

T.R.

**GEBZE TECHNICAL UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES**

**STUDIES ON TESTING POLYPHOSPHATE DEFICIENT
STREPTOMYCES COELICOLOR AS A HOST FOR HOMOLOG
AND HETEROLOGOUS EXPRESSION OF VALUABLE PRODUCTS**

**BUSE ÇINAR
A THESIS SUBMITTED FOR THE DEGREE OF
MASTER OF SCIENCE
DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS**

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THESIS SUPERVISOR
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T.C.
GEBZE TEKNİK ÜNİVERSİTESİ
FEN BİLİMLERİ ENSTİTÜSÜ

**POLİFOSFAT YOKSUNU STREPTOMYCES
COELICOLOR'UN DEĞERLİ ÜRÜNLERİN
HOMOLOG VE HETEROLOG İFADELERİ
İÇİN KONAK OLARAK TEST EDİLMESİ
ÇALIŞMALARI**

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MOLEKÜLER BİYOLOJİ VE GENETİK ANABİLİM DALI

DANIŞMANI
PROF. DR. SEDEF TUNCA GEDİK

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SUMMARY

Streptomyces are Gram-positive, filamentous soil-dwelling bacteria with high G-C content. Recent studies indicated that they have a high potential to produce more variety of secondary metabolites thanks to their cryptic biosynthetic gene clusters in their genomes. A previous study carried out in our laboratory showed that the deletion of polyphosphate kinase gene (*ppk*), responsible for polyphosphate biosynthesis, increased the production of actinorhodin in both *Streptomyces coelicolor* strains. So, the deletion of the *ppk* gene created *S. coelicolor* mutant strain with a strong endogenous antibiotic production capacity. In the scope of the present study, we investigated whether the *S. coelicolor* Δppk strain could also produce primary metabolites in high amounts or be a possible prototype as a heterologous expression host. For that reason, we have selected cholesterol oxidase to determine the effect of *ppk* deletion on primary metabolism and the 8-demethyl-tetracenomycin (8-DMTC), an anti-tumor agent, to study the potential of Δppk strain as a host for the heterologous expression. We have successfully cloned the putative cholesterol oxidase gene of *S. coelicolor*, but the effect of polyphosphate deficiency on enzyme production couldn't be determined because of the lack of substrate (cholesterol) that must be supplemented into the medium. In the case of heterologous expression experiments, the 8-DMTC expressing recombinant *S. coelicolor* Δppk strain produced slightly more 8-DMTC than the recombinant wild type. Besides, we have noticed morphological changes in the colony formation of the recombinants caused by the production of 8-DMTC. The recombinant *S. coelicolor* wild type and Δppk strains can not form colonies, pellets or clumps. Moreover, they can not produce pigmented antibiotics and do not sporulate, unlike wild type and Δppk . Our study provided valuable information to the literature since the interesting morphological changes of the *S. coelicolor* colonies caused by the expression of 8-DMTC have not been reported before.

Key Words: Δppk , heterologous expression, *Streptomyces* colony morphology, pellet disaggregation, 8-DMTC.

ÖZET

Streptomyces'ler Gram-pozitif, filamentli, DNA'larında yüksek G-C oranına sahip toprak bakterileridir. Yakın zamanlı çalışmalar genomlarındaki kriptik biyosentetik gen kümeleri sayesinde çok daha çeşitli ikincil metabolit üretme potansiyeline sahip olduklarını göstermiştir. Laboratuvarımızda yürütülen bir çalışmada, genomdan polifosfat üretiminden sorumlu olan polifosfat kinaz (*ppk*) geninin silinmesiyle hem *Streptomyces coelicolor*'te aktinorhodin antibiyotiğinin üretiminin arttığı gösterilmiştir. Böylece *ppk* geninin silinmesi, güçlü bir endojen antibiyotik üreticisi olan bir *S. coelicolor* mutant suşu oluşturmuştur. Çalışmamız kapsamında, *S. coelicolor* Δppk suşunun birincil metabolitleri de daha çok üretip üretmeyeceğini veya heterolog ifade konağı olarak olası bir prototip olup olmayacağını araştırdık. Bu nedenle, birincil metabolizmayı test etmek için kolesterol oksidazı, *S. coelicolor* yaban tip ve Δppk 'daki heterolog ifadeyi test etmek ve karşılaştırmak için de bir anti-tümör ajanı olan 8-demetil tetrasenomisini (8-DMTC) seçtik. Deneylerin sonucunda, *S. coelicolor*'ın olası kolesterol oksidaz geni başarıyla klonlandı ancak besiyerine eklenmesi gereken substrat (kolesterol) olmadığından polifosfat eksikliğinin enzim üretimine etkisi belirlenemedi. Heterolog ekspresyon deneylerinde, *S. coelicolor* Δppk rekombinant suşun yaban tip rekombinantına göre 8-DMTC'yi biraz daha fazla ürettiği gözlemlendi. Bunun yanında, rekombinantların koloni formasyonunda 8-DMTC üretiminden kaynaklanan morfolojik değişiklikler olduğu tespit edildi. Rekombinant *S. coelicolor* yaban tip ve Δppk suşlarının koloni, pelet ya da topak oluşturmadıkları, ayrıca yaban tip ve Δppk suşun aksine pigmentli antibiyotikleri üretmedikleri ve sporlanamadıkları gözlemlendi. 8-DMTC'nin üretilmesi sebebiyle *S. coelicolor* kolonilerindeki ilginç morfolojik değişiklikler daha önce rapor edilmemiş olduğundan çalışmamız literatüre değerli bilgiler sağlamıştır.

Anahtar Kelimeler: Δppk , heterolog ifade, *Streptomyces* koloni morfolojisi, pellet disagregasyonu, 8-DMTC.

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TABLE OF CONTENTS

	<u>Page</u>
SUMMARY	v
ÖZET	vi
ACKNOWLEDGMENTS	vii
TABLE OF CONTENTS	viii
LIST of ABBREVIATIONS and ACRONYMS	xi
LIST of FIGURES	xiii
LIST of TABLES	xiv
1. INTRODUCTION	1
1.1. The Aim, Contribution and The Original Value of The Thesis	1
2. POLYPHOSPHATE MOLECULE	3
3. STREPTOMYCETES	5
3.1. Life Cycle	5
3.2. Secondary Metabolism	6
3.2.1. Phosphate Mechanism in Secondary Metabolite Production	7
4. BIOSYNTHETIC GENE CLUSTERS	9
4.1. Activation of Biosynthetic Gene Clusters	9
4.2 Heterologous expression	10
5. MATERIAL AND METHODS	12
5.1. Materials	12
5.1.1. Media	12
5.1.2. Chemicals	13
5.1.3. Solutions	13
5.1.4. Enzymes	14
5.1.5. DNA Kits and Markers	14
5.1.6. Microorganisms	14
5.1.7. Plasmids and Cosmids	15
5.1.8. Primers	17
5.1.9. Antibiotics	18
5.1.10. Devices	19

5.2. Methods	19
5.2.1. Plasmid Isolation from <i>E. coli</i>	19
5.2.2. Primer Design	19
5.2.3. Amplifying the Gene of Interests via PCR	20
5.2.4. Agarose Gel Electrophoresis	21
5.2.5. Gel Clean-Up Kit	21
5.2.6. Restriction Enzyme Digestion	22
5.2.7. Ligation	22
5.2.8. Preparation of <i>E. coli</i> Competent Cells	22
5.2.9. MiniPrep Plasmid Isolation	22
5.2.10. Chemical Transformation of <i>E. coli</i> Cells	23
5.2.11. Isolation of Cosmids	23
5.2.12. Transformation of ET12567/pUZ8002 cells via Electroporation	24
5.2.13. Harvesting <i>Streptomyces</i> Spores	24
5.2.14. <i>Streptomyces</i> Conjugation	25
5.2.15. Genomic DNA Isolation	26
5.2.16. Detection and Characterisation of 8-DMTC	26
5.2.17. Confocal Laser Scanning Microscopy Analysis (CLSM)	27
6. RESULTS	28
6.1. Effect of Polyphosphate Kinase Deficiency on Primary Metabolism of <i>S. coelicolor</i>	30
6.1.1. Amplifying the Cholesterol Oxidase Gene	28
6.1.2. Cloning the Cholesterol Oxidase Gene into pSPG Plasmid	29
6.2. Potential of $\Delta appk$ Strain as a Host for Heterologous Expression	30
6.2.1. Transfer of cos16F4 into <i>Streptomyces</i> Strains	31
6.2.2. Detection of the 8-DMTC Compound via HPLC	33
6.3. Morphological Analysis of <i>Streptomyces</i> Recombinants Harboring cos16F4	35
6.4. Analysis of <i>Streptomyces</i> Recombinants Harboring cos16F4 with CLSM	37
6.4.1. Confocal Analysis of <i>S. coelicolor</i> A3(2) Recombinants Grown in Liquid	37
6.4.2 Confocal Analysis of <i>S. coelicolor</i> A3(2) Recombinants in Liquid Culture during the first 24 h	38
6.4.3 Confocal Analysis of <i>S. coelicolor</i> A3(2) Recombinants Grown on Agar	41
6.5. Construction of cos16F4/ Δelm	41
6.5.1. Confocal Analysis of <i>S. coelicolor</i> A3(2) cos16F4/ Δelm Recombinants	42

6.6. Treatment of WT <i>S. coelicolor</i> A3(2) Strain with Extracellular 8-DMTC	46
7. DISCUSSION	46
REFERENCES	52
BIOGRAPHY	58

LIST of ABBREVIATIONS and ACRONYMS

Abbreviations Explanations and Acronyms

8-DMTC	: 8-demethyl-tetracenomycin
α	: Alfa
Δ	: Delta
μL	: Microlitre
μM	: Micromolar
Apr	: Apramycin
BamHI	: <i>Bacillus amyloliquefaciens</i> H endonuclease
bp	: Base pair
CaCl_2	: Calcium chloride
CDA	: Calcium-dependent antibiotic
CLSM	: Confocal laser scanning microscopy
EDTA	: Ethylenediaminetetraacetic acid
h	: Hour
kb	: kilobase
KCl	: Potassium chloride
KOAc	: Potassium acetate
L	: Litre
LB	: Luria-Bertani broth
M	: Molar
Mg	: Milligram
Mg^{+2}	: Magnesium ion
mL	: Millilitre
mM	: Millimolar
NaCl	: Sodium chloride
NaOH	: Sodium hydroxide
NdeI	: <i>Neisseria denitrificans</i> endonuclease I
PCR	: Polymerase chain reaction
PI	: Propidium iodide
SDS	: Sodium dodecyl sulfate

SpeI : *Sphaerotilus* sp. endonuclease I
Tris : Tris (Hydroxymethyl) aminomethane

LIST of FIGURES

<u>Figure No :</u>	<u>Page</u>
2.1: Different polyP groups.	3
3.1: <i>Streptomyces</i> life cycle.	6
5.1: Markers used for the study.	14
5.2: The <i>elm</i> cluster that is responsible for the biosynthesis of 8-DMTC.	17
5.3: The map of pKC505.	17
5.4: The map of pSPG.	17
6.1: The possible cholesterol oxidase gene obtained by PCR reaction .	29
6.2: BLAST analysis of the possible cholesterol oxidase gene.	29
6.3: pSPG plasmid and cholesterol oxidase gene digested via <i>NdeI</i> and <i>SpeI</i> .	30
6.4: Confirmation of recombinant pSPG plasmid isolated from DH5 α .	30
6.5: Confirmation of ET/pUZ/cos16F4 transformants with PCR.	32
6.6: Confirmation of cos16F4 insertion into chromosomal DNA.	33
6.7: HPLC analysis of 8-DMTC.	34
6.8: Comparison of <i>S. coelicolor</i> strains' chromatograms.	34
6.9: Macroscopic view of <i>S. coelicolor</i> A3(2) WT, Δ <i>appk</i> and their recombinants.	35
6.10: Confocal images of <i>S. coelicolor</i> WT strains and their recombinants.	37
6.11: Confocal images of <i>S. coelicolor</i> Δ <i>appk</i> strains and their recombinants.	38
6.12: Confocal analysis of <i>S. coelicolor</i> A3(2) strains.	39
6.13: Confocal analysis of <i>S. coelicolor</i> A3(2) strains on agar.	41
6.14: Confirmation of DH5 α /cos16F4/ Δ <i>elm</i> transformants.	42
6.15: Confirmation of ET/pUZ/cos16F4/ Δ <i>elm</i> with <i>BamHI</i> digestion.	42
6.16: The morphological analysis of <i>S. coelicolor</i> wild type + cos16F4/ Δ <i>elm</i> .	43
6.17: Effect of external 8-DMTC on <i>S. coelicolor</i> A3(2) in R2YE broth.	44
6.18: Confocal images of single colonies of <i>S. coelicolor</i> A3(2) samples.	45

LIST of TABLES

<u>Table No:</u>	<u>Page</u>
5.1: Media and their ingredients.	12
5.2: Solutions and their contents.	13
5.3: The bacterial strains used in this study.	15
5.4: Properties of cosmids and plasmids.	16
5.5: Primer sequences.	18
5.7: PCR reaction components and conditions.	21

1. INTRODUCTION

Streptomyces family take an important place in medicine, biotechnology, and pharmaceutical area thanks to their highly active secondary metabolism. They produce anti-tumor, antibacterial, antifungal agents and two-third of the antibiotics available commercially. In addition to the secondary metabolites that were identified previously, 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 can not be expressed or detected in their natural hosts under standard fermentation conditions, therefore they need to be activated. The heterologous expression is one of the most efficient ways among the activation methods of BGCs.

Secondary metabolism is affected by many crucial mechanisms of the cell, such as the developmental cycle, the programmed cell death process (PCD), phosphate metabolism, etc. Polyphosphate kinase (PPK) enzyme plays roles in phosphate metabolism by catalyzing the biosynthesis of polyphosphate (polyP) polymer which has very important functions in every living cell. Previous studies demonstrated that the deletion of the *ppk* gene caused an increase in actinorhodin production in *S. lividans* and *S. coelicolor*, thereby proving the importance of polyP also in secondary metabolism.

1.1. The Aim, Contribution and The Original Value of The Thesis

In our study, we aimed to investigate the effect of *ppk* deletion on the primary metabolism of *S. coelicolor*, and also to investigate the potential of *S. coelicolor* Δppk strain as host for the production of secondary metabolites. Cholesterol oxidase is a primary metabolite that is responsible for degrading cholesterol as a carbon source for the cell. We have selected this enzyme to test how the primary metabolism is affected by the deletion of the *ppk* gene because of not only its crucial duty in carbon metabolism but also its anti-tumor properties against breast and urothelial tumors. To evaluate the potential of *S. coelicolor* Δppk strain as a heterologous host, an anti-tumor agent from *Streptomyces olivaceus* strain Tü 2353, 8-DMTC, has been selected.

