



# Heterologous expression of 8-demethyl-tetracenomycin (8-dmtc) affected *Streptomyces coelicolor* life cycle

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## Abstract

Heterologous hosts are highly important to detect the expression of biosynthetic gene clusters that are cryptic or poorly expressed in their natural hosts. To investigate whether actinorhodin-overproducer *Streptomyces coelicolor*  $\Delta$ *ppk* mutant strain could be a possible prototype as a heterologous expression host, a cosmid containing most of the *elm* gene cluster of *Streptomyces olivaceus* Tü2353 was integrated into chromosomes of both *S. coelicolor* A3(2) and  $\Delta$ *ppk* strains. Interestingly, it was found that the production of tetracyclic polyketide 8-demethyl-tetracenomycin (8-DMTC) by recombinant strains caused significant changes in the morphology of cells. All the pellets and clumps were disentangled and mycelia were fragmented in the recombinant strains. Moreover, they produce neither pigmented antibiotics nor agarase and did not sporulate. By eliminating the *elm* biosynthesis genes from the cosmid, we showed that the morphological properties of recombinants were caused by the production of 8-DMTC. Extracellular application of 8-DMTC on *S. coelicolor* wild-type cells caused a similar phenotype with the 8-DMTC-producing recombinant strains. The results of this study may contribute to the understanding of the effect of 8-DMTC in *Streptomyces* since the morphological changes that we have observed have not been reported before. It is also valuable in that it provides useful information about the use of *Streptomyces* as hosts for the heterologous expression of 8-DMTC.

**Keywords** *Δppk* · Heterologous expression · *Streptomyces* colony morphology · Pellet disaggregation · 8-DMTC

## Introduction

*Streptomyces* family takes an important place in medicine, biotechnology, and pharmaceutical area because of their ability to produce highly active secondary metabolites including two-thirds of the known antibiotics, pigments, herbicides, anti-tumor, and anti-inflammatory agents [1]. Secondary metabolite production is a well-timed process that is triggered by differentiation and correlated with growth throughout the complex life cycle of *Streptomyces*. In liquid culture, favorable conditions trigger the growth of vegetative mycelium (MI) from the spore and expansion with polar growth unlike most unicellular bacteria [2]. During exponential growth, vegetative hyphae form

branches made of compartments that are separated by peptidoglycan cross-walls and also by cross-membranes without peptidoglycan [3]. These extended vegetative hyphae form pellets whose centers are going to suffer from the first round of programmed cell death (PCD) as a next step [4]. Some compartments of vegetative mycelia die off via PCD and then both the viable and dead compartments stay in the same hyphae [5]. Furthermore, alive compartments that survived from the first round of PCD differentiate into multinucleated, secondary metabolite-producing MII mycelia as the second stage [1]. *Streptomyces* strains produce pellets and clumps in liquid cultures but they generally cannot differentiate into aerial mycelia (late MII) or sporulate in liquid media, although there are exceptions such as *Streptomyces venezuelae* [6].

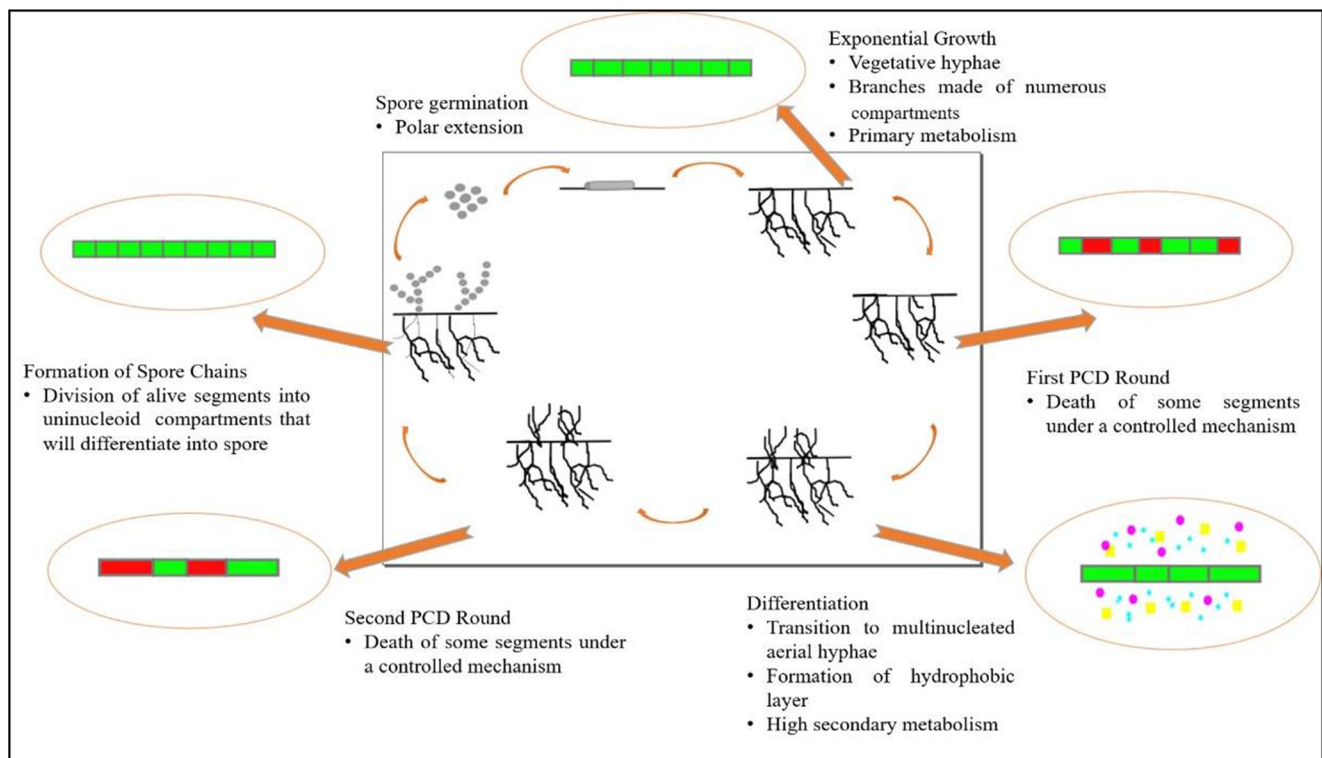
On solid media, aerial mycelium (late MII) grows through the air with the help of a hydrophobic protein layer that allows it to grow out of the aqueous environment [7]. Aerial hyphae undergo the second round of the PCD and then the remaining viable multinucleated compartments divide into several uninucleoid segments that will differentiate into spore chains as the third stage. Figure 1 summarizes the life cycle of *Streptomyces* on solid media. In short, even though the molecular triggers and

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**Fig. 1** *Streptomyces* life cycle. Red and green colors represent dead and alive compartments, respectively. Purple-, blue-, and yellow-colored shapes represent different secondary metabolites produced by MIII mycelia

functions of the PCD mechanism in *Streptomyces* have not been illuminated completely, it is crystal clear that these PCD rounds play a key role in the differentiation process of the life cycle and exhibit an undeniable sign of bacterial multicellularity under the name of *Streptomyces* mycelium [5].

