

T.R.
GEBZE TECHNICAL UNIVERSITY
GRADUATE SCHOOL

**TRANSCRIPTIONAL ANALYSIS OF DEVELOPMENTAL GENES OF
ANTIBIOTIC OVERPRODUCER RECOMBINANT *STREPTOMYCES*
*COELICOLOR***

SUDE KARA

**A THESIS OF MASTER OF SCIENCE
DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS**

ADVISOR: PROF. DR. SEDEF TUNCA GEDİK

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GEBZE TEKNİK ÜNİVERSİTESİ
LİSANSÜSTÜ EĞİTİM ENSTİTÜSÜ

YÜKSEK ANTİBİYOTİK ÜRETİCİSİ REKOMBİNANT
***STREPTOMYCES COELICOLOR*'UN GELİŞİM GENLERİNİN**
TRANSKRİPSİYONEL ANALİZİ

SUDE KARA

YÜKSEK LİSANS TEZİ
MOLEKÜLER BİYOLOJİ VE GENETİK ANABİLİM
DALI

DANIŞMAN: PROF. DR. SEDEF TUNCA GEDİK

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ABSTRACT

The Lon protease, which is highly conserved across species, performs important functions in every organism. In *Streptomyces*, Lon is part of the HspR/HAIR heat shock response regulon. In other bacterial species, it is involved in a wide range of cellular events, including pathogenicity, encapsulation, motility, DNA repair, sporulation, UV stress response, heat shock, starvation response and antibiotic resistance.

Streptomyces, one of the most prolific producers of antibiotics today, has a life cycle that is unique among bacterial species. The life cycle begins with the germination of spores and continues with the reproduction of vegetative mycelia in a suitable environment. The colony, which is subjected to stress conditions such as high temperature, inappropriate pH, nutrient starvation, and interspecific competition, first differentiates into aerial hyphae and then forms spores that are resistant to environmental conditions. During the transition phase, as vegetative hyphae differentiate into aerial hyphae, the colony undergoes two programmed cell deaths, during which secondary metabolite production occurs. Secondary metabolite production, which is closely related to differentiation, is known to be triggered by stress in *Streptomyces*.

Within the scope of a previous study carried out by our laboratory group, the *lon* gene was integrated into the genome of *S. coelicolor* A3(2) and the recombinant strain obtained was found to be a high antibiotic producer. In this study, considering the relationship between antibiotic production and differentiation, transcriptional analysis of the developmental genes of the high antibiotic producer recombinant strain was performed by RT-qPCR. At the 16th h of growth, the change in the expression level of 32 genes involved in developmental cycle (genes responsible for aerial mycelium formation and sporulation, and genes that express the proteins of hydrophobic cover-chaplin and rodlin, divisome, regulators, synthesis and degradation of c-di GMP) was determined. The study was then focused on 12 of these 32 genes at the 48th and the 72nd hours of growth. Compared to the wild type strain, genes involved in sporulation (*whi*) were overexpressed in the early stages of development in the recombinant cell, while the expression of genes responsible for the formation of aerial hyphae (*bld*) was generally low in the early stages of development and increased later. The findings of this study are significant in demonstrating that Lon directly or indirectly affects cellular differentiation in *Streptomyces*, and that this is one of the factors contributing to a notable increase in antibiotic production.

Keywords: *Streptomyces*, Antibiotics, Lon Protease, Cell Differentiation.

ÖZET

Türler arasında yüksek oranda korunmuş olan Lon proteaz, bütün organizmalarda önemli işlevleri yerine getirir. *Streptomyces*'te Lon, HspR/HAIR ısı şoku yanıt regülasyonunun bir parçasıdır. Diğer bakteri türlerinde, patojenite, kapsülleme, hareketlilik, DNA onarımı, sporülasyon, UV stres yanıtı, ısı şoku, açlık yanıtı ve antibiyotik direnci dahil olmak üzere çok çeşitli hücresel olaylarda görev alır.

Günümüzde en önemli antibiyotik üreticilerinden biri olan *Streptomyces*, bakteri türleri arasında benzersiz bir yaşam döngüsüne sahiptir. Yaşam döngüsü sporların çimlenmesi ile başlar ve uygun bir ortamda vejetatif misellerin oluşması ile devam eder. Yüksek sıcaklık, uygun olmayan pH, besin açlığı ve türler arası rekabet gibi stres koşullarına maruz kalan koloni, önce hava hiflerine farklılaşır ve ardından çevresel koşullara dayanıklı sporlar oluşturur. Geçiş aşamasında, vejetatif hifler hava hiflerine farklılaşırken, koloni iki programlı hücre ölümü geçirir ve bu aşamada ikincil metabolit üretimi gerçekleşir. Farklılaşma ile yakından ilişkili olan sekonder metabolit üretiminin *Streptomyces*'lerde stres tarafından tetiklendiği bilinmektedir.

Laboratuvar grubumuz tarafından daha önce gerçekleştirilen bir çalışma kapsamında *lon* geni *S. coelicolor* A3(2) genomuna entegre edilmiş ve elde edilen rekombinant suşun yüksek antibiyotik üreticisi olduğu tespit edilmiştir. Bu çalışmada, antibiyotik üretimi ve farklılaşma arasındaki ilişki göz önünde bulundurularak, yüksek antibiyotik üreticisi rekombinant suşun gelişim genlerinin transkripsiyonel analizi RT-qPCR ile gerçekleştirilmiştir. Büyümenin 16. saatinde, gelişim döngüsünde yer alan 32 genin (hava miselyum oluşumu ve sporülasyondan sorumlu genler ve hidrofobik kılıf-chaplin ve rodlin, divizom, düzenleyiciler, c-di GMP sentez ve yıkım proteinlerini ifade eden genler) ifade düzeyindeki değişim belirlenmiştir. Çalışmada daha sonra büyümenin 48. ve 72. saatlerinde bu 32 genden 12'sine odaklanılmıştır. Yaban suşla karşılaştırıldığında, sporülasyonda rol oynayan genlerin (*whi*) rekombinant hücrede gelişimin erken aşamalarında aşırı ifade edildiği, hava hiflerinin oluşumundan sorumlu genlerin (*bld*) ifadelerinin ise gelişimin erken aşamalarında düşük olup daha sonra arttığı tespit edilmiştir. Bu çalışmanın sonuçları, Lon'un *Streptomyces*'te hücresel farklılaşmayı doğrudan veya dolaylı olarak etkilediğini ve bunun antibiyotik üretiminde dikkat çekici bir artışa neden olan faktörlerden biri olduğunu göstermesi açısından önemlidir.

Anahtar Kelimeler: *Streptomyces*, Antibiyotik, Lon Proteaz, Hücre Farklılaşması.

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LIST OF SYMBOLS AND ABBREVIATIONS

α	: Alfa
Δ	: Delta
σ	: Sigma
μL	: Microliter
Act	: Actinorhodin
Apr	: Apramycin
ATP	: Adenosine triphosphate
bp	: Base pair
bld	: Bald
CaCl	: Calcium chloride
CDA	: Calcium-dependent antibiotic
CCR	: Carbon Catabolite Repression
c-di-GMP	: 3'-5'-cyclic diguanylic acid
EDTA	: Ethylenediaminetetraacetic acid
h	: Hour
GYM	: Glucose/Yeast/Malt
kb	: Kilobase
L	: Liter
M	: Molar
Mg	: Miligram
Mg^{+2}	: Magnesium ion
mL	: Millilitre
mM	: Millimolar
NaCl	: Sodium chloride
NaOH	: Sodium hydroxide
PCR	: Polymerase chain reaction
ppGpp	: Guanosine tetraphosphate
pppGpp	: Guanosine pentaphosphate
Red	: Undecylprodigiosin
RNA	: Ribonucleic acid
rpm	: Revolutions per minute
RT-qPCR	: Quantitative reverse transcription polymerase chain reaction
Tris	: Tris (Hydroxymethyl) aminomethane
TSA	: Tryptic soy agar
TSB	: Tryptic soy broth
whi	: White

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1. INTRODUCTION

Streptomyces are gram-positive, filamentous bacteria that are remarkable for the secondary metabolites they produce. Their secondary metabolites can be used as antiviral, cytotoxic, antitumor, antihypertensive, immunosuppressive, and antifungal agents and this species is the largest producer of antibiotics in use [1].

The *Streptomyces* life cycle began with spore germination. Subsequently, the germination stage is followed by the formation of substrate/ vegetative hyphae. The differentiation process is pivotal in the *Streptomyces* life cycle. When the environmental conditions are not favorable for the organism, substrate mycelium goes on differentiation which is triggered by stress factors such as nutrient depletion, pH, and temperature. This differentiation turns vegetative mycelium into aerial mycelium and then forms spores that are resistant to environmental stress. Programmed cell death (PCD) occurs during *Streptomyces*'s life cycle and it is essential for the transition of the mycelia form. The phase during which mycelia differentiate into one another is referred to as the transition phase and secondary metabolite is produced during this phase. It has been demonstrated that common control mechanisms regulate secondary metabolite production and differentiation [2], [3].

Lon protease, which is highly conserved among species, is a protein-digesting enzyme involved in many cellular processes. Studies have shown that Lon is involved in sporulation and differentiation in some bacterial species. For example, the sporulation process in *Bacillus subtilis* is regulated by Lon [4]. In a growth environment unsuitable for sporulation, such as low pH, Lon has been demonstrated to inhibit sporulation by degrading σ^H , which is responsible for regulating the transcription of the *spoA* gene. In *Myxococcus xanthus* and *Vibrio parahaemolyticus*, Lon has been shown to play a role in the developmental cycle and differentiation [5], [6]. In *Streptomyces*, LON is part of the HspR/HAIR heat shock response regulon. In the study conducted by our laboratory group, it was found that the recombinant strain obtained by integrating two extra copies of the *lon* gene into the genome of *Streptomyces coelicolor* became a high producer of antibiotics [7]. The recombinant strain produced 34 times more actinorhodin and 9 times more undecylprodigiosin than the wild-type strain. Transcriptional analyses revealed that the expression of stress-related genes, including

relA and *sigB*, was up-regulated in the recombinant strain [8]. According to the data obtained, the addition of the extra *lon* gene to the genome caused stress and triggered antibiotic production. Considering that the developmental cycle of *Streptomyces* and the production of secondary metabolites are related, and the effect of stress on development, it is thought that the developmental genes of the recombinant strain may be affected. To date, no evidence for an effect of *lon* on development has been reported in *Streptomyces*.

1.1. The Aim and Original Value of The Thesis

In the previous studies in our laboratory, the recombinant strain Sco-pRA*lon* was generated by integrating two copies of the *lon* gene into the *S. coelicolor* A3(2) chromosome via the integrative pRA vector. This recombinant strain is known to be an antibiotic overproducer. The objective of this thesis was to perform transcriptional analyses of the developmental genes of this high antibiotic producer recombinant strain. The results will demonstrate how the *lon* gene, which is known to induce cellular stress and increase production of secondary metabolites, affects developmental processes. This study will therefore elucidate the relationship between the antibiotic production, stress and the developmental process in the recombinant strain. Understanding the relationship between antibiotic production and development may facilitate the improvement of the production efficiency of these metabolites by providing insight into the underlying processes of secondary metabolite biosynthesis. In addition, this thesis will attempt to elucidate the effect of Lon protease, known to be involved in differentiation and sporulation in other bacteria, on *Streptomyces* growth.

2. LON PROTEASE

Protein balance is essential for maintaining cellular viability and homeostatic stability, as well as the ability to adapt to changing environmental conditions. Living organisms require specific proteins throughout their life cycles, a situation which necessitates both synthesis and degradation of proteins. Proteolysis is crucial for the elimination of misfolded, toxic, accumulated, or dysfunctional proteins. Proteolysis plays a pivotal role in the adaptation of organisms to diverse growth conditions, the rapid response to environmental stress, and the resumption of normal cell growth once the stress factor has been removed (Figure 2.1) [9]. The major proteases that carry out proteolysis in prokaryotes belong to the AAA⁺ family of proteins (ATPases Associated with a variety of cellular Activities). The Lon proteases, Clp proteases (ClpAP, ClpXP, and ClpYQ), and membrane-bound HflB (FtSH) proteases are members of this protein family, and a widely conserved member of this family is the Lon protease. Lon is a conserved protein that has been identified in bacteria and human [10]. Its first discovery was in *Escherichia coli* [11].

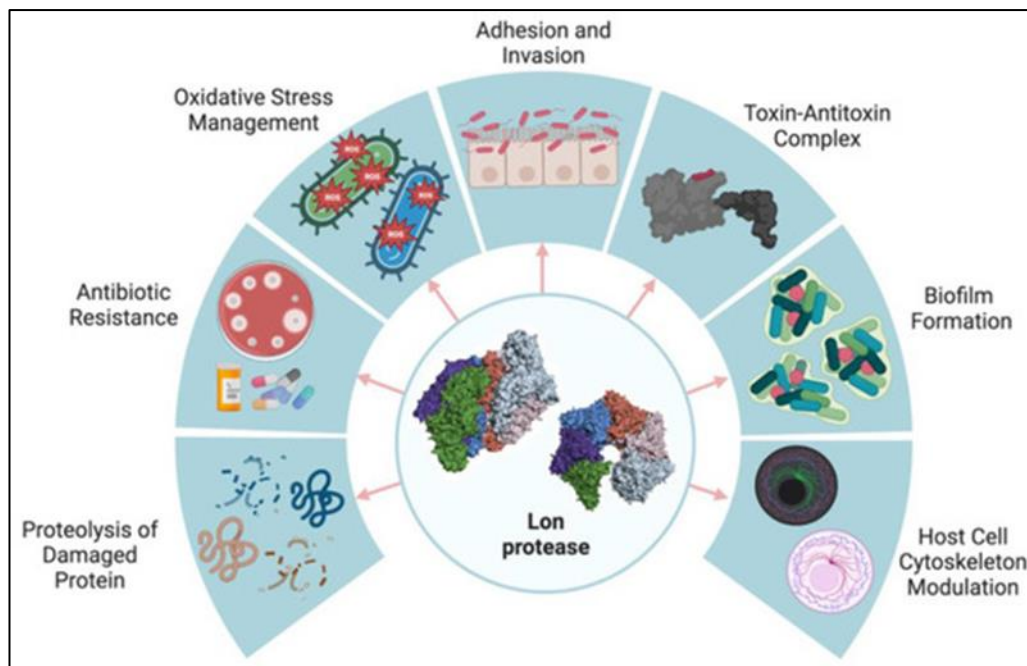


Figure 2.1: Cellular processes that Lon protease involved [9].

Lon proteolytically digests its substrates by forming a barrel-shaped complex consisting of an ATPase and a peptidase domain. Short peptide sequences, known as degradation tags or degrones, in substrates, are recognised by an extended N-terminal domain of the Lon protease [12]. Although Lon is highly conserved between species, its substrates are organism specific. This renders the identification of Lon's targets challenging. Lon proteases are involved in different cellular processes across species [9]. Lon is involved in DNA replication and repair in bacteria [13]. In *E. coli*, *lon* mutants exhibited a failure in cell division due to the inability of the cytoplasmic concentration of SulA to decrease [14]. Studies have demonstrated that Lon plays a role in regulating cellular processes associated with virulence factors, encapsulation, and motility. It has been demonstrated that it plays a role in the pathogenicity mechanisms of several bacterial species, including *Pseudomonas aeruginosa*, *Salmonella Typhimurium*, *Yersinia species*, *Brucella abortus*, and *E. coli* [14].

Lon proteases play an important role in the life cycle of bacteria and have been shown to regulate differentiation. Sporulation in *B. subtilis* is caused by activation of the SpoA transcription factor. In an unsuitable condition for sporulation, *B. subtilis* LonA negatively affects the transcription of the *spoA* gene. LonA inhibits sporulation by degrading σ^H , which is responsible for regulating the transcription of the *spoA* gene [15]. Tojo et al. showed that the *lonD* gene (*bsgA*) plays an important role in the developmental cycle of *M. xanthus* and is active in sporulation [4]. In *V. parahaemolyticus*, the Lon protease is known to be involved in the regulation of cell differentiation [5]. RcsA, a transcriptional activator of genes responsible for capsule synthesis in *E. coli* cells under normal growth conditions, is known to be degraded by Lon [6].

The inactivation of Lon in various species has been shown to result in sensitivity to stress. Furthermore, Lon has been identified as a key player in the SOS response, UV sensitivity, sporulation, heat shock, and stress responses [9]. The expression of Lon is triggered under various stress conditions. In the study by Kirthika et al. on *S. typhimurium*, it was observed that *lon* mutants exhibited a reduced capacity to cope with free radicals on oxidative stress; it was determined that Lon plays a pivotal role in response to other abiotic stressors, including cold, acidic and osmotic conditions [16]. *E. coli* studies show that Lon expression is upregulated under high salt, oxidative stress [17], and high-temperature conditions [18]. Osmotic stress stimulates Lon

expression in phytopathogen *Dickeya dadantii* [19]. In a study by Zeinert et al. with *Caulobacter crescentus*, it was demonstrated that Lon controls deoxyribonucleoside triphosphate (dNTP) pools through the transcription factor CcrM during stress [20]. In *S. coelicolor*, the deletion of the DnaK chaperone machinery resulted in the negative feedback regulation of *lon* and *clpB*. In contrast, Lon and heat shock molecular chaperones (DnaK and GroES/ GroEL1/ GroEL2) were found to be synergistically upregulated under heat stimulation. In *Streptomyces lividans*, Lon was confirmed to belong to the HspR (heat shock) protein regulator [21]. The observation that Lon functions in a free form as (HspR) in the presence of high temperatures suggests that the excessive production of Lon protease may be toxic to the bacteria. However, studies have demonstrated that Lon overexpression affects cell metabolism in different ways [9]. Lon negatively regulates the antibiotic production of pyoluteorin in *Pseudomonas fluorescens* Pf-5 [22]. However, overexpression of the *lon* gene in *B. thuringiensis* has been shown to increase endotoxin production and resistance to UV exposure, and osmotic and heat shock stress [23]. Furthermore, overexpression of the *lon* gene in *S. coelicolor* resulted the strain to become an over antibiotic producer. The two extra copies of *lon* in the genome caused a 34-fold increase in actinorhodin production and a 9-fold increase in undecylprodigiosin production in the recombinant strain [7].

3. *STREPTOMYCES*

The genus *Streptomyces*, the largest in the phylum *Actinobacteria*, comprises Gram-positive bacteria with a high GC content, ranging from 69 to 78%. The *Streptomyces* species can be found in a wide range of terrestrial and marine environments [24]. They exhibit physiological characteristics that are comparable to those of fungi, including a distinctive life cycle that involves the formation of different hyphae and results in sporulation. Differentiation plays a fundamental role in their life cycle, which, is conducive to the production of valuable secondary metabolites. Approximately two-thirds of the antibiotics currently in use have been derived from actinomycetes, with approximately 80% of these derived from the genus *Streptomyces*. A total of 279 new natural metabolites were discovered from *Streptomyces* spp. just between 2015 and 2020. The majority of these metabolites exhibited significant antibiotic activity [1].

The antibiotics produced by *Streptomyces* are highly diverse, encompassing aminoglycosides, anthracyclines, glycopeptides, β -lactams, macrolides, ansamycins, nucleosides, peptides, polyenes, polyesters and tetracyclines [25]. As an intermediate species, *Streptomyces* are also in close competition with fungi in their habitats. Therefore, *Streptomyces* are the source of numerous bioactive compounds with antifungal properties. They possess bioactive components that are utilized in industrial and agricultural applications, including the production of pesticide, antioxidants, plant growth promoters, and herbicidal agents. This is due to their close interaction with other species, which is a consequence of their habitats. Several pharmaceuticals, including antiviral, cytotoxic, antitumor, antihypertensive, and immunosuppressive agents, can be derived from *Streptomyces* metabolites [26]. In addition to those in use, it is thought that there are still many bioactive components waiting to be discovered in *Streptomyces* and that the presence of cryptic smBGCs (secondary metabolite biosynthetic gene clusters) may be a significant factor in this regard. The necessity for the discovery of new antibiotics, due to the emergence of resistant bacteria, the rapid increase in diseases such as cancer, and the investigation of *Streptomyces*, which may be a source of drugs for all of these, is promising for the future [1].

3.1. *Streptomyces coelicolor* A3(2)

S. coelicolor A3(2) is the most well-studied genus of *Streptomyces*. It has a single linear chromosome of 8.6 Mbp, encoding 7800 genes, in addition to two episomes: the linear plasmid SCP1 and the circular plasmid SCP2 [27]. It is a valuable model organism because mutations affecting secondary metabolite production are readily apparent. This strain produces pigmented secondary metabolites, including blue-colored actinorhodin (Act) and red prodigiosin (Red). Qualitative demonstration of antibiotic production is possible through macro-observation [28]. In addition, the strain produces two other well-known antibiotics, calcium-dependent antibiotic (CDA) and methylomycin (Mmy) [29]. A total of 20 gene clusters related to the production of secondary metabolites have been identified in *S. coelicolor* [30] indicating the existence of new bioactive components that could be used industrially or clinically.

4. LIFE CYCLE OF *STREPTOMYCES*

Streptomyces are unique bacteria due to the production of rich secondary metabolites and their distinct life cycle which remarkably relies on differentiation. *Streptomyces* growth is more similar to fungi than typical bacterial growth because of its filamentous structures. Their life cycle includes different mycelial formation and sporulation, they can be uni-genomic or multinucleated depending on the stage of differentiation [2].

