>AvrII</i> Hyg ^R , Amp ^R	⁵¹
pQM5062	Plasmid containing <i>eGFP</i> Tn5062	⁵²
pNG3-1731compl	pNG3 plasmid containing the ORF of <i>SCO1731</i>	This study
I11.2.G06	I11 cosmid carrying I11.2.G06 transposant	⁴¹

4