Studies have shown that the *Streptomyces* genome has many cryptic biosynthetic gene clusters (BGCs) which have a great possibility to synthesize new drugs and agents. However, these BGCs usually cannot be expressed or detected in their natural hosts under standard fermentation conditions; therefore, they need to be activated by using different methods such as changing cultivation conditions, inducing stress response, and genetic manipulations (expression of BGCs in heterologous hosts, random mutagenesis, regulation of gene expression, ribosomal engineering, using elicitors) [8, 9]. The heterologous expression is one of the most efficient ways among the activation methods of BGCs.

Many groups have preferred *Streptomyces* as heterologous hosts for the production of specific secondary metabolites, such as *Streptomyces albus* J1074 for fredericamycin [7], *Streptomyces lividans* 1326 for capreomycin [10] and viomycin [11], and a deletion mutant of *Streptomyces avermitilis* (SUKA) for streptomycin production [12]. *S. coelicolor* is one of the most popular options among heterologous hosts since it has been well-studied to date and its regulatory mechanisms are mostly understood. Furthermore, large gene clusters can be inserted into its genome and the expression of these clusters can be controlled by

defined genetic tools (promoters, activators, etc.) [13]. In addition to wild-type *S. coelicolor* strain, many genetically developed *S. coelicolor* strains have been constructed in previous studies such as *S. coelicolor* CH999 for producing polyketides [14], *S. coelicolor* M512 for the regulation of actinorhodin and prodiginine production [15], *S. coelicolor* for the erythronolide PKS [14], *S. coelicolor* 1146 for production of congocidine [16].

In this study, tetracyclic polyketide 8-demethyl-tetracenomycin (8-DMTC) was heterologously expressed in both *S. coelicolor* A3(2) wild type and *S. coelicolor* A3(2)  $\Delta$ *ppk* strains and the effect of this agent on the life cycle of *Streptomyces* was determined. *S. coelicolor*  $\Delta$ *ppk* strain cannot synthesize polyphosphate kinase enzyme (PPK) which catalyzes the biosynthesis of polyphosphate polymer [17]. 8-DMTC inhibits the proliferation of murine L1210 leukemia cells as an anti-tumor agent [18]. It also acts against the Gr<sup>+</sup> bacteria by intercalating with their DNA, although the mechanism of the antibacterial activity has not been elucidated in detail yet [18–20].

## Materials and methods

### Microorganisms and growth conditions

The bacteria used in this study are shown in Table 1. *Streptomyces* strains were grown at 30 °C in R2YE, MS,

**Table 1** The bacterial strains used in this study

Bacterial strains	Genotypic properties	Reference
<i>E. coli</i> DH5 $\alpha$	F– <i>recA1</i> , <i>endA1</i> , <i>gyrA96</i> , <i>thi-1</i> , <i>hsdR17</i> (rK–, mK+), <i>sup44</i> , <i>relA1<math>\lambda</math>–, (<math>\sigma</math>80 dLacZAM15), D(<i>lacZYA-argF</i>)U169</i>	[21]
<i>E. coli</i> ET12567/pUZ8002	<i>dam</i> , <i>dcm</i> , <i>hsdS</i> , <i>cat</i> , <i>tet/tra</i> , <i>neo</i> , <i>RP4</i>	[2]
<i>E. coli</i> DH5 $\alpha$ +cos16F4ie/ $\Delta$ <i>elm</i>	Recombinant cells that carry cos16F4ie without <i>elm</i> genes	This study
<i>E. coli</i> ET12567/pUZ8002 +cos16F4ie/ $\Delta$ <i>elm</i>	Recombinant cells that carry cos16F4ie without <i>elm</i> genes	This study
<i>S. coelicolor</i> A3(2)	Wild type	[22]
<i>S. coelicolor</i> A3(2) $\Delta$ <i>ppk</i>	<i>ppk</i> -deleted mutant cell	[17]
<i>S. coelicolor</i> A3(2) +cos16F4ie	Recombinant cells that carry cos16F4ie	This study
<i>S. coelicolor</i> A3(2) $\Delta$ <i>ppk</i> +cos16F4ie	Recombinant cells that carry cos16F4ie	This study
<i>S. coelicolor</i> A3(2) +cos16F4ie/ $\Delta$ <i>elm</i>	Recombinant cells that carry cos16F4ie without <i>elm</i> genes	This study

and TBO media, and *E. coli* strains were grown at 30 °C or 37 °C in Luria broth and Luria agar. *E. coli* ET12567/pUZ8002 was used to transfer cosmid DNA to *Streptomyces* by conjugation. After conjugation, wild-type cells were grown on MS+ MgCl<sub>2</sub> agar while  $\Delta$ *ppk* mutant cells were grown on R2YE plates since they do not grow on MS agar. In order to normalize the inoculation size between strains, we used equal amount of mycelium since recombinant strains carrying cos16F4ie cosmid were not able to sporulate. Specific antibiotics were used to select the cos16F4ie recombinants. Although cos16F4ie carries apramycin, erythromycin, and tetracycline resistance genes, we only used apramycin (50  $\mu$ g/ml) and erythromycin (50  $\mu$ g/ml) as selective agents since our experiments showed that tetracycline antibiotic cannot inhibit the growth of wild-type *S. coelicolor* on agar. Nalidixic acid (25  $\mu$ g/ml) was used to inhibit *E. coli* cells after conjugation.

### Plasmids, cosmids, and DNA methods

Cosmids and plasmids used in this study are listed in Table 2. cos16F4ie cosmid was a kind gift from Dr. Jose A. Salas and Dr. Carmen Mendez (Universidad de Oviedo, Spain). Cosmid and plasmid isolations, restriction enzyme digestions, and transformation of *E. coli* were performed according to Sambrook et al. [25]. Primers used in the PCR reactions were prepared according to the *elm*MII gene (forward primer: 5' TTCCTCGTCCATGACCTCAC3'; reverse primer: 5' TCATACGTAGTCGATCTC3') present on the cos16F4ie cosmid. Chromosomal DNA isolation from *Streptomyces*

cells, transformation of *Streptomyces*, and intergeneric conjugation with *E. coli* were carried out according to Kieser et al. [26].

### Detection and characterization of 8-DMTC

For the detection of 8-DMTC, high-performance liquid chromatography (HPLC) analysis was optimized and performed according to the study of Heide et al. (2006) [27]. Wild-type,  $\Delta$ *ppk* mutant, and their recombinants harboring cos16F4ie cosmid were cultured in 100 ml R2YE at 30 °C, at 180 rpm for 48 h. Then, 5 ml of these pre-cultures was used to inoculate 100 ml fresh R2YE and incubated at 30 °C, at 180 rpm for 5 days. At the end of incubation, 15 ml of each culture was centrifuged and their supernatants were adjusted to pH 3.5 with formic acid and extracted with 5 ml ethyl acetate. C18 Inertsil ODS-3V (4.6  $\times$  250 mm, 5  $\mu$ m, GL Sciences) column was used to detect the presence of 8-DMTC in the extracts. In the elution program, a linear gradient from 25 to 100% acetonitrile as solvent B in 0.1% phosphoric acid (in water) as solvent A was followed at a flow rate of 1 ml/min for 28 min. UV detection of the peaks was performed at 386 nm [27].