When uni-genomic *Streptomyces* spores encounter a favorable environment, they initiate the first stage of their life cycle (Figure 4.1), known as germination. This process involves the regulation of various cellular activities such as the synthesis of initial molecules, energy supply to the cell, and the emergence of the germ tube [31]. With the onset of germination, the cell needs to exchange substances with the external environment and begin to hydrolyze the spore sheath [32] which causes the loss of hydrophobic structure, and the subsequent elongation of the germ tube inside the spore, and this is followed by the formation of the substrate mycelium. The direction of cell growth is critical for *Streptomyces*, and vegetative hyphae exhibit apical-type extension by branching horizontally and growing rapidly towards the mycelial tips. Early substrate hyphae, known as MI, are uni-genomic and contain compartments. Through undergoing PCD, the compartmental structure of MI cells is disrupted, and the colony becomes multinucleated. The hyphae in this state are known as MII [33]. This hypha is still in vegetative form. While cell growth continues vegetatively, *bld* genes are expressed with changing environmental conditions and substrate mycelia differentiate into aerial hyphae, the beginning of the reproductive structure [34]. During this differentiation, *Streptomyces* produce valuable secondary metabolites [35]. The aerial hyphae in the MII form contain a hydrophobic sheath and the colony grows vertically, it extends into the air. Although the mycelial type has changed, the aerial hyphae are multinucleated, and the cell is still in the MII phase. The aerial hyphae begin to differentiate into spore chains whereby the expression of sporulation genes, *whi*, and then, mature uni-genomic spores are formed with septation [35].

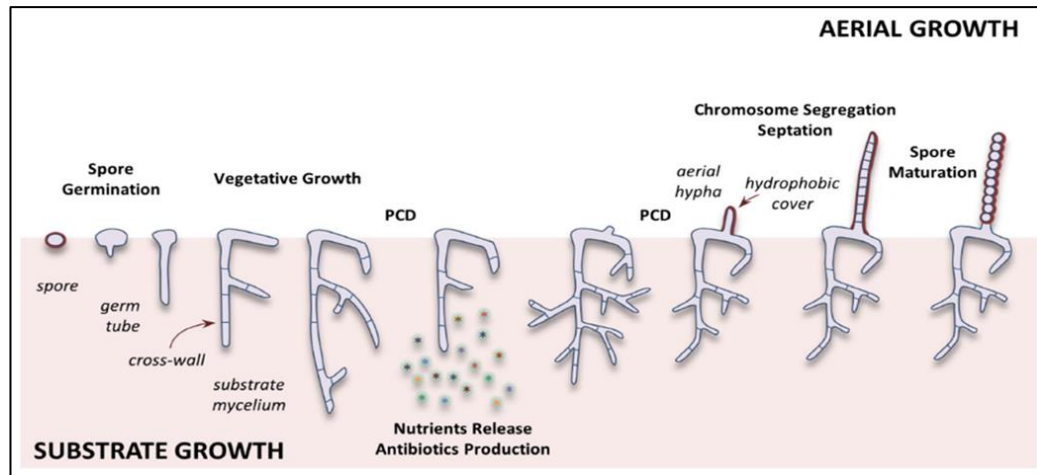


Figure 4.1: *Streptomyces* life cycle [36].

4.1. Germination

The process of germination in *Streptomyces*, which is the least studied phase of its life cycle, is not yet fully understood. In 1978, Hardisson et al. classified the germination process of *Streptomyces* into three distinct stages: darkening, swelling, and emergence of the germ tube [37]. During the dark phase, the organism uses the energy stored in its spore reserves, such as trehalose and polyphosphates (volutin), eliminating the need for an external energy source. Unlike the source of energy, the organism requires the presence of exogenous divalent cations such as Ca^{2+} , Mg^{2+} , or Fe^{2+} . Swelling is a process that requires only carbon intake from outside the organism, whereas germ tube emergence requires carbon and nitrogen intake. Hydrolysis of the spore sheath is an important event for germination, and dormant spores were found to already have hydrolase enzymes [38], [39].

The study conducted by Strakova et al. in 2012 revealed that chaperones are stored in the dormant spore in addition to hydrolases. During the first 30 minutes of germination, chaperone production was shown to be significantly increased. Additionally, the study revealed that the synthesis of antioxidants such as TrxA and Trx4A, commenced within the initial 30 minutes, thereby protecting the spore from oxidative stress and toxic agents [40]. It can be hypothesized that the spores that emerge from their dormant state are primarily trying to maintain the homeostasis that they will later establish. Genes related to development are expressed during germination. *bldG*, which controls the activation of *sigH* - an important sigma factor-

and *bldD*, a master regulator for development, are expressed during germination. In addition, proteins related to cytoskeleton formation during growth and development, such as DivIVA, FilP, and FtsZ, are synthesized at germination. While laboratory studies demonstrated that the germ tube commenced formation at 5.5 h [40], Manteca and Sanchez revealed that *Streptomyces* germination in forest soil started after 7 days and lasted up to 21 days [41]. In its natural habitat, germination is a long process.

4.2. Vegetative Growth

Following germination, *Streptomyces* cells undergo vegetative growth. During this phase of the life cycle, cells branch out with a focus on multiplication, forming new hyphae. These structures, called vegetative or substrate hyphae, grow by apical extension. Apical growth is not mediated by the cytoskeletal protein MreB, as is the case in rod-like bacteria such as *E. coli*. While the growth of *Streptomyces* hyphae is similar to that of fungal growth, it is notable that the proteins involved in growth are prokaryotic [42]. The growth is dependent upon the identification of hyphae tips, which are known as polarisomes. The coiled-coil protein DivIVA serves to mark the hyphae tips and to facilitate the arrival of other cytoskeletal proteins at the designated sites [43]. An increased gradient of DivIVA triggers branching and apical growth. The depletion of DivIVA has been observed to have chaotic consequences for cell growth, indicating that this gene is essential for vitality. In partial depletion of DivIVA, problems in cell growth and cell shape have been reported. Conversely, when this gene is overexpressed, hyphae are shorter and thicker, with an oval and swollen shape, growth is slow and there are more disrupted cells. [44].

Another crucial element in the process of apical growth is Scy (*Streptomyces* cytoskeletal element), which is a bacterial homolog of eukaryotic cytoskeletal proteins. Scy plays a role in the localization of DivIVA and also controls cell wall synthesis [45]. FilP, another cytoskeleton protein involved in apical growth, is recruited to the apical region by DivIVA, but they are not colocalized [46]. The *Streptomyces* polarisation mechanism is regulated by AfsK, a eukaryotic-type Ser/Thr protein kinase, through DivIVA. AfsK is localized at hyphae tips and phosphorylates DivIVA, thereby providing bidirectional regulation (Figure 4.2) [47], [48]. Although during vegetative growth FtsZ is involved in the formation of lateral cell walls, in *ftsZ*

mutant vegetative growth can be completed. These mutants have long hyphae lacking septa, which demonstrates that the lateral cell wall is not essential for the colony [49].

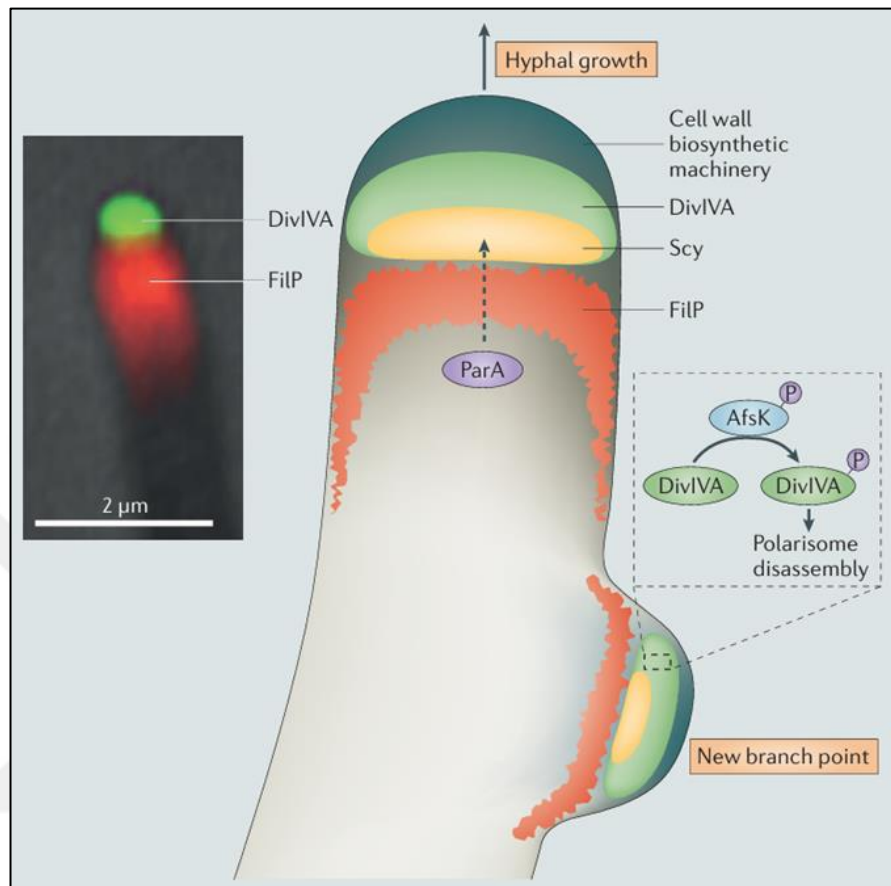


Figure 4.2: *Streptomyces* hyphal growth [48].

Substrate mycelia can exist in two distinct phases, designated as MI and MII. Firstly, uni-genomic MI hyphae with a compartmentalized structure are the predominant form in the colony. Although septation is present in these hyphae, the MI septa are thinner than the cell wall and have different permeability [41], [50]. It was demonstrated that the MI state transitions to the MII state between 20 h and 36 h under laboratory conditions, whereas it persists for 21 days in *Streptomyces* cultivated under conditions analogous to the natural environment [41]. This indicates that MI hyphae are the dominant form in the natural environment throughout the life cycle. Although the subsequent transition from the uni-genomic to the multi-nucleate form provides an advantage in terms of nutrition and biochemical signalling, genetic errors that spread throughout the colony can threaten the organism's existence. This is in support of the hypothesis that MI hyphae are the predominant mycelia. [51]. MI hyphae differentiate

into MII hyphae with a multinucleate structure, which serves as the basis of the reproductive structure in response to environmental signals such as nutrient depletion, pH, and temperature.

The formation of the MII structure is mediated by programmed cell death (PCD) and developmental arrest [52]. The observed state of PCD and differentiation leads to the production of secondary metabolites. This production, initiated during this transient phase between MI and MII, is carried out by secondary mycelia [53]. Following this phase, the secondary mycelia are coated with a hydrophobic sheath and begin to form aerial hyphae (Figure 4.3) [53]. A study by Yagüe et al. compared the transcriptomic data of MII/MI (MII24 h/MI16 h, MII48 h/MI16 h and MII72 h/MI16 h). The results demonstrated that genes related to primary metabolite production were upregulated in primary mycelia, while genes related to secondary metabolite production and differentiation were upregulated in secondary mycelia. The transcriptomic data of secondary mycelia collected at the 24th h, 48th h and 72nd h exhibited 95% similarity [3].

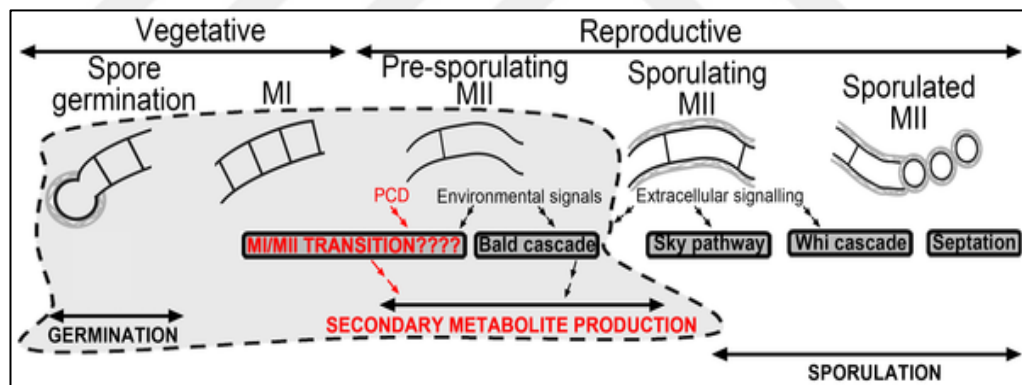


Figure 4.3: The PCD and the production of secondary metabolites during the differentiation of *Streptomyces* mycelia [53].

The genes involved in the formation of aerial hyphae in *Streptomyces* life cycle have been identified and named *bld* because mutations of these genes result in a bald phenotype in which aerial hyphae are defective [54]. The maintenance of vegetative growth is regulated by the transcriptional repression of differentiation genes by the DNA-binding BldD protein, which is expressed by the *bldD* gene [55], [56].

BldD is a transcriptional regulator that negatively controls the expression of *bld* genes involved in the formation of aerial hyphae and *whi* genes associated with sporulation

[56]. BldD has been demonstrated to repress the transcription of genes that play a pivotal role in development, such as *whiG*, *bldM*, and *bldA* [58]. Furthermore, it represses its own expression by binding to its own promoter [59]. Hengst et al. identified 167 genes with binding sites for *bldD*. The genes include *bldA*, *bldC*, *bldH/adpA*, *bldM*, *bldN*, *ssgA*, *ssgB*, *ftsZ*, *whiB*, *whiG*, *smeA*-SSF, which are responsible for morphological differentiation; *nsdA*, *leuB*, and *leuC*, which are responsible for metabolism; *sigH*, which is responsible for the synthesis of a sigma factor involved in the regulation of stress responses. Furthermore, the growth of *bldD* null mutants is notably slow, and genes related to primary metabolism are downregulated [60]. BldD mutants, like other *bld* mutants, display a bald phenotype devoid of aerial hyphae and are unable to produce antibiotics on culturing minimal media [58], [59].

BldD targets include genes involved in energy storage, cell division, cell wall modification, signaling pathways, antibiotic production, proteolytic and regulatory functions. This evidence demonstrates that BldD is at the beginning of the developmental pathway and is a master regulator [60]. The control of these master regulator on genes is achieved through the binding of the extremely rare c-di-GMP (3',5'-cyclic diguanilic acid) tetramer to the BldD dimer [61]. c-di-GMP, which is not produced in archaea or eukaryotes, is a common second messenger in bacteria. c-di-GMP is synthesized by diguanylate cyclase (DGCs), and its degradation is mediated by c-di-GMP-specific phosphodiesterases (PDEs) [62]. In *S. coelicolor*, high levels of c-di-GMP delay sporulation, while low levels of c-di-GMP accelerate sporulation [63]. Overexpression of the diguanylate cyclase gene (*cdgB*) has been observed to inhibit actinorhodin production, while both overexpression and deletion have been shown to inhibit aerial mycelium formation [64]. Hull et al. demonstrated that the mutation of the cyclic di-GMP phosphodiesterases, RmdA and RmdB, delayed sporulation and altered colony morphology [65]. As a result of environmental signaling, the effect of c-di-GMP and BldD complex on target genes is eliminated and the cell undergoes differentiation (Figure 4.4) [66], [67].

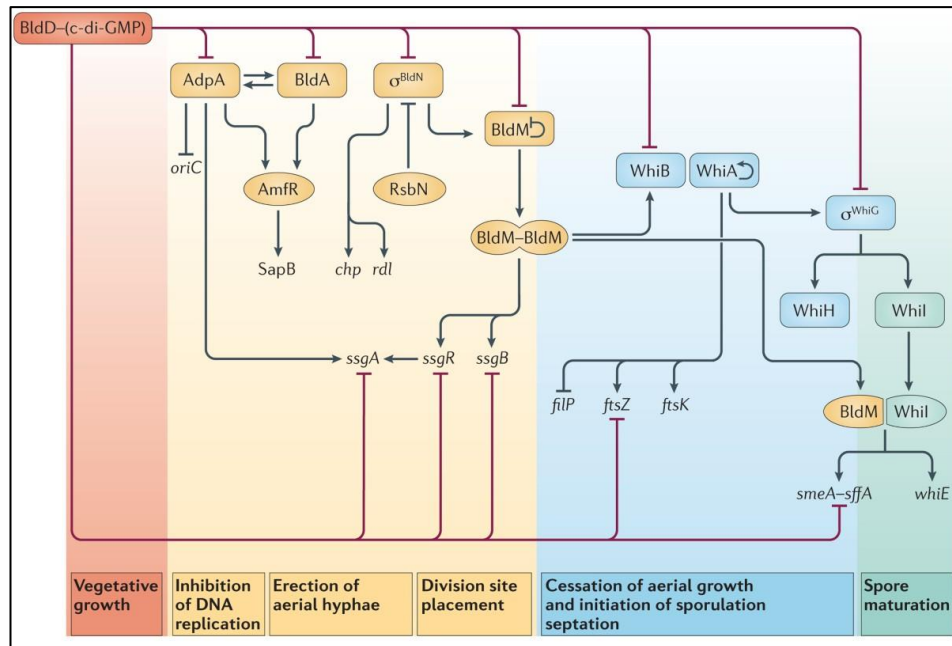


Figure 4.4: Genes involved in *Streptomyces* life cycle and their control by BldD/c-di-GMP complex [67].

4.3. Aerial Mycelium Formation

Secondary mycelia, which are formed during vegetative phase, continue to grow until they undergo another PCD process. During the PCD, cell debris is dispersed, and the colony environment becomes nutrient-rich. These nutrients are utilized by the differentiating cells. Essentially, the colony itself serves as its energy source [68]. The cell waste products released into the environment may be attractive to other organisms as a food source, and the production of secondary metabolites at this stage of differentiation may be intended to protect the colony from external factors [69]. Following the process of PCD, a hydrophilic sheath begins to form around the secondary mycelia [70]. Concurrently, the *bld* genes facilitate the requisite preparations for the formation of aerial hyphae. The common phenotype resulting from a mutation in *bld* genes is bald colonies lacking or defective aerial hyphae [71]. However, this mutant phenotype is observed in cells grown on minimal media, and the defective phenotype is reversible in colonies grown on a non-glucose medium such as mannitol. This indicates that *bld* genes are linked to carbon catabolite repression (CCR), which may permit the cell to continue to grow in nutrient excess, rather than differentiate and produce antibiotics [72]. Moreover, the majority of *bld* mutants are unable to produce antibiotics. *bld* genes are involved in a hierarchical extracellular

signaling cascade. When mutant *bld* cells are grown in proximity to wild-type colonies without direct contact, the mutant phenotype is observed to disappear [73].

BldG is a pleiotropic anti-anti-sigma factor with a complex role in the *Streptomyces*'s life cycle. It interacts with 15 anti-sigma factors. It activates five sigma factors involved in stress response and differentiation. Furthermore, it is involved in the activation of *sigF* and *sigH*, which play a pivotal role in differentiation. σ^H plays a dual role in the osmotic stress response and differentiation [74]. While cell growth is affected in σ^H mutants under high osmotic conditions, morphological differences are observed in mutants. *sigH* mutant cells exhibit partial differences in pigmentation and undifferentiated aerial hyphae with rare septation [75]. *sigH* expression can be regulated through four different promoters, and sigma factor activity is negatively regulated post-transcriptionally by the anti-sigma factor UshX/PrsH [76].

The *bldA* gene, which synthesizes tRNA required for UUA, a rare leucine codon in *Streptomyces* with a high GC ratio, plays a crucial role in the development and antibiotic production of this organism. Transcription of TTA codons in the genome is contingent upon the presence of functional *bldA* [77]. *bldA* mutants of *S. coelicolor* display deficiencies in the formation of aerial hyphae and in the production of actinorhodin, undecylprodigiosin (red), and methylenomycin. The ActII-ORF4, *redZ*, *mmyB*, and *mmfL* genes contain the TTA codon and are part of the actinorhodin, undecylprodigiosin, and methylenomycin gene clusters, respectively. *Streptomyces griseus bldA* mutants were also unable to produce streptomycin [78]. One of the targets of *bldA* is the developmental gene *bldH* (*adpA*), which contains a TTA codon. The expression of *bldA* is necessary for the transcription of *bldH*, whereas *bldH* is required for *bldA* transcription. As is the case with other *bld* mutants, *bldH* mutants are unable to form aerial hyphae and are defective in antibiotic production [79]. In *S. coelicolor*, *str1* (encoding *Streptomyces* trypsin inhibitor), *clpP1* (gene encoding ClpP1, an ATP-dependent protease that affects morphological and physiological differentiation), *ramR* (regulator of *ram* genes that regulate the production of SapB, a hydrophobic protein), *ssgA*, which encodes a small acidic protein essential for sporulation (septum formation) were identified as target genes of *adpA* [80]. The *bldN* gene, which is responsible for the expression of chaplins that form the hydrophobic sheath, was also identified as the target gene of *adpA* in both *S. griseus* and *S. coelicolor* [81], [82].

The *bldN* gene is responsible for the expression of the extracytoplasmic function (ECF) sigma factor. This sigma factor is involved in the regulation of the expression of the chaplin and rodlin genes, which are involved in hydrophobic sheath formation. Unlike other *bld* genes, *bldN* is not placed in a hierarchical step. Furthermore, unlike other mutants, the bald phenotype is observed in *bldN* mutants regardless of the medium content. The transcription of this gene is regulated indirectly or directly by *bldH* and *bldG* in *S. coelicolor* [82]. The BldD-(c-di-GMP) complex represses the *bldN* expression during vegetative growth. Additionally, sigma factor activity is also controlled post-transcriptionally [83]. The synthesized sigma factor is retained by the anti-sigma factor RsbN until an external signal is received.

A study by Bibb et al. with *S. venusealla* revealed that the sigma factor and anti-sigma factor form a stable complex and that the null mutant of the anti-sigma factor leads to precocious sporulation. In this study, it was shown that anti-sigma *rsbN* is located 300 bp downstream of the *bldN* gene and can be regulated via the *bldN* promoter. This situation differs from the general relationship between ECF sigma factors and anti-sigma factors. Nevertheless, *bldN* and *rsbN* are co-expressed, indicating that the expression of the anti-sigma factor may be mediated by two different promoters, one within the *bldN* gene region and the other downstream of the *bldN* stop codon. This suggests that the expression of the anti-sigma factor may occur independently of *bldN* [84]. The *bldN* promoter was found to be sequence conserved in *S. coelicolor*, and *bldN* and *rsbN* are conserved and adjacent in each of the 28 publicly available *Streptomyces* genome sequences with the intergenic gap. It is also known that *rsbN* is a *bldD* target gene in *S. coelicolor*. The absence of chaplin and rodlin expression in *bldN* mutants indicates that these genes are directly controlled by BldN. Another direct target of the sigma factor BldN is the *bldM* promoter [84].