A proper, high-producer heterologous host may provide many advantages for the production of BGCs of the *Streptomyces* genome. In that way, our study contributes to biotechnology, medicine, and pharmaceutical area since *S. coelicolor* Δppk strain has the potential to be a promising heterologous host on the course to discovering new drugs. Moreover, to the best of our knowledge the morphological changes in *Streptomyces* colonies because of the 8-DMTC expression, have never been reported before. Thus this study provides some clues for the impact of 8-DMTC on colony morphology and consolidates the importance of colony morphology in secondary metabolism in *Streptomyces*.

2. POLYPHOSPHATE MOLECULE

Inorganic polyphosphate (polyP) is a linear polymer consisting of hundreds of orthophosphate residues. These residues are interconnected by high-energy phosphoanhydride bonds [1]. PolyP is stable against extreme conditions, such as a wide range of temperature, pH values, and oxidants, making it possible to find polyP in all living things from the volcanic areas to the depths of the oceans [2]

PolyP is a stable polymer that functions as a phosphate reservoir and energy source for the cell. According to their chemical structures, three different groups of polyP exist in the cell: cyclic condensed phosphates (metaphosphates), linear polyphosphates (linear metaphosphates), ultraphosphates (branched polyphosphates) (Figure 2.1) [3].

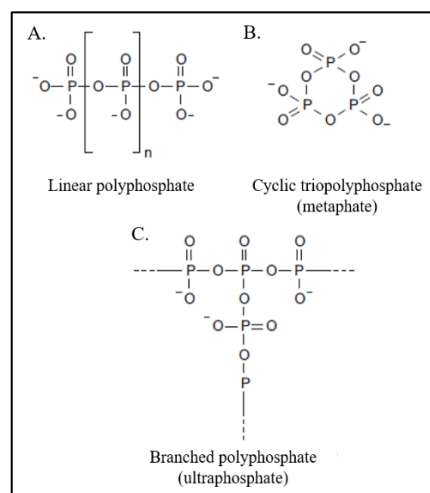
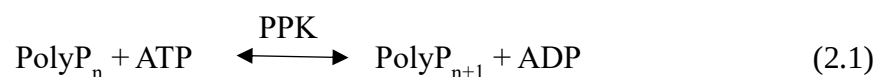


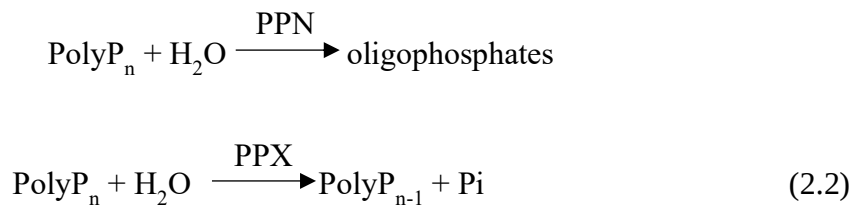
Figure 2.1: Different polyP groups.

In prokaryotes and some microbial eukaryotes, polyphosphate synthesis is carried out by the polyphosphate kinase 1 (PPK1) enzyme, a member of the phosphotransferase's superfamily. PPK catalyzes the transfer of energy-rich phosphate from ATP to the polyP chain [3].



Most bacteria have one more polyP kinase besides PPK1. Unlike PPK1, polyP kinase 2 (PPK2) can catalyze the reaction with GTP or ATP [2]. However, PPK2 is also responsible for the synthesis of GTP. Moreover, it catalyzes the GTP synthesis reaction 75-fold more than polyP synthesis. Therefore, it acts as a polyP-degrading enzyme instead of a polyP synthesis [4].

polyP polymers are degraded by endopolyphosphatase (PPN) and exopolyphosphatase (PPX) enzymes. PPN cleaves long polyP chains into shorter ones without releasing an inorganic phosphate, on the other hand, PPX degrades polyP by hydrolyzing and cleaving the terminal phosphate from the chain [3].



Depending on the cellular localization, polyP carries out numerous tasks in many crucial processes [1], [3]: membrane ion transport, ATP substitution, channel for DNA entry, cation chelation, buffer against alkali ions, motility, virulence, and persistence, stress response, cell signaling, cell energy, phosphorus reservoir, cell acclimation to nutrient-deficiency, developmental regulation, applications in industry: as antibacterial agents, ATP source, and insulating fibers. The roles of polyphosphate in all these processes carry the PolyP to a very important point for both prokaryotic and eukaryotic organisms.

3. STREPTOMYCETES

Streptomyces are Gram-positive, filamentous soil-dwelling bacteria with a high G-C percentage in their DNA. Although *Streptomyces* family can be found both in water and soil habitats, most strains of *Streptomyces* prefer the soil ecosystem. As an environment, soil harbors nutritionally and biologically many different organisms. The crucial features of *Streptomyces* such as the ability to produce a broad spectrum of secondary metabolites and having a highly complex life cycle put them in an important place among other organisms in the soil ecology and also in biotechnology, medicine, and pharmaceutical area [5].

3.1. Life Cycle

The complex life cycle of *Streptomyces* is comprised of three main stages (Figure 3.1). As a sporogenic microorganism, the first stage of development is spore germination. In favorable conditions, vegetative hyphae (MI) grow out from spore and extend by polar growth unlike most of the unicellular bacteria [6]. During exponential growth, vegetative hyphae form branches made of multinucleated compartments that are separated by peptidoglycan cross-walls and also by cross-membranes without peptidoglycan [7]. Extension of vegetative hyphae throughout the media is followed by the first round of programmed cell death (PCD). Some compartments of vegetative hyphae die off via PCD and then both the viable and dead compartments stay in the same hyphae [8]. Furthermore, alive compartments that survived from the first round of PCD differentiate into multinucleated, secondary metabolite-producing aerial hyphae (MII) as the second stage. Aerial mycelium grows through the air with the help of a hydrophobic protein layer that allows it to grow out of the aqueous environment [9]. In this stage, morphological changes triggered by the PCD are accompanied by metabolic changes as well. Aerial mycelium stage has an active secondary metabolism, unlike the vegetative stage which is focused on primary metabolism [10]. Aerial hyphae undergo the second round of the PCD and then remaining viable multinucleated compartments divide into several uninucleoid segments that will differentiate into spore chains as the third stage. Even though the molecular triggers and 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 [8].

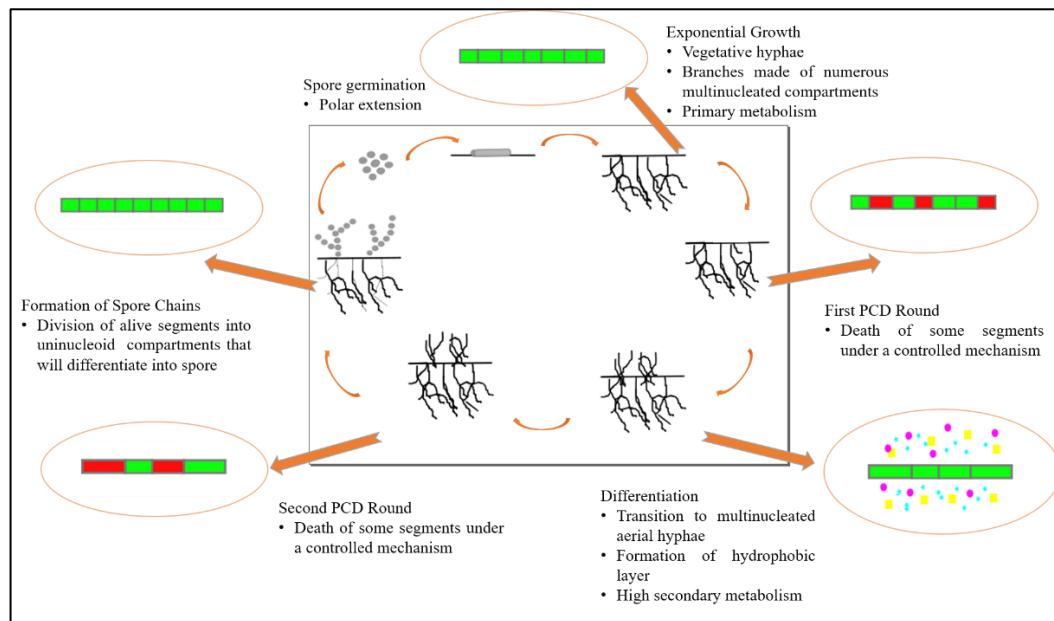


Figure 3.1: *Streptomyces* life cycle.

3.2. Secondary Metabolism

Streptomyces have been studied widely not only because of the curiosity in their complex life cycle but also the broad spectrum of the secondary metabolites that are produced alongside developmental changes. They have great potential in biotechnology, medicine, and pharmaceutical area due to their ability to produce secondary metabolites including two-thirds of the known antibiotics, pigments, herbicides, anti-tumor, and anti-inflammatory agents [10].

Secondary metabolite production is a well-timed process that is triggered by differentiation and correlated with growth throughout the life cycle. The formation of the aerial hyphae from vegetative mycelium as a result of the programmed cell death round is accompanied by the secondary metabolite production. Though, the mechanism that relates antibiotic production with differentiation has not completely enlightened yet. There are some studies that link the antibiotic production to various mechanisms of the cells. The transcription factor DasR which is known to control the sugar phosphotransferase system and import several carbon sources, relates the carbon availability to secondary metabolism and antibiotic production in *Streptomyces* [11].

Another mechanism that is closely related to antibiotic production is the stringent response. A study has shown that the deletion of the RelA enzyme which is responsible for alarmone synthesis in the stringent response, causes a failure in producing actinorhodin and undecylprodigiosin antibiotics under nitrogen-limited conditions [12]. In addition to these mechanisms, the morphological control also affects antibiotic production. Different morphologies such as loose aggregates or dense clumps can be observed in various *Streptomyces* cultures and these differences in the morphology influence antibiotic production [6].

The whole genome of *Streptomyces coelicolor* A3(2) which is a model organism, was sequenced by Bentley and his group in 2002 [5]. In addition to the previous information that *S. coelicolor* can produce the actinorhodin (aromatic polyketide antibiotic), red-pigmented oligopyrrole prodiginine antibiotics (RED), and the nonribosomal peptide CDA [13], [14]; a further 18 biosynthetic gene clusters, which can synthesize new secondary metabolites, have been identified in the genome of this organism [5]. These studies have revealed that both *S. coelicolor* A3(2) and its derivative M145 strain (lacking its two plasmids SCP1 and SCP2) have a high potential as a producer of natural products.

3.2.1. Phosphate Mechanism in Secondary Metabolite Production

Streptomyces produce many different antibiotics under phosphate-deficient conditions. The negative effect of phosphate on different antibiotics had been proved by many research groups. The antibiotic production in *Streptomyces* is strictly controlled by a two-component PhoR-PhoP system which also controls the alkaline phosphatase gene (*phoA*) [15]. Phosphate can regulate antibiotic production in transcriptional and post-transcriptional ways. Phosphate control (PC) sequences where are located at upstream of the phosphate-regulated *Streptomyces* promoters control the expression at transcriptional levels. Sequence analysis showed that these PC sequences are highly similar to phosphate boxes (*pho* etc.) which play main roles in the two-component system [16]. Further studies focused on phosphate mechanism to identify the regulatory components of the control process.

In 2002, Chouayekh and Virolle proved the importance of polyphosphate kinase (PPK) in the negative regulation of antibiotic production by deleting the *ppk* gene of *S. lividans*. *ppk* deletion mutants of *S. lividans* showed an induced actinorhodin

production under phosphate limited conditions. This induction was caused by the active transcription of the *actII-ORF4*, pathway-specific activator gene, in the Δppk mutant strain of *S. lividans* [17]. Another study of the same group has shown that the expression of *ppk* was higher under phosphate-limitation and was under the control of the two-component system PhoR/PhoP [18]. Proteomic analysis of the Δppk mutant strain of *S. lividans* TK24 has revealed more information about the role of PPK enzyme in the regulation of antibiotic production. The analysis showed that Triacylglycerol (TAG) content was higher in wild type strain than Δppk . TAG which takes place in fatty-acid storage of the cell, shares the same precursor, acyl-CoA, with actinorhodin. Apparently, the disruption of the PPK enzyme induced the degradation of TAG and provide more acyl-CoA precursors for actinorhodin production. So that, a relation between antibiotic production and lipid storage was enlightened by this proteomic analysis [19].

In a study carried out in our laboratory, the *ppk* gene was deleted in both *S. coelicolor* A3(2) and M145 strains. The actinorhodin production was found to be 8.5-times more in A3(2) Δppk mutant than A3(2) wild type and 2.6-times more in M145 Δppk mutant than M145 wild type strain. Moreover, *S. coelicolor* strains that produced more antibiotics have been shown to have less TAG content, thereby proving the relation between antibiotic production and lipid storage previously shown in *S. lividans*.

4. BIOSYNTHETIC GENE CLUSTERS

The secondary metabolites such as antibiotics, anti-microbial and anti-tumor agents produced by *Streptomyces* family provides many benefits to the scientific world. However, the problem of antibiotic resistance in bacteria remains and continues to increase day by day. Commercially available antibiotics lose their effectiveness against bacteria due to the excessive and wrong antibiotic usage. Bacterial strains resistant to known antibiotics cause serious problems in the pharmaceutical field and threaten human health. Therefore, the discovery of new antibiotics becomes vital in the present day [20].

In laboratories, numerous secondary metabolites are produced by the *Streptomyces* family. However, the desperate need for the discovery of the new antibiotics leads the scientist to the depths of the *Streptomyces* genome, to cryptic regions. Genome mining studies have revealed that the *Streptomyces* genome contains several biosynthetic gene clusters (BGCs) that are not expressed under standard fermentation conditions [21]. Thus, either their functions are undefined, or production levels are only slightly detectable in their natural hosts [22]. In both cases, these biosynthetic gene clusters have a high potential to be the new drug sources waiting to be discovered. Thus, these cryptic BGCs have further strengthened the importance of *Streptomyces* in medical and pharmaceutical fields.

4.1. Activation of Biosynthetic Gene Clusters

Studies with biosynthetic gene clusters could be divided into two main categories according to their purposes; to understand the functions of cryptic BGCs and to obtain the product of BGCs in high yields. The basic but also critical step of each category is the activation of the clusters.

There are many factors affecting the production of the secondary metabolites in *Streptomyces* and hence their activation mechanisms. Cryptic gene clusters are silent under the laboratory culture conditions, so changing the cultivation conditions may be a way of activating gene clusters. Secondary metabolism, on the other hand, is strictly related to life cycle stages, as noted earlier. Any manipulation to the differentiation process has a large activation potential in biosynthetic gene clusters [22]. Even the morphological changes, such as pellet and clump formation or disaggregation are

known to affect antibiotic production [23]. Moreover, the activation may be in the natural host by manipulating its genome or regulatory mechanisms, or it may also be in a heterologous host designed for production. By considering all these factors and variabilities, many different activation methods have been defined previously [22], [23]; changing cultivation conditions, inducing stress response, genetic manipulations; expression of BGCs in heterologous hosts, random mutagenesis, regulation of gene expression, ribosomal engineering, elicitor.