### Confocal laser scanning microscopy analysis

To prepare the samples for confocal analysis, the LIVE/DEAD Bac-Light Bacterial Viability Kit (L-7012) was used. One milliliter of the culture was centrifuged at 13,000 rpm for 5 min. After discarding the supernatant,

**Table 2** Properties of cosmids and plasmids

Plasmid/cosmids	Properties	Size	Reference
pUZ8002	RK2 derivative with defective oriT (aph)	55695 bp	[23]
cos16F4ie	Integrative cosmid, carries most of the <i>elm</i> gene cluster	49800 bp	[24]
cos16F4ie/ $\Delta$ <i>elm</i>	Integrative cosmid, without <i>elm</i> gene cluster	22000 bp	This study

cells were washed with 1 ml dH<sub>2</sub>O twice to get rid of any medium leftover that might interact with nucleic acid stains. Meanwhile, a dye mix made of 1.5 µl of each fluorescent nucleic acid stain (SYTO 9-green, propidium iodide (PI)-red) in 1 ml dH<sub>2</sub>O was prepared. The non-permeable nucleic acid stain PI could label only dead cells containing a damaged membrane, while permeable SYTO 9 fluorescent nucleic acid dye could label both live cells containing an intact membrane and dead cells containing a damaged membrane. Since PI has a higher nucleic acid affinity than SYTO 9, it causes a reduction in SYTO 9 stain when it enters the dead cells through damaged membranes. After centrifugation, the pellet was dissolved with 200 µl of the dye mixture and incubated in the dark at least for 10 min. Twenty microliters of the mix was deposited on a clean glass slide and observed under the confocal microscope, at a wavelength 488 and 568 excitation and 530 (green) or 630 (red) emission.

## Results

### Potential of $\Delta ppk$ strain as a host for heterologous expression

*elm* gene cluster of *Streptomyces olivaceus* Tü2353 [18] was used to determine the potential of *S. coelicolor*  $\Delta ppk$  strain as a host for heterologous expression. The cos16F4ie cosmid harbors most of not only the *elm* gene cluster but also  $\Phi$ C31 integrase and *attP* for integration into the *attB* site of the *Streptomyces* chromosomal DNA and *oriT* region for conjugal transfer from *E. coli*. This cosmid was first transferred into methylation deficient *E. coli* ET12567/pUZ8002 cells, and the presence of cosmid in transformants was confirmed via PCR (Fig. 2a).

Then, one of the *E. coli* transformants was used to transfer integrative cosmid cos16F4ie into *S. coelicolor* A3(2) and  $\Delta ppk$  mutant strains via conjugation. Production of 8-DMTC by the recombinant strains harboring cos16F4ie was seen in HPLC chromatograms (Fig. 2b, c). As we can see in Fig. 2d,  $\Delta ppk$  + cos16F4ie, and wild type + cos16F4ie produce nearly the same amount of 8-DMTC. The slight increase in 8-DMTC production in  $\Delta ppk$  + cos16F4ie strain might be negligible. So, it is possible to say that  $\Delta ppk$  strain was not better than wild strain for heterologous expression of 8-DMTC. Interestingly, while carrying out our experiments, we realized that none of the recombinants produces any actinorhodin in R2YE broth (Fig. 3a, b) or on R2YE agar (Fig. 3c). So we wanted to analyze the recombinant strains in detail by using a confocal microscope.

### Confocal analysis of recombinant strains expressing 8-DMTC

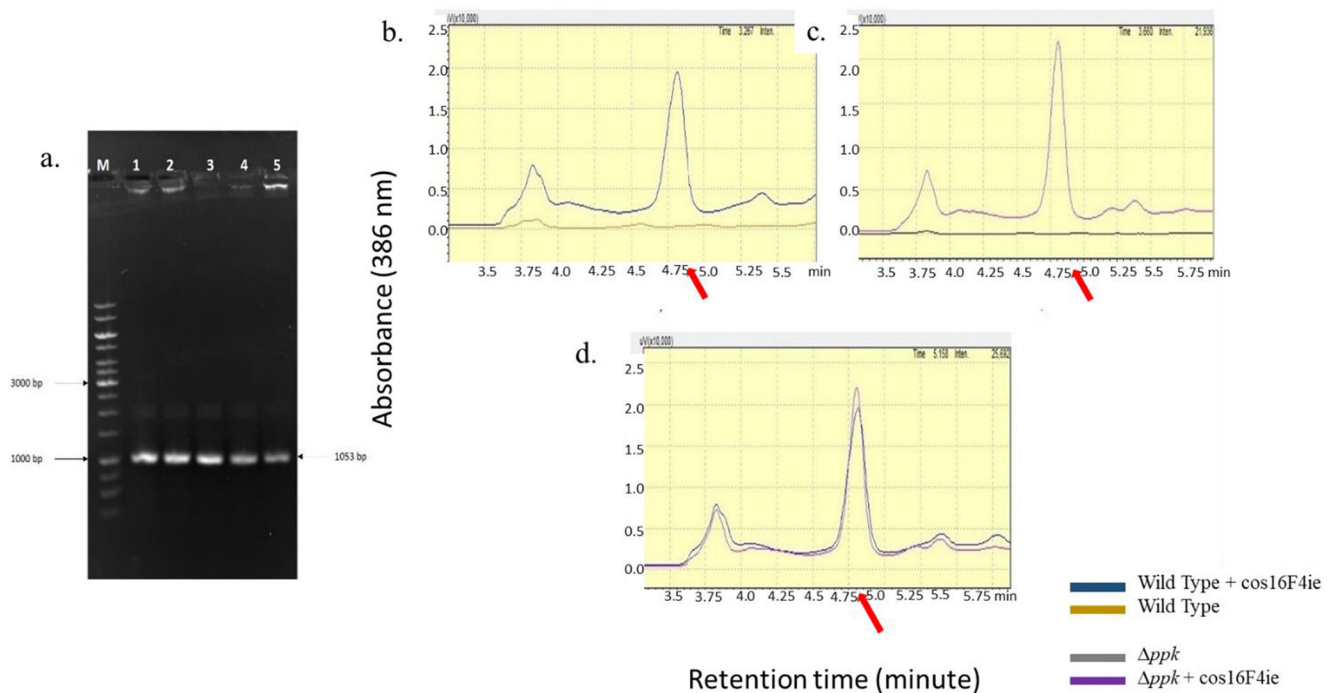
For confocal analysis, dead cells containing a damaged membrane were labeled with nucleic acid stain PI (red) and alive cells containing an intact membrane were labeled by SYTO 9 (green). Wild-type cultures show the properties of a classical *Streptomyces* life cycle (Fig. 4a). After 24-h incubation, the control cultures have already passed through the first programmed cell death round and then differentiated into secondary mycelia as we can see the non-compartmentalized hyphae spreading out of the colony center. The center of the colony is stained red with PI as expected since the death round starts in the center. The rest of the colony is alive at this hour of growth. Through the incubation, the dead cell rate is increasing until 72 h. Then, DNA of the dead segments degraded, and alive cells start to arise again thus increasing the viability of the colonies until 96 h. After that, the dead cell rate increases again so that, at the end of 120-h incubation, most of the culture consists of dead mycelia segments. During this 120-h life cycle, actinorhodin production correlates with secondary mycelia differentiation as expected.