Two promoters of the *bldM* gene have been identified, one of them being a direct *bldN* target. Consequently, although *bldM* expression is controlled by BldN, independent expression is also possible. *bldM* mutants exhibit a bald phenotype similar to that observed in other *bld* mutants and are members of the extracellular signal cascade [85]. The amino acid sequence of BldM is 100% conserved within the species. A study by Al-Bassam et al. in *S. venusealla* revealed that BldM is a direct transcriptional activator for 131 genes [86]. In the initial stages of aerial hyphae formation, BldM functions as a homodimer. However, during sporulation, it forms a heterodimer with

another orphan response regulator, WhiI, which is involved in sporulation and acts as a transcriptional activator. Although it is known that transcriptional factors in eukaryotes can function as heterodimers, this is a rare in bacteria. The observation that the genes of the *bldM* null mutant and the *whiI* null mutant that are downregulated during sporulation are identical suggests that the working activity of WhiI is directly depend on BldM. This implies that WhiI may be a helper protein of BldM [29], [86]. The target genes and activity of BldM indicate that it plays a crucial role in both aerial hyphae formation and sporulation.

4.3.1. Hydrophobic Sheath Formation

Three hydrophobic proteins related to development have been identified in *Streptomyces*: SapB, chaplins (Chp), and rodlin (Rdl). SapB, a small hydrophobic protein secreted into the environment, enables the aerial hyphae to overcome surface tension more easily and with less energy [87]. It forms a hydrophobic film on the hyphae surface. It is not produced in all mutants, except those with *bldN* and *bldM* mutations. If the Bld cascade is complete as a result of environmental signals, SapB production occurs, leading to the formation of aerial hyphae. SapB, a lantibiotic-like protein, is produced by the *ramS* gene and finalized by modification with an enzyme synthesized by the *ramC* gene, which is similar to lantibiotic modification enzymes [88]. The synthesis of this protein is initiated by the activation of the *ramCSAB* operon by a response regulator synthesized by *ramR*. The *ram* genes have been identified in *Streptomyces* and have been abbreviated as Rapid Aerial Mycelium. *ramS* null mutants do not produce SapB, whereas the addition of SapB rescues the mutant phenotype [88], [89]. Furthermore, the addition of SC3, a fungal hydrophobin, also rescues the phenotype of *ramS* mutants. This evidence is significant in demonstrating the fungal similarity of *Streptomyces*. Nevertheless, the addition of the surfactants produced by *Bacillus* and *Pseudomonas* were unable to fulfill the same function [90].

The hyphae that extend upwards into the air are coated with a hydrophobic sheath. This sheath, known as the rodlet layer, is formed by the combination of Chaplin and Rodlin and closely resembles the structure found on the surface of fungal hyphae. The presence of rodlin is not necessary for the formation of the hydrophobic sheath; in the absence of rodlin, the micelles are covered with fine fibrils consisting of chaplins.

In *S. coelicolor*, there are eight chaplins, and the Δchp ABCDEFGH strain is completely unable to form air hyphae. Interestingly, mutants not only lack the chaplin fibrils but also the rodlet layer. This indicates that rodlinins are not functional without chaplin in the formation of the hydrophobic sheath [88], [91], [92].

4.4. Sporulation

Once sufficient aerial biomass has been produced, aerial hyphae growth is arrested, followed by the differentiation of aerial hyphae into sporogenic structures and simultaneous septation of multinucleate aerial hyphae into unigenomic spore chains. The mature spores, which are covered with a spore sheath that is resistant to ambient conditions, are then dispersed and remain dormant until they find a suitable environment and undergo germination. Sporulation is regulated by so-called *whi* genes. These developmental genes are referred to as *whi* because mutations in these genes result in the production of white colonies that are devoid of the spore pigment. In these mutations, sporulation is unable to occur properly.

The presence of longer undifferentiated aerial hyphae in *whiA* and *whiB* mutants compared to wild type suggests the existence of mechanisms regulated by these proteins, although the signaling mechanism involved in the arrest of aerial hyphae growth and sporulation is not fully understood. It is noteworthy that *whiA* and *whiB* mutations result the same phenotype. Studies have demonstrated that the products of these genes function in concert, with the absence of one resulting in the dysfunction of the other [93]. WhiA is a protein widely distributed among Gram-positive bacteria. Its N-terminal domain exhibits homology to the eukaryotic homing endonuclease, but it is not active [94]. WhiA functions as both an activator and repressor of gene expression. The growth of aerial hyphae occurs through the polarisome mechanism, with an apical-type extension similar to that observed in vegetative hyphae. The expression of the *filP* which is involved in the polarisome mechanism is repressed by WhiA, which also results in the termination of aerial hyphal growth. In contrast, the expression of proteins required for septation, including FtsZ, is activated by WhiA. It is also postulated that WhiA is involved in the regulation of *whiG* expression [95].

whiB is activated by the *bldM* homodimer during the onset of differentiation and WhiB is an actinomycete-specific protein. Furthermore, it is a founding member of a class of

proteins known as the Wbl (Whi B-like) family. Septation failure has also been observed in addition to aerial hyphae that continue to grow in *whiA* and *whiB* mutants. The mutants lack septation and exhibit uncondensed DNA [93], [95].

The sporulation-specific sigma factor WhiG initiates the transcription of two essential *whi* genes, *WhiH* and *WhiI*, during the early stages of sporulation, and plays a pivotal role in sporulation [96]. The study conducted by Gallagher et al. demonstrated that overexpression of *whiG* in *S. venusealla* resulted in hypersporulation and the null mutant exhibited undifferentiated aerial hyphae. Furthermore, they found that overexpression of *whiI* and *whiH* in *whiG* null mutants caused hyper sporulation like that observed with *whiG* overexpression. Also, this study revealed that σ^{WhiG} is post-translationally controlled by an anti-sigma factor designated RsiG. The binding of the sigma factor and anti-sigma factor in the correct conformation and with greater efficacy is facilitated by c-di-GMP. A reduction in the c-di-GMP concentration results in the dissociation of the σ^{WhiG} and the RsiG, and the subsequent expression of other sporulation genes (Figure 4.5) [97]. The effect of this secondary messenger on sporulation and the maintenance of vegetative growth by *bldD* demonstrates the global effect of c-di GMP in the *Streptomyces* life cycle.

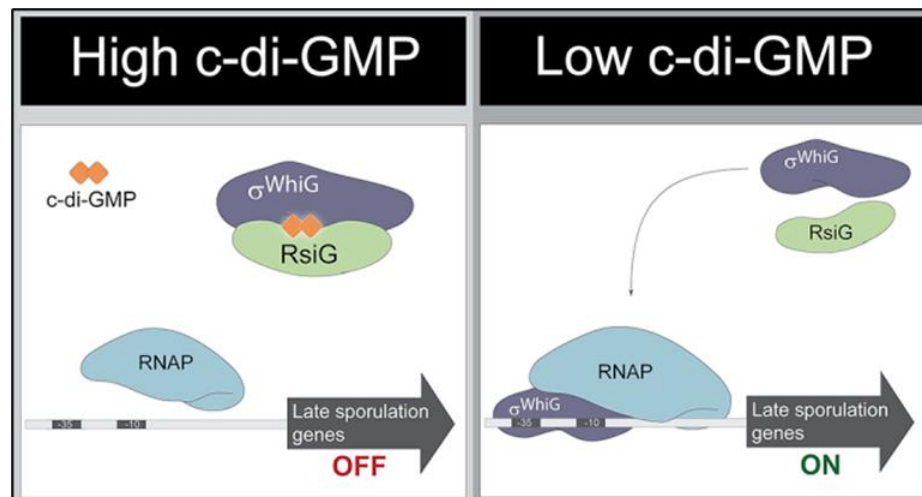


Figure 4.5: Control of σ^{WhiG} activation with RsiG and c-di-GMP [97].

whiH encodes a member of the GntR family of transcription factors, yet the specific target genes remain unknown. *WhiH* mutants exhibit irregular sporulation septation, although sporulation septa are observed in some segments, resulting in the formation

of abnormally separated elongated compartments containing multiple copies of the chromosome [98]. This mutant phenotype suggests that WhiH is required for septation.

WhiI, which is not thought to have a function independent of BldM, is involved in the differentiation of aerial hyphae into mature spores. The *SmeA-SffA* operon and *WhiE* locus involved in sporulation septa are activated by the BldM-WhiI heterodimer [99]. The *WhiE* involved in late sporulation is responsible for spore pigment production. The *whiE* promoter is one of the σ^F targets. [100].

S. coelicolor SigF, one of the nine close homologs of SigB in *B. subtilis* is involved in the late stages of sporulation. The spores resulting from the mutation exhibit septation yet contain uncondensed DNA and display pigmentation deficiencies. The spore shape is distorted, and the spore wall is thinner. In the mutant spore chain, defective spores are separated from the spore chain more difficultly compared to the wild type strain. *sspA* is another target gene of SigF in addition to the *whiE*. *SspA* is not expressed in the *sigF* null mutant. The *sspA* null mutant, which is thought to play a role in septum formation and spore maturation, exhibits irregular sporulation septation and altered spore shape. This mutant phenotype is similar to the *sigF* mutant phenotype, while the malformation is characterized differently for both mutants. There is no detectable transcription of *sigF* in *whiA*, B, G, H, I, or J mutants [101], [102].

4.4.1. Septation and Spor Wall Forming

The conversion of multinucleate aerial hyphae into uni-genomic spores necessitates the presence of a fully functional mechanism. During the sporulation stage, septation occurs almost simultaneously in a synchronized manner, resulting in the formation of spore chains, each containing a single copy of chromosome. The septation observed during sporulation differs from that observed in the early stages of development where the formation of cross walls is irregular and random. However, in the sporulation stage, the septums are regular and evenly spaced.

Z-rings, which are formed by the accumulation of tubulin homolog FtsZ protein, serve as scaffolds. In the long aerial hyphae, multiple FtsZ ladders, comprising up to 100 septa, are produced. *ftsZ* transcription is essential for sporulation. Although null mutant of *ftsZ* cells retain viability, they are unable to sporulate [51]. The overexpression of *ftsZ* in null mutants of the "early" *whi* regulatory genes *whiA*, *whiB*,

whiG, *whiH*, *whiI*, and *whiJ* can result in the rescue of the mutant phenotype [103]. In other bacterial species, the localization of FtsZ is regulated by a negative effect, with Min proteins localizing to the poles and FtsZ proteins accumulating at the equator of the cell. In *Streptomyces*, however, the formation of the Z-ring in long hyphae is positively controlled by SsgA and SsgB proteins, which are unique to sporulating actinobacteria and localize FtsZ proteins. SsgA activates sporulation-specific cell division and binds to the division segments, after which SsgB localizes to these segments to recruit FtsZ and initiate sporulation polymerization (Figure 4.6) [29], [104], [105], [106].

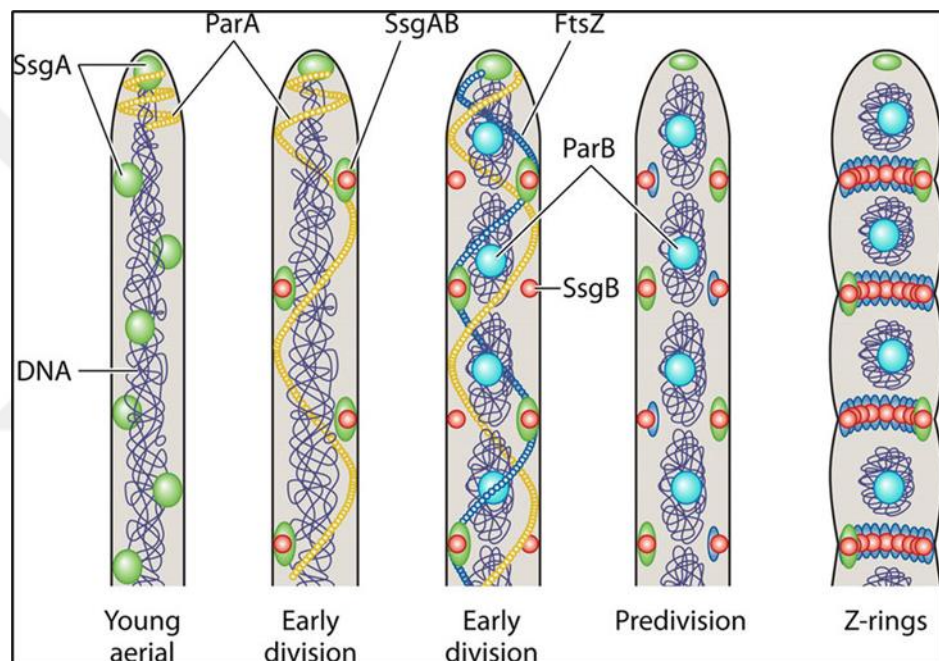


Figure 4. 6: *Streptomyces* septation [106].

CrgA is another protein that affects Z-ring assembly. The mutation of this sporulation-specific cell division protein, which is specific for actinomycetes, has different effects in different species. CrgA mutation usually leads to problems in septation, although it has different effects cross to species. In *S. coelicolor*, a mutant strain of *crgA* displays early differentiation and the early production of the blue antibiotic actinorhodin [29], [106]. The equal distribution of chromosomes during septation is mediated by the chromosome-partitioning proteins ParA and ParB. The FtsK, FtsK-like SffA, and SmeA proteins play a role in the segregation of multiple chromosome copies into the spore compartments. For the spores to mature, the spore wall must be formed to

withstand harsh environmental conditions, such as heat, drying, and high osmolarity. In bacteria, a cytoskeletal protein, MreB, is involved in the formation of rod shape. However, MreB is involved in spore wall synthesis in *Streptomyces*.

The synthesis of the *S. coelicolor* A3(2) thickened spore envelope is depend on the *Streptomyces* spore wall synthesis complex SSSC, a multiprotein complex comprising proteins that direct peptidoglycan synthesis (MreBCD, PBP2, Sfr, RodZ) and cell wall glycopolymer synthesis (PdtA). Furthermore, two eukaryotic serine/threonine protein kinases (eSTPK), PkaI and PkaH, are also present within the complex and are responsible for phosphorylating MreC [107].



5. SECONDARY METABOLITES AND GROWTH

The genus *Streptomyces* is notable for the valuable bioactive secondary metabolites it produces. These molecules are of great interest to the pharmaceutical and biotechnology industries and have the potential to be employed in clinical trials. Secondary metabolite production overlaps with PCD and secondary mycelia formation in their life cycle. *Streptomyces* that undergo two times of programmed cell death during their lifecycle may be more susceptible to environmental hazards. The transformation of compartmentalized primary mycelia into multinucleate secondary mycelia by PCD confers an advantage upon the organism for nutrient transport and signaling pathways, but also it provides some disadvantages. The rapid growth of aerial hyphae and the rapid replication of genetic material can also facilitate the occurrence of genetic errors, which may subsequently spread to the entire colony. In addition, the cell debris released by PCD provides a nutrient source for *Streptomyces*, while also providing food for other organisms. It is no coincidence that the colony, which may be more vulnerable during differentiation, produces secondary metabolites that can be used to its advantage. The antibiotics produced by these organisms protect other organisms, while their pigmented secondary metabolites are thought to protect the colony against UV radiation.

For *Streptomyces*, secondary metabolite production and cell development are intertwined and affect each other. Depletion of nutrients is one of the main factors that triggers cell differentiation and encourages the cell to sporulate. DasR, a global regulator of the GntR family, is a transcriptional repressor of *actII-ORF4* and *redZ*. During PCD, the environment becomes enriched in N-acetylglucosamine (GlcNAc) due to the disintegration of cell walls. The presence of GlcNAc in the environment during nutrient scarcity inhibits DasR repressing effect of antibiotic biosynthetic gene clusters [108]. The control of antibiotic production by GlcNAc-dependent nutritional signaling provides evidence for a relationship between differentiation and secondary metabolite production. Another example that supports the relationship between nutrient deprivation and differentiation in conjunction with antibiotics is the stringent response. This system, which is stimulated by uncharged tRNAs in starvation, initiates the synthesis of alarmones (p)ppGpp from GTP and ATP via RelA or SpoT. The accumulation of alarmones within the cell may influence several cellular processes, including growth,

adaptation, secondary metabolism, survival, persistence, cell division, motility, biofilm formation, development, competence, and virulence [109]. It is known that metabolic stress occurs in a *relA* null mutant *S. coelicolor* that is unable to synthesize ppGpp, which impairs differentiation [110]. In addition, the control of polyphosphate metabolism plays an important role in *Streptomyces* development. The repressive effect of BldD, the master regulator of the life cycle, is provided by the complex it forms with c-di-GMP, while the activity of WhiG, an important transcriptional activator for sporulation, is also controlled by c-di-GMP. As the production of ppGpp and c-di-GMP is linked to the metabolism of GTP, it reveals the relationship between the polyphosphate mechanism, stress response and development (Figure 5.1) [111].

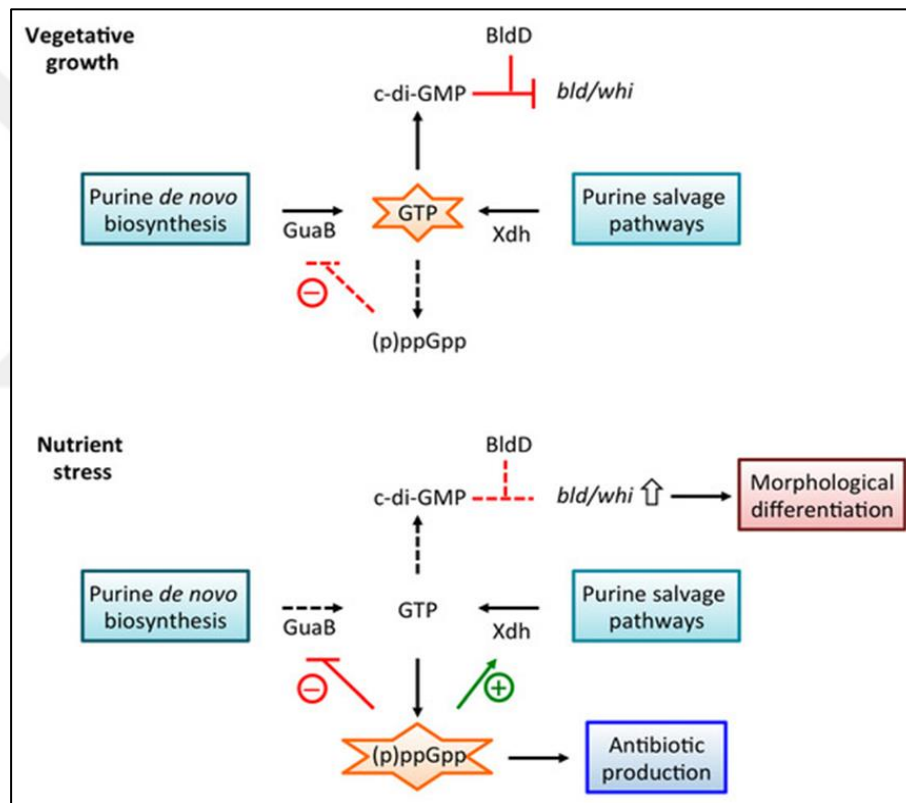


Figure 5.1: Effect of polyphosphate metabolism on *Streptomyces* differentiation [111].

The results of numerous studies indicate that mutations affecting differentiation, or the production of secondary metabolites exert a reciprocal influence upon each other. Bacterial hormones are defined as signaling molecules that act in small concentrations within bacteria. Several bacterial hormones have been identified in *Streptomyces*, with γ -butyrolactones being the most prevalent within the species. The γ -butyrolactone synthase ScbA is responsible for the biosynthesis of *S. coelicolor* butanolides (SCBs).

In the study by D'Alia et al., the *scbA* gene was mutated in *S. coelicolor*. In the mutant cells, differences in the expression of antibiotic gene clusters and the expression of genes related to development were observed [112]. A study by Mao et al. revealed that the degradation of SigT, an ECF σ factor involved in the negative regulation of cell differentiation, is dependent on ClpP protease. The study also demonstrated that both antibiotic production and differentiation are observed at an early stage of *sigT* mutation, while both processes are delayed in the case of overexpression of the gene [113]. Another study shows that in the Sco4117, defined as the extracytoplasmic function (ECF) sigma factor, knockout strain the production of actinorhodin and CDA was found to be reduced, with the formation of aerial mycelia and sporulation being delayed [114]. The disruption of Sco2140, which belongs to the leucine-responsive regulatory proteins (Lrps) family, resulted in a significant reduction in the production of actinorhodin and CDA antibiotics and caused delays in the formation of aerial mycelia [115].

In the previous studies conducted in our laboratory, a recombinant Sco-pRA_{lon} strain was obtained by the addition of two copies of *lon* gene to the *S. coelicolor* A3(2) strain via the genome-integrative pRA plasmid. In the recombinant strain, actinorhodin production increased by 34-fold, while undecylprodigiosin production increased by 9-fold [7]. Furthermore, an increase in the expression of stress-related genes, including *relA* and *sigB*, was observed in the recombinant strain [8]. It was postulated that the additional *lon* gene in the genome triggered the production of antibiotics by inducing cellular stress. As seen in the sample studies mentioned above, mutations that impact antibiotic production in *Streptomyces* also influence developmental processes. It has been demonstrated that the Lon protease plays a role in the developmental processes of other bacteria. Given the established relationship between stress and antibiotic production, it was postulated that the development of the recombinant strain may have been affected. In this thesis, it was aimed that a transcriptional analysis of the development-related genes of the over-antibiotic producer Sco-pRA_{lon} strain was conducted using RT-qPCR. The impact of Lon on antibiotic production and its effect on development were also examined. It is also known that plasmids used in recombinant studies can affect the cell metabolism. Therefore, to prove whether the transcriptional changes were due to Lon and to see the effect of the plasmid alone, the developmental genes of the Sco-pRA strain containing the empty plasmid were also analyzed in the scope of this thesis.