4.2 Heterologous expression

The increasing necessity of both the discovery of new antibiotics and then obtaining these products in high yields becomes more important lately. Since the cryptic secondary metabolites are produced poorly in their natural hosts, the search for meeting the need is directed to more suitable expression hosts. Since the secondary metabolite production is under complex control and regulation mechanisms, heterologous hosts should be closely related to the natural organism, thus ensuring compatibility between systems. The genome of the heterologous hosts has to be fully sequenced, manipulation techniques have to be well-studied and their regulation mechanisms should be described in detail [21]. Therefore, the selection of the heterologous host, taking into account all the requirements, is an important step towards high secondary metabolite production.

Previous studies have shown that *Streptomyces* strains are naturally the most suitable heterologous hosts. Most of the *Streptomyces* strains are well-studied organisms whose genomes are fully sequenced. As well as being able to naturally produce many secondary metabolites, they can also be genetically engineered based on the characteristics expected from a host for specific productions [24]. Many groups have preferred *Streptomyces* as heterologous hosts for production of specific secondary metabolites, such as *Streptomyces albus* J1074 for fredericamycin [25], *S. lividans* 1326 for capreomycin [26] and viomycin [27], a deletion mutant of *S. avermitilis* (SUKA) for streptomycin production [28]. *S. coelicolor*, in particular, is one of the most popular options among heterologous hosts due to its beneficial properties. *S. coelicolor* has been well-studied to date, so its chromosome is fully sequenced, 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, activator, etc.) [24]. In addition to wild type *S. coelicolor* strain, genetically developed many *S. coelicolor* strains have been constructed in previous studies such as; *S. coelicolor* CH999 for producing polyketides [29], *S. coelicolor* M512 for the regulation of actinorhodin and prodiginine production [30], *S. coelicolor* for the erythronolide PKS [29], *S. coelicolor* 1146 for production of congocidine [31].

In the scope of the previous studies in our laboratory, our group formed *S. coelicolor* mutant strain by the deletion of the polyphosphate kinase gene (*ppk*). This *S. coelicolor* Δppk strain can produce 8.5-times more endogenous antibiotic actinorhodin than wild type. Since the Δppk mutant strain is a strong producer of endogenous antibiotics, the aim of this study is to investigate the effect of PPK deficiency on the primary metabolism and to determine whether the *S. coelicolor* Δppk strain could be a possible prototype strain for heterologous expression. For this purpose, the putative cholesterol oxidase enzyme which belongs to primary metabolism is chosen to study the effect of PPK deficiency. The role of cholesterol oxidase in the primary metabolism is to utilize cholesterol as a carbon source in the media [32]. This enzyme has also anti-tumor properties. It inhibits breast tumor growth [33], urothelial tumor growth [34] by attacking the membrane cholesterol of the tumor cell-lines since the cholesterol content of the membrane is higher in tumor cells. The importance of cholesterol oxidase in medical treatments increases the need for producing and obtaining this enzyme in high amounts.

To investigate the potential of *S. coelicolor* Δppk strain as a heterologous host. 8-demethyl-tetracenomycin (8-DMTC) from *Streptomyces olivaceus* strain Tü 2353, is selected. 8-DMTC is an anti-bacterial and anti-tumor agent. It acts against the Gr⁺ bacteria by intercalating with their DNA however the mechanism of the antibacterial activity has not been elucidated in detail yet [35], [36], [37]. As an anti-tumor agent, 8-DMTC inhibits the proliferation of murine L1210 leukemia cells [35].

5. MATERIAL AND METHODS

5.1. Materials

5.1.1. Media

All media that were used in this study and their ingredients are shown in Table 5.1. Every medium was sterilized via autoclave at 121°C for 20 minutes unless otherwise specified.

Table 5.1: Media and their ingredients.

MEDIA	
Luria Bertani (LB) Broth-800 ml • 8 g NaCl • 8 g Peptone • 4 g Yeast Extract	R2YE Broth-800 ml • 103 g Sucrose • 0.25 g K₂SO₄ • 10.12 g MgCl₂.6H₂O • 10 g Glucose • 5 g Yeast Extract • 0.1 g Casaminoacids
Mannitol Salt (MS) Agar-600 ml • 12 g Mannitol • 12 g Soy-bean Flour • 12 g Agar -MS agar was autoclaved twice at 121 °C for 20 minutes. -10 mM MgCl₂ was added separately.	-22 g agar was added into the medium for R2YE Agar. -The following substances must be added before using R2YE; • 10 ml KH₂PO₄ (0.5%) • 80 ml CaCl₂.2H₂O (3.68%) • 15 ml L-proline (20%) • 100 ml TES Buffer (5.73%) • 2 ml Trace Elements Solution - 200 mg ZnCl₂ - 1000 mg FeCl₃.6H₂O - 50 mg CuCl₂.2H₂O - 50 mg MnCl₂.4H₂O - 50 mg Na₂B₄O₇.10H₂O - 50 mg (NH₄)₆Mo₇O₂₄.4H₂O • 5 ml NaOH (1N)
Tryptic Soy Broth (TSB)-800 ml • 24 g Tryptic Soy Broth • 12 g agar was added into the medium for Tryptic Soy Agar (TSA).	
TBO Agar-800 ml • 16 g Oat Bran • 16 g Tomato Paste • 20 g Agar	
YEME Broth-800 ml • 2.4 g Yeast Extract • 4 g Peptone • 2.4 g Malt Extract • 8 g Glucose • 272 g Sucrose	2xYT Broth-800 ml • 12.8 g Peptone • 8 g Yeast Extract • 4 g NaCl

5.1.2. Chemicals

The following chemicals were used in the experiments of the thesis; Peptone (Bacto™, Merck), NaCl (Sigma-Aldrich), Yeast extract (Bacto™), Agar (Merck), NaOH (Merck), SDS (Merck), Tris (Merck), EDTA (Merck), D (+) Glucose (Merck), KOAc (Sigma Aldrich), Isopropanol (Riedel de Haen), Agarose (Sigma Aldrich), Sucrose (Merck), CaCl₂ (Merck), KCl (Riedel de Haen), Acetic Acid (Riedel de Haen), 99.5% Ethanol (Riedel de Haen), Tryptic soy broth (Merck), K₂HPO₄ (Merck), K₂SO₄ (Merck), TES Buffer (Applichem), Mannitol (Merck), Bacto Casaminoacid (BD), EDTA (Merck), 84-88% Glycerol (Sigma Aldrich), Phenol (Merck), Formic Acid (Merck), Ethyl Acetate (Merck), Acetonitrile (Merck), Phosphoric Acid (Merck).

5.1.3. Solutions

The solutions used in this study were prepared as shown in Table 5.2.

Table 5.2: Solutions and their contents.

SOLUTIONS	
Solution I (+4° C) • 25 mM Tris (pH: 8.0) • 10 mM EDTA (pH: 8.0) • 50 mM Glucose Solution II • 0.2 N NaOH • 1% SDS Solution II must be prepared freshly.	TfbI Solution (+4 °C) • 5 M KOAc • 1 M MnCl₂ • 1 M KCl • 1 M CaCl₂ • 75% Glycerol TfbII Solution (+4 °C) • 1 M Na-MOPS • 1 M CaCl₂ • 1 M KCl • % 75 Glycerol CTAB/NaCl: • 10% CTAB • 0.7 M NaCl 50x TAE • 50 mM EDTA • 2 M Tris • 1 M Glacial Acetic Acid
Solution III (+4° C) • 0.2 N • 60% 5 M KOAc • 11.5% Glacial Acetic Acid Tris-EDTA (TE) Buffer • 10 mM Tris • 1 mM EDTA CIA • 24:1 Chloroform: Isoamyl alcohol	

5.1.4. Enzymes

Lysozyme (Sigma), RNase, Phusion DNA Polymerase (Thermoscientific), *NdeI* Restriction Endonuclease (Fermantas), *SpeI* Restriction Endonuclease (Fermantas), *BamHI* Restriction Endonuclease (Fermantas), *EcoRI* Restriction Endonuclease (Roche), T4 DNA Ligase (NEB).

5.1.5. DNA Kits and Markers

For Confocal Laser Scanning Microscopy (CLSM) analysis, cells were stained with the LIVE/DEAD Bac-Light Bacterial Viability Kit (L-7012).

The PCR Clean-Up and Gel Extraction Kit (MN) was used for both purifications of plasmids and PCR products from agarose gel. During agarose gel electrophoresis, the markers used for distinguishing DNA molecules based on their sizes are presented in Figure 4.1.

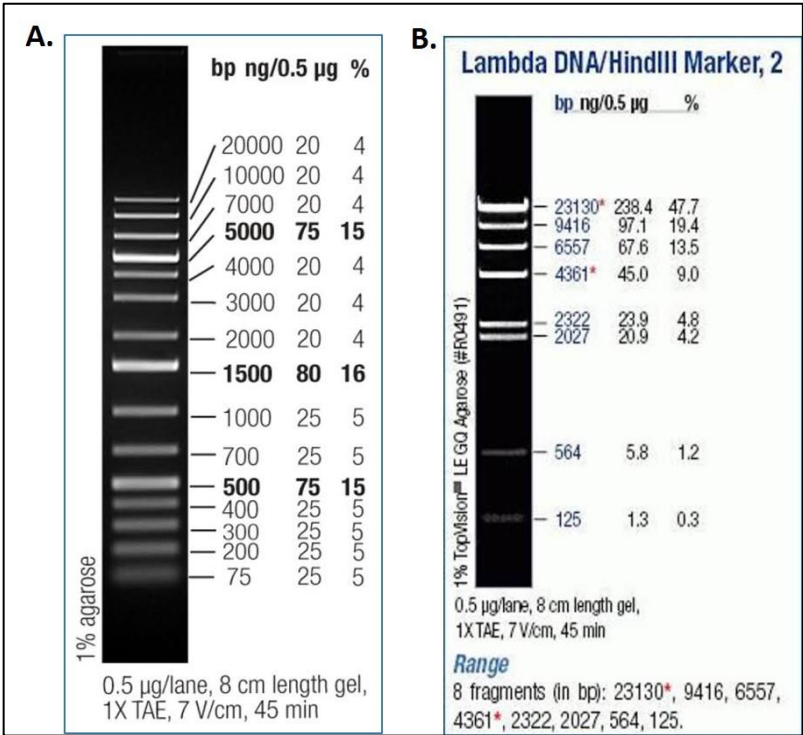


Figure 5.1: Markers used for the study. A: GeneRuler 1 kb DNA Ladder (Thermo, SM0311) B: Lambda DNA/HindIII Marker (Thermo, SM0101).

5.1.6. Microorganisms

Bacterial strains used in this study are listed in Table 5.3.

Table 5.3: The bacterial strains used in this study.

Bacterial Strains	Genotypic Properties	Reference
<i>E. coli</i> ET12567/pUZ8002	<i>dam, dcm, hsdS, cat, tet/ tra, neo, RP4</i>	[6]
<i>S. coelicolor</i> A3(2) and M145	Wild type	[38]
<i>S. coelicolor</i> A3(2) and M145 + cos16F4	Recombinant cells that carry cos16F4	This Study
<i>S. coelicolor</i> A3(2) +cos16F4/Δ<i>elm</i>	Recombinant cells that carry <i>elm</i> deleted cos16F4 derivative	This Study
<i>S. coelicolor</i> A3(2) and M145 Δ<i>ppk</i>	<i>ppk</i> deleted mutant cell	[39]
<i>S. coelicolor</i> A3(2) and M145 Δ<i>ppk</i> + cos16F4	Recombinant cells that carry cos16F4	This study
<i>E. coli</i> DH5α +cos16F4/Δ<i>elm</i>	Recombinant cells that carry <i>elm</i> deleted cos16F4 derivative	This study
<i>E. coli</i> ET12567 +cos16F4/Δ<i>elm</i>	Recombinant cells that carry <i>elm</i> deleted cos16F4 derivative	This study
<i>E. coli</i> DH5α + pSPG + Cholesterol Oxidase	Recombinant cells that carry pSPG + CO	This Study

5.1.7. Plasmids and Cosmids

The cosmids and plasmids used for this study are listed in Table 5.4.

Table 5.4: Properties of cosmids and plasmids.

NAME	Resistance	Size	Reference
cos16F4	Apramycin Erythromycin Tetracyclin	49800 bp	[40]
cos16F4 <i>Δelm</i>	Apramycin	22000 bp	This study
pUZ8002	Kanamycin	70 kb	[41]
pSPG	Apramycin Ampicilin	8149 bp	[42]

Elloramycin (*elm*) cluster (Figure 5.2) which is responsible for the production of 8-DMTC is carried on the cosmid cos16F4. This cosmid is an integrative derivative of apramycin-resistant pKC505 (Figure 5.3). A 6.9 kb *SpeI* DNA fragment from pFL1139 was cloned into the unique *XbaI* restriction site. This fragment consists of a conjugative *oriT*, the tetracycline and erythromycin resistance cassettes, the site-specific recombination *attP* site, the integrase gene (*int*) of Φ C31. The Φ C31 integrase is a site-specific, serine recombinase which inserts the required fragment into the 34 bp *attB* site in the host genome by unidirectional recombination. In the *S. coelicolor* genome, there is a main, identified *attB* site inside a chromosome condensation protein-encoding gene (SCAC2.06c). There are also several pseudo-*attB* sites however, the insertion of the fragment into the main *attB* site is 300 times more possible than into the pseudo sites [43].

Table 5.5: Primer sequences. *Nde*I and *Spe*I restriction sites are underlined.

Primers	Sequences	Length
Cholesterol Oxidase F Primer	5' <u>GGAATTCCATATGGAATTC</u> CGTGCCCCAGGATGCAT 3' <i>Nde</i> I Restriction Site	36 bp
Cholesterol Oxidase R Primer	5' <u>GACTAGTC</u> TCTACTTCTTCGGCGGCACCGT 3' <i>Spe</i> I Restriction Site	29 bp
<i>elmMII</i> F primer	5' TTCCTCGTCCATGACCTCAC 3'	20 bp
<i>elmMII</i> R primer	5' TCATACGTAGTCGATCTC 3'	18 bp
<i>elmD</i> F primer	5' CTCATCGAAGACCCTTAC 3'	18 bp
<i>elmD</i> R Primer	5' GGCTCGGAGTTCACATCT 3'	19 bp

5.1.9. Antibiotics

Antibiotics used throughout the study are given in Table 5.6.

Table 5.6: Antibiotics and their concentrations for the selection of microorganisms.