On the other hand, the confocal images of wild type containing cos16F4ie in its genome show a completely different morphology as can be seen in Fig. 4b. The colony structure is totally disrupted in all recombinant strains with cos16F4ie. All mycelia are disentangled and fragmented hence causing a lack of pellet and clump formation in the cultures. Moreover, these short fragments of mycelia cannot be distinguished whether they are primary or secondary since they do not show any specific properties. The morphology of the recombinant culture does not correlate with the life cycle nor the incubation time. Dead and live fragments are randomly present in the cultures, independent from incubation time.

The general morphology of  $\Delta ppk$  colonies displays the classical life cycle characteristics with an intact colony formation (Fig. 4c). The central area of the colony consists of dead cells, while the outer zone is formed from alive mycelia at 24 and 48 h. The death rate is increasing until 72 h, then decreasing again until 120 h. Alive cells arise from dead segments between 72 and 120 h. The only difference between the morphologies of wild-type and  $\Delta ppk$  strain is the spread of mycelia around the  $\Delta ppk$  colonies. When we check the cos16F4ie recombinants of the  $\Delta ppk$  strain, the morphology is the same as the cos16F4ie harboring wild-type strain (Fig. 4d). They are deficient in colony formation and mycelia continuity. Dismantled mycelia parts consist of dead and alive cells without any correlation with incubation intervals.

Thus far, our findings showed that cos16F4ie recombinants display atypical morphology with the fragmentation of mycelia and lack of pellet formation during incubation from 24 to 120 h. However, we did not examine the cultures with CLSM within the first 24 h. So, our findings do not explain yet



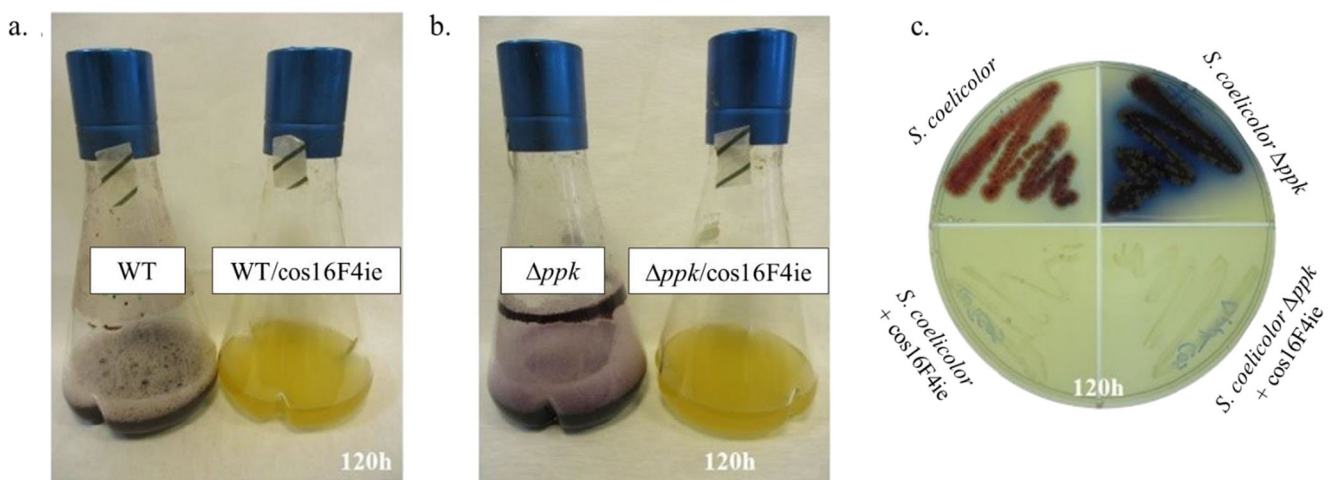


**Fig. 2** Confirmation of *E. coli*+cos16F4ie transformants with PCR by using *elmMII* (1053 bp) gene specific primers (a). Comparison of HPLC chromatograms of *S. coelicolor* A3(2) wild type/wild type +

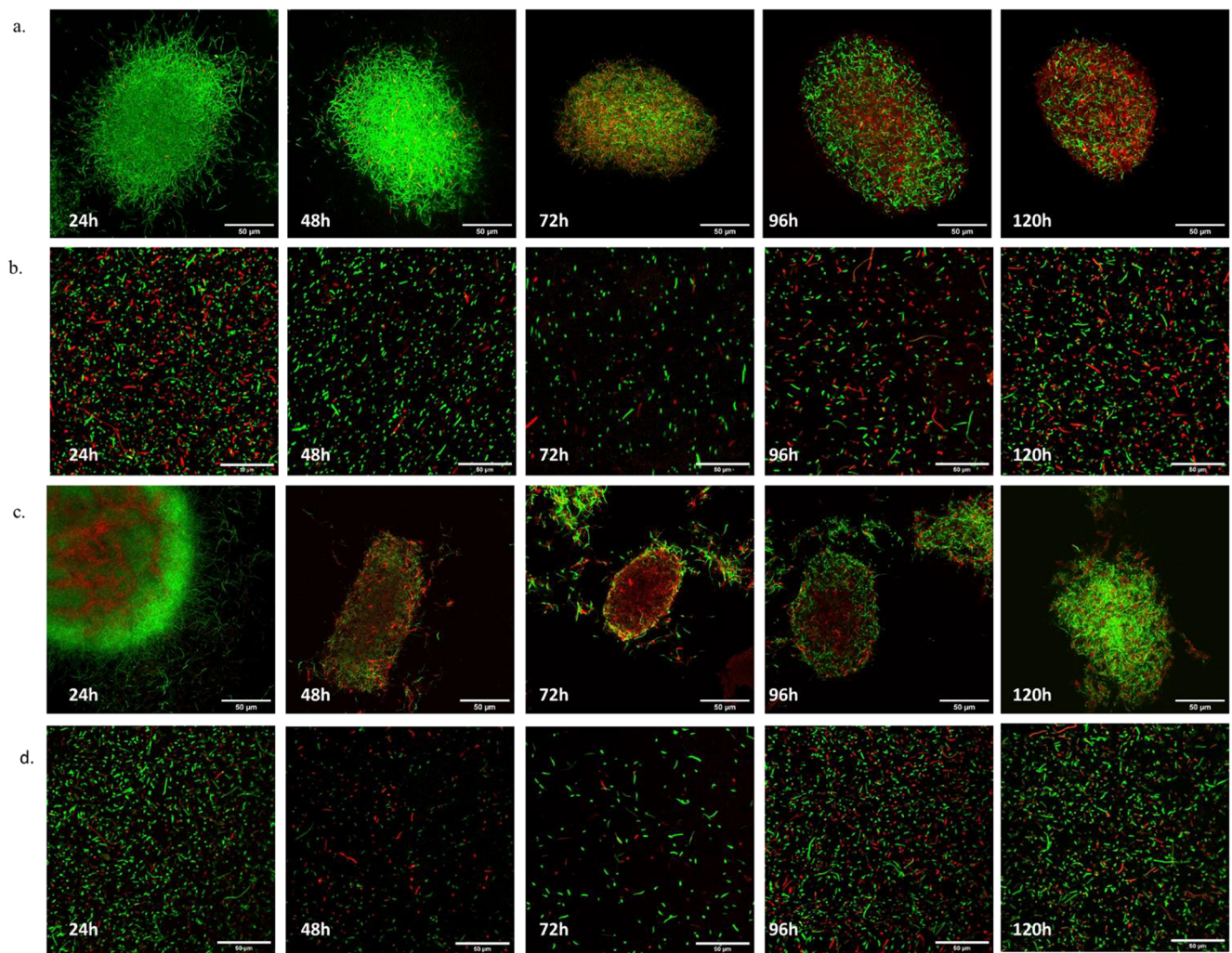
cos16F4ie (b),  $\Delta ppk/\Delta ppk$  + cos16F4ie (c), wild type + cos16F4ie/ $\Delta ppk$  + cos16F4ie (d). The red arrow shows 8-DMTC with 4.8-min retention time