6. MATERIAL AND METHODS

6.1. Materials

6.1.1. Devices

4°C Refrigerator (Ariston), -20°C freezer (Ariston), -80°C freezer (Thermo, Forma - 86°C ULT Freezer), Ice Machine (BAR-LINE BF85), Horizontal Electrophoresis System (Bio-rad), Power Supply (Bio-rad, PowerPac Basic), Centrifuge (Heraeus, Labofuge 400R), Centrifuge (BECKMAN COULTER, Microfuge 22R), Autoclave (HIRAYAMA, HICLAVE HVE-50), pH Meter (Hana Instruments, pH 211 Microprocessor pH Meter), Sterile Cabinet (Heraeus, HP48), Incubator (BINDER, 9010-0078), Incubator (Heraeus, D-63450 Heraeus), Shaking Incubator (Edmund Bühler, KS-15), Water Bath (TECHNE, TE-10A Tempette), UV Imager (Azure Biosystems), Microwave Oven (Samsung), PCR Instrument (Applied Biosystems), StepOnePlus™ Real-Time PCR System (Applied Biosystems), 'NanoDrop Lite' Spectrophotometer (Thermo Scientific).

6.1.2. Chemicals

The following chemicals were used in the experiments of the thesis; D (+) Glucose (Merck), Yeast extract (Bacto™), Malt Extract, Agar (Merck), Tryptic soy broth (Merck), RedSafe (Intron), Agarose (Sigma Aldrich), NaOH (Merck), HCl, Tris (Merck), EDTA (Merck), Acetic acid (Riedel de Haen), 99.5% Ethanol (Riedel de Haen), 84-88% Glycerol (Sigma Aldrich).

6.1.3. Enzymes

Taq DNA polymerase (Thermo Scientific), Lysozyme (Sigma), and Deoxyribonuclease I (Thermo Scientific) enzymes were used under the conditions specified by the manufacturer.

6.1.4. Kits and Biomarkers

The NucleoSpin RNA Plus isolation kit (Macherey-Nagel, Germany) was used for total RNA isolation. The iScript cDNA synthesis kit (Bio-rad, USA) synthesized cDNA from the isolated RNA samples. SYBR Green qPCR Master Mix (Bio-Rad, USA) was used for RT-qPCR, gene expression analysis. GeneRuler 100 bp DNA Ladder (SM0311, Thermo Scientific) was used to determine the integrity of RNAs obtained by isolating total RNA. GeneRuler 1 kb DNA Ladder (SM0241, Thermo Scientific) was used to quantify the size of the DNA bands obtained from PCR. The markers used are presented in Figure 6.1.

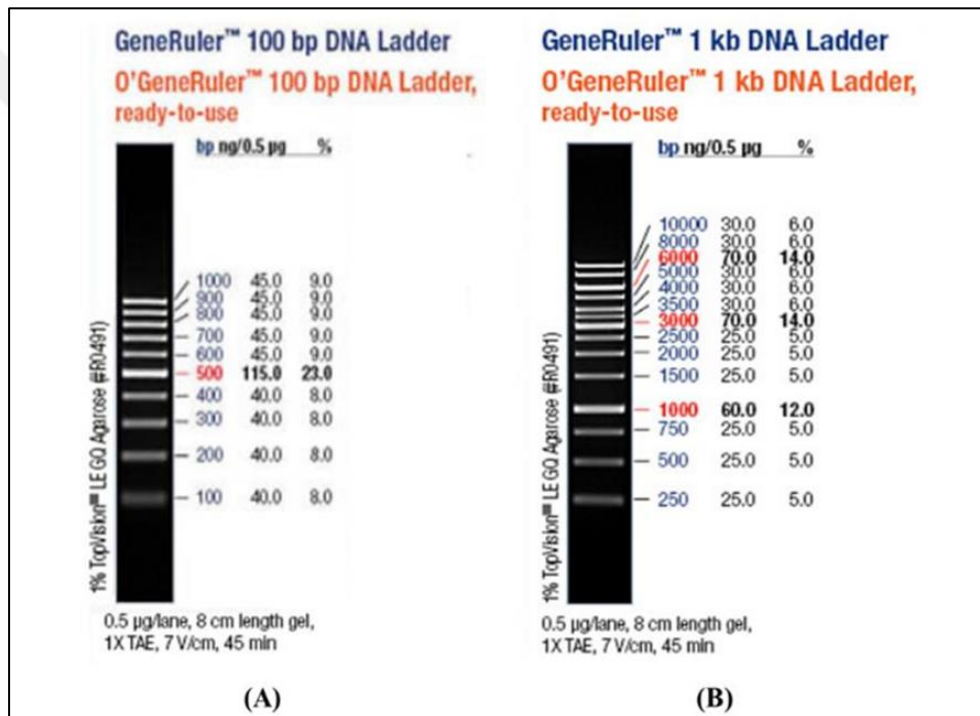


Figure 6.1: Molecular DNA markers used in the study. A) 100 bp Molecular DNA Marker (SM0311, Thermo Scientific); B) 1 kb Molecular DNA Marker (SM0241, Thermo Scientific).

6.1.5. Antibiotic and Media

6.1.5.1. Antibiotics

Apramycin (Apr) was used to grow recombinant strains. 50 mg/ml stock antibiotic was prepared using distilled water as a solvent and added to the media at a ratio of 50 (µg/ml) during the experiments.

6.1.5.2. Media

The Tryptic Soy Broth (TSB) as a pre-culture, TBO agar for sporulation, Tryptic Soy Agar (TSA) as a control for purity of the harvested spores, and Glucose/Yeast/Malt (GYM) medium for RNA isolation were used and they were prepared by the methodology outlined below.

Tryptic Soy Broth (TSB)

30 g TSB medium (Oxoid) was weighed and dissolved in dH₂O to a final volume of 1 L. Then the medium is autoclaved at 121 °C for sterilization for 20 minutes.

Tryptone Soy Agar (TSA)

- 1 L TSB medium
- 15 g Agar

One liter of TSB medium is prepared and divided into bottles containing 1.5% agar. Then sterilization is performed by autoclaving at 121 °C for 20 minutes.

TBO Agar

- Oat Bran
- Tomato Paste
- Agar

20 g tomato paste, 20 g oats, and 25 g agar were mixed and adjusted to a final volume of 1 L with dH₂O, autoclaved at 121°C for 20 min.

Glucose/Yeast/Malt (GYM) medium

- Glucose

- Yeast extract
- Malt extract
- Agar

4 g glucose, 4 g yeast, and 10 g malt extract were weighed and dissolved in 900 ml dH₂O. The pH was adjusted to 7.2, and 20 g agar was added to the mixture. After that, the final volume was completed to 1000 ml with dH₂O and autoclaved at 121°C for 20 min.

To compare the data obtained in this work with the results of a recombinant strain obtained in another study using the pSPG*lon* plasmid which has a strong glycerol-inducible promoter in front of *lon* gene, 5% glycerol was added to all media except TBO. Sterilized glycerol is added to the sterile liquid media at a concentration of 5% before inoculation. The same procedure is employed for solid media, with the addition of glycerol just before pouring the media into petri dishes.

6.1.6. Solutions

The solutions used in this study are listed in Table 6.1.

Table 6. 1: The solutions.

Solutions	Ingredients
Tris-EDTA (TE) Buffer	• 10 mM Tris-HCl (pH 8) • 1 mM EDTA (pH 8)
Tris- Acetate -EDTA (TAE) (50X)	• 242 g Tris Base • 57.1 ml Glacial acetic acid • 100 ml 0.5 M EDTA (pH 8)

6.1.7. Microorganisms

The bacterial strains used in this study and their genetic properties are listed in Table 6.2.

Table 6.2: The bacterial strains used in this study.

Bacterial Strains	Genotypic Properties	Reference
<i>S. coelicolor</i> A3(2) (Sco)	Wild type	[117]
<i>S. coelicolor</i> A3(2) + pRA _{lon} (Sco-pRA _{lon})	Strain that carries pRA _{lon} recombinant plasmid	[7]
<i>S. coelicolor</i> A3(2) + pRA (Sco-pRA)	Strain carrying pRA plasmid	[7]

6.1.8. Plasmids

The recombinant strains, (Sco-pRA, Sco-pRA_{lon}) used in this study; were created by the pRA plasmid which is an *E. coli-Streptomyces* shuttle vector derived from pSET152 (Figure 6.2). This integrative vector carries an apramycin resistance gene (*aac*(3) IV) and enables the insertion of a single copy of a gene to be transferred into the chromosome. The recombinant plasmid carrying the gene wanted to be integrated can be inserted into the attB region (approximately 34 bp) in the *Streptomyces* genome through the ϕ C31 int/attP region in the pRA vector [117]. In a previous study in our laboratory, it was found that the pRA-*lon* vector is integrated into the genome of *S. coelicolor* A3(2) +pRA_{lon} at two different sites: firstly, into the SCO3798 gene encoding a putative chromosome condensation protein, and secondly into the SCO3793 gene encoding a hypothetical protein. Hence, the *S. coelicolor* A3(2) +pRA_{lon} strain has two extra copies of the *lon* gene (unpublished data).

6.1.9. Primers

The *hrdB* primers used in the PCR reaction are listed in Table 6.3. The housekeeping genes *hrdB* and *gyrA* primers used as reference in RT-qPCR, and developmental genes of *Streptomyces* used in RT-qPCR are shown in Table 6.4 and Table 6.5, respectively.

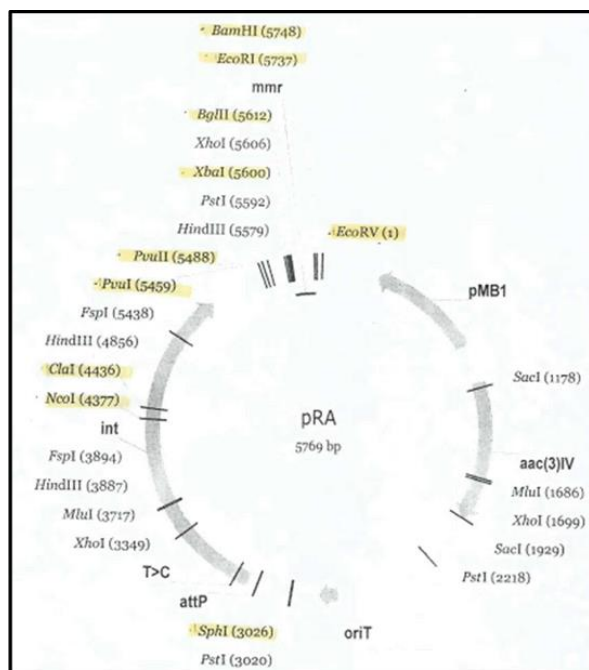


Figure 6.2: pRA shuttle vector (5769 bp).

Table 6.3: *hrdB* primer for PCR used in this study.

Gene	Oligonucleotide sequence 5' 3'	Amplicon Size (bp)
<i>hrdB</i> -F	CTCTTCCTGGACCTCATCCA	543 bp
<i>hrdB</i> -R	GTACACCTTGCCGATCTCGT	

Table 6.4: *hrdB* and *gyrA* primer for RT-qPCR, used in this study.

Gene	Oligonucleotide sequence 5' 3'	Amplicon Size (bp)
<i>hrdB</i> -F	TGCCTTCCTGGACCTCAT	86 bp
<i>hrdB</i> -R	GAACCTGTAGCCCTTGGTGTAG	
<i>gyrA</i> -F	GGTACACCGAGTGCAAGAT	89 bp
<i>gyrA</i> -R	GTCGTAGTTGTCCGTGAAGT	

Table 6.5: Primers of developmental genes for RT-qPCR, used in this study.

Developmental Gen	Amplicon Size (bp)	Sequences 5'-->3'	Developmental Gen	Amplicon Size (bp)	Sequences 5'-->3'
<i>bld D</i>	20	F -- GGTGACTACAACGGCAAGGT	<i>ssgB</i>	20	F -- AGACCGTCGAGTGGGTCTTC
	19	R -- GGCGACTGGTCGTAGATGA		19	R -- AGCTGAGAGCGATGCACAC
<i>bldA</i>	19	F -- GGATGGTGGAATGCAGACA	<i>divIVA</i>	20	F -- CTTCCTCGATGAGGTCGAAG
	17	R -- CGGAGCCGGACTTGAAC		20	R -- CGCATGTTCTGCTGGTTCT
<i>bldN</i>	20	F -- TACGACCAGTACAGCGACAC	<i>parA</i>	20	F -- GACGATCCTCTCACCATGT
	20	R -- GGTAGGTGAAGTGCCGATG		19	R -- TAGGAGAGGGCTCCACTTGA
<i>bldM</i>	20	F -- CTGATTCTGATGGACGTGCG	<i>parB</i>	20	F -- AGAGATCGTGACCCTCATGG
	20	R -- CGACGGTGAGCATGATGATG		20	R -- GAATCCACGGTGATCTTGC
<i>adpA /bldH</i>	20	F -- CGTACCTCGACAGGTCTTT	<i>mreB</i>	20	F -- CTGCCAATACCGCCAGTC
	20	R -- GTCTCCACGTCGAACTGCTC		20	R -- GGCACCCTTGACGTACAC
<i>bldG</i>	20	F -- GACTTCCTCGACTCCACAGG	<i>pdtA</i>	20	F -- GTGAACAACACCACCACAA
	20	R -- GAGGTGTGAATGGGGAACAC		20	R -- ATCTGGAGGTAGGGCGTGTC
<i>whiG</i>	20	F -- ACCGTCGTCACGCTGTACTA	<i>dasR</i>	20	F -- GCTGCAACTCACCTCGTACA
	20	R -- ACCGACTTGGTGTGGATCTG		20	R -- GTGATGTAGCCGATGTCCAG
<i>whiH</i>	19	F -- AGGAGATCGGACAGCGTTT	<i>sigF</i>	20	F -- TACAGCAACCTCACGAGTC
	20	R -- ATCCAGCAGGTTCCAGTCAC		20	R -- CCTCAACCGCTGGAACTAC
<i>WhiE</i>	20	F -- ACTACCAGGTGACCGTCGAG	<i>sigH</i>	20	F -- GTGAGATCAAGCGGCACTTC
	19	R -- AAGGTGTGAAACCGTCGT		20	R -- CTCCGAGATCGACAGCTTCT
<i>whiA</i>	19	F -- CAGCGGTGAAGAGCGAGAT	<i>ramR</i>	20	F -- ATGGTTCGTATTGCCGTGGT
	20	R -- CCCGAAGATCTCCAGGATGT		20	R -- GTCGTCGGATCTGAGCAGTT
<i>chpD</i>	19	F -- AACGTCATCCAGGTTCCCG	<i>ramC</i>	20	F -- AAGCCGCTCAACACGTTCTT
	20	R -- GTTCAGCAGACCAACGACGT		20	R -- AACAGGTGCAGGTGCTTGAA
<i>chpA</i>	17	F -- GGCAACGAGTGCGAGAA	<i>crgA</i>	20	F -- GTCACGTATCCGAAGAAGG
	18	R -- CGAAGTCGAGGACGAGGA		20	R -- AACCGTCGGTGACGTAGAAG
<i>rdlA</i>	19	F -- TCGAGGGCAGCTGAACAA	<i>cdgE</i>	18	F -- TCAAGCGGGTGAACGACA
	20	R -- TTGATGTCCTGGATCGGCAC		19	R -- GCGAGCAGACAGAACTCGT
<i>rdlB</i>	20	F -- TCATCCAGGGCTCGTTCAAC	<i>cdgC</i>	20	F -- GGCATCCTCTACAACGTGCT
	20	R -- CGTTGGAGGACAGGATCGAG		20	R -- GCGGAAGTGGTTCTCTTCT
<i>ftsZ</i>	20	F -- CCTCGCAGTCATCAAAGTCA	<i>rmdB</i>	20	F -- AACTCGAGATCACGGAGCAC
	19	R -- AGACCGACCTCGATCATCC		20	R -- AGTCGTCCAGGGACATCTTG
<i>ssgA</i>	18	F -- AGCTCTCTTCCGCATCC	<i>rmdA</i>	19	F -- CCTGGAGAAAGCCCTGAAC
	18	R -- GTCTCCGGGCAGATGAAA		20	R -- CTGTCGTTGACGGTCTTGAA

6.2. Method

6.2.1. Transcriptional Analysis of Developmental Genes of Sco, Sco-pRA and Sco-pRA_{lon}

6.2.1.1. Collection of Cell Pellets

Spores of Sco, Sco-pRA, and Sco-pRA_{lon} cells were cultivated in 5% glycerol-TSB medium at 30 °C for two days. Concurrently, GYM medium was poured into Petri dishes, and unbleached baking paper, which will serve as cellophane discs, were placed on them after being sterilized. These papers were initially subjected to microwave irradiation in boiling water at a wavelength of 600 for six minutes after they were autoclaved at a temperature of 120 °C for 20 minutes. It is important and necessary to place cellophane or similar structures between the medium and the cells to be cultured to obtain pure, agar-free cell pellets of *Streptomyces* species because they have an agarose activity and mycelial life cycle. Equal wet-weight cell pellets obtained from pre-cultures were inoculated into GYM media. After inoculation, the cell pellets were scraped from the medium using a sterile toothpick for total RNA isolation at 16th, 48th, and 72nd hours of growth and collected into Eppendorf tubes. Cell pellets were dissolved in 1 ml TE buffer and were partitioned into Eppendorf tubes as 200 µl aliquots. These tubes were centrifuged at 11.000 rpm for 20 minutes at +4 °C and then washed twice with TE buffer under similar centrifugation conditions. Finally, the pellets were frozen with liquid nitrogen and stored at -80 °C for later use.

6.2.1.2. RNA Isolation

RNA isolations were performed from Sco, Sco-pRA, and Sco-pRA_{lon} cell pellets collected at 16th, 48th, and 72nd hours of growth. To perform total RNA isolation, the cell pellet must first be dissolved with TE buffer and subjected to lysozyme. For this purpose, the cell pellets were incubated at 37 °C for 90 minutes with ~ 200 µl TE buffer and ~ 70 µl lysozyme (10 mg/ml). During incubation, Eppendorfs were gently shaken every half hour. Following the incubation, RNA isolation was carried out using the "NucleoSpin RNA Plus" isolation kit (MN) by the protocol recommended by the manufacturer company by following the steps below.

- 350 μ L of lysis buffer (LBP) was added to the lysed cell pellets and incubated for 4-5 minutes.
- To remove genomic DNA from the cell lysate, it was transferred to the column specified by the kit (NucleoSpin® gDNA Removal Column) and centrifuged at 8.117 x rpm for 30 seconds and the column was removed. This column retains unwanted DNA.
- 100 μ L BS (RNA-binding solution) was added to the centrifuged liquid and kept for 1-2 minutes.
- The mixture treated with the binding solution was transferred to the kit-specific column (NucleoSpin® RNA Plus Column), which allows the RNA to bind, and centrifuged at 8.117 x rpm for 15 seconds. The liquid obtained at this stage is waste and the RNA is retained in the column.
- After centrifugation, the column was transferred to a new collection tube and washed three times. First, 200 μ L of WB1 (wash buffer number 1) was added to the column and centrifuged at 8.117 x rpm for 15 seconds. Then 600 μ L of WB2 (wash buffer number 2) was added and the same procedure was repeated. Finally, 250 μ L of WB2 was added and the column was centrifuged at 8.117 x rpm for 2 min.
- After washing, the column was taken to nuclease-free Ependorfs, and 30 μ L RNase-free H₂O was added and centrifuged at 8.117 x rpm for 1 minute. After repeating this process, RNA isolation was completed.

All incubation procedures were carried out at room temperature as recommended by the kit. Approximately 60 μ L of RNA solution was obtained as a result of isolation. The concentrations and OD 260/280 ratios of the isolated RNAs were measured with the "NanoDrop Lite" spectrophotometer.

6.2.1.3. Agarose Gel Electrophoresis

Agarose gel electrophoresis was employed to ascertain the integrity of the RNA. A high-density 1.6% (w/v) agarose gel was prepared to facilitate the visualization of the RNA bands. For this purpose, 0.8 g agarose was added to 50 ml 1x TAE and dissolved in a microwave oven. Following cooling, 2.5 μ L RedSafe was added to the gel and poured into the tray with a well comb. Once solidified, samples containing 6x Loading Dye were diluted to 1x as the final concentration of the loading dye and loaded into

the wells, after which electrophoresis was performed under 90V current for 40-45 minutes. The resulting bands were visualized under UV.

6.2.1.4. DNase Treatment

The risk of DNA contamination during RNA isolation is very high due to the nature of prokaryotic genetic material, which can lead to unwanted results in RT-qPCR. To eliminate this contamination, RNA samples were incubated with deoxyribonuclease I enzyme. The components of the reaction are described in Table 6.6. All reaction components were mixed and incubated at 37 °C for 1 h and 20 minutes. DNase was inhibited by adding 1 µl of 50 mM EDTA to the reaction tube and incubating at 75 °C for 10 minutes. This process is necessary to prevent cDNA, which is obtained in the later stages of the experiment, degradation with DNase.

Table 6.6: DNase Treatment.

REACTION COMPONENTS	PROCEDURE
6000 ng RNA Sample 10 µl Reaction Buffer 1 µl DNase Complete 100 µl dH ₂ O	The reaction components were mixed and incubated at 37 °C for 70 minutes.
1 µl EDTA	Incubated at 75 °C for 10 min for DNase inhibition

6.2.1.5. Control of Genomic DNA Contamination in Isolated RNAs

To confirm the success of the DNase treatment, DNase-treated RNA samples were used as a template in a PCR reaction with Taq DNA polymerase enzyme and primers specific to the *hrdB* gene. The *Sco* genomic DNA was used as a positive control, while a template-free reaction was set up as a negative control (Non-Template Control). The PCR was carried out as recommended by the manufacturer. Reaction detail was shown in Table 6.7. The PCR products were visualized with agarose gel electrophoresis to ascertain the presence of DNA contamination. At this stage, the appearance of bands

in reactions that used RNA as a template indicated the potential for genomic DNA contamination. In such instances, the process of DNase treatment was repeated.