ANTIBIOTIC	Stock Concentration (mg/ml)	Final Concentration for <i>Streptomyces</i> (µg/ml)	Final Concentration for <i>E. coli</i> (µg/ml)
Apramycin (in dH ₂ O)	50	50	50
Kanamycin (in dH ₂ O)	50	50	50
Chloramphenicol (in dH ₂ O)	25	-	25
Erythromycin (in 96% Ethanol)	50	50	50
Nalidixic Acid (in 0.15 M NaOH)	25	-	25

5.1.10. Devices

Centrifuges (Heraeus, Labofuge 400R and Beckman Coulter, Microfuge 22R), horizontal electrophoresis system (BIO_RAD, Mini-Sub® Cell GT Cell), power supply (BIO-RAD, PowerPac™), refrigerator +4 °C (Ariston), freezer -20 °C (Ariston), freezer -80 °C (Thermo, Forma -86 °C ULT Freezer), ice-maker (BAR-LINE BF85), autoclave (Hirayama, Hiclave HVE-50), pH-meter (Az Instruments, 86505), water-bath (Weightlab Instruments, WF-6SB22), sterile cabin (Tez-San), oven (Binder), incubator (Wisecube), shaking incubator (Edmund Bühler, KS-15), microwave oven (Kumtel), PCR thermal cycler (Applied Biosystems), UV imaging system (Azure Biosystems, c600), UV spectrophotometer (SHIMADZU, UV-1800), HPLC (SHIMADZU) with an Inertsil ODS-3V (4.6x250 mm, 5 µm, GL Sciences) column, confocal microscopy (ZEISS, LSM 880, Airyscan).

5.2. Methods

5.2.1. Plasmid Isolation from *E. coli*

pSPG was isolated according to Sambrook et al (1989). 100 ml overnight *E. coli* + pSPG culture was divided into two 50 ml and centrifugated at 4000 rpm for 15 minutes. 800 µl Solution I was added onto the pellet, vortexed until pellet dissolved, then incubated at RT for 5 min. The mix was partitioned into Eppendorf tubes as 200 µl aliquots. 400 µl Solution II (prepared freshly) was added and incubated on ice for 5 min. After that, 300 µl Solution III was added, mixed gently and incubated on ice for 10 min. After centrifugation for 20 min, 0.6 volume isopropanol was added to the supernatant, incubated at RT for about an hour. The mix was centrifuged for 30 min. The pellet was dissolved in 25 µl dH₂O and each 25 µl was united to reach 100 µl final volume. 10 µl RNase (10 mg/ml) was added onto 100 µl mix. The mix was incubated at 37° C for 30 min. After that, the MN PCR and Gel Purification Clean-Up Kit instructions were followed. The plasmid was collected from the colon with 50 µl dH₂O and stored at -20° C.

5.2.2. Primer Design

For cloning cholesterol oxidase gene (NO: SCO4781) of *S. coelicolor* A3(2) genome, primers were designed with consideration of criteria as the length, G-C ratio,

the melting temperature (T_m), dimer forming possibility, etc. *NdeI* and *SpeI* restriction enzyme sites suitable for the pSPG plasmid were added to the forward and reverse primers, respectively. Other pairs of primers were designed for the *elmMII* and *elmD* genes which are located in the cos16F4. So that the insertion of the cosmid into the *S. coelicolor* genome could be confirmed via PCR.

5.2.3. Amplifying the Gene of Interests via PCR

The suitable primer pair shown in Table 5.5 were used to amplify the cholesterol oxidase gene (NO: SCO4781) of the *S. coelicolor* A3(2) genome. The PCR reaction components and conditions are listed in Table 5.7.

To confirm the integration of cos16F4 to chromosomal DNA of *Streptomyces*, two pairs of primers were designed; *elmD* and *elmMII*. However, neither of the primers could amplify their target genes with genomic DNA as a template although they worked with cos16F4 itself. *Streptomyces* has a large genome with a high G-C ratio, that's why PCR efficiency is low. To overcome this difficulty, we fragmented the genome with a restriction endonuclease which does not cut our target *elmMII* gene. *EcoRI* restriction endonuclease was selected for fragmentation. Firstly, the whole genome was digested with *EcoRI* according to the manufacturer's recommendation. The possible fragments (~19 kb) that contained *elmMII* gene were extracted from agarose gel via MN Gel and PCR Clean-Up kit. Then, these purified fragments were used as a template for the confirmation PCR. So that, the *elmMII* gene was amplified from recombinants' chromosomal DNA, thereby confirmed the cos16F4 insertion.

Table 5.7: PCR reaction components and conditions.

PCR Components	Cycle Conditions
1x final conc. of Q5 5x Reaction Buffer 1x final conc. of Q5 5x High G-C Enhancer 0.2 mM final conc. of dNTP 0.6 µM final conc. of F CO Primer 0.6 µM final conc. of R CO Primer 2.5 ng <i>S. coelicolor</i> A3(2) genome 0.25 µl Q5 Polymerase Add dH ₂ O to 25 µl reaction volume	Initial Denaturation: 98° C/5' Denaturation: 98° C/30" Annealing: 70° C/30" Extension: 72° C/60" Final Extension: 72° C/3' Final: +4° C/∞ 35 Cycle
1x final conc. of Q5 5x Reaction Buffer 1x final conc. of Q5 5x High G-C Enhancer 0.2 mM final conc. of dNTP 0.6 µM final conc. of F <i>elmMII</i> Primer 0.6 µM final conc. of R <i>elmMII</i> Primer 2.5 ng Recombinant <i>S. coelicolor</i> genome 0.25 µl Q5 Polymerase Add dH ₂ O to 25 µl reaction volume	Initial Denaturation: 98° C / 30" Denaturation: 98° C / 10" Annealing: 67.5° C/30" Extension: 72° C /45" Final Extension: 72° C/2' Final: +4° C/∞ 35 Cycle

5.2.4. Agarose Gel Electrophoresis

Agarose gel electrophoresis was performed to differentiate nucleic acids by size. For the preparation of 0.8% (w/v) agarose gel, 0.4 g agarose was added into 50 ml 1x TAE and dissolved in the microwave. After cooling 2.5 µl RedSafe was added into the gel and it was poured into the tray with a well comb. After solidification, samples with 6x Loading Dye (final concentration of loading dye: 1x) were loaded into wells and run under 90V current for 40-45 minutes. Bands were visualized under UV.

5.2.5. Gel Clean-Up Kit

PCR products and restriction enzyme reactions were purified from agarose gel via MN Gel and PCR Clean-Up kit. The protocol provided by the manufacturer was followed.

5.2.6. Restriction Enzyme Digestion

The purified gene of interests, plasmid and cosmids were digested with proper restriction enzymes for cloning and confirmation steps. The reaction conditions such as buffers, concentrations, and durations were applied as the manufacturer's recommendation.

5.2.7. Ligation

The ligation protocol of Sambrook et al (1989) was followed [44]. The concentrations of vector and insert were calculated based on the equation (5.1).

$$insert\ (ng) = \frac{vector\ (ng) \times insert\ (kb)}{vector\ (kb)} \quad (5.1)$$

5.2.8. Preparation of *E. coli* Competent Cells

E. coli cells were inoculated into 10 ml LB broth, incubated at 37 °C, at 180 rpm. 1% of the pre-culture was inoculated into fresh 50 ml LB and incubated at 37 °C, at 180 rpm. When the OD₆₀₀ value of the culture reached 0.4, cells were centrifuged at +4 °C, 4500 rpm for 10 min. The pellet was dissolved in 40 ml TfbI solution and incubated on ice for 10 min, then centrifuged at +4 °C, 4500 rpm for 8 min. The pellet was dissolved in 1 ml TfbII. 50 µl cell mix was aliquoted into Eppendorf tubes, frozen in liquid nitrogen and stored at -80° C.

5.2.9. MiniPrep Plasmid Isolation

To screen the transformant colonies, the MiniPrep plasmid isolation of Holmes et al (1981) was followed [45]. 1 ml *E. coli* cell culture was grown overnight. The culture was centrifuged at +4 °C, at 13000 rpm for 3 minutes. The supernatant was discarded and 250 µl STET + lysozyme (final concentration: 1 mg/ml) mix was added to the pellet and vortexed until it was dissolved. The mix was incubated in boiling water for 15 min, then centrifuged at +4 °C, at 13000 rpm for 15 min. The white pellet was discarded with a sterile toothpick. 250 µl isopropanol was added to the supernatant and then incubated at room temperature for 15 minutes. After incubation, the mix was centrifuged at 13000 rpm for 15 min. Pellet was dissolved in 20 µl dH₂O.

5.2.10. Chemical Transformation of *E. coli* Cells

The chemical transformation protocol of Sambrook et al (1989) was followed to transform *E. coli* DH5 α and ET12567/pUZ8002 cells with ligation products. 3 μ l of ligation product was mixed with 50 μ l competent cells and incubated on ice for 25 minutes. Then the cells were heat-shocked at 42 °C for 45 seconds, then incubated on ice for 2 minutes. After incubation on ice, 600 μ l sterile LB was added onto the cells and incubated at 37 °C, at 180 rpm for an hour. Then 100 μ l of the cells were plated out on the selective LB agar plates, incubated at 37 °C overnight.

5.2.11. Isolation of Cosmids

Cosmids were isolated from *E. coli* DH5 α and ET12567/pUZ8002 cells according to Sambrook et al [44]. *E. coli* cells harbouring cosmid were inoculated into 100 ml LB containing apramycin and incubated overnight at 37 °C, at 180 rpm. The culture was centrifuged at 4500 rpm, +4 °C for 20 minutes. 5 ml Solution I (TEG) was added to the pellet and vortexed. Then, the lysozyme was added to the mixture (final concentration: 10 mg/ml). The mixture was kept on ice for 10 minutes. 10 ml Solution II was added slowly, kept on ice for 10 minutes and stirred gently every 2 minutes. 7.5 ml Solution III was added, kept on ice for 10 minutes and stirred gently every 2 minutes. The mixture was centrifuged at 4500 rpm, +4 °C for 30 minutes. The supernatant was taken to another falcon with the help of a sterile gauze bandage. 12 ml isopropanol was added onto supernatant and stirred, then kept at room temperature for 15 minutes. The mixture was centrifuged, and the pellet was dried and dissolved with 2 ml dH₂O. The mix was taken into a falcon, 2 ml of 5M LiCl was added and then incubated on ice for 15 minutes. After incubation, the mix was centrifuged at 4500 rpm for 15 minutes. The supernatant was taken into another falcon and 10 ml 100% ethanol was added onto. The mixture was incubated at -80 °C for 1 hour and then centrifuged at 4500 rpm, +4 °C for 15 minutes. The pellet was dried and dissolved with 200-400 μ l dH₂O. 10 μ l RNase (10 mg/ml) was added and incubated at 37 °C for 30 minutes. The mixture was taken into Eppendorf tubes. Then phenol/CIA extraction was performed as follows. Firstly, ½ volume phenol and ½ volume CIA were added, after mixing it was centrifuged at 13000 rpm for 5 minutes. The upper site was taken gently to a new Eppendorf. This step was repeated again, and later extraction was repeated with only 1 volume CIA. The upper phase was taken to a new Eppendorf,

mixed with 3 volume 100% ethanol and incubated at -80 °C for an hour. After that, the mixture was centrifuged at 13000 rpm, +4 °C for 30 minutes. As a final step, the pellet was dissolved in 25 µl dH₂O.

5.2.12. Transformation of ET12567/pUZ8002 cells via Electroporation

The electroporation method was used for the transformation of *E. coli* ET12567/pUZ8002 cells with cos16F4 cosmid. Before starting the process, the electroporator cuvettes and Eppendorf tubes containing 2 ml sterile LB broth were cooled at +4 °C. After that, 2 µl of cosmid was added onto 50 µl of electrocompetent ET12567/pUZ8002 cells on ice. Cell-cosmid mix was put into a sterile 0.2 cm electroporator cuvettes immediately. The conditions for electroporation were; 200 Ω, 2.5 µF, 2.5 kV. After the process, 700 µl sterile, cold LB was added into cuvette directly. All mixture was taken into a sterile Eppendorf tube after pipetting well and then incubated at 37 °C, at 180 rpm for 90 minutes. Subsequently, 100 µl of cells were plated on LB agar containing apramycin and incubated at 37 °C overnight. After incubation, large colonies were selected since the possibility to have more cosmid is higher with the larger colonies. 500 µl of the recombinant cell culture was mixed with 500 µl of sterile, cold 40% glycerol, and stored at -80 °C.

5.2.13. Harvesting *Streptomyces* Spores

Streptomyces WT and *Δppk* cells were inoculated into 50 ml TSB or R2YE+Apramycin broth respectively and then they were incubated at 30 °C for 48 hours. After incubation, culture was centrifuged at 3500 rpm for 20 minutes. The supernatant was discarded, and the pellet was dissolved in residual 5 ml supernatant. 300 µl of the mix was spread onto TBO containing antibiotics. Plates were incubated at 30 °C for 10 days for sporulation. 5 ml cold, sterile 40% glycerol was placed onto agar plates. Spores were scratched from the agar surface via sterile cotton swaps. The spore-glycerol solution was collected into a sterile falcon and then centrifuged at 4500 rpm, at +4 °C for 20 min. After that, the supernatant was discarded, and the pellet was dissolved in residual supernatant. Lastly, 200 µl spore suspension was aliquoted into Eppendorf tubes and stored at -80 °C.

Serial dilutions up to 10^{-8} were prepared to calculate the initial number of spores. 100 μ l ($V_{\text{inoculation}}$) of each dilution were spread onto TSA agar with antibiotics. Plates were incubated at 30 °C for 3-4 days. After incubation, grown colonies were counted and the initial number of spores was calculated based on the following equation (5.2):

$$\text{Initial number of spores (cfu/ml)} = \frac{\text{colony number} \times \text{dilution factor} \times V_{\text{total}}}{V_{\text{inoculation}}} \quad (5.2)$$

5.2.14. *Streptomyces* Conjugation

For transfer of cos16F4 cosmid from *E. coli* cells to *S. coelicolor* wild type and Δppk cells, the conjugation protocol of Gust et al (2002) was followed [46]. ET/pUZ/cos16F4 cells were inoculated into fresh LB broth containing apramycin, kanamycin, and chloramphenicol and grown overnight at 37 °C, at 180 rpm. After incubation, 1 ml of the overnight culture was inoculated into 50 ml fresh LB broth containing the same antibiotics above and then incubated at 37 °C, at 180 rpm until the OD₆₀₀ of the culture reached 0.4. The culture with 0.4 OD was washed with LB twice to get rid of any antibiotics. After discarding the supernatant, the pellet was resuspended in residual liquid (approximately 50 μ l). While washing the ET/pUZ/cos16F4 cells, *S. coelicolor* WT or Δppk spores (10^8) were added to 500 μ l 2xYT broth on ice, and heat-shocked at 50°C for 10 minutes then left to cool down to room temperature. 500 μ l heat-shocked *S. coelicolor* spores and 500 μ l ET/pUZ/cos16F4 suspension were mixed and centrifuged at 13000 rpm for 1 minute. After discarding the supernatant, the pellet was dissolved in the residual 50 μ l liquid. A serial dilution including 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} in a total 200 μ l volume was prepared. 100 μ l of each dilution of *S. coelicolor* WT or Δppk was plated on MS or R2YE agar, respectively. Plates were incubated at 30 °C for 20 hours. After incubation, plates were overlaid with desired antibiotics in 1 ml sterilised dH₂O and incubated at 30 °C.