whether disentanglement happens after the colonies have been formed within the first 24 h or that the recombinants are not capable of forming colony structure at all. Therefore, confocal analysis of A3(2) wild type and its recombinant strain was performed for 24 h at 6-h intervals. Figure 5a shows wild-type colony morphology during 24-h incubation. In the first image, the colony suffers from the first round of programmed cell death. After the first PCD round, the viable compartments of the primary mycelia come together and transform into the long segmented, multinucleated secondary mycelia. The first

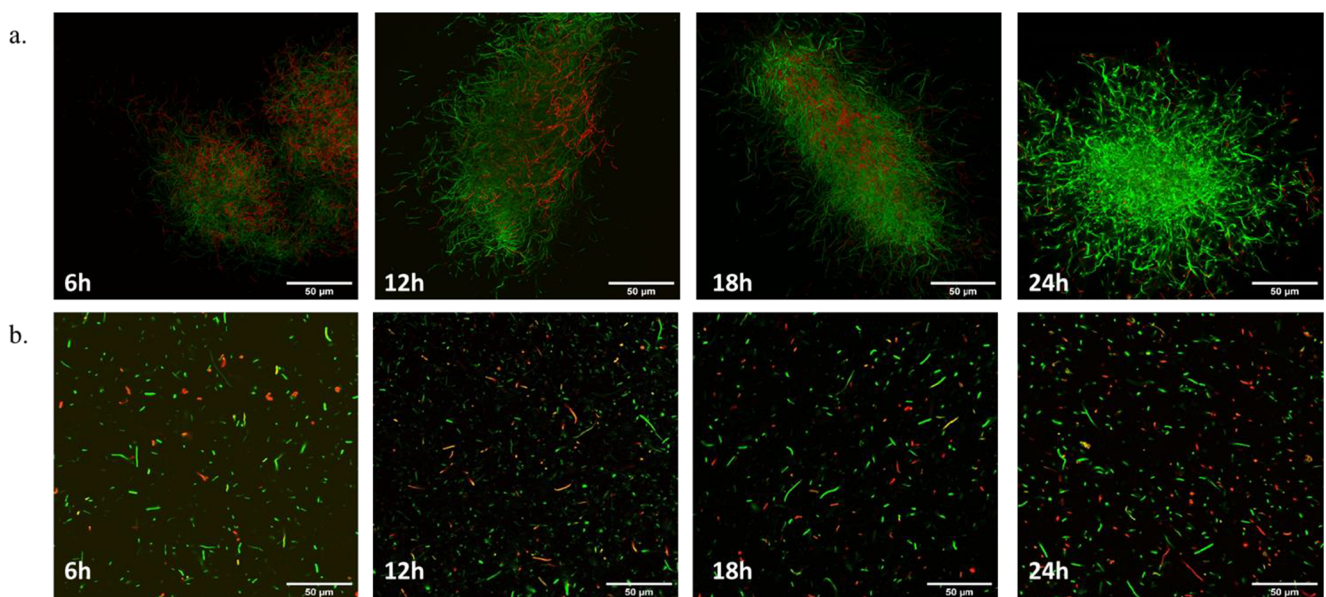
image shows a snap of this transition process at the 6th hour. The dead cell rate in the colony is high because of the PCD. Both primary and secondary mycelia exist in the colony at the same hour. At 12 h, the dead cell rate has decreased since the dead parts of the colony have been used as a nutrient and genetic material source for the formation of new ones. There are fewer primary mycelia in the colony at 12 h, most of the colony consists of secondary mycelia. At 18 h, the center of the colony is dead while the outer zone is alive with secondary mycelia. Finally, the colony at 24 h mostly consists of viable



**Fig. 3** Macroscopic view of *S. coelicolor* A3(2) wild type and wild type + cos16F4ie (a),  $\Delta ppk$  and  $\Delta ppk$  + cos16F4ie (b) in R2YE broth and on R2YE agar (c) after 120-h incubation



**Fig. 4** Confocal images of *S. coelicolor* A3(2) wild type (a), wild type + cos16F4ie (b),  $\Delta ppk$  (c), and  $\Delta ppk$  + cos16F4ie (d) strains during 120-h incubation



**Fig. 5** Confocal analysis of *S. coelicolor* A3(2) wild type (a) and wild type + cos16F4ie (b) strains during the 24-h incubation



secondary mycelia. On the other hand, the confocal images of the recombinant samples do not show any sign of pellet and clump formation (Fig. 5b).

According to all these findings, we can conclude that the recombinant strains exhibit a completely different morphology than *S. coelicolor* WT and  $\Delta ppk$ . 8-DMTC-producing recombinant strains do not form colonies, do not exhibit the cell cycle steps, do not produce colored antibiotics (actinorhodin and undecylprodigiosin), and do not have agarase activity (data not shown). They have fragmented mycelia during the all growth cycle. Furthermore, the inoculation of the recombinant cells on the TBO agar shows that these strains cannot sporulate at all on this medium (data not shown).

### Construction of *cos16F4ie/Δelm*

All recombinant strains displayed the same morphological changes in the name of colony structure, lack of pellets, and clumps. We wanted to investigate whether the reason for this altered morphology is the insertion of the cosmid into the genome or the expression of the *elm* cluster. For this purpose, the *elm* cluster was deleted from *cos16F4ie* by cutting it with *Bam*HI restriction enzyme since the *elm* cluster (24.2 kb) had been cloned into the unique *Bam*HI site in PKC505 as noted earlier. Moreover, the *elm* cluster has several *Bam*HI restriction sites inside. So that, when we digested *cos16F4ie* with the *Bam*HI, the *elm* cluster was digested at many sites and cut out from *cos16F4ie* while the rest of the cosmid (22 kb) was linearized (Fig. 6a). The 22-kb band was obtained from agarose gel and then self-ligated. *E. coli* DH5 $\alpha$  cells were transformed with the ligation products. The possible transformants were confirmed with cosmid isolation, and then *Bam*HI digestion (Fig. 6b). Then, *E. coli* ET12567/pUZ8002 cells were transformed with *cos16F4ie/Δelm* and again confirmed with *Bam*HI digestion (Fig. 6c). Finally, *cos16F4ie/Δelm* was transferred to *S. coelicolor* A3(2) cells via conjugation with *E. coli* ET12567+*cos16F4ie/Δelm*.