Table 6.7: PCR reaction components and procedure.

REACTION COMPONENTS	PROCEDURE
2.5 µl 10X Taq Buffer	Initial Denaturation: 95 °C/1'
2 µl 25 mM MgCl ₂	
2.5 µl dNTP mixture (10 mM)	Denaturation: 95 °C/15" Annealing: 59.5 °C/30" Extension: 72 °C/30" 40 cycle
1.25 µl Primer F (10 µM)	
1.25 µl Primer R (10 µM)	
1 µl RNA as a Template	Final Extension: 72 °C/3' Final: +4 °C/∞
1 µl Taq DNA polymerase	
13.5 µl Nuclease-free dH ₂ O	

6.2.1.6. The cDNA Synthesis

The cDNA was synthesized from the RNA samples, which were ensured to be free of DNA contamination, using the "iScript cDNA Synthesis Kit" (Bio-rad, USA). By the instructions provided in the kit, an appropriate quantity of RNA was added to the PCR tubes, ensuring that each RNA sample concentration was 600 ng with spectrophotometric measurement. Subsequently, 4 µl of 5X reaction mixture (comprising oligo dT and random hexamer primer) was added, and the final volume was completed to 19 µl with nuclease-free dH₂O. Samples were pre-treated by incubation at 70°C for 5 minutes and then placed on ice for 2-3 minutes before the addition of reverse transcriptase enzyme. This pre-treatment was conducted to prevent the formation of primary dimer and hairpin structures. Following this, 1 µl of iScript reverse transcriptase enzyme was added to each tube. The reaction mixture, with a final volume of 20 µl, was incubated by the procedure outlined in Table 6.8 and cDNA synthesis was completed. The cDNA was diluted with 20 µl of nuclease-free dH₂O. The objective of this step was to increase the amount of usable cDNA solution obtained in a single reaction. To ascertain whether dilution affected the cDNA efficiency, RT-qPCR reactions were set up using 40 µl of cDNA solution prepared with a total of 600 ng RNA and 20 µl of undiluted cDNA solution, which is the reaction volume of the

kit prepared with 300 ng RNA. Since the data from the two RT-qPCR results were consistent, it was concluded that there was no negative effect of dilution. cDNA synthesis was carried out in 40 µl as mentioned above.

Table 6.8: cDNA Synthesis Kit's reaction components and procedure.

REACTION COMPONENTS	PROCEDURE
10 µl RNA Sample 4 µl 5X reaction mix [oligo(dT) and random hexamer primer] 5 µl Nuclease-free dH ₂ O	Incubating at 70 °C for 5 minutes Keeping on ice for 2-3 minutes
1 µl iScript reverse transcriptase	25 °C/5' 46 °C/60' 85 °C/5'

6.2.1.7. Primer Synthesis

The primers used in this thesis were designed using the Primer 3 program and the Primer BLAST program of the National Center for Biotechnology Information (NCBI). The criteria considered in primer design are as follows.

- Low homodimer formation rates of the primers. Forward (F) and reverse (R) primers could form homodimers by themselves
- Low heterodimer formation rates of the primers. F and R primers could form heterodimers with each other
- Melting Temperature
- Primer length
- G-C content

Each designed primer pair was subjected to analysis using the OligoAnalyzer Tool (IDT) and Primer Select under DNA Star software. Gradient PCR was conducted to determine the optimal binding temperature for the primers.

Gradient PCR was carried out at varying temperatures (57.5°C, 60°C, 62.5°C) with Taq DNA polymerase, as per the manufacturer's instructions, using Sco genomic DNA as the template.

6.2.1.8. Reverse Transcription-quantitative Polymerase Chain Reaction (RT-qPCR)

In relative quantitation studies, *gyrA* and *hrdB* genes were used as reference genes. The changes in gene expression of Sco-pRA, and Sco-pRA_{lon} cells were calculated by the $2^{-\Delta\Delta CT}$ formula [118], comparing with the wild-type Sco strain. SYBR Green qPCR Master Mix (Bio-rad, USA) and StepOnePlus™ Real-Time PCR System (Applied Biosystems) were used in RT-qPCR studies which were performed by the reaction volume and cycling conditions recommended by the kit (Table 6.9).

Table 6.9: SYBR Green qPCR Master Mix components and RT-qPCR procedure.

REACTION COMPONENTS	PROCEDURE
10 µl SYBR Green super mix (2X)	Initial Denaturation: 95 °C/1'
3 µl cDNA (30 ng)	Denaturation: 95 °C/15"
0.8 µl Primer F (10 µM)	Annealing: 59.5 °C/30"
0.8 µl Primer R (10 µM)	Extension: 60 °C/30"
8.4 µl Nuclease-free dH ₂ O	Melting curve: 55-95 °C
	Measurement at each 0.1 °C change

The expression levels of the developmental genes of Sco, Sco-pRA, and Sco-pRA_{lon} were quantitatively determined by SYBR Green-based real-time PCR analysis. To determine a general profile, 32 genes were analyzed at the 16th h. 32 genes listed in Table 6.10. Afterward, the study focused on 12 genes and analyzed their expression levels at 48th and 72nd hours. These 12 genes were selected based on their roles in the developmental cycle and the results of the 16th h expression analysis. These genes are *bldD*, *bldN*, *bldM*, *adpA*, *whiA*, *whiG*, *whiH*, *chpA*, *rdlA*, *ftsZ*, *sigF*, *ssgB*.

Table 6.10: Target genes and the cellular events they are involved in.

Aerial Mycelium Formation <i>bld</i> Gene	Sporulation <i>whi</i> Gene	Hydrophobic Cover Chaplin and Rodlin	Divisome	Regulators	Synthesis and Degradation of c-di GMP
<i>bldD</i> SCO1489	<i>whiA</i> SCO1950	<i>chpD</i> SCO2717	<i>ftsZ</i> SCO2082	<i>dasR</i> SCO5231	<i>cdgE</i> SCO4931
<i>bldA</i>	<i>whiE</i> SCO5316	<i>chpA</i> SCO2716	<i>ssgA</i> SCO3926	<i>sigF</i> SCO4035	<i>cdgC</i> SCO5511
<i>bldN</i> SCO3323	<i>whiG</i> SCO5621	<i>rdlA</i> SCO2718	<i>ssgB</i> SCO1541	<i>sigH</i> SCO5243	<i>rmdB</i> SCO5495
<i>bldM</i> SCO4768	<i>whiH</i> SCO5819	<i>rdlB</i> SCO2719	<i>divIVA</i> SCO2077	<i>ramR</i> SCO6685	<i>rmdA</i> SCO0928
<i>adpA</i> SCO2792			<i>parA</i> SCO3886	<i>ramC</i> SCO6681	
<i>bldG</i> SCO3549			<i>parB</i> SCO 3887	<i>crgA</i> SCO3854	
			<i>mreB</i> SCO 2451		
			<i>pdtA</i> SCO2578		

6.2.2. Harvesting of *Streptomyces* Spores

- Spores of Sco, Sco-pRA, and Sco-pRA_{lon} were inoculated into 100 ml TSB+Apramycin at 30 °C for 2 days as a preculture.
- End of the incubation, culture was centrifuged at 3.500 rpm for 20 minutes.
- Following centrifugation, a portion of the supernatant is removed and collected as a pellet. At this stage, approximately 5-10 ml of supernatant is required to dissolve the cells.
- 300 µl of the mix was spread onto TBO containing antibiotics and incubated at 30 °C for 10 days for sporulation.
- After the incubation, sterile 40% 5 ml glycerol was added onto a plate for scratching spores from the agar surface via sterile cotton swaps.
- The spore-glycerol solution was collected into a sterile falcon and then centrifuged at 4.500 rpm, at +4 °C for 20 min.
- The vast majority of the supernatant was removed, and the pellet was dissolved in the residual supernatant.
- Lastly, 250 µl spore suspension was aliquoted into labeled Eppendorf tubes and stored at -80 °C.

Spores were serially diluted (10^2 - 10^8) and incubated in TSA at +30 °C for 4 days. After the incubation, grown colonies were counted and the number of spores was calculated with the given equation (6.1).

$$\text{Number of Spores } \left(\frac{\text{cfu}}{\text{mL}} \right) = (\text{colony number} \times \text{dilution factor}) \times \left(\frac{V \text{ total}}{V \text{ inoculation}} \right)$$

(6.1)

7. RESULTS

7.1. Transcriptional Analysis of Developmental Genes of Antibiotic Over-Producer Sco-pRA_{lon} Mutant Strain

Among the bacterial kingdoms, *Streptomyces* is characterized by its unique life cycle and production of secondary metabolites. Increasing the production of these secondary metabolites and bringing them into industrial and clinical use is of great value. Previous work in our laboratory has demonstrated that having two extra copies of the *lon* gene in the Sco-pRA_{lon} genome enhances antibiotic production. Secondary metabolites are produced when *Streptomyces* differentiate to form aerial hyphae. Given the strong correlation between secondary metabolite production and differentiation, understanding the life cycle of *Streptomyces* will shed light on understanding of secondary metabolite production.

In this study, transcriptional analysis of developmental genes of recombinant Sco-pRA_{lon} strain, a high antibiotic producer, was performed. The expression of 32 (*bldD*, *bldG*, *bldA*, *bldH*, *bldN*, *bldM*, *chpA*, *chpD*, *rdlA*, *rdlB*, *ramR*, *ramC*, *whiA*, *whiG*, *whiH*, *whiE*, *ftsZ*, *ssgA*, *ssgB*, *parA*, *parB*, *divIVA*, *mreB*, *pdtA*, *dasR*, *crgA*, *sigF*, *sigH*, *cdgE*, *cdgC*, *rmdA*, and *rmdB*) developmental genes identified through literature searches as critical for the *Streptomyces* life cycle, was analyzed via RT-qPCR using 16th h RNA samples isolated from the wild type (Sco), Sco-pRA, and Sco-pRA_{lon} strains. The next stage of the study focused on 12 (*bldD*, *bldH*, *bldN*, *bldM*, *chpA*, *rdlA*, *whiA*, *whiG*, *whiH*, *ftsZ*, *ssgB*, *sigF*) of these genes that were considered significant and analyzed the changes in gene expression at 48th and 72nd hours of growth.

7.1.1. Determination of Optimum Binding Temperature for Primers

Primers were synthesized for 32 genes and, according to the results of gradient PCR, their optimum binding temperature was found to be 60°C. Figure 7.1 shows the representative results of the experiment carried out on three primer pairs specific for *adpA*, *parB* and *ramC* genes.

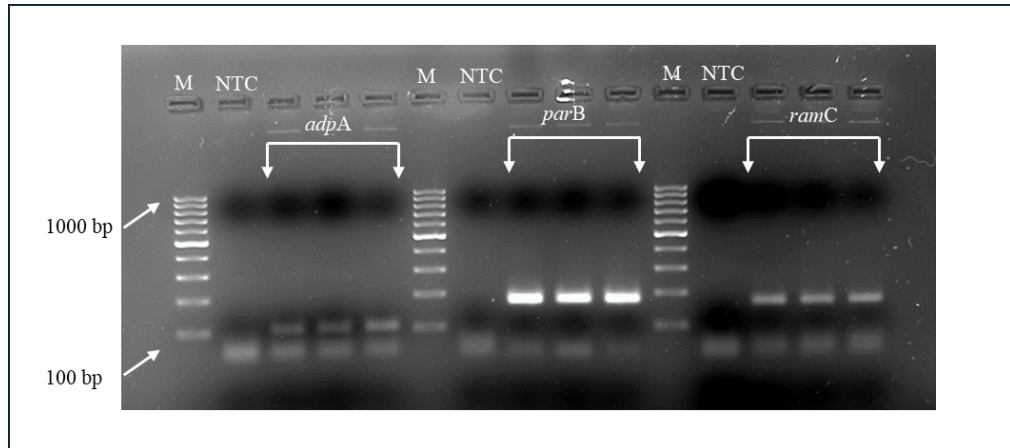


Figure 7.1: Gradient PCR results using genomic DNA as a template with primers prepared for RT-qPCR. M: 100 bp molecular DNA marker, NTC; non template control. PCR products of *adpA*, *parB* and *ramC* genes obtained at binding temperatures of 57.5°C, 60°C, 62.5°C.

7.1.2. RNA Isolation from Sco, Sco-pRA and Sco-pRA_{lon} Cells

Sco, Sco-pRA, and Sco-pRA_{lon} cells were grown on a GYM solid medium and total RNA was isolated from samples taken at 16th, 48th, and 72nd hours of growth. After the isolation, the RNA samples were run on 2% agarose gel to confirm their integrity, and the results indicated successful isolation as shown in Figure 7.2.

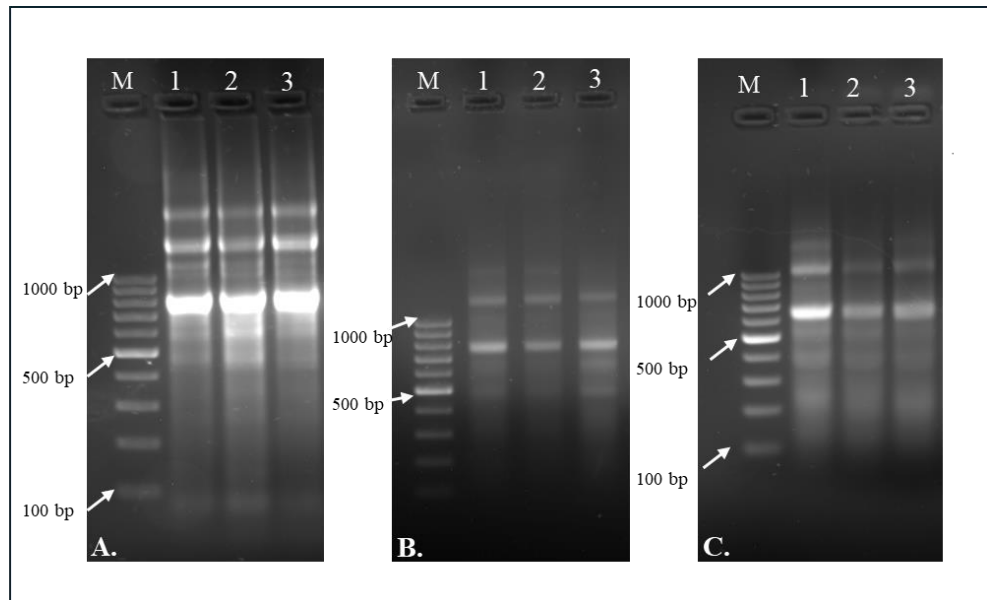


Figure 7.2: Total RNA isolated from Sco (lane 1), Sco-pRA (lane 2) and Sco-pRA_{lon} (lane 3) cells; M: marker, **A.** 16th h RNA samples **B.** 48th h RNA samples **C.** 72nd h RNA samples.

7.1.3. DNase Treatment of RNA Sample

The isolated RNAs were incubated with DNase enzyme and tested for the presence of genomic DNA contamination. This control was conducted by using RNA samples as a template in a PCR reaction established with the Taq polymerase enzyme, which can only amplify DNA. In this reaction, Sco genomic DNA as a positive control template and primers specific to the *hrdB* gene were used. The absence of bands in the PCR reaction using NTC (non-template control) and RNA samples as templates indicated that there was no genomic DNA contamination, and that the DNase treatment was successful (Figure 7.3).

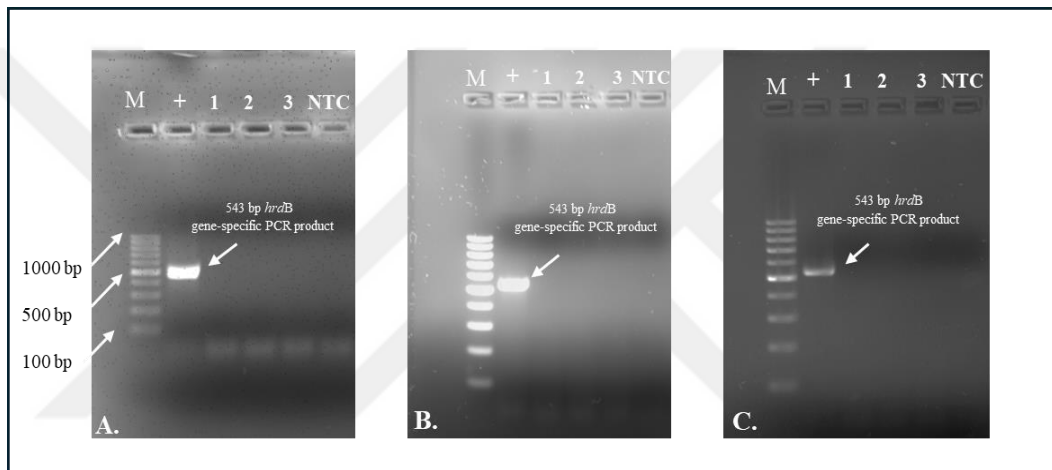


Figure 7.3: PCR results of RNA samples of Sco (lane 1), Sco-pRA (lane 2), and Sco-pRA_{lon} (lane 3) after DNase treatment **A.** 16th h RNA samples **B.** 48th h RNA samples **C.** 72nd h RNA samples M; marker, +: positive control, NTC; non template control.

Two biological replicates of RNA samples from Sco, Sco-pRA, and Sco-pRA_{lon} cells, grown for 16th, 48th and 72nd hours, were used to synthesize cDNAs. Transcriptional analysis of the genes was performed using these cDNAs and the housekeeping genes *gyrA* and *hrdB* as references. To determine the change in gene expression, the average of the Cp (Crossing point) values was calculated for each sample. The relative quantification analysis was applied to the samples using the formula $2^{-\Delta\Delta CT}$, and the change in gene expression was calculated by comparing the data of recombinant Sco-pRA and Sco-pRA_{lon} strains with the data of wild-type Sco.

7.1.4. Gene Expression Analysis with 16th RNA Samples

The expression changes of the 32 transcriptionally analyzed genes in Sco-pRA and Sco-pRA_{lon} compared to the wild type strain at the 16th h are given in Table 7.1. The fold ratios for the increase and decrease in gene expression are shown by schematizing with heatmap (Figure 7.4).

A general decrease in the expression of *bld* genes was observed in Sco-pRA_{lon} compared to the wild type strain, whereas a 2.5-fold increase in *bldD* expression was observed. The expression of *whi* genes in Sco-pRA_{lon} showed an unexpected increase at the 16th h. Expression differences were also observed for some genes in the Sco-pRA strain, containing the empty plasmid used as a control, although the expression changes in Sco-pRA were not as dramatic as in Sco-pRA_{lon} and were considered to be random.

Table 7.1: 16th h gene expression changes.

	Genes	Sco-pRA Expression level at 16 th h	Sco-pRA _{lon} Expression level at 16 th h
Aerial Mycelium	<i>bldD</i>	Non-significant	2.5-fold Increase
	<i>bldA</i>	2-fold Increase	2.6-fold Decrease
	<i>bldN</i>	1.6-fold Decrease	8-fold Decrease
	<i>bldM</i>	1.2-fold Decrease	3.4-fold Decrease
	<i>adpA /bldH</i>	1.2-fold Decrease	1.3-fold Increase
	<i>bldG</i>	1.4- fold Decrease	1.4- fold Decrease
Sporulation	<i>whiG</i>	2.9-fold Increase	11-fold Increase
	<i>whiH</i>	Non-significant	3-fold Increase
	<i>whiE</i>	1.6-fold Decrease	2.7-fold Increase
	<i>whiA</i>	1.2-fold Decrease	1.5-fold Increase
Hydrophobic Cover	<i>chpD</i>	Non-significant	1.6-fold Increase
	<i>chpA</i>	Non-significant	Non-significant
	<i>rdlA</i>	Non-significant	2.8-fold Increase
	<i>rdlB</i>	Non-significant	Non-significant
Divisome and Segregation	<i>ftsZ</i>	Non-significant	1.6-fold Increase
	<i>ssgA</i>	1.2-fold Decrease	1.4-fold Decrease
	<i>ssgB</i>	1.6-fold Increase	2-fold Decrease
	<i>divIVA</i>	1.5-fold Decrease	3-fold Decrease
	<i>parA</i>	2.3-fold Decrease	1.4-fold Increase
	<i>parB</i>	1.5-fold Decrease	Non-significant
	<i>mreB</i>	Non-significant	Non-significant
	<i>pdtA</i>	Non-significant	2-fold Increase
Regulators	<i>dasR</i>	Non-significant	1.1-fold Increase
	<i>sigF</i>	1.2-fold Increase	4.7-fold Increase
	<i>sigH</i>	1.2-fold Decrease	1.4-fold Decrease
	<i>ramR</i>	Non-significant	2.7-fold Decrease
	<i>ramC</i>	1.4-fold Decrease	Non-significant
	<i>crgA</i>	Non-significant	2-fold Increase
c-di GMP Metabolism	<i>cdgE</i>	1.2-fold Increase	1.6-fold Increase
	<i>cdgC</i>	Non-significant	3.3-fold Decrease
	<i>rmdA</i>	2.5-fold Decrease	2.7-fold Decrease
	<i>rmdB</i>	1.4-fold Decrease	1.6-fold Decrease

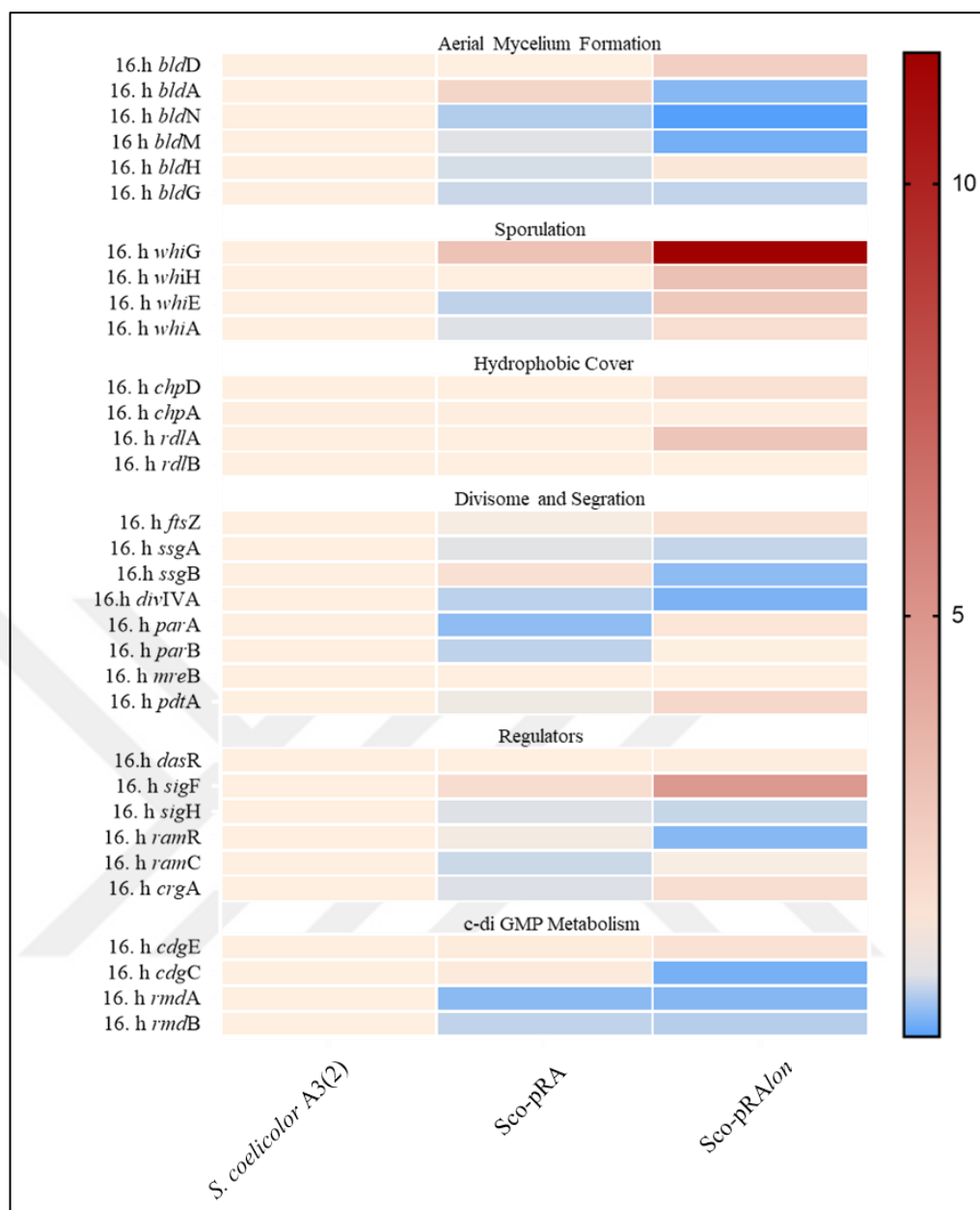


Figure 7.4: Representation of fold difference of 16th h gene expression change with heat map.