Conjugants were selected on R2YE agar containing apramycin, erythromycin, and nalidixic acid. This step was repeated several times to avoid any *E. coli* contamination. The grown culture was checked under a light microscope to ensure that culture was not contaminated. After incubation at 30 °C, the streak plate method was repeated with one of these isolated colonies. One colony was selected and inoculated

into R2YE broth with apramycin and erythromycin, incubated at 30 °C, at 180 rpm for 3 days. After checking the purity of the culture under a light microscope, glycerol stock of the grown culture was prepared with cold 40 % glycerol.

5.2.15. Genomic DNA Isolation

100 ml fresh YEME broth plus antibiotics was inoculated with seed culture, incubated at 30 °C, at 180 rpm for 48 hours. Then the culture was divided into two sterile falcons (50 ml for each) and centrifuged at +4°C, at 4500 rpm for 20 minutes. After discarding the supernatant, the pellet was dissolved in 8.5 ml TE via vortex and incubated for 1-2 minutes in the presence of lysozyme (final concentration: 2 mg/ml). After the addition of 500 µl 20% SDS and 500 µl RNase (final concentration: 10 mg/ml), the mixture was incubated at 37 °C for an hour. Then 1500 µl 5M NaCl and 1200 µl CTAB/NaCl were added onto the mix, then incubated at 65 °C for 25 minutes. After that, ½ volume phenol and ½ volume CIA was added and centrifuged at +4 °C for 15 minutes. The upper phase was taken into a new falcon and 1 volume of CIA was added. The centrifugation step was repeated. 0.6 volume isopropanol was added onto the upper phase and centrifuged at 4°C, at 4500 rpm for 30 minutes. Pellet was dissolved with 2 ml TE buffer and divided into two Eppendorf tubes. Phenol/CIA treatment was performed twice. After phenol/CIA treatments, the upper phase was extracted with 1 volume of CIA only. 3 volume of 100% ethanol was added onto the upper phase and incubated at -80 °C for an hour. After centrifugation for 30 minutes, the supernatant was discarded, and the pellet was dried, dissolved in 25 µl dH₂O and stored at -20°C.

5.2.16. Detection and Characterisation of 8-DMTC

For the detection of 8-DMTC, High-Performance Liquid Chromatography (HPLC) analysis was performed with both wild type and recombinant cells of *S. coelicolor*. Wild type, Δppk mutant and their recombinants harbouring cos16F4 cosmid were cultured in 100 ml R2YE broth plus antibiotics at 30 °C, at 180 rpm for 48 hours. After 48 hours, 5 ml of these pre-cultures were used to inoculate 100 ml fresh R2YE broth plus antibiotics and incubated at 30 °C, at 180 rpm for 5 days. At the end of 5-day incubation, 15 ml of each culture was centrifuged, their supernatants were adjusted to pH 3.5 with formic acid and extracted with 5 ml ethyl acetate. C18

Inertsil ODS-3V (4.6x250 mm, 5 μ 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 minutes. UV detection of the peaks was performed at 386 nm.

5.2.17. Confocal Laser Scanning Microscopy Analysis (CLSM)

During 5-day incubation, the growth of the *Streptomyces* cultures was analysed at regular time intervals. To prepare the samples for microscopy, the LIVE/DEAD Bac-Light Bacterial Viability Kit (L-7012) was used. 1 ml of the culture was centrifuged at 13000 rpm for 5 minutes. After discarding the supernatant, cells were washed with 1 ml dH₂O twice to get rid of any medium leftover that might interact with nucleic acid stains. Meanwhile, a dye mix made of 1.5 μ l of each fluorescent nucleic acid stain (SYTO 9-green, propidium iodide (PI)-red) in 1 ml dH₂O 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 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 μ l of the dye mixture and incubated in the dark at least for 10 minutes. 20 μ l 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.

To follow the growth of solid cultures, the seed culture in R2YE broth containing proper antibiotics was incubated at 30 °C, at 200 rpm for 48 h. After incubation, cultures were centrifuged at 4500 rpm for 20 minutes. The pellet weights of WT, *Δ ppk* and their cos16F4 recombinants were equalized and dissolved in 5 ml of supernatant. 100 μ l of the cell mix was spread out on R2YE agar and incubated at 30 °C. For visualization of the colonies, a small agar cube was cut out and the top of the cube was covered with 200 μ l of the dye mix, incubated in the dark for 10 minutes at least. After incubation, a slim slice was vertically cut out from the cube using a razor blade and deposited on a clean glass slide, to be observed under the confocal microscope, at previously described conditions.

6. RESULTS

Pioneering studies of Chouayekh ve Virolle (2002) showed that the deletion of polyphosphate kinase causes an increase in actinorhodin production, an endogenous antibiotic in *S. lividans* TK24A [17]. A similar effect of *ppk* was also seen in *S. coelicolor*. Polyphosphate kinase deletion mutants of *S. coelicolor* A3(2) and M145 were shown to produce 8.5 and 2.6 times more actinorhodin than the wild type, respectively.

In this study, we aimed to investigate whether *S. coelicolor* Δppk mutants can also produce an endogenous primary metabolite more than wild type strain and to determine the potential of Δppk strains as a host for heterologous expression. First, a putative gene encoding cholesterol oxidase enzyme was cloned into a multiple copy vector (pSPG) and the recombinant vector was planned to be used to transform *S. coelicolor* Δppk and wild type cells. Later, the *elm* gene cluster, which is responsible for the production of an anti-tumor and anti-bacterial agent, 8-DMTC, was inserted into the genome of *S. coelicolor* A3(2) and M145 Δppk and wild type cells.

6.1. Effect of Polyphosphate Kinase Deficiency on Primary Metabolism of *S. coelicolor*

In order to determine the effect of PPK deficiency on primary metabolism, we have selected the putative cholesterol oxidase gene (SCO4781) of *S. coelicolor* because of its antitumor activity.

6.1.1. Amplifying the Cholesterol Oxidase Gene

The putative cholesterol oxidase gene was amplified from *S. coelicolor* A3(2) genome via PCR reaction (Figure 6.1). 1809 bp fragment was purified from agarose gel and sequenced (BM Labosis, Ankara). The data were analyzed via the bioinformatics program BLAST and the results confirmed that the obtained fragment was cholesterol oxidase gene. The result of BLAST analysis is given in Figure 6.2.

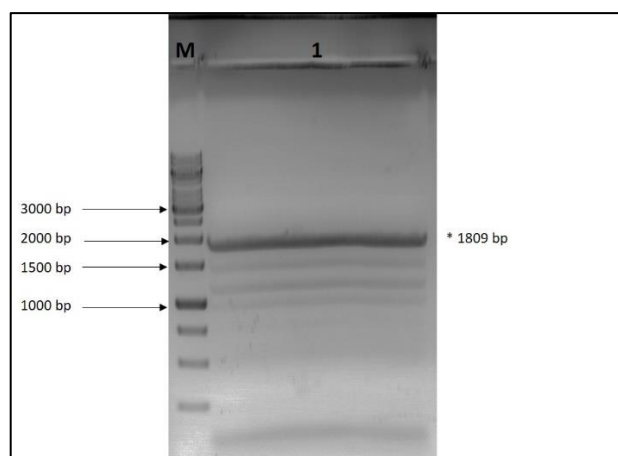


Figure 6.1: The possible cholesterol oxidase gene (*1809 bp) obtained by PCR reaction (1). M: GeneRuler 1 kb DNA Ladder.

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Streptomyces coelicolor A3(2) complete genome; segment 18/29
Sequence ID: [AL939121.1](#) Length: 292100 Number of Matches: 1

Range 1: 123281 to 123756 GenBank Graphics

Score	Expect	Identities	Gaps	Strand
852 bits(461)	0.0	471/476(99%)	0/476(0%)	Plus/Plus
Query 6	TACCAAGTCTCTCGTGGGATCCGGCTTCGGCGGCTCCGCTCTCCGCCCTCCGCCTCACC	65		
Sbjct 123281	TACGACGTCTCTCGTGGGATCCGGCTTCGGCGGCTCCGCTCTCCGCCCTCCGCCTCACC	123340		
Query 66	GAGAAGGGCTACCGCGTCGGCGTCTGGAGGCCGGCCGCTTTCACCCGGGAGTCGCTG	125		
Sbjct 123341	GAGAAGGGCTACCGCGTCGGCGTCTGGAGGCCGGCCGCTTTCACCCGGGAGTCGCTG	123400		
Query 126	CCCAAGAACTCCTGGGACCTGAAGAACTACCTCTGGGCGCCGAAGCTCGGCATGTTTCGGC	185		
Sbjct 123401	CCCAAGAACTCCTGGGACCTGAAGAACTACCTCTGGGCGCCGAAGCTCGGCATGTTTCGGC	123460		
Query 186	ATCCAGCGCATCCACCTGCTCGGGAATGTATGGTCTTGGCAGGCGCCGGGTCGGCGGC	245		
Sbjct 123461	ATCCAGCGCATCCACCTGCTCGGGAATGTATGGTCTTGGCAGGCGCCGGGTCGGCGGC	123520		
Query 246	GGCTCGCTCAACTACGCCAACACCTGTACGTGCCGCCAAGCCCTTCTTCAACGACCCC	305		
Sbjct 123521	GGCTCGCTCAACTACGCCAACACCTGTACGTGCCGCCAAGCCCTTCTTCAACGACCCC	123580		
Query 306	CAGTGGCGCGGCATCACCAGCTGGCACGAGGAATCAGCGCTACTACGACAGGCCCGG	365		
Sbjct 123581	CAGTGGCGCGGCATCACCAGCTGGCACGAGGAATCAGCGCTACTACGACAGGCCCGG	123640		
Query 366	CGCATGCTCGGGGTCGGGCTCAACCCGACGATGACCCCTTCAACGTCACCTGAAGGCC	425		
Sbjct 123641	CGCATGCTCGGGGTCGGGCTCAACCCGACGATGACCCCTTCAACGTCACCTGAAGGCC	123700		
Query 426	GCCGCGGATCGGATGGGCTGCGGCGACACCTTCACATGGCCCCGGTCGGGCTCTT	481		
Sbjct 123701	GCCGCGGATCGGATGGGCTGCGGCGACACCTTCACATGGCCCCGGTCGGGCTCTT	123756		

Figure 6.2: BLAST analysis of the possible cholesterol oxidase gene.

6.1.2. Cloning the Cholesterol Oxidase Gene into pSPG Plasmid

For expression of cholesterol oxidase gene in *Streptomyces* strains, high-copy number, *Streptomyces* shuttle vector pSPG (8149 bp) was used. The pSPG plasmid and the gene of interest were prepared via restriction endonuclease digestion. Figure 6.3 shows the cholesterol oxidase gene and pSPG plasmid digested with *NdeI* and *SpeI* restriction enzymes. More than one ligation reaction was set up with different vector: insert ratios.

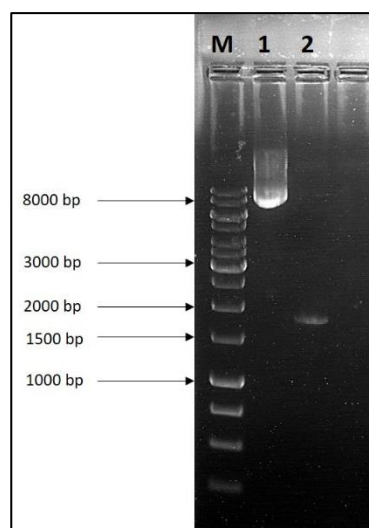


Figure 6.3: pSPG plasmid (1) and cholesterol oxidase gene (2) digested via *Nde*I and *Spe*I restriction enzymes. M: GeneRuler 1 kb DNA Ladder.

E. coli DH5 α cells were transformed with these ligation products. Then, DH5 α colonies were screened via the MiniPrep plasmid isolation technique. The recombinant plasmid obtained from DH5 α cells was confirmed via PCR reaction with cholesterol oxidase primers (Figure 6.4).

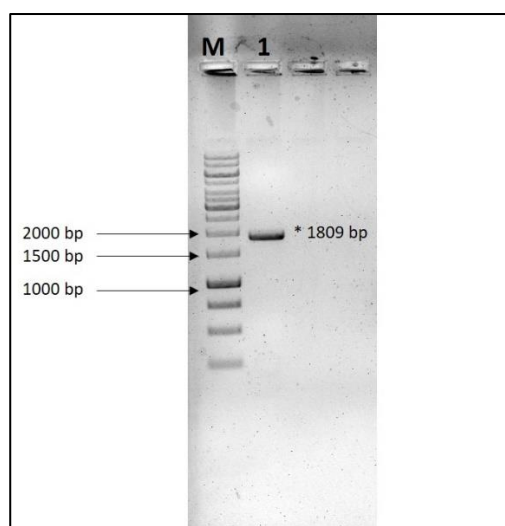


Figure 6.4: Confirmation of pSPG + cholesterol oxidase plasmid isolated from transformed *E. coli* DH5 α strain via PCR. M: GeneRuler 1 kb DNA Ladder.

6.2. Potential of Δ *appk* Strain as a Host for Heterologous Expression

8-DMTC is a tetracyclic polyketide produced by *Streptomyces olivaceus* Tü2353 [35]. It is an anti-tumor and anti-microbial agent [37]. 8-DMTC is synthesized by the

elm gene cluster found in the cos16F4 cosmid. cos16F4 is an integrative cosmid, carrying not only the *elm* cluster but also integrase and *attP* for integration into chromosomal DNA, *oriT* region for conjugal transfer from *E. coli*, erythromycin and apramycin resistance genes. cos16F4 was transferred into *S. coelicolor* wild type and Δ *ppk* strains to assess the potential of Δ *ppk* strain as a heterologous host for 8-DMTC production.

6.2.1. Transfer of cos16F4 into *Streptomyces* Strains

cos16F4 cosmid was a kind gift from Dr. Jose A Salas and Dr. Carmen Mendez (Universidad de Oviedo, Spain). We aimed to express *elm* genes in wild type and mutant *S. coelicolor* Δ *ppk* strains to compare their heterologous expression potentials. As a first step, cos16F4 was transferred into *E. coli* ET12567/pUZ8002 cells via electroporation protocol. Methylation deficient *E. coli* ET12567/pUZ8002 cells are able to transfer plasmids through conjugation thanks to their *tra* genes on the pUZ plasmid. After the transformation of *E. coli* ET12567/pUZ8002 cells with cos16F4, the possible transformants were screened via cosmid isolation technique and the presence of cosmid was confirmed via PCR. *elmMII* (1053 bp) gene on cos16F4 was amplified with *elmMII* primers and the results confirmed that transformants carry the cosmid (Figure 6.5).