### Confocal analysis of *S. coelicolor* + *cos16F4ie/Δelm* recombinants

*S. coelicolor* A3(2) wild type and wild type + *cos16F4ie/Δelm* cells were analyzed by confocal microscope to examine the effect of *elm* deletion on the colony morphology of recombinants. For analysis, 24<sup>th</sup> hour was selected since a whole colony structure with a dead central part and mostly alive secondary hyphae could be seen in wild-type cultures at this hour. As can be seen in Fig. 7a, *cos16F4ie/Δelm* culture is colored indicating that it produced pigmented antibiotics as wild type did. *cos16F4ie/Δelm* colonies exhibited the same morphological characteristics as wild-type colonies. The first round of programmed cell death has already occurred since we can see dead central part and alive secondary mycelia (Fig. 7b). More importantly, *cos16F4ie/Δelm* culture

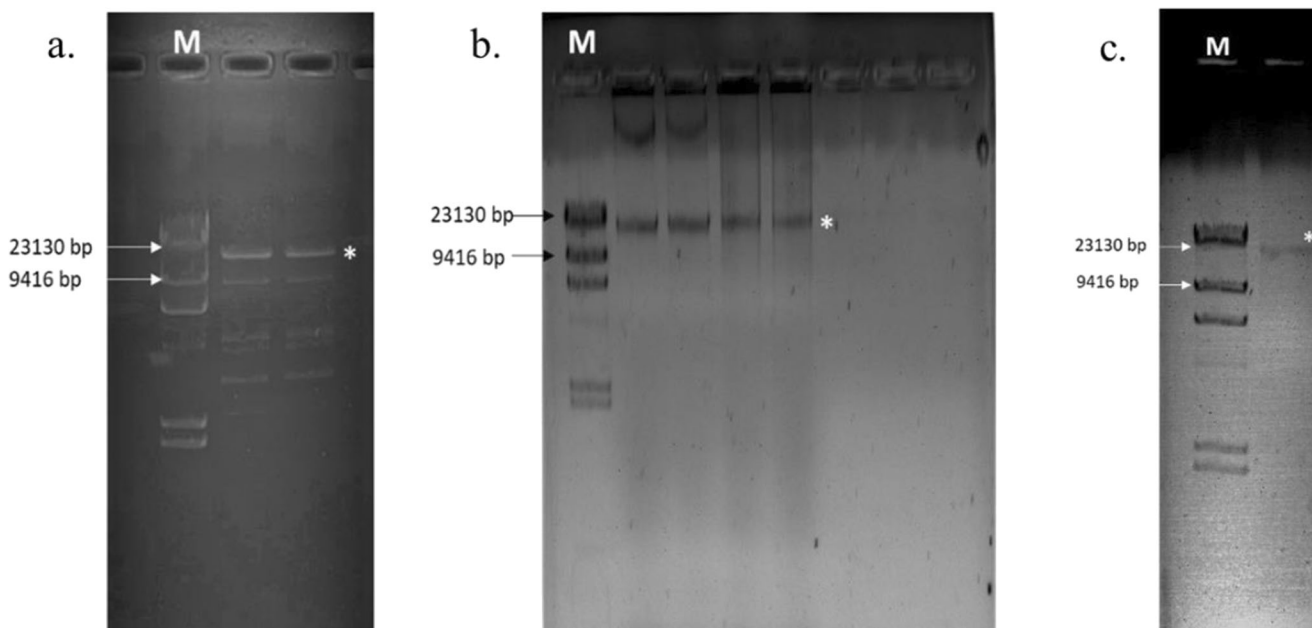
forms pellets, clumps, and wild-type-like colony formation. Its mycelia are not fragmented at all as we can see in Fig. 7c, the area between the separate colonies was clean. Moreover, recombinant cells showed agarase activity on R2YE agar and sporulated on TBO agar (data not shown). These findings prove that the fragmentation of mycelia and lack of colony formation of recombinant cultures were all caused by the expression of the *elm* cluster in *cos16F4ie*, not the insertion of the cosmid into the genome.

### Treatment of wild-type *S. coelicolor* A3(2) strain with 8-DMTC-containing supernatant

Our results showed that the expression of the *elm* cluster causes a deficiency in colony structure and clump-pellet formation in *S. coelicolor*. We wanted to determine the effect of the supernatant that contains 8-DMTC on *S. coelicolor* A3(2) wild type. Therefore, *S. coelicolor* A3(2) wild-type cells were treated by 8-DMTC-containing supernatant of the recombinant (*S. coelicolor* A3(2) + *cos16F4ie*) culture at the beginning of fermentation. At one trial, the supernatant of the recombinant strain was filtered to prevent any cell transfer from the recombinant culture. However, there was no difference between the effects of filtered or non-filtered supernatants after 24-h incubation (Fig. 8c, d, f, g).

Firstly, the color of the cultures in the flask was checked for actinorhodin production (Fig. 8a). The control wild-type sample was dark blue as a sign of high colored antibiotic production. However, 8-DMTC-treated samples were slightly colored which means the 8-DMTC compound decreased the production levels of pigmented antibiotics. It is known that the production of actinorhodin by secondary mycelial cells is directly proportional to the pellet structure and even the size. As explained in detail below, it is possible that as 8-DMTC slows down the growth rate and prevents pellet formation, antibiotic production has also decreased.

The detailed confocal analysis showed that the wild-type sample grows well and forms many colonies (Fig. 8b). The mycelia of these colonies are secondary as we expected to see at the 24th hour. The long compartments are signified as secondary mycelia. There are very few disconnected mycelia between colonies (Fig. 8e). On the other hand, the samples treated with supernatant with 8-DMTC exhibit a different profile. There are fewer colonies in the sample, and the mycelia of these colonies are primary mycelia at the 24th hour instead of secondary mycelia. The primary mycelia can be easily differentiated from the secondary mycelia since the primary mycelia compartmentalized into several short segments with cross-membranes in addition to peptidoglycan cell walls (Fig. 8f, g) that is atypical for *Streptomyces* growth because normally primary mycelia are detectable only in the first 10 h [3]. That means the 8-DMTC application decreases the growth rate of wild-type culture so that the treated culture could not reach the secondary mycelia phase at 24 h. Moreover, in the



**Fig. 6** Confirmation of *E. coli* DH5 $\alpha$ +cos16F4ie/ $\Delta$ elm transformants. *Bam*HI digestion of cos16F4ie (a); cos16F4ie/ $\Delta$ elm (\*: 22 kb) isolated from DH5 $\alpha$  transformants (b). Confirmation of *E. coli* ET12567/