7.1.4.1. Result of Expression Levels of *bld* Genes at 16th h of Growth

The differentiation of vegetative hyphae into aerial hyphae during the development of *Streptomyces* is controlled by the *bld* genes. The phenotype of these genes is characterized by the formation of bald colonies, in which the development of aerial hyphae is either absent or defective. Most of *bld* mutants display a distinct pattern of antibiotic production, which differs from that observed in the wild type strain.

Expression changes of the *bld* genes -*bldD*, *bldH*, *bldN*, *bldM*, *bldA*, *bldG*- were analyzed.

bldD gene product is a master regulator for the *Streptomyces*'s development. BldD represses approximately 170 genes during vegetative growth. The results of the expression levels of the *bldD* gene indicated a 2.5-fold increase in the Sco-pRA_{lon} strain compared to the Sco strain at 16th h. In the Sco-pRA strain, the level of *bldD* gene expression did not exhibit a statistically significant change at the 16th h of development (Figure 7.5).

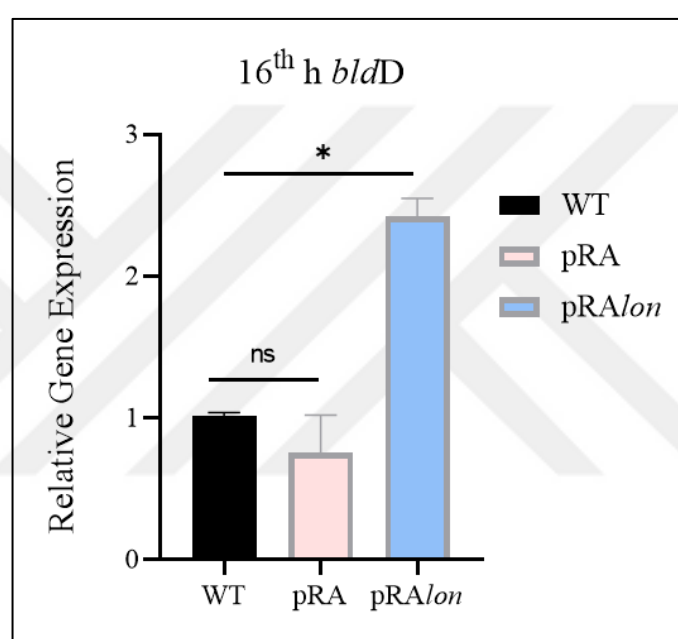


Figure 7.5: The differences in the expression of the *bldD* genes in the Sco-pRA_{lon} and the Sco-pRA strains at the 16th h in comparison to Sco strain. The "*" sign indicates the statistically significant results ($p < 0.05$).

The gene *bldH* (*adpA*) serves as a crucial global regulator of development and antibiotic production. The analysis of *bldH* gene expression revealed an upregulation of 1.3-fold at the 16th h in the Sco-pRA_{lon} strain compared to the Sco strain. In the Sco-pRA strain, the level of *bldH* gene expression was reduced by 1.2-fold at the 16th h (Figure 7.6).

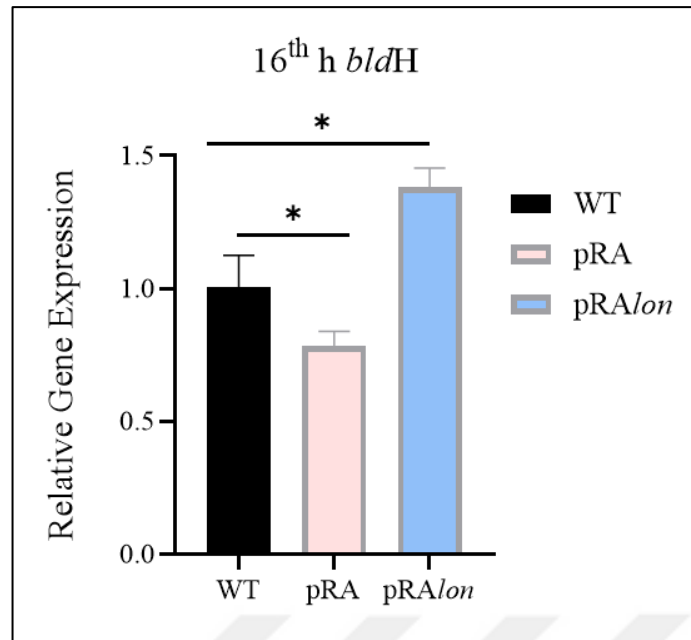


Figure 7.6: The differences in the expression of the *bldH* genes in the Sco-pRA_{lon} and the Sco-pRA strains at the 16th h in comparison to Sco. The "*" sign indicates the statistically significant results ($p < 0.05$).

The gene *bldN* encodes an RNA polymerase σ factor, which regulates the expression of *chp*, *rdl* and *bldM*. The expression of the *bldN* gene was reduced by 8-fold, in the Sco-pRA_{lon} strain compared to the Sco strain at the 16th. Similarly, in the Sco-pRA strain, the *bldN* gene expression was downregulated by 1.6-fold at the 16th h (Figure 7.7).

The *bldM* gene encodes a transcriptional factor that is involved in the formation of aerial hyphae as a homodimer, and it is involved in sporulation as a heterodimer form with WhiI. The expression of the *bldM* gene decreased by 3.4-fold at the 16th h in the Sco-pRA_{lon} strain compared to the Sco strain. In the Sco-pRA strain, the level of *bldM* gene expression was reduced by 1.2-fold at the 16th h (Figure 7.8).

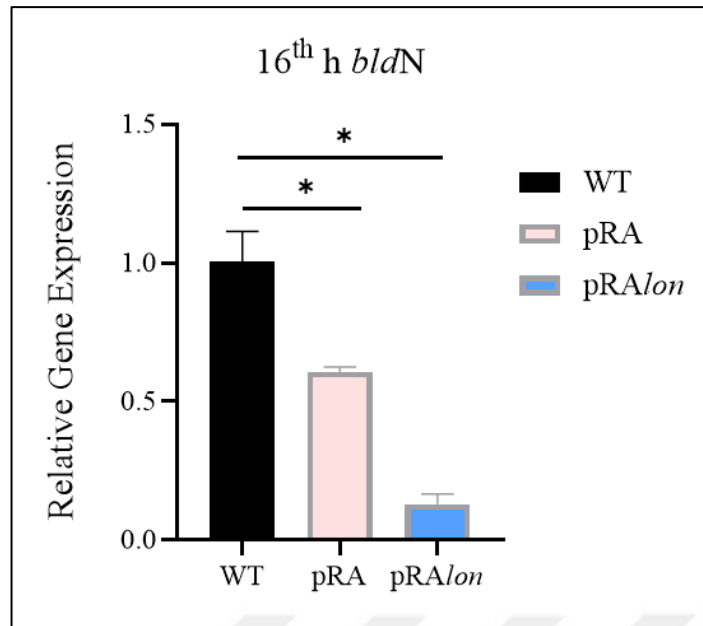


Figure 7.7: The differences in the expression of the *bldN* genes in the Sco-pRA_{lon} and the Sco-pRA strains at the 16th h in comparison to Sco strain. The "*" sign indicates the statistically significant results (p<0.05).

The *bldM* gene encodes a transcriptional factor that is involved in the formation of aerial hyphae as a homodimer, and it is involved in sporulation as a heterodimer form with WhiI. The expression of the *bldM* gene decreased by 3.4-fold at the 16th h in the Sco-pRA_{lon} strain compared to the Sco strain. In the Sco-pRA strain, the level of *bldM* gene expression was reduced by 1.2-fold at the 16th h (Figure 7.8).

The gene expression levels of the global regulator *bldA*, which provides tRNA for the rare leucine codon UUA, were calculated at the 16th h (Figure 7.9). The *bldA* expression levels of Sco-pRA were found to be 2-fold upregulated, while those of Sco-pRA_{lon} exhibited a 2.6-fold decrease compared to the Sco strain.

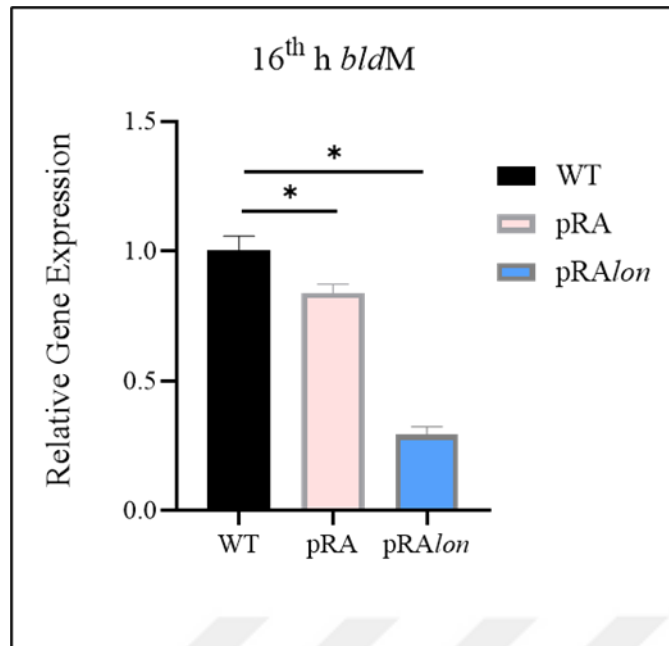


Figure 7.8: The differences in the expression of the *bldM* gen in the Sco-pRA_{lon} and the Sco-pRA strains at the 16th h in comparison to Sco strain. The "*" sign indicates the statistically significant results ($p < 0.05$).

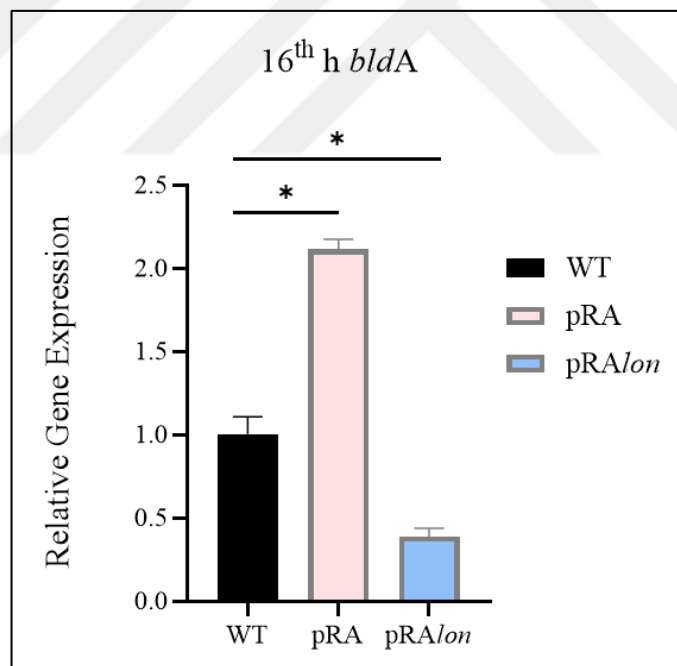


Figure 7.9: The differences in the expression of the *bldA* gen in the Sco-pRA_{lon} and the Sco-pRA strains at the 16th h in comparison to Sco strain. The "*" sign indicates the statistically significant results ($p < 0.05$).

BldG is a pleiotropic anti-anti-sigma factor whose targets include sigma factors related to stress response and differentiation. The gene expression levels of *bldG* at 16th h were calculated (Figure 7.10). The expression levels of both Sco-pRA and Sco-pRA_{lon} decreased by 1.4-fold compared to the Sco strain.

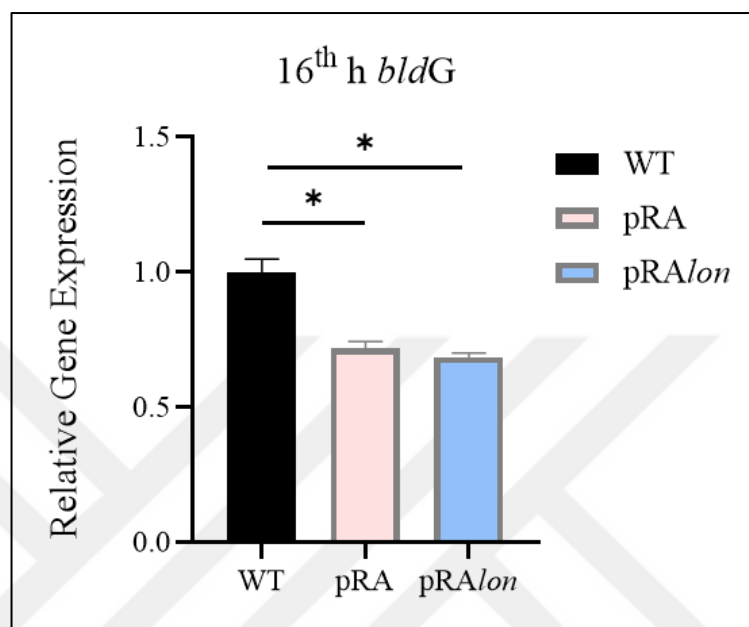


Figure 7.10: The differences in the expression of the *bldG* genes in the Sco-pRA_{lon} and the Sco-pRA strains at the 16th h in comparison to Sco strain. The "*" sign indicates the statistically significant results ($p < 0.05$).

7.1.4.2. Result of Expression Levels of *whi* Genes at 16th h of Growth

The sporulation of *Streptomyces* is under the control of the *whi* genes. The disruption of these genes results in the formation of defective, spore pigment-deprived spores. Conversely, the overexpression of these genes leads to the premature onset of sporulation. The changes in the expression levels of the *whi* genes (*whiA*, *whiG*, *whiH*, *whiE*) investigated in the study are presented in detail below.

whiA, belonged in the 'early' *whi* regulatory genes. It plays a role in halting the expansion of airtel hyphae and septation. *whiA* gene expression level increased by 1.5-fold at 16th h in the Sco-pRA_{lon} strain compared to Sco. In the Sco-pRA strain, the level of *whiA* gene expression was reduced by 1.2-fold at 16th h (Figure 7.11).

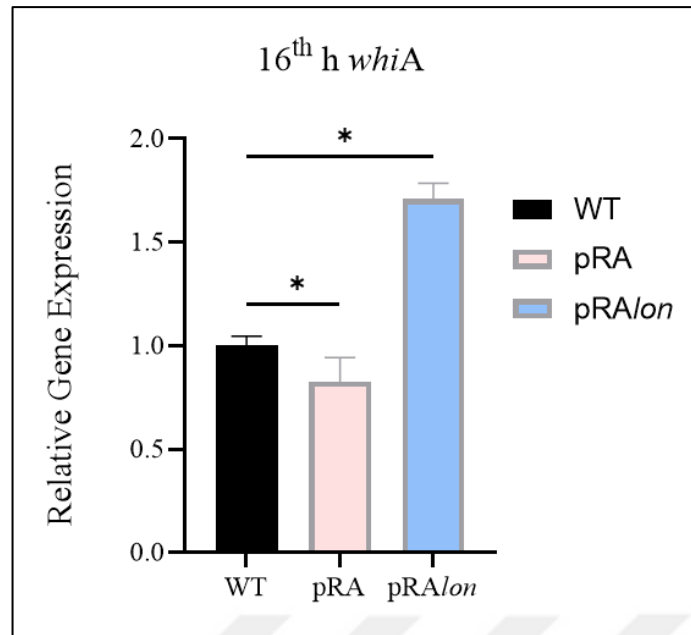


Figure 7.11: The differences in the expression of the *whiA* genes in the Sco-pRA_{lon} and the Sco-pRA strains at the 16th h in comparison to Sco strain. The "*" sign indicates the statistically significant results ($p < 0.05$).

whiG is involved in the early stage of sporulation and is responsible for the expression of an RNA polymerase sigma factor (σ^{whiG}). This sigma factor is activated expression of important sporulation genes such as *whiI* and *whiH*. *whiG* gene expression level was increased 11-fold at 16th h in the Sco-pRA_{lon} strain compared to Sco. In the Sco-pRA strain, *whiG* gene expression increased 2.9-fold at the 16th h (Figure 7.12).

The *whiH* is essential for the regular multi-sporulation septation of aerial hyphae of Sco and for the expression of some late sporulation genes. In the Sco-pRA_{lon} strain, *whiH* gene expression was increased by 3-fold at the 16th h within comparison to Sco. In the Sco-pRA strain, *whiH* gene expression was not statistically significantly changed at the 16th h (Figure 7.13).

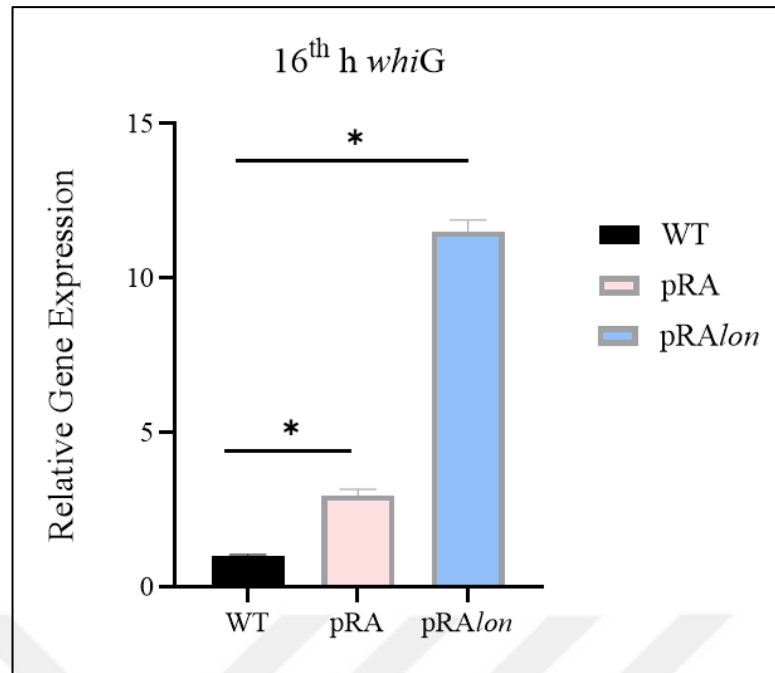


Figure 7.12: The differences in the expression of the *whiG* genes in the Sco-pRA_{lon} and the Sco-pRA strains at the 16th h in comparison to Sco strain. The "*" sign indicates the statistically significant results ($p < 0.05$).