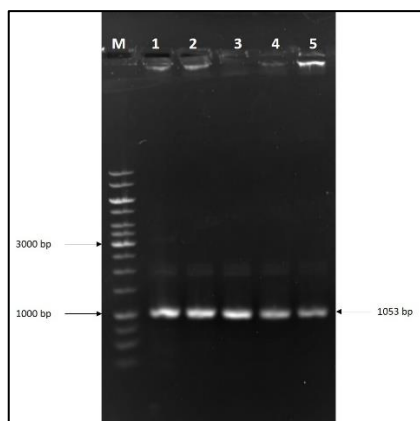


Figure 6.5: Confirmation of ET/pUZ/cos16F4 transformants with PCR. *elmMII* (1053 bp) gene on cos16F4 isolated from transformants was amplified.

cos16F4 was transferred into *S. coelicolor* A3(2) and M145 wild type and $\Delta appk$ mutant strains via conjugation with recombinant *E. coli* ET/pUZ cells. After conjugation, wild type cells were inoculated onto MS+MgCl₂ plates while $\Delta appk$ mutant cells were inoculated onto R2YE plates since they do not grow on MS agar. After 16 hours of incubation, plates were overlaid with selective antibiotics for the recombinants carrying cos16F4 and nalidixic acid for eliminating *E. coli* cells. cos16F4 carries apramycin, erythromycin and tetracycline resistance genes however we only used apramycin and erythromycin as selective agents. Because our experiments showed that tetracycline antibiotic can not inhibit wild type *S. coelicolor* growth on agar, so it was not efficient to use it for selection. cos16F4 is an integrative cosmid with its site-specific recombination *attP* site and integrase gene. So, the integration of cos16F4 to the *attB* site of *Streptomyces* chromosomal DNA was confirmed via PCR using *elmMII* primers. As an example, Figure 6.6 shows the result of PCR for *S. coelicolor* M145 $\Delta appk$ is given as an example.

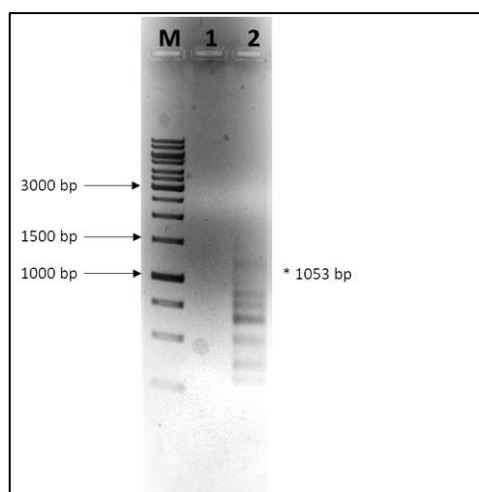


Figure 6.6: Confirmation of cos16F4 insertion into chromosomal DNA. PCR results of *S. coelicolor* M145 Δppk (1) and its cos16F4 recombinant (2); *elmMII* gene (*1053 bp) is observed in the recombinant sample. M: GeneRuler 1 kb DNA Ladder.

6.2.2. Detection of the 8-DMTC Compound via HPLC

8-DMTC compound can be detected via the HPLC method. The analysis was performed on the protocol of Salas et. al [47]. The samples taken from 5-day old R2YE cultures were analyzed with HPLC analysis. Figure 6.7 shows the chromatograms of *S. coelicolor* A3(2) wild type (A), wild type + cos16F4 (B), Δppk (C) and Δppk + cos16F4 (D) strains. The red arrow shows the possible peak of 8-DMTC with 4.8 min retention time. The retention time of the same compound may be variable based on different HPLC protocols and column properties [47], [48], [49]. Therefore, we determined the possible peak of 8-DMTC by comparison with wild type and recombinant strains and according to the previous study of Salas et al. The peak which was absent in the chromatograms of wild type and Δppk , but present in the chromatograms of the recombinants is likely the 8-DMTC. Figure 6.8 shows comparisons of WT and WT + cos16F4 (A), Δppk and Δppk + cos16F4 (B), WT + cos16F4 and Δppk + cos16F4 chromatograms. The red arrow indicates the 8-DMTC with 4.8 min retention time as expected. As we can see in Figure 6.8 C, Δppk + cos16F4 and wild type + cos16F4 produce nearly the same amount of 8-DMTC. The slight increase in 8-DMTC production in Δppk + cos16F4 strain might be negligible.

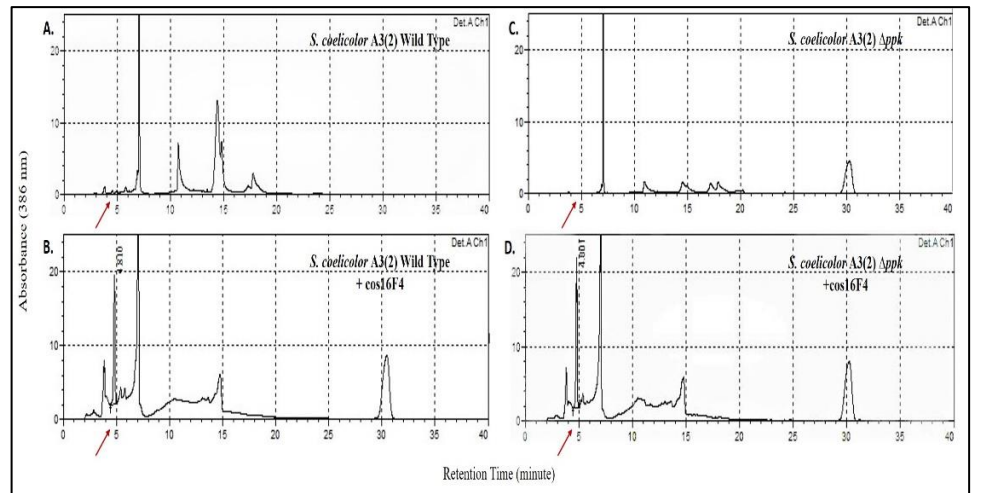


Figure 6.7: HPLC analysis of the biosynthesis of 8-DMTC. *S. coelicolor* A3(2) A: Wild Type B: Wild Type + cos16F4 C: Δppk D: Δppk + cos16F4.

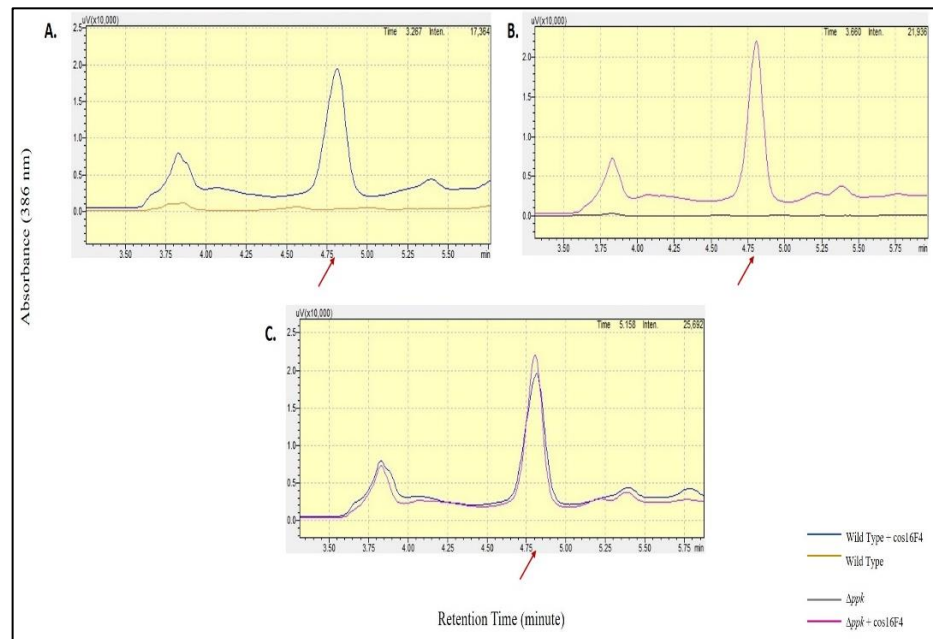


Figure 6.8: Comparison of *S. coelicolor* strains' chromatograms. A: Wild Type/Wild Type + cos16F4 B: $\Delta ppk/\Delta ppk$ + cos16F4 C: Wild Type + cos16F4/ Δppk + cos16F4.

6.3. Morphological Analysis of *Streptomyces* Recombinants Harboring cos16F4

Normally, wild type and Δppk strains produce blue-pigmented actinorhodin which makes the culture dark blue at the end of 120 h fermentation. While carrying out our experiments with cos16F4 recombinants of *S. coelicolor* A3(2) wild type and Δppk strains, we realized that none of the recombinants produce any actinorhodin in R2YE broth or on R2YE agar (Figure 6.9).

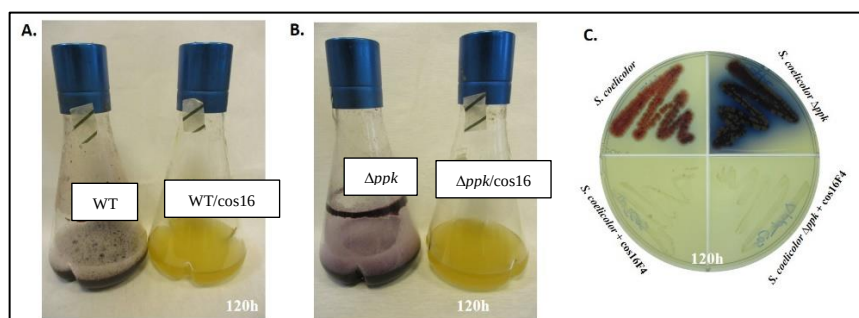


Figure 6.9: Macroscopic view of *S. coelicolor* A3(2) wild type and its cos16F4 recombinant (A), Δppk and its cos16F4 recombinant (B) strains in R2YE broth and on R2YE agar (C) after 120-hour incubation.

6.4. Analysis of *Streptomyces* Recombinants Harboring cos16F4 with Confocal Laser Scanning Microscopy

6.4.1. Confocal Analysis of *S. coelicolor* A3(2) Recombinants Grown in Liquid Medium

Since the macroscopic views of recombinant strains were different from the wild type profile, the colony formation was also investigated under the confocal microscope. For CSLM analysis, samples were stained with the LIVE/DEAD Bac-Light Bacterial Viability Kit. The kit includes the non-permeable nucleic acid stain PI (red) which labels only dead cells containing a damaged membrane and the permeable SYTO 9 (green) fluorescent nucleic acid dye that labels live cells containing an intact membrane. Figure 6.10. A and C shows the wild type colonies of A3(2) and M145, respectively, during 120 h incubation in R2YE broth. Wild type cultures show the properties of a classical *Streptomyces* life cycle. 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 noncompartmentalized hyphae spreading out of the colony center [8]. 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 (green stained with SYTO9) 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 till 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-hour life cycle, actinorhodin production correlates with secondary mycelia differentiation as expected since secondary metabolism is related to the developmental stages of *Streptomyces* [49]. On the other hand, the confocal images of cos16F4 containing A3(2) and M145 recombinants show a completely different colony morphology and physiology as can be seen in Figure 6.10. B and D, respectively. The colony structure is totally disrupted in recombinant cultures. All mycelia are disentangled and fragmented hence causes 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 don't show any specific properties. The morphology of the recombinant culture does not correlate with the life cycle or the incubation time. Dead and live fragments are randomly present in the cultures, independent from incubation time.

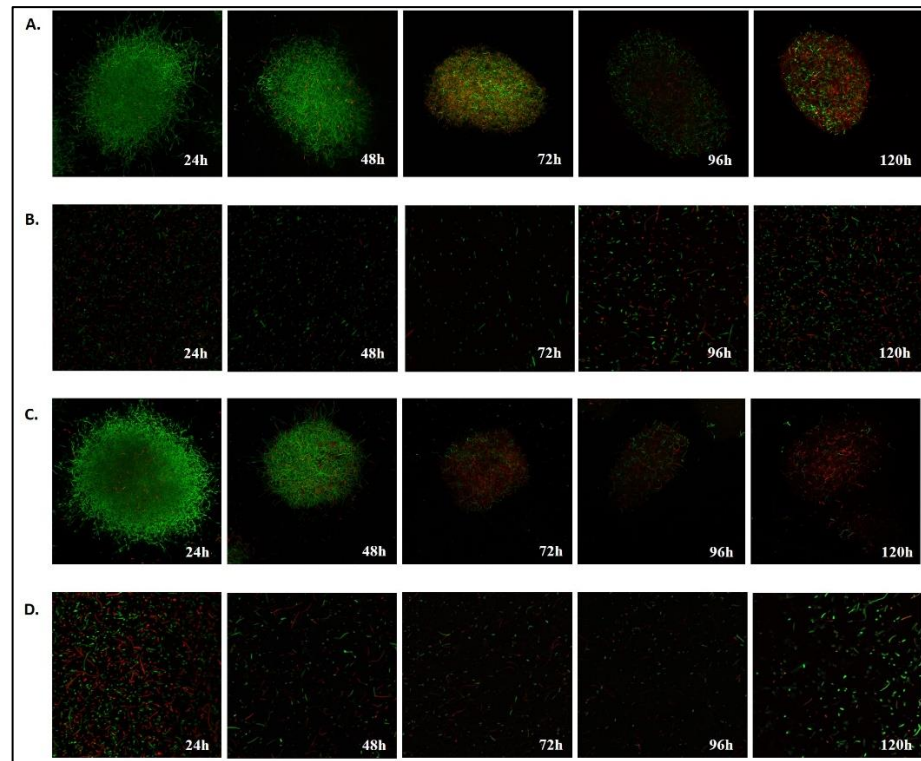


Figure 6.10: Confocal images of *S. coelicolor* A3(2) wild type (A), A3(2) + cos16F4 (B) and M145 wild type (C), M145 + cos16F4 (D) strains during 120-hour incubation.

Figure 6.11. A and C show the *S. coelicolor* A3(2) and M145 Δppk strain's morphology in R2YE broth during 120 h incubation. The general morphology of Δppk colonies displays the classical life cycle characteristics with an intact colony formation. 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 Δppk strain is the spread of mycelia around the Δppk colonies. When we check the cos16F4 recombinants of the A3(2) (Figure 6.11. B) and M145 (Figure 6.11. D) Δppk strains, the morphology is the same as the cos16F4 recombinant of the wild type strains (Figure 6.10. B, D). They are deficient in colony formation and mycelia continuity. Dismantled mycelia parts consist of dead and alive cells without any correlation with incubation intervals.