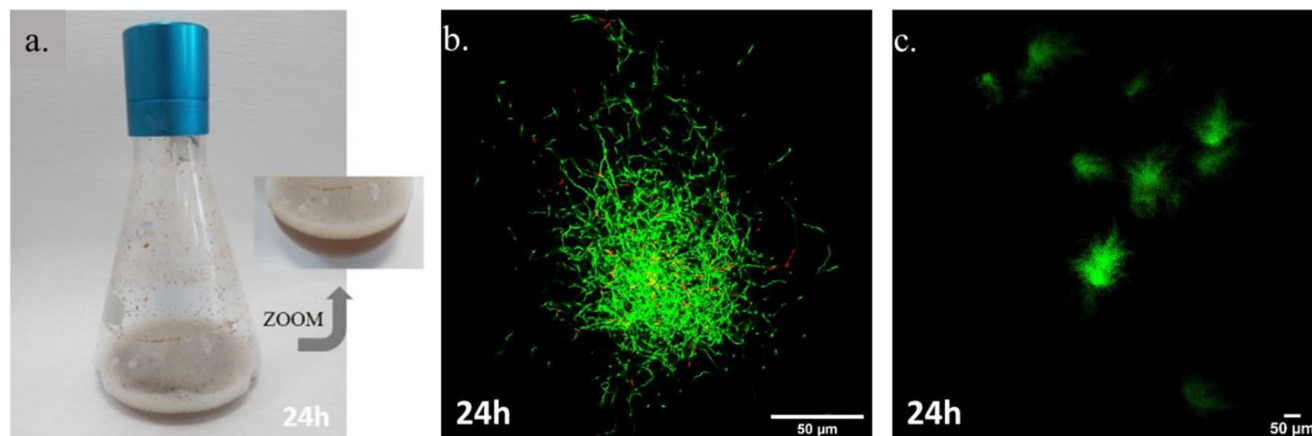
pUZ8002+cos16F4ie/ $\Delta$ elm transformants with *Bam*HI digestion (c). \*:cos16F4ie/ $\Delta$ elm (22 kb), M: Lambda DNA/*Hind*III Marker

space between colonies, there are many disentangled and fragmented mycelia parts unlike the wild-type sample (Fig. 8f, g). 8-DMTC treatment dismantled the mycelia and disrupted the colony structure and pellet formation. In short, extracellular application of 8-DMTC caused the same morphological results as in the case of recombinant strains that express it.

## Discussion

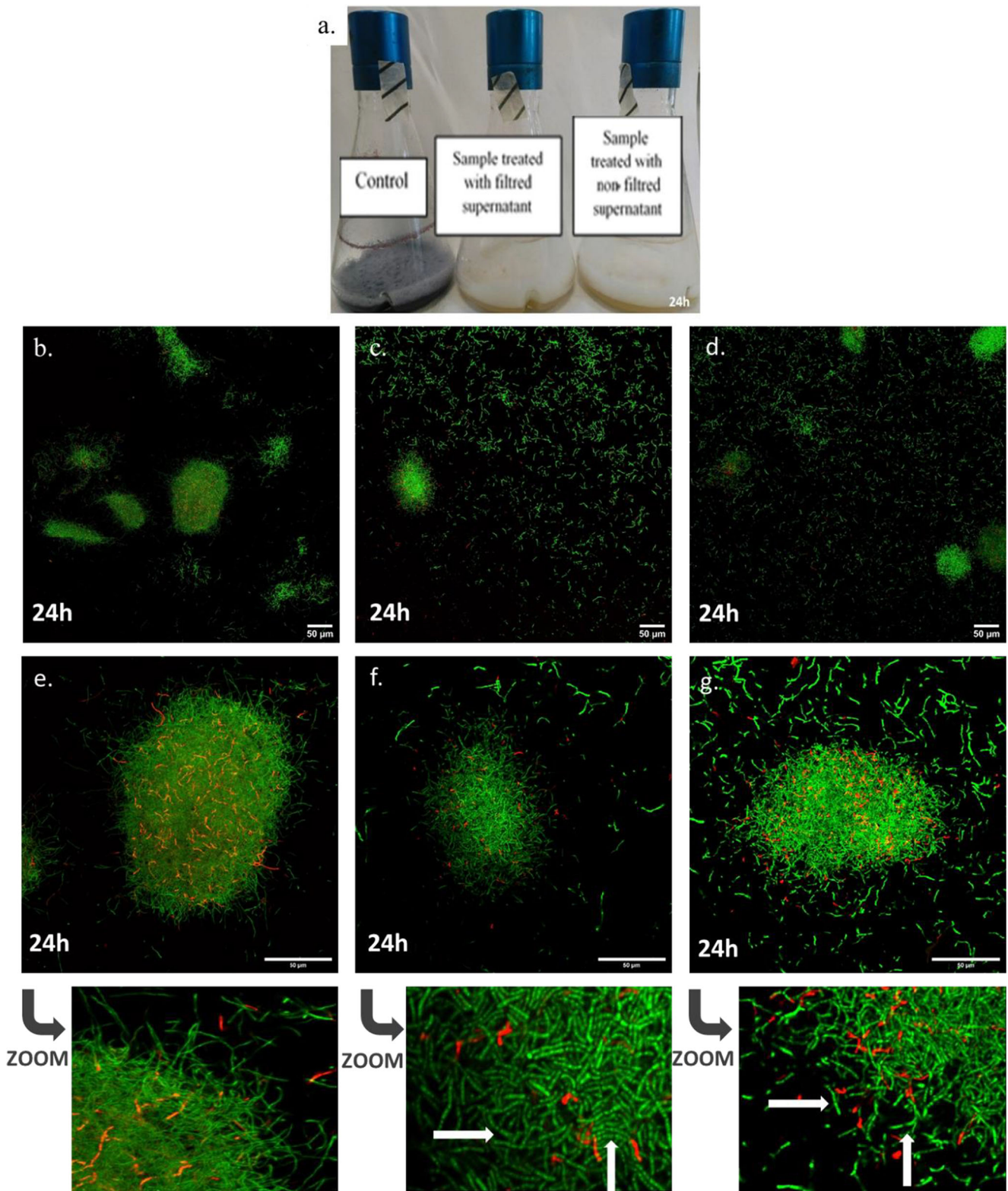
In the scope of this study, we wanted to evaluate the potential of actinorhodin-overproducer *S. coelicolor*  $\Delta$ ppk strain [17] as a heterologous host for the production of an

anti-tumor and antibacterial agent 8-DMTC [18–20]. *S. coelicolor* A3(2) wild type and  $\Delta$ ppk cells were transformed with cos16F4ie which carries most of the *elm* gene cluster encoding 8-DMTC. Production of 8-DMTC by recombinant strains was detected by using HPLC. The 8-DMTC peak with 4.8-min retention time was present only in the chromatograms of recombinant of both *S. coelicolor* A3(2) wild-type and  $\Delta$ ppk strains. There was a small difference between the production levels of 8-DMTC of wild type + cos16F4ie and  $\Delta$ ppk + cos16F4ie.  $\Delta$ ppk recombinant strain produced slightly more 8-DMTC than wild-type recombinant strain. However, this difference was not significant enough to announce  $\Delta$ ppk strain as a heterologous host at least for this metabolite.



**Fig. 7** The morphological analysis of *S. coelicolor* wild type + cos16F4ie/ $\Delta$ elm strain. The macroscopic view (a), confocal (b), and tile scan (c) images of *S. coelicolor* wild type + cos16F4ie/ $\Delta$ elm in R2YE broth at 24 h





**Fig. 8** Effect of supernatant that contains 8-DMTC on *S. coelicolor* A3(2) morphological differentiation in R2YE broth. Macroscopic view of actinorhodin (blue color) production of control and 8-DMTC-treated culture at 24-h (a); CLSM analysis of *S. coelicolor* A3(2) (b, e); treated

with filtered supernatant containing 8-DMTC (c, f) and treated with non-filtered supernatant containing 8-DMTC (d, g). White arrows indicate the compartments of primary mycelia

During the fermentation process of wild type, *Δppk*, and their cos16F4ie recombinants for HPLC analysis, we have noticed some abnormalities in the appearance of the recombinants' culture in R2YE broth and also on R2YE agar. Normally, the *Streptomyces* wild-type and *Δppk* strains produce blue-pigmented actinorhodin and red-pigmented undecylprodigiosin antibiotics. However, the cos16F4ie recombinants did not produce any colored antibiotics.