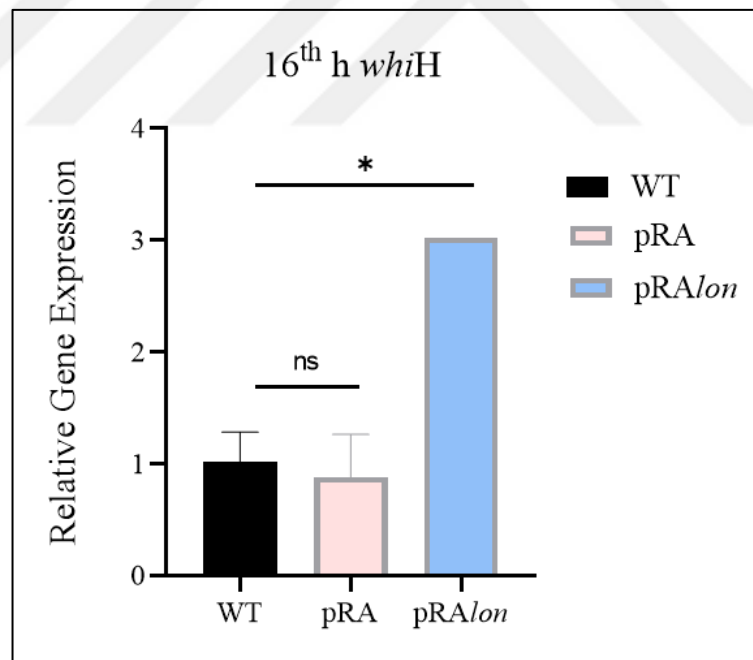


Figure 7.13: The differences in the expression of the *whiH* genes in the Sco-pRA_{lon} and the Sco-pRA strains at the 16th h in comparison to Sco strain. The "*" sign indicates the statistically significant results ($p < 0.05$).

whiE, which is responsible for the formation of spore pigments, belongs to late sporulation genes. Comparative analysis of gene expression levels at 16th h revealed downregulation of Sco-pRA by 1.6-fold and up-regulation of Sco-pRA_{lon} expression by 2.7-fold relative to the Sco strain (Figure 7.14).

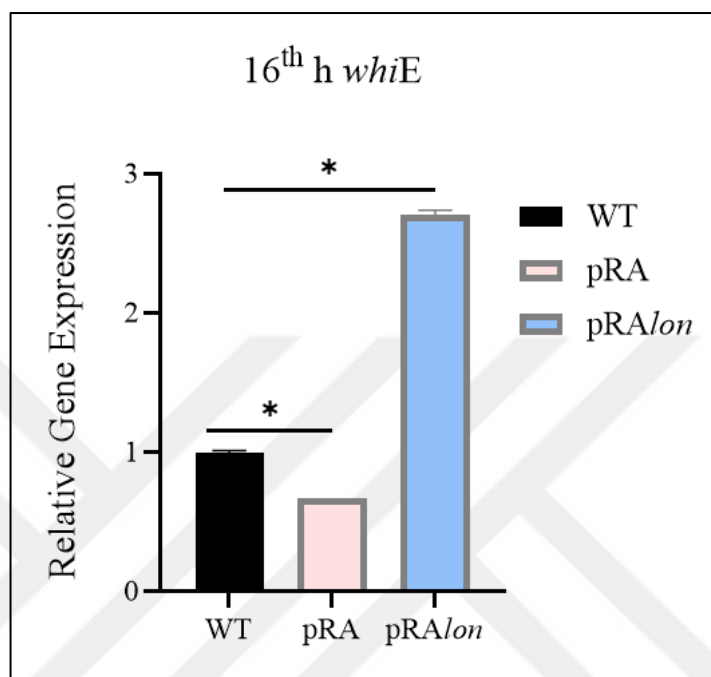


Figure 7.14: The differences in the expression of the *whiE* genes in the Sco-pRA_{lon} and the Sco-pRA strains at the 16th h in comparison to Sco strain. The "*" sign indicates the statistically significant results ($p < 0.05$).

7.1.4.3. Result of Expression Levels of Genes Involved in c-di-GMP Synthesis and Degradation in Recombinant Strain at 16th h of Growth

c-di-GMP is a secondary messenger involved in the control of differentiation, forming a complex with the master regulator BldD. c-di-GMP synthesis is mediated by diguanylate cyclase. In this study, gene expression levels at 16th h for two different diguanylate synthase enzymes, *cdgC*, and *cdgE*, were analyzed. In comparison to the Sco strain, *cdgC* expression was not significantly changed in Sco-pRA but was found to be down-regulated by 3.3-fold in Sco-pRA_{lon}. Conversely, *cdgE* expression was found to be up-regulated by 1.2 and 1.6-fold in Sco-pRA and Sco-pRA_{lon}, respectively (Figure 7.15).

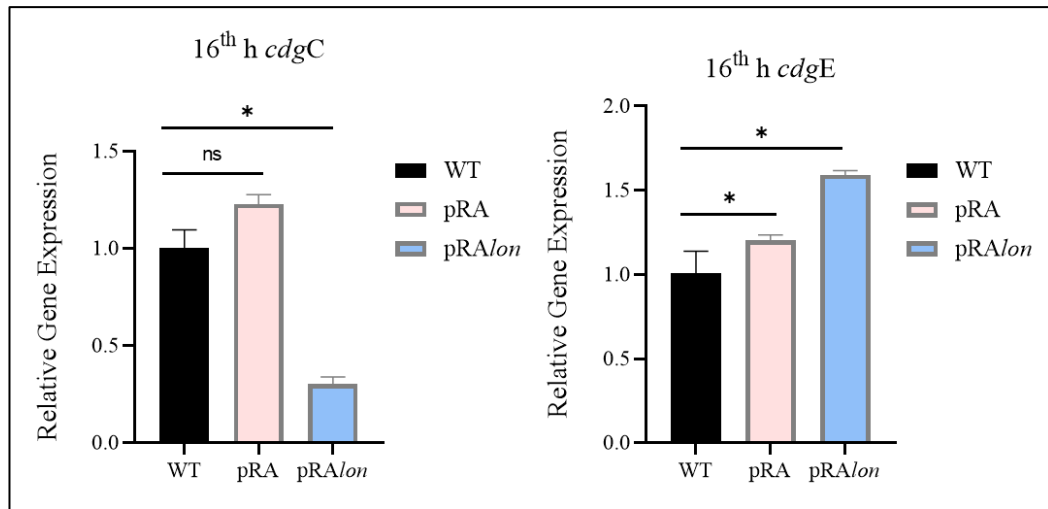


Figure 7.15: The differences in the expression of the *cdgC* and *cdgE* genes in the Sco-pRA_{lon} and the Sco-pRA strains at the 16th h in comparison to Sco strain. The "*" sign indicates the statistically significant results (p<0.05).

The degradation of the c-di-GMP enzyme is conducted by specific phosphodiesterases. In this study, we analyzed the 16th h gene expression levels of *rmdA* and *rmdB* genes involved in the synthesis of RmdA and RmdB phosphodiesterase to obtain information about the degradation of secondary messenger. In comparison to the Sco strain, the *rmdA* expression was found to be down-regulated by 2.5 and 2.7-fold in Sco-pRA and Sco-pRA_{lon}, respectively. Similarly, the *rmdB* expression was observed to be down-regulated by 1.4 and 1.6-fold in Sco-pRA and Sco-pRA_{lon}, respectively at 16th h (Figure7.16).

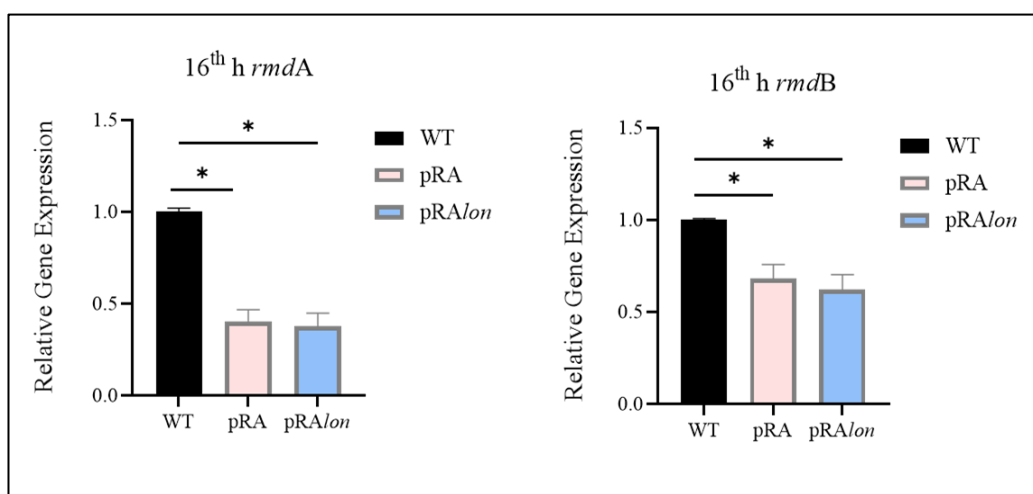


Figure 7.16: The differences in the expression of the *rmdA-rmdB* genes in the Sco-pRA_{lon} and the Sco-pRA strains at the 16th h in comparison to Sco strain. The "*" sign indicates the statistically significant results (p<0.05).

7.1.4.4. Result of Expression Levels of Genes Responsible for Hydrophobic Sheath Formation at 16th h of Growth

As vegetative hyphae differentiate into aerial hyphae, the mycelia become covered with a hydrophobic sheath. This coat then forms the basis of the spore coat. This sheath, consisting of 8 chaplins and 2 rodlin, protects the mycelia against surface tension and disintegration.

RdlA and RdlB are rodlin proteins located in the rodlet layer that form the hydrophobic sheath in the *S. coelicolor*'s aerial hyphae. The *rdlA* gene expression level increased 2.8-fold at the 16th h, in the Sco-pRA_{lon} strain compared to the Sco strain. A comparison of the *rdlA* gene expression levels of the Sco-pRA strain with the Sco strain revealed no statistically significant differences at the 16th h. There was no significant change in *rdlB* expression in both strains at 16th h (Figure 7.17). The change in expression of recombinant strains was calculated according to Sco.

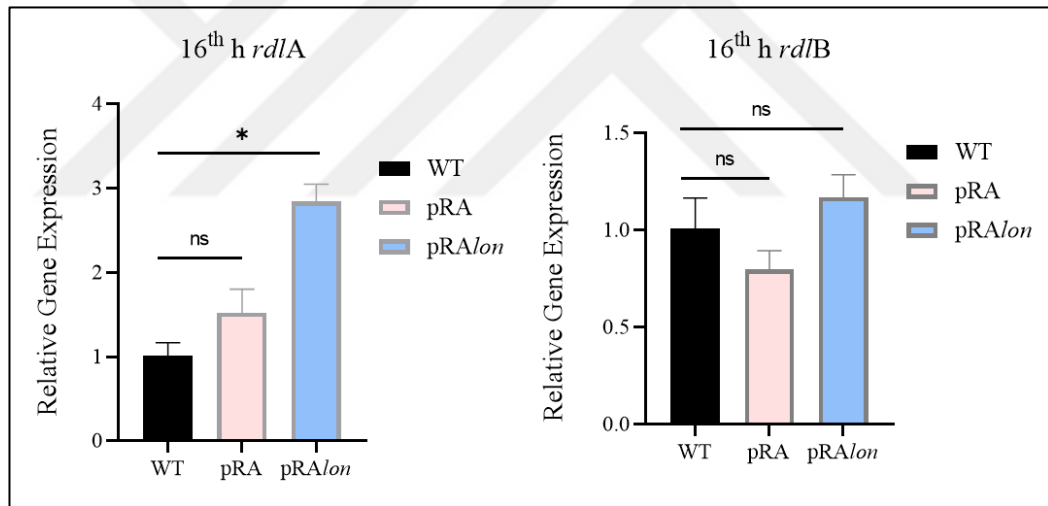


Figure 7.17: The differences in the expression of the *rdlA* and *rdlB* genes in the Sco-pRA_{lon} and the Sco-pRA strains at the 16th h in comparison to Sco strain. The "*" sign indicates the statistically significant results ($p < 0.05$).

In addition to rodlin, another component of the hydrophobic sheath is chaplains. The formation of the hydrophobic sheath is dependent on the presence of chaplains (ChpA-H) they are crucial. At the 16th h, the expression of *chpD* was not significantly altered in Sco-pRA, whereas in Sco-pRA_{lon}, the expression level increased by 1.6-fold in comparison to Sco (Figure 7.18).

The results of the analysis of *chpA* gene expression levels revealed no statistically significant differences between the Sco-pRA_{lon} and Sco strains at 16th h. The *chpA* gene expression levels of the Sco-pRA strain revealed no statistically significant differences at the 16th h (Figure 7.18).

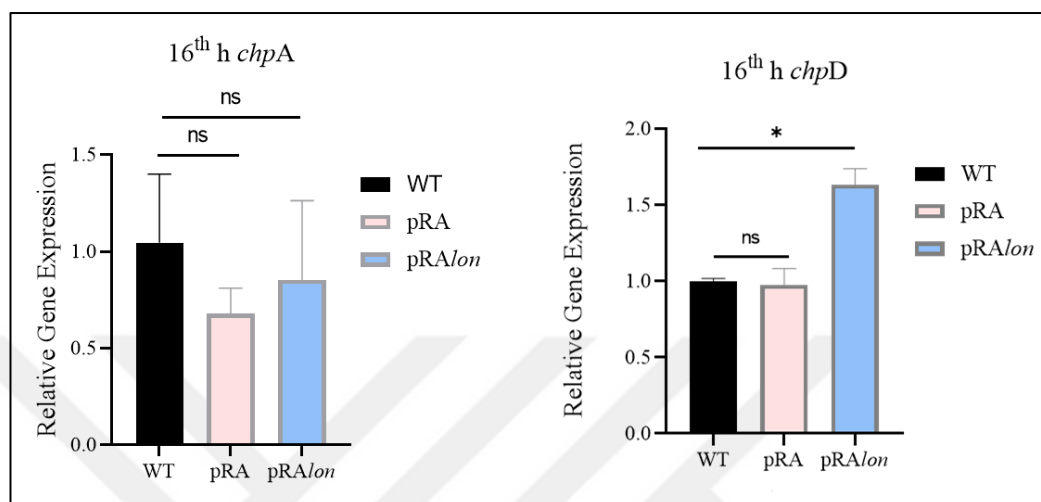


Figure 7.18: The differences in the expression of the *chpA* and *chpD* genes in the Sco-pRA_{lon} and the Sco-pRA strains at the 16th h in comparison to Sco strain. The "*" sign indicates the statistically significant results (p<0.05).

7.1.4.5. Result of Expression Levels of Divisome and Septation Genes at 16th h of Growth

Cell division and septation are crucial processes during *Streptomyces* development. The growth of hyphae and the concomitant septation of multinucleate hyphae to form unigenomic spores are orchestrated by a complex system comprising numerous molecules. The present thesis comprises a transcriptional analysis of the *divIVA*, *ftsZ*, *parA*, *parB*, *ssgA*, *ssgB*, *parB*, *ssgA*, *ssgB* genes, with the objective of investigating the changes in genes related to the regulation of cell division, chromosome segregation and septation in recombinant strains.

The DivIVA is a crucial element in the polarizom machinery for the growth of hyphae. The *divIVA* expression levels of both Sco-pRA and Sco-pRA_{lon} were found to be 1.5 and 3-fold downregulated, respectively, compared to the Sco strain at the 16th h (Figure 7.19). The cytoskeletal protein MreB, which plays a role in determining the shape of rod-shaped bacteria, does not appear to have a similar function in *Streptomyces*.

However, it is known to be involved in the formation of the spore wall. The expression level of *mreB* did not change significantly at the 16th h for both Sco-pRA and Sco-pRA_{lon} (Figure 7.19).

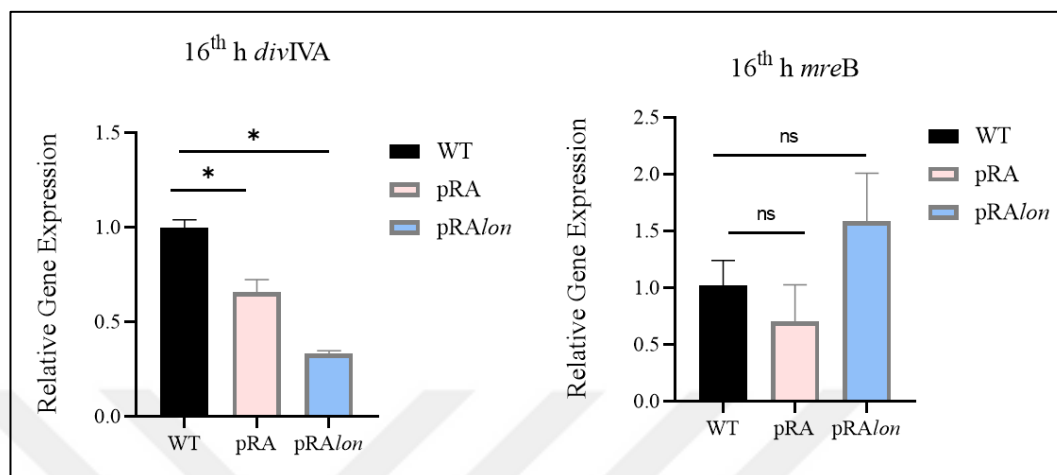


Figure 7.19: The differences in the expression of the *divIVA* and *mreB* genes in the Sco-pRA_{lon} and the Sco-pRA strains at the 16th h in comparison to Sco strain. The "*" sign indicates the statistically significant results ($p < 0.05$).

FtsZ, is expressed by *ftsZ* gene, is involved in Z-ring formation, and it is essential in sporulation septation. It is also functional for cross-wall formation, but it is not essential for vegetative growth. *ftsZ* gene expression was upregulated by 1.6-fold in the Sco-pRA_{lon} strain at 16th h compared to the wild-type Sco strain. The expression of the *ftsZ* gene in the Sco-pRA strain did not exhibit a significant change at the 16th h (Figure 7.20).

The actinomycete-specific proteins SsgA and SsgB are essential for sporulation and localize FtsZ at spore septation. In recombinant strains, Sco-pRA and Sco-pRA_{lon}, *ssgA* gene expression decreased by 1.2 and 1.4-fold, respectively, at 16th h compared to Sco. The *ssgB* gene expression level was decreased 2-fold at 16th h in the Sco-pRA_{lon} strain. In the Sco-pRA strain, *ssgB* gene expression increased 1.6-fold at the 16th h (Figure 7.21).

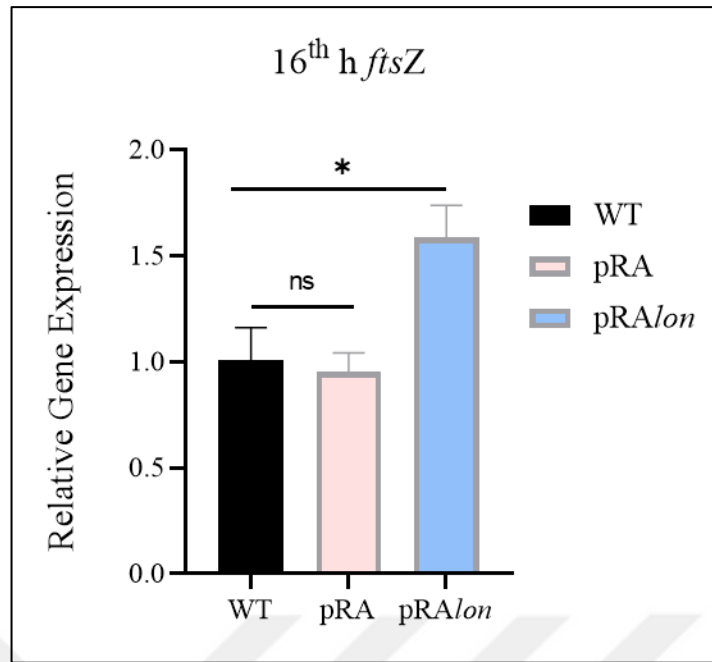


Figure 7.20: The differences in the expression of the *ftsZ* genes in the Sco-pRA_{lon} and the Sco-pRA strains at the 16th h in comparison to Sco strain. The "*" sign indicates the statistically significant results (p<0.05).

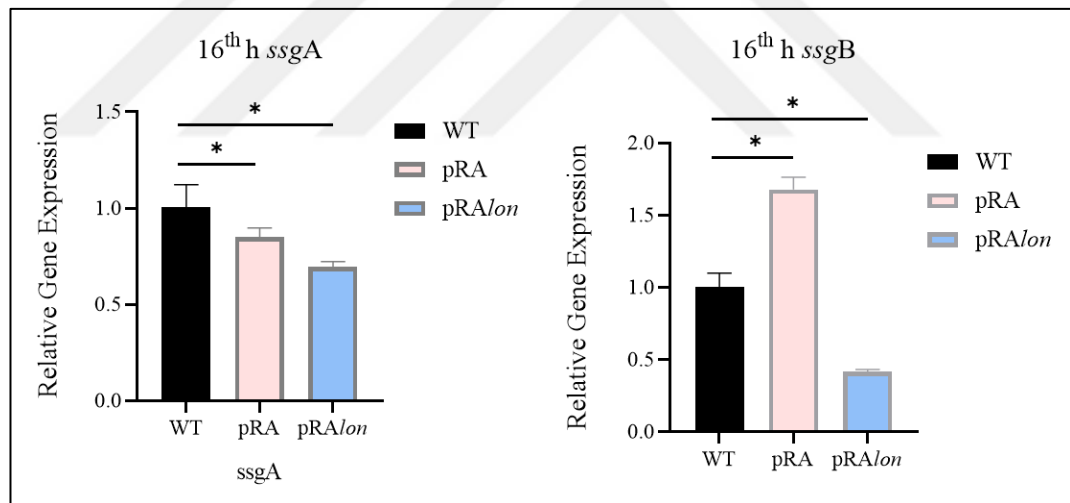


Figure 7.21: The differences in the expression of the *ssgA* and *ssgB* genes in the Sco-pRA_{lon} and the Sco-pRA strains at the 16th h in comparison to Sco strain. The "*" sign indicates the statistically significant results (p<0.05).

The chromosome partitioning proteins ParA and ParB are involved in ensuring that DNA is evenly distributed in unigenomic spore chains. Compared to the Sco strain, *parA* expression was downregulated 2.3-fold in Sco-pRA and upregulated 1.4-fold in Sco-pRA_{lon}; *parB* expression was downregulated 1.5-fold in Sco-pRA and showed no significant change in Sco-pRA_{lon} at 16th h (Figure 7.22).

Polydiglycosylphosphate Transferase (PdtA) is a component of the *Streptomyces* spore wall-synthesizing complex (SSSC), and it is crucial for proper sporulation. There was no significant change in *pdtA* expression in Sco-pRA at 16th h, while in Sco-pRA_{lon} there was an increase of 2-fold compared to Sco (Figure 7.23).