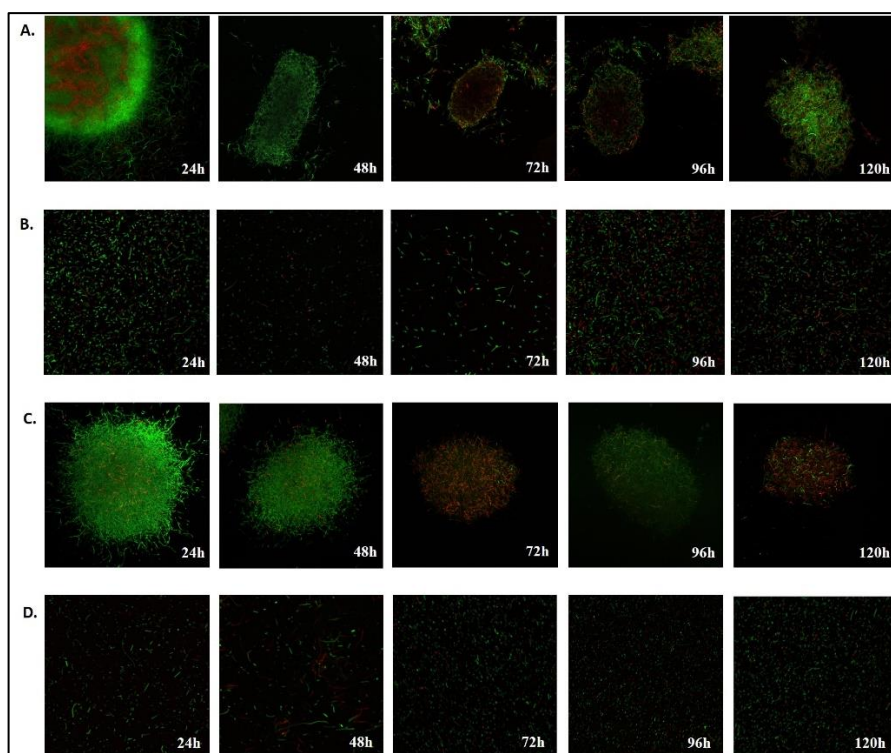


Figure 6.11: Confocal images of *S. coelicolor* A3(2) Δ ppk (A), its cos16F4 recombinant (B) and M145 Δ ppk (C), its cos16F4 recombinant (D) strains during 120-hour incubation.

6.4.2. Confocal Analysis of *S. coelicolor* A3(2) Recombinants in Liquid Culture during the first 24 h of Incubation

Thus far, our findings showed that cos16F4 recombinants display atypical morphology with the fragmentation of mycelia and lack of pellet formation during incubation from 24 h to 120 h. However, we did not examine the cultures with CLSM within the first 24 h. So, our findings do not explain yet 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 6.12. A shows the A3(2) 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 h. Dead cell rate (red-stained with PI) 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 consist 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 secondary mycelia like described above for A3(2) 120 h incubation samples. On the other hand, the confocal images of the recombinant samples do not show any sign of colony formation (Figure 6.12. B). The morphology of the recombinant culture is quite similar to the 120 h incubation samples. It means that the hypothesis that colonies were formed within the first 24 h and then they were disrupted, was wrong. Since we haven't observed any colony formation even at 6 h, it is safe to say that the cos16F4 recombinants lack pellet and clump due to their incapability to form colonies from the beginning of growth.

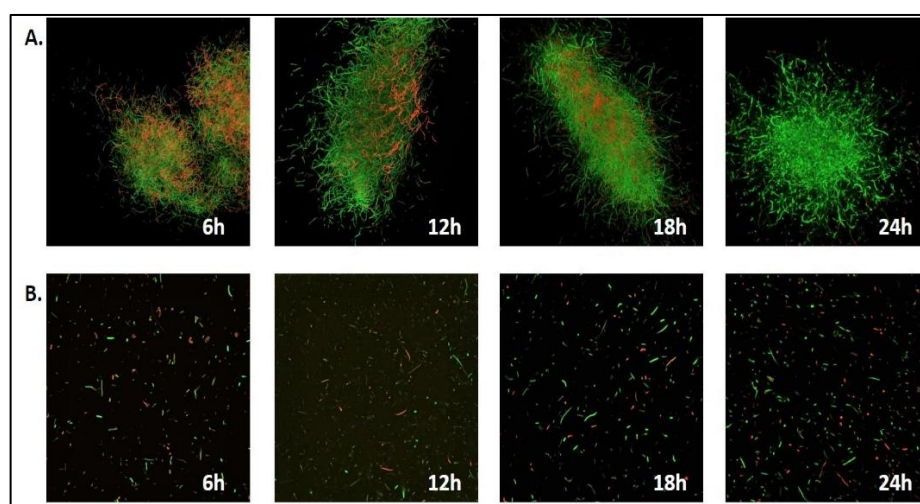


Figure 6.12: Confocal analysis of *S. coelicolor* A3(2) wild type (A) and A3(2) + cos16F4 (B) strains during the 24-hour incubation

6.4.3. Confocal Analysis of *S. coelicolor* A3(2) Recombinants Grown on Solid Medium

For the next step, we investigated the morphology of the *S. coelicolor* wild type cos16F4 recombinants grown on solid medium (R2YE) at different times during 24 h incubation. R2YE is a rich media so that the developmental cycle of *Streptomyces* occurs rapidly on R2YE agar. The first round of the PCD process and the transition from substrate hyphae to aerial hyphae can be observed within the first 24 h. WT and Δppk colonies on R2YE agar during 24 h incubation are shown in Figure 6.13. A and C, respectively. At 12 h, the dominant stage of WT colonies is substrate mycelia

growing and branching throughout the agar. The transition from the substrate to aerial mycelia has just occurred since we can see the young and few aerial hyphae rise up from the agar by breaking the surface tension thanks to their hydrophobic protein layer. For Δppk surface culture at 12 h, the substrate mycelia are denser than WT colonies. Similarly, the Δppk colonies have more aerial hyphae grown on the surface than the WT colonies. Most of the mycelia of both WT and Δppk colonies are alive (stained green by SYTO9) at 12 h. At 18 h, the traces of the first round of the PCD process are visible at the central zone of both WT and Δppk colonies. After the PCD round, live segments turn into MII mycelia. With this morphological and chemical transformation, aerial hyphae have become denser for both cultures. Moreover, the agarase activity of *S. coelicolor* is clearly visible for both cultures at 18 h. The colonies are located themselves inside the agar by forming a cavity via their agarase activity. Finally, the 24 h images were taken from the smear part of the surface culture instead of colonies themselves so the focus of the images is not the substrate mycelia but aerial mycelia. The aerial hyphae of both WT and Δppk grow longer at the end of 24 h incubation. The difference between the WT and Δppk cultures is the growth rate of the colonies. As it is seen in each image, Δppk colonies grow and get denser more quickly than WT ones. Moreover, since more aerial mycelia appear in Δppk colonies than WT ones at the same hour, it is safe to assume that the differentiation process of Δppk culture is performed faster than the WT culture. We already know that Δppk cells produce actinorhodin 8.5 times more than WT cells. So it is logical that the higher pace of growth and differentiation correlates with higher production of actinorhodin in Δppk culture.

Figure 6.13. B and D show the confocal images of 8-DMTC producing recombinants of WT and Δppk strains, respectively. When we compare the colonies of recombinants and the WT, Δppk strains, there is no similarity. The recombinants do not form colonies as WT strains do. There are no substrate or aerial mycelia zones, the hyphae fragments disperse randomly. The rate of alive and dead segments do not correlate with incubation time. Furthermore, the 8-DMTC producing recombinant strains do not show an agarase activity since there is no cavity where the cells located.

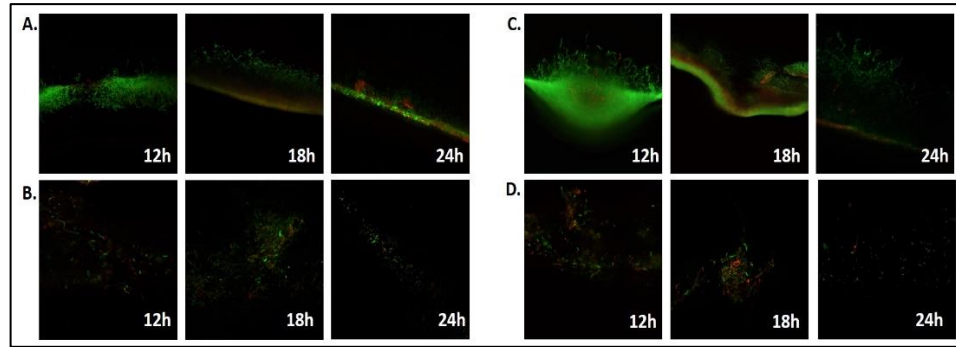


Figure 6.13: Confocal analysis of *S. coelicolor* A3(2) wild type (A), Δppk (B) and their cos16F4 recombinants (C, D) incubated on R2YE agar for 24h.

According to all these findings, we can conclude that the recombinant strains exhibit completely different morphology than *S. coelicolor* WT and Δppk . 8-DMTC producing recombinant strains do not form colonies, do not exhibit the cell cycle steps, do not produce actinorhodin and do not have agarase activity. They have fragmented mycelia during the all growth cycle. Furthermore, the inoculation of the recombinant cells on the TBO (sporulation) agar shows that these strains can not sporulate at all. The strain difference A3(2), M145 wild type or Δppk , incubation time, media type do not change the effect of the presence of the cos16F4 in the chromosome of the cells.

6.5. Construction of cos16F4/ 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, a recombinant strain of *S. coelicolor* without *elm* cluster was constructed. To delete the *elm* cluster from cos16F4, the *Bam*HI restriction enzyme was used since *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 cos16F4 with the *Bam*HI restriction enzyme, the *elm* cluster was digested at many sites and cut out from cos16F4 while the rest of the cosmid (22 kb) was linearized (Figure 6.14. A). The 22 kb band was obtained from agarose gel and then self-ligated. *E. coli* DH5 α cells were transformed with these ligation products. The possible transformants were confirmed with cosmid isolation, and then *Bam*HI digestion (Figure 6.14. B). After confirmation, *E. coli* ET12567/pUZ8002 cells were transformed with cos16F4/ Δelm . Then again, the ET/pUZ/cos16F4/ Δelm transformant was confirmed with *Bam*HI digestion (Figure

6.15). Finally, *cos16F4/Δelm* was transferred to *S. coelicolor* A3(2) cells via conjugation with ET/pUZ/ *cos16F4/Δelm*.

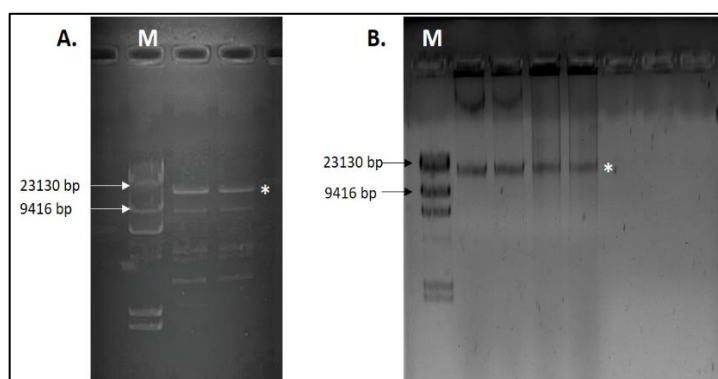


Figure 6.14: Confirmation of DH5 α /*cos16F4/Δelm* transformants. A: *Bam*HI digestion of *cos16F4* B: *cos16F4/Δelm* (*:~22 kb) isolated from DH5 α transformants. M: Lambda DNA/*Hind*III Marker

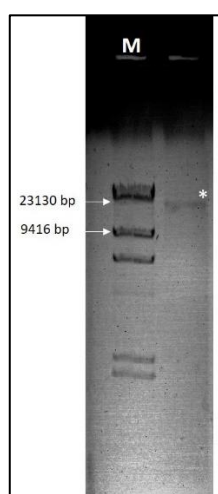


Figure 6.15: Confirmation of ET/pUZ/*cos16F4/Δelm* transformants with *Bam*HI digestion. *: *cos16F4/Δelm* (~22 kb) *cos16F4* (M: Lambda DNA/*Hind*III Marker)

6.5.1. Confocal Analysis of *S. coelicolor* A3(2) *cos16F4/Δelm* Recombinants

S. coelicolor A3(2) wild type and A3(2) + *cos16F4/Δelm* cells were analyzed under a confocal microscope to examine the effect of *elm* deletion on the colony morphology of recombinants. For analysis, 24th 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 Figure 6.16. A *cos16F4/Δelm*

culture is colored indicating that it produced pigmented antibiotics as wild type did. *cos16F4/Δelm* colonies exhibited the same morphological characteristics with wild type colonies. The first round of programmed cell death has already occurred since we can see dead central part and alive secondary mycelia (Figure 6.16. B). More importantly, *cos16F4/Δelm* culture forms pellets, clumps and wild type-like colony formation. Its mycelia are not fragmented at all as we can see in Figure 6.16. C, the area between the separate colonies are clean. These findings prove that the fragmentation of mycelia and lack of colony formation of recombinant cultures were caused by the expression of the *elm* cluster in *cos16F4*, not the insertion of the cosmid into the genome.

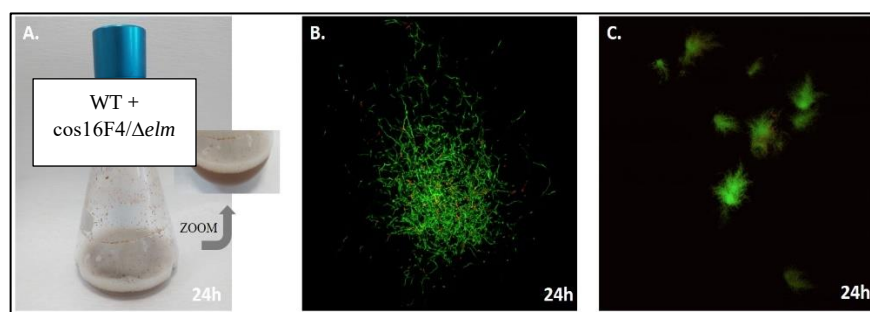


Figure 6.16: The morphological analysis of *S. coelicolor* wild type + *cos16F4/Δelm* strain. A: The macroscopic view (A), confocal (B) and tile scan (C) images of *S. coelicolor* wild type + *cos16F4/Δelm* in R2YE broth at 24 h.

6.6. Treatment of Wild Type *S. coelicolor* A3(2) Strain with 8-DMTC Containing Supernatant

The antimicrobial effect of 8-DMTC against Gr⁺ bacteria was shown by other groups, and it is known that 8-DMTC intercalates with DNA, however, its activation mechanism and how it affects the cell have not been enlightened completely [35], [36], [37]. 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, the supernatant of the *S. coelicolor* A3(2) WT/*cos16F4* recombinant culture which includes 8-DMTC, was applied to the *S. coelicolor* A3(2) WT culture at the beginning of fermentation to see whether the extracellular application of 8-DMTC compound will affect colony formation and cause fragmentation in the cultures. After

24-hour incubation, samples were checked under the confocal microscope. 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 effect of filtered or non-filtered supernatants.

Firstly, the color of the cultures in the flask was checked for the actinorhodin production (Figure 6.17). The control wild type sample was dark blue as a sign of high actinorhodin production. However, 8-DMTC treated samples were slightly pink which means the 8-DMTC compound decreased the production levels of actinorhodin. Confocal images of 8-DMTC treated cells were totally different than the wild type (Figure 6.17).