The cos16F4ie recombinants of *S. coelicolor* A3(2) wild type and *Δppk* were analyzed with confocal laser scanning microscopy (CLSM) to understand whether any changes were present in the morphology of the recombinants' colony structure. Colonies of A3(2) wild-type and *Δppk* strains were intact without fragmentation and most parts of the colonies were alive within the first 48 h. The death ratio was increasing until 72 h. Between 72 and 96 h, alive segments were growing again. Then, most of the segments died up to 120 h. The confocal images of the cos16F4ie harboring recombinants of both A3(2) wild-type and *Δppk* strains showed that the colony structure was totally disrupted, with no correlation between the incubation time and the alive-death cell ratio. Dead and viable fragments of mycelia were randomly spread around the culture.

Confocal analysis of A3(2) wild type and its recombinant strain was performed for 24 h at 6-h intervals to determine whether disentanglement happens after the colonies have been formed within the first 24 h or that the recombinants are not capable of forming colony structure at all. Confocal images showed that the recombinant strain never exhibited a healthy colony formation, pellets, and clumps.

The antibiotic production is closely related to pellet and clump formation in *Streptomyces*. Previous studies showed that pellet formation is essential for antibiotic production in *S. coelicolor* [1, 8]. So, the reason for the lack of ability to produce pigmented antibiotics might be the lack of pellet and clump formation of the cos16F4ie recombinants.

Although unlikely, another reason for the cease in antibiotic production could be the presence of a partial IclR family regulator gene (GenBank: CAP12614.1) that lies in the *elm* cluster. Generally, IclR family regulators are known to be transcriptional repressors that bind to the specific sequences of promoters or operators [28]. A study based on DoxR protein which belongs to IclR family regulators has shown that DoxR expression in *S. coelicolor* inhibited the expression of pigmented actinorhodin or undecylprodigiosin antibiotics. Moreover, the transcriptional analysis has demonstrated that there was no expression of *actII-ORF4*, the regulator of actinorhodin biosynthetic gene cluster, and less expression of *redD*, the regulator of undecylprodigiosin biosynthetic gene cluster, in DoxR-expressing *S. coelicolor* [29]. Furthermore, another group has targeted an IclR family regulator NdgRyo, to reveal new secondary metabolites in *Streptomyces youssoufiensis*. The removal of repression by

the deletion of *ndgRyo* has led to the discovery of a new fatty acid amide [30]. Considering these findings, the partial IclR family regulator located in the *elm* cluster may play a repressing role in the production of actinorhodin and undecylprodigiosin in *S. coelicolor* A3(2) wild type+cos16F4ie and *Δppk*+cos16F4ie strains. Further study is needed to prove this possibility.

cos16F4ie is an integrative cosmid; the  $\Phi$ C31 integrase enzyme is responsible for the integration of the cosmid into the *attB* site of the *S. coelicolor* genome [31]. The reason for the strange developmental pattern of the recombinant strains could be the disruption of the genome caused by the integration of the cosmid itself or it could be the expression of 8-DMTC compound encoded by *elm* cluster. To answer this question, we have deleted the whole *elm* cluster including partial IclR family regulator gene from cos16F4ie, and then transformed *S. coelicolor* A3(2) wild-type cells with this cos16F4ie/ $\Delta elm$  construct. At first sight, *S. coelicolor* wild type + cos16F4ie/ $\Delta elm$  cells produced pigmented antibiotics after 24-h incubation in R2YE broth. Moreover, they showed agarase activity on R2YE agar and sporulate on TBO agar. Confocal analysis showed that *S. coelicolor* wild type + cos16F4ie/ $\Delta elm$  strain could form colonies similar to wild-type ones. At 24 h, most of the colony was alive, only the center was suffering from PCD. The only change in features of cos16F4ie/ $\Delta elm$  recombinant from wild type was the delayed growth. Normally, wild-type colonies have already passed the first PCD round by 24 h, though there were few primary mycelia present in the colony, most of them have transformed to secondary mycelia. However, *S. coelicolor* wild type + cos16F4ie/ $\Delta elm$  colonies contained more primary mycelia than secondary ones. That means the growth was slower than wild-type colonies. Nonetheless, *S. coelicolor* wild type + cos16F4ie/ $\Delta elm$  showed quite similarities with *S. coelicolor* wild-type colonies. Thereby, we concluded that the deficiency in colony morphology and production of pigmented antibiotics was not caused by the insertion of the cosmid but the expression of *elm* cluster, thereby the production of 8-DMTC.

We have also searched whether the extracellular addition of 8-DMTC causes deformation of *Streptomyces* colonies as we observed in our cos16F4ie recombinant strains. So that, *S. coelicolor* wild-type colonies were treated with the supernatant of *S. coelicolor* wild type + cos16F4ie culture which contained 8-DMTC. We have performed the treatment with both filtered and non-filtered supernatant to avoid any transfer of cell or fragmented mycelia from the recombinant culture. After 24 h of the treatment, we have observed that the pigmented antibiotic production was lower in 8-DMTC-treated wild-type culture than that in the untreated ones. When the colony structure of 8-DMTC-treated wild-type culture was checked under confocal microscopy, we found that it had fewer colonies than wild-type culture. These few colonies were formed from primary mycelia indicating that the growth

was delayed. Furthermore, the area between the colonies was full of fragmented and disentangled mycelia. The CLSM analysis proved that 8-DMTC causes fragmentation of mycelia and disentanglement of colonies, thereby causing a lack of pellet formation. We could not explain why recombinants suffer from the anti-microbial effect of 8-DMTC since they contain a resistance gene in the *elm* gene cluster. Further research is needed to determine if the resistance gene is expressed or not in these recombinant strains.

To the best of our knowledge, the morphological changes that we have observed in *Streptomyces* that expresses 8-DMTC have not been reported before. Moreover, the results of this study provide useful information about the use of *Streptomyces* as hosts for the heterologous expression of this tetracyclic polyketide.

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**Author contribution** In order to recognize the authors' participation, we highlight each individual contribution: S.T. designed the study and supervised the experiments, B.C. and Z.D. contributed in confocal analysis of *S. coelicolor* and all recombinant strains expressing 8-DMTC, and B.C. made also contribution in the construction of cos16F4ie/ $\Delta$ elm and treatment of wild-type *S. coelicolor* A3(2) strain with 8-DMTC-containing supernatant. All these authors have substantial contributions to the final manuscript and approved this submission. All authors are aware of the order of authorship and that no further change in authorship will be performed after submission, except those previously authorized by the editor-in-chief.

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## Declarations

**Ethics approval** Not applicable.

**Consent to participate** Not applicable.

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**Conflict of interest** The authors declare no competing interests.

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