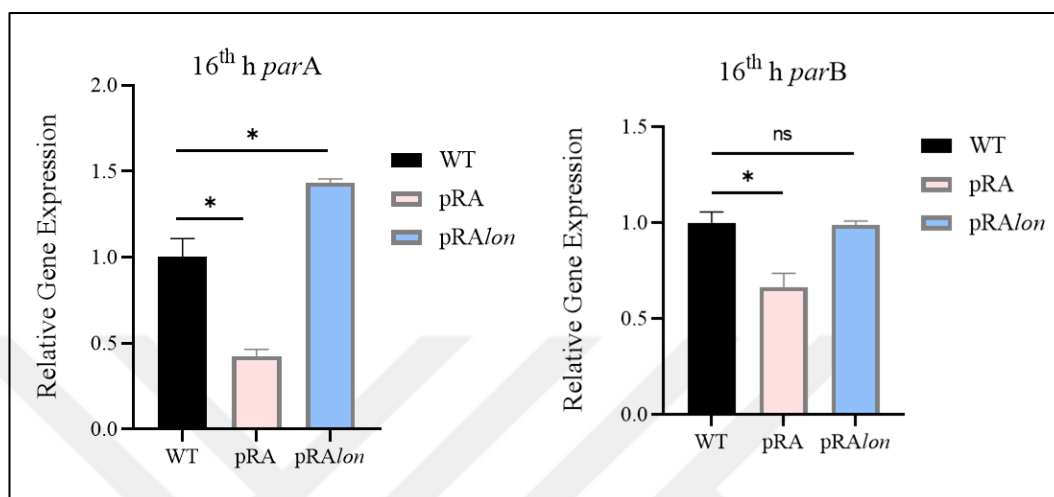


Figure 7.22: The differences in the expression of the *parA* and *parB* genes in the Sco-pRA_{lon} and the Sco-pRA strains at the 16th h in comparison to Sco strain. The "*" sign indicates the statistically significant results ($p < 0.05$).

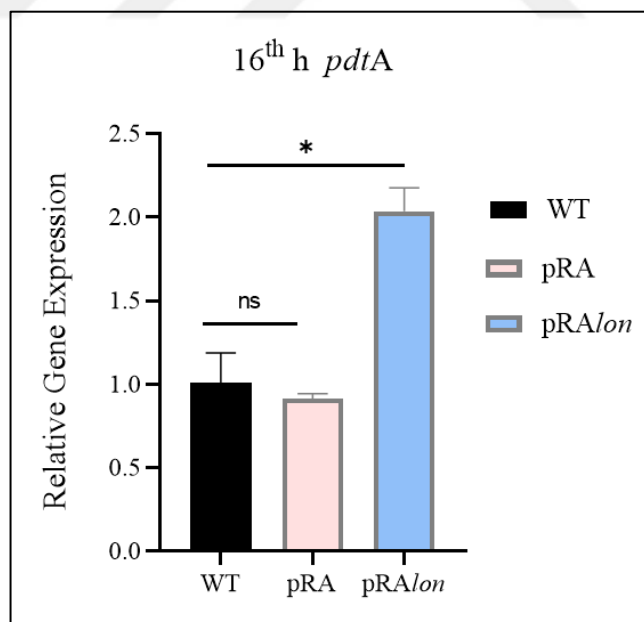


Figure 7.23: The differences in the expression of the *pdtA* gene in the Sco-pRA_{lon} and the Sco-pRA strains at the 16th h in comparison to Sco strain. The "*" sign indicates the statistically significant results ($p < 0.05$).

7.1.4.6. Result of Expression Levels of Regulator Genes at 16th h of Growth

During *Streptomyces* development, numerous molecules exert an indirect or direct influence on the regulation of differentiation. The expression of genes encoding regulators such as *dasR*, *crgA*, *ramR*, *ramC*, and sigma factors *sigH* and *sigF* were examined, and they were classified as regulators in this thesis study.

The actinomycete-specific protein CrgA is responsible for regulating the timing of the onset of reproductive growth and also inhibiting septation in sporulation. There was no significant change in *crgA* expression in Sco-pRA; however, in Sco-pRA_{lon}, *crgA* expression was observed to increase by 2-fold compared to Sco at 16th h (Figure 7.24).

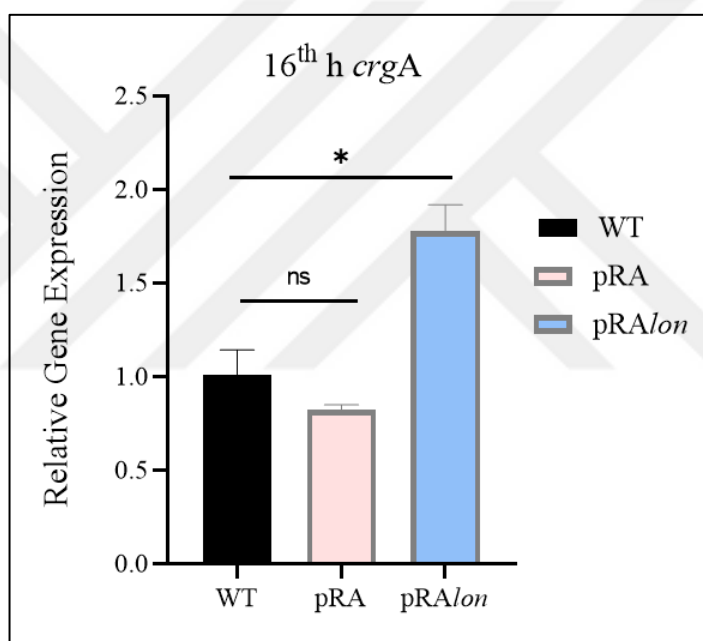


Figure 7.24: The differences in the expression of the *crgA* gene in the Sco-pRA_{lon} and the Sco-pRA strains at the 16th h in comparison to Sco strain. The "*" sign indicates the statistically significant results ($p < 0.05$).

DasR, which derepress antibiotic production during nutrient depletion, is a significant example of the relationship between differentiation and antibiotic production. The 16th h gene expression profile of *dasR* demonstrated that the expression levels were not significantly altered in Sco-pRA and Sco-pRA_{lon} was upregulated by 1.1-fold. A comparison of the recombinants was conducted with the Sco strain, as the other expression analysis (Figure 7.25).

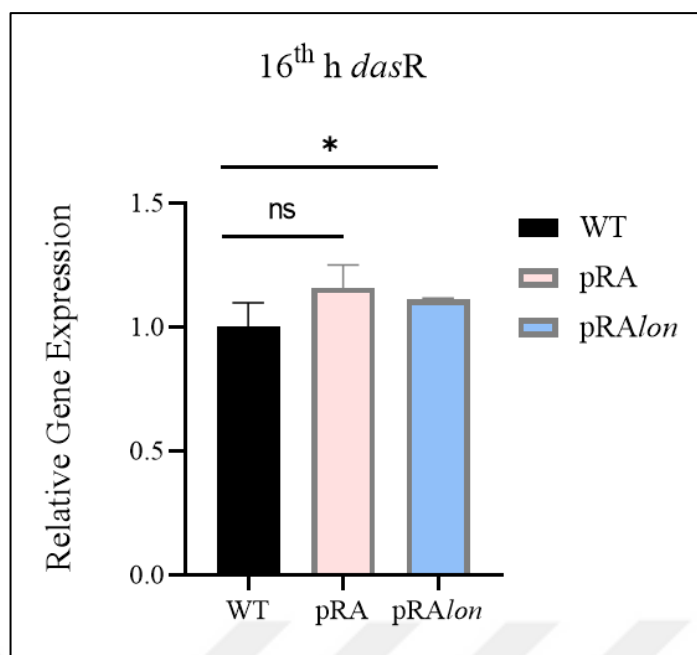


Figure 7.25: The differences in the expression of the *dasR* gene in the Sco-pRA_{lon} and the Sco-pRA strains at the 16th h in comparison to Sco strain. The "*" sign indicates the statistically significant results (p<0.05).

The ramCSABR operon, which is required for the formation of aerial hypha and responsible for SapB production and transport, is regulated by an activator expressed by *ramR*. At the 16th h, *ramR* expression was not significantly changed in Sco-pRA, whereas in Sco-pRA_{lon} the expression level was downregulated by 2.7-fold compared to Sco. In contrast, *ramC* expression decreased by 1.4-fold in Sco-pRA and it was not significantly altered in Sco-pRA_{lon} at 16th h (Figure 7.26).

SigH plays a dual role in the osmotic stress response and differentiation. The *sigH* expression levels of both Sco-pRA and Sco-pRA_{lon} were found to be 1.2 and 1.4-fold downregulated, respectively, in comparison to the Sco strain at the 16th h (Figure 7.27).

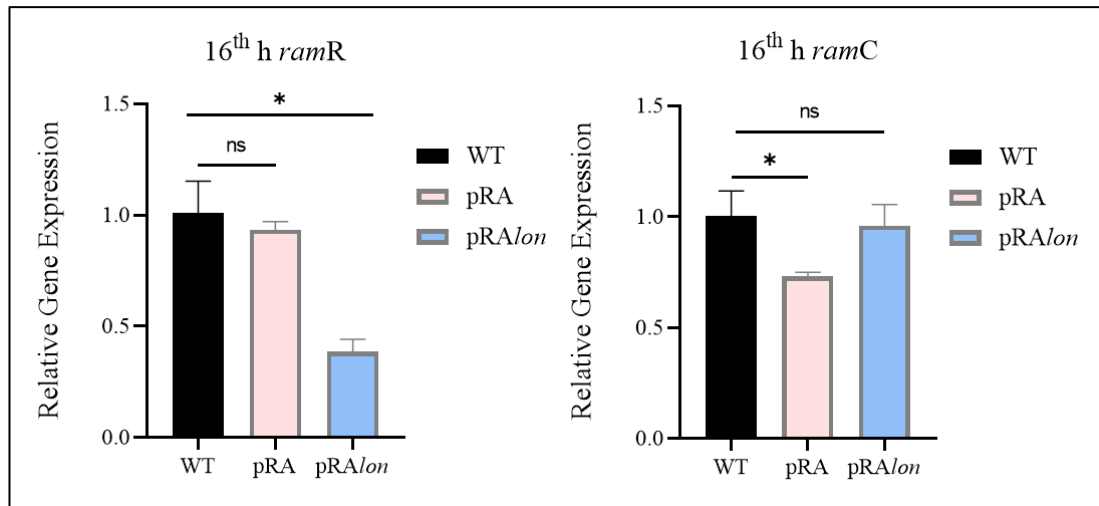


Figure 7.26: The differences in the expression of the *ramR* and *ramC* genes in the Sco-pRA_{lon} and the Sco-pRA strains at the 16th h in comparison to Sco strain. The "*" sign indicates the statistically significant results ($p < 0.05$).

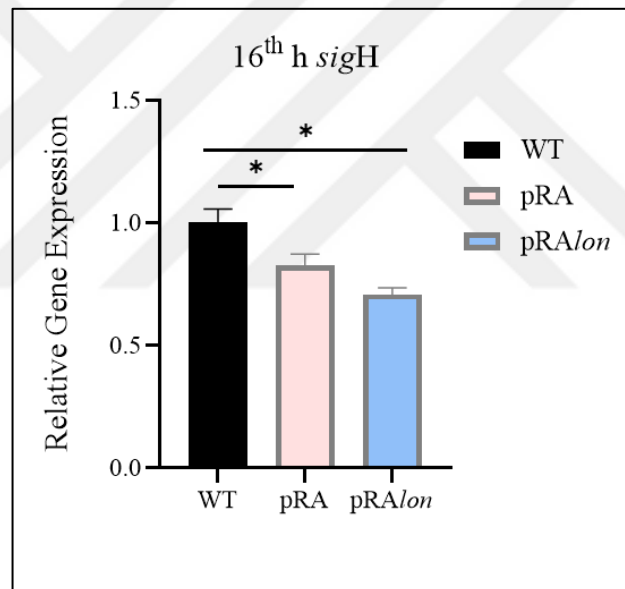


Figure 7.27: The differences in the expression of the *sigH* genes in the Sco-pRA_{lon} and the Sco-pRA strains at the 16th h in comparison to Sco strain. The "*" sign indicates the statistically significant results ($p < 0.05$).

The *sigF* gene is responsible for the expression of σ^F , which is involved in the late stage of sporulation, and it activates the late sporulation gene. *whiE* is an important target for σ^F . The expression of the *sigF* gene was found to be upregulated 4.7-fold in the Sco-pRA_{lon} strain compared to the wild-type Sco strain at 16th h. In the Sco-pRA strain, the *sigF* gene expression was upregulated by 1.2-fold at the 16th h (Figure 7.28).

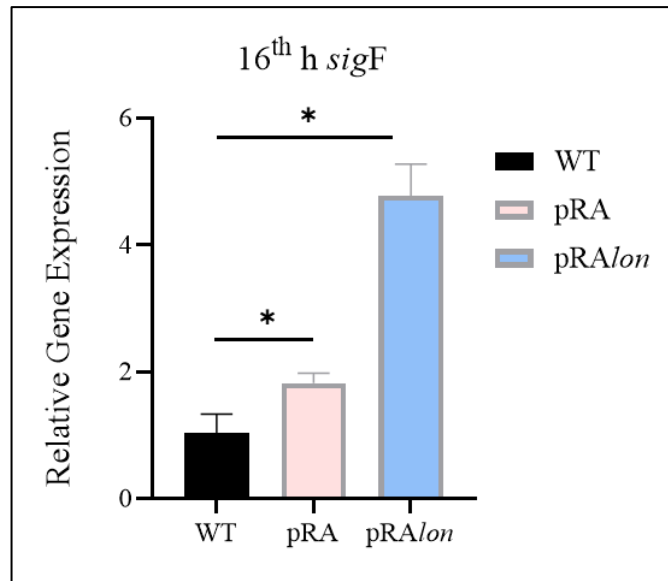


Figure 7.28: The differences in the expression of the *sigF* genes in the Sco-pRA_{lon} and the Sco-pRA strains at the 16th h in comparison to Sco strain. The "*" sign indicates the statistically significant results (p<0.05).

7.1.5. Gene Expression Analysis with 48th and 72nd Hours RNA Samples

According to the 16th h gene expression results, 12 of the 32 genes were selected and the changes in gene expression of these genes at 48th h and 72nd h of development were analyzed. These genes are *bldD*, *bldN*, *bldM*, *adpA*, *whiA*, *whiG*, *whiH*, *chpA*, *rdlA*, *ftsZ*, *sigF*, *ssgB*. The changes in the gene expression of these genes at the 48th h and 72nd h are elucidated (Table 7.2). The changes in gene expression of these genes at the 16th h are given again in conjunction with the data at the 48th h and 72nd h to provide a clear basis for comparison and evaluation of the gene expression changes that occur at three different stages of development.

Table 7.2: A. 48th h gene expression changes **B.** 72nd h gene expression changes.

A.

Gene	Sco-pRA Expression level at 48 th h	Sco-pRA _{lon} Expression level at 48 th h
<i>bldD</i>	3-fold Decrease	2.1-fold Decrease
<i>bldN</i>	1.7-fold Decrease	2-fold Decrease
<i>bldM</i>	Non-significant	2-fold Increase
<i>adpA /bldH</i>	Non-significant	5.8-fold Increase
<i>whiG</i>	Non-significant	1.6-fold Increase
<i>whiH</i>	1.9-fold Increase	1.6-fold Increase
<i>whiA</i>	Non-significant	2.5-fold Decrease
<i>chpA</i>	Non-significant	5.5-fold Increase
<i>rdlA</i>	4.9-fold Increase	8-fold Increase
<i>ssgB</i>	1.6-fold Decrease	2.3-fold Increase
<i>ftsZ</i>	Non-significant	1.7-fold Increase
<i>sigF</i>	2-fold Decrease	Non-significant

B.

Gene	Sco-pRA Expression level at 72 nd h	Sco-pRA _{lon} Expression level at 72 nd h
<i>bldD</i>	Non-significant	1.7-fold Decrease
<i>bldN</i>	Non-significant	1.7-fold Decrease
<i>bldM</i>	Non-significant	Non-significant
<i>adpA /bldH</i>	Non-significant	Non-significant
<i>whiG</i>	1.8-fold Decrease	2.5-fold Decrease
<i>whiH</i>	1.7-fold Decrease	2.8-fold Decrease
<i>whiA</i>	Non-significant	2.3-fold Decrease
<i>chpA</i>	Non-significant	1.6-fold Decrease
<i>rdlA</i>	Non-significant	2.3-fold Decrease
<i>ftsZ</i>	2-fold Decrease	2-fold Decrease
<i>ssgB</i>	Non-significant	Non-significant
<i>sigF</i>	Non-significant	1.8-fold Increase

The results of the expression levels of the *bldD* gene, which is a master regulator for development, indicated a 2.5-fold increase in the Sco-pRA_{lon} strain compared to the wild type strain at 16th h. However, at 48th h and 72nd h, the levels decreased by 2.1 and 1.7 times, respectively. In the Sco-pRA control strain, the level of *bldD* gene expression did not exhibit a statistically significant change at either the 16th h or 72nd h, while a 3-fold decrease was observed at the 48th h (Figure 7.29).

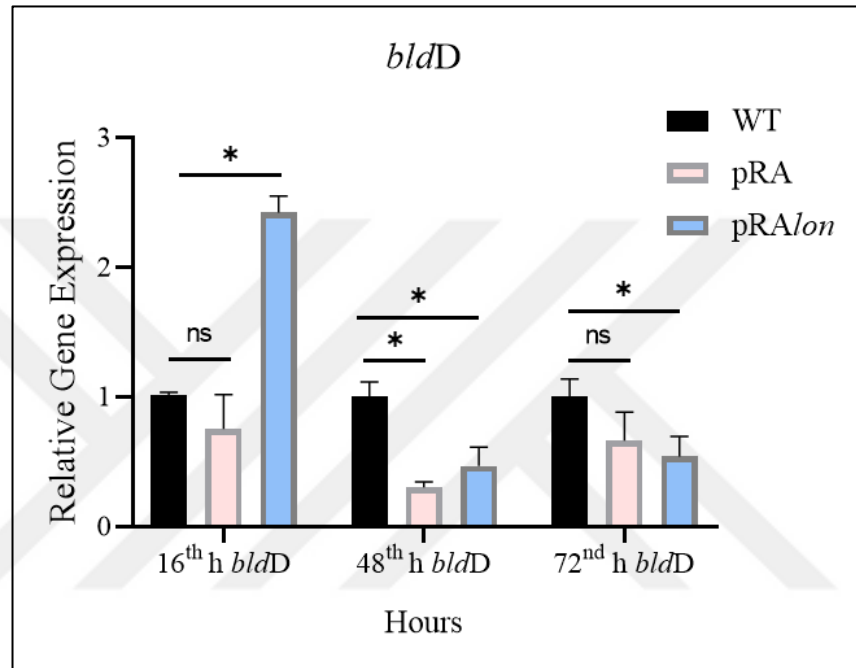


Figure 7.29: The differences in *bldD* gene expression in recombinant strains at 16th, 48th and 72nd hours. The "*" sign indicates the statistically significant results ($p < 0.05$).

The analysis of *bldH* gene expression revealed a significant upregulation of 1.3 and 5.8-fold at the 16th and 48th h, respectively, for the Sco-pRA_{lon} strain compared to the wild type strain. There was no statistically significant change in gene expression at 72nd h. In the Sco-pRA strain, the level of *bldH* gene expression was reduced by 1.2-fold at the 16th h, but *bldH* gene expression were not statistically significant change at either the 48th h or 72nd h (Figure 7. 30).

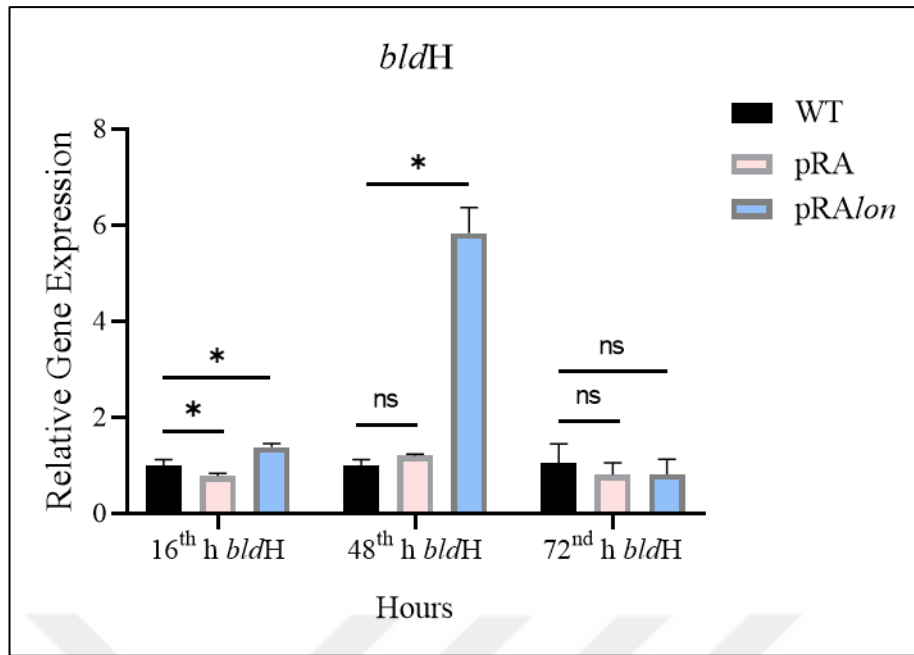


Figure 7.30: The differences in *bldH* gene expression in recombinant strains at 16th, 48th and 72nd hours. The "*" sign indicates the statistically significant results ($p < 0.05$).

In the Sco-pRA_{lon} strain, the expression levels of the *bldN* gene were significantly reduced compared to the wild-type strain. The expression of the *bldN* gene was reduced by 8-fold, 2-fold, and 1.7-fold in the