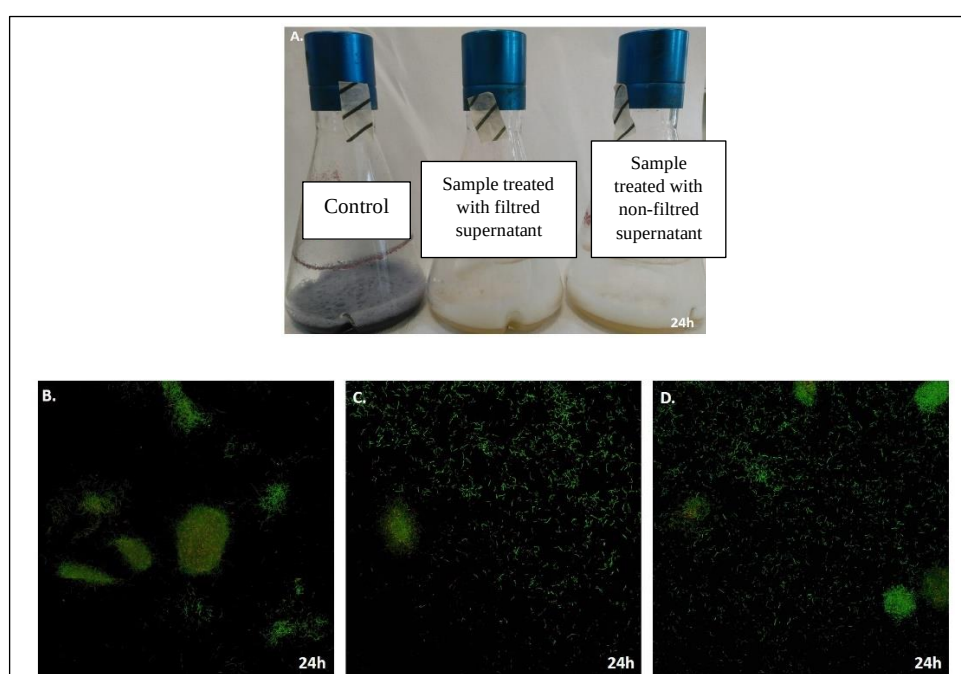


Figure 6.17: Effect of supernatant that contains 8-DMTC on *S. coelicolor* A3(2) morphological and physiological differentiation in R2YE broth. Macroscopic view of actinorhodin (blue color) production of control and 8-DMTC treated culture at 24-hour (A). CLSM analysis of *S. coelicolor* A3(2) (B), treated with filtered supernatant containing 8-DMTC (C) and treated with non-filtered supernatant containing 8-DMTC (D)

The detailed confocal analysis showed that the wild type sample grows well and forms many colonies. The mycelia of these colonies are secondary mycelia 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 (Figure 6.18. A). On the other hand, the samples treated with supernatant with 8-DMTC exhibits a

different profile. There are fewer colonies in the sample and the mycelia of these colonies are primary mycelia at 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 (Figure 6.18. B, C) That is atypical for *Streptomyces* growth because normally primary mycelia are detectable only in the first 10 h [7]. 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 space between colonies, there are many disentangled and fragmented mycelia parts unlike the wild type sample (Figure 6.18. B, C). 8-DMTC treatment dismantles the mycelia and disrupts the colony structure and thus pellet formation.

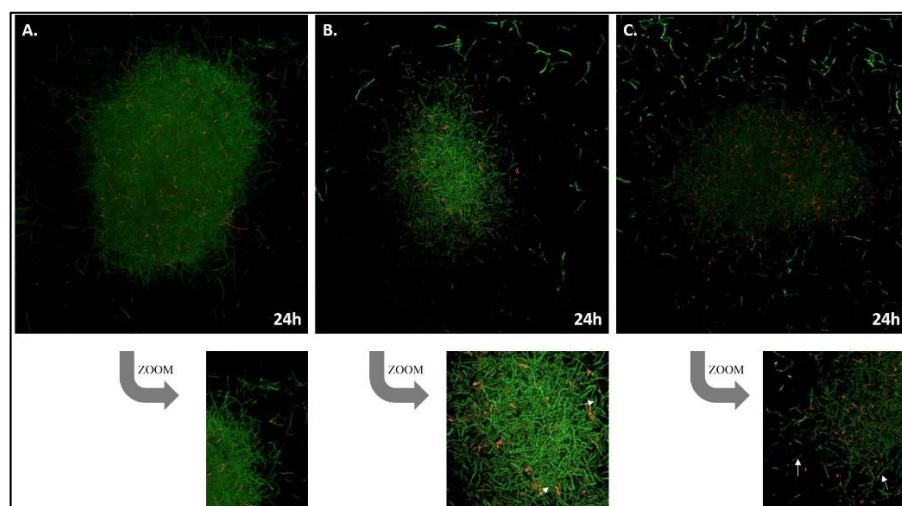


Figure 6.18: Confocal images of single colonies of *S. coelicolor* A3(2) samples after a 24-hour incubation in the presence of 8-DMTC containing supernatant. A: wild type B: treated with filtered supernatant containing 8-DMTC (at 0-hour) wild type culture C: treated with non-filtered supernatant containing 8-DMTC (at 0-hour) wild type culture. White arrows indicate the compartments of primary mycelia.

7. DISCUSSION

Polyphosphate kinase (PPK) is an enzyme catalyzing the biosynthesis of polyphosphate polymer which plays important roles in every living cell. The deletion of PPK in *S. lividans* has caused an increase in the production of an endogenous antibiotic, actinorhodin [17]. A similar effect of PPK was also seen in *S. coelicolor*. A previous study in our laboratory has shown that the deletion of the *ppk* gene in *S. coelicolor* A3(2) and M145 increased the actinorhodin production 8.5 and 2.6 fold, respectively. Each study has proven the inducing effect of *ppk* deletion on secondary metabolism.

In the first part of this thesis, we aimed to investigate the effect of *ppk* deletion on the primary metabolism of *S. coelicolor*. For that purpose, the cholesterol oxidase gene belonging to primary metabolism was selected. Cholesterol oxidase is responsible for the degradation of cholesterol which is used as the carbon source for the cell [32]. This activity also provides anti-cancer properties to cholesterol oxidase. It can inhibit the growth of breast and urothelial tumors by degrading the cholesterol component of the tumor cells' membranes [33], [34]. Hence, producing cholesterol oxidase at high levels would contribute to the medical field. The cloning steps of cholesterol oxidase gene from *Streptomyces* genome to pSPG, high-copy number shuttle vector, and then transforming *E. coli* DH5 α cells with the recombinant pSPG were accomplished. However, for detecting the expression and activity of cholesterol oxidase in *Streptomyces* cell, the media must contain cholesterol as the carbon source [50]. Unfortunately, the price of the cholesterol which was available commercially was too high to purchase with the budget of the BAP therefore, we couldn't continue the cholesterol oxidase experiments in *Streptomyces*.

The aim of the second part of the thesis was to investigate whether *S. coelicolor* Δppk mutant strain could be a high-producer host for heterologous expression. The heterologous expression and heterologous hosts are highly important to detect and produce biosynthetic gene clusters that are cryptic or poorly expressed in their natural hosts. To test the potential of *S. coelicolor* Δppk mutant strain as a heterologous host, we have selected an anti-tumor and anti-bacterial agent from *S. olivaceus* strain Tü 2353; 8-DMTC which is encoded by *elm* cluster carried on an integrative cosmid cos16F4. 8-DMTC has known to inhibit the proliferation of murine L1210 leukemia cells and also inactivate the Gr⁺ bacteria by intercalating with their chromosomal DNA

although the details of the action mechanism of the antibacterial activity have not been enlightened yet [35], [36], [37].

The *S. coelicolor* A3(2) wild type and Δppk cells were transformed with cos16F4 which carries 8-DMTC encoding *elm* cluster. 8-DMTC produced by recombinant strains was detected by using the HPLC [47]. The 8-DMTC peak with 4.8 min retention time was present in the chromatograms of recombinant of both *S. coelicolor* A3(2) wild type and Δppk strain. On the other hand, wild type and Δppk strains didn't have the 8-DMTC peak in their chromatograms. We determined the possible retention time of 8-DMTC based on the absence and presence of the peak in the chromatograms of wild type, Δppk , and their recombinants, and based on previous studies [47]. However, for the future works, it could be further proved via the semi-preparative HPLC method [51]. This method allows us to collect the fractions of determined retention times, so that these fractions can be analyzed and identified via Liquid Chromatography-Mass Spectrometer (LC-MS), Nuclear Magnetic Resonance (NMR) or Fourier-Transform Infrared (FTIR) spectrometry.

There was a small difference between the production levels of 8-DMTC of wild type + cos16F4 and Δppk + cos16F4. Δppk recombinant strain produced slightly more 8-DMTC than wild type recombinant strain. However, to decide whether this difference is significant, we need a standard curve based on the chromatogram of pure 8-DMTC so that the area under the peaks of recombinants could be calculated and compared based on this curve. Only then, the difference between the 8-DMTC production levels can be assessed as statistically significant or not.

During the fermentation process of wild type, Δppk and their cos16F4 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 cos16F4 recombinants did not produce any colored antibiotics.

The cos16F4 recombinants of *S. coelicolor* A3(2), M145 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) and M145 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 72-96 h, alive segments were growing again.

Then most of the segments died up to 120 h. The confocal images of the cos16F4 recombinants of A3(2) and M145 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 mycelia fragments were randomly spread around the culture. Furthermore, there was no difference between the morphology of the cos16F4 recombinants of A3(2) and M145 strains. These findings proved that the morphology of the recombinants was independent of Δppk mutation or the strain difference.

The antibiotic production is closely related to pellet and clump formation in *Streptomyces*. Previous studies showed that the pellet formation is essential for actinorhodin production in *S. coelicolor* [22], [10]. In our case, the cos16F4 recombinants do not produce actinorhodin and when their culture is checked via confocal analysis, there is no sign of colony formation. So, the reason for the lack of ability to produce actinorhodin might be the lack of pellet and clump formation of the cos16F4 recombinants. Another reason for the cease in antibiotic production could be the presence of 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 [52]. 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* in R2YE [53]. 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 [54]. 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), M145 wild type+cos16F4 and Δppk +cos16F4 strains. Further study is needed to prove this possibility.

Up to this point, the experiments we have carried out showed that the recombinant strains did not display a classical developmental cycle during 120 h incubation, did not have a colony structure. The colonies were disentangled and

mycelia were fragmented in the recombinants. As a next step, 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. This result indicated that the recombinant strain is not able to form colonies, pellets, and clumps.

The results given so far were belonging to the *Streptomyces* cultures grown in broth. However, *Streptomyces*' growth on solid media differs from broth cultures. During incubation in liquid media, the primary mycelia lifespan passes quickly in the first 6 hours, so aerial mycelia is the dominant stage for most of their life cycle [55]. On the other hand, solid cultures exhibit the typical life cycle steps. The primary mycelia inside the agar and the hydrophobic coated aerial mycelia on the surface of the agar could be observed at the same time. Moreover, *S. coelicolor* sporulates at the final stage of the developmental cycle on agar. Also, the agarase activity can be easily noticed on agar since the degradation of agar causes a decrease in viscosity of the solid media [56] and forms a cavity where the colonies located.

Confocal analysis of solid cultures has also demonstrated that cos16F4 recombinants displayed lack of pigmented antibiotics production, deficiency in colony morphology, fragmentation and disentanglement in mycelia independent from the duration of the incubation (24 h or 120 h), strain difference (A3(2) or M145) or deletion of *ppk*. Moreover, the recombinant strains did not show any agarase activity and did not sporulate on solid media.

cos16F4 is an integrative cosmid; the Φ C31 integrase enzyme is responsible for the integration of the cosmid into the *attB* site of the *S. coelicolor* genome [43]. 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 *elm* cluster from cos16F4, and then transformed *S. coelicolor* A3(2) wild type cells with this cos16F4/ Δelm construct. At first sight, *S. coelicolor* wild type + cos16F4/ Δelm cells produced pigmented-antibiotics after 24 h incubation in R2YE broth. Moreover, they showed agarase activity on R2YE agar and sporulate on TBO agar. To check their colony morphology, the confocal analysis was performed. *S. coelicolor* wild type + cos16F4/ Δelm 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 cos16F4/ Δelm recombinant from wild type was the delayed growth. Normally, wild type colonies have already passed the first PCD round by 24 hours, though there are few primary mycelia present in the colony, most of them have transformed to secondary mycelia. However, *S. coelicolor* wild type + cos16F4/ Δelm colonies contained more primary mycelia than secondary ones. That means the growth was slower than wild type colonies. Nonetheless, *S. coelicolor* wild type + cos16F4/ Δelm showed quite similarities with *S. coelicolor* wild type colonies. Thereby, we concluded that the deficiency in colony morphology and production of pigmented antibiotics were not caused by the insertion of the cosmid but the expression of *elm* cluster, thereby the production of 8-DMTC.

As noted earlier, 8-DMTC is an anti-tumor and antibacterial agent that is known to act against Gr⁺ bacteria by intercalating with their DNA [35], [36], [37]. Although, there were several studies about 8-DMTC characterization and purification, the antimicrobial effect of 8-DMTC was not studied deeply. In this study, we have searched whether the extracellular addition of 8-DMTC causes deformation of *Streptomyces* colonies as we observed in our cos16F4 recombinant strains. So that, *S. coelicolor* wild type colonies were treated with the supernatant of *S. coelicolor* wild type + cos16F4 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 the untreated ones. When the colony structure of 8-DMTC treated wild type culture was checked under confocal microscopy, there were fewer colonies than wild type culture had. 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 the 8-DMTC compound caused fragmentation and deficiency in the colony formation. Altogether these results indicate that the 8-DMTC compound itself decreases the growth rate, disrupts the pellet formation and causes fragmentation of mycelia. These results give a clue for the impact of 8-DMTC action in *S. coelicolor*; 8-DMTC causes fragmentation of mycelia, disentanglement of colonies, thereby causing a lack of pellet formation. However, since 8-DMTC recombinants contain a resistance gene in the *elm*

gene cluster, it does not explain why they suffer from the anti-microbial effect of 8-DMTC.

As a summary, the recombinant *S. coelicolor* wild type + cos16F4 and Δppk + cos16F4 strains produce 8-DMTC as detected by HPLC. The 8-DMTC production levels of Δppk + cos16F4 strain are slightly more than wild type + cos16F4, however, the absence of a standard makes it impossible to decide whether this difference is significant or not. Hence the potential of *S. coelicolor* Δppk as a heterologous host is still promising but needs further investigation.

On the other hand, the focus of the study showed a tendency to understand the morphological changes occurred during 8-DMTC production in the recombinant cells. The findings have determined following come ups. cos16F4 carrying *S. coelicolor* A3(2), M145 wild type and Δppk strains can not produce pigmented antibiotics (actinorhodin and undecylprodigiosin) in broth or on agar. They do not have agarase activity, and they do not sporulate on solid media. The recombinants can not form colonies. All the pellets and clumps are disentangled and mycelia are fragmented. Thereby, they do not display any feature of the developmental cycle of *Streptomyces*. The deficiency in the colony formation is caused by the expression of *elm* cluster carried on the cos16F4. Extracellular application of 8-DMTC against *S. coelicolor* wild type causes a similar phenotype with the cos16F4 carrying recombinants strains. Since the morphological changes that we have observed and investigated have never been reported before, our findings contribute to the understanding of the effect of 8-DMTC in *Streptomyces*. Moreover, this thesis is a pioneer for the next studies to understand the importance of colony formation beside pellets and clumps in antibiotic production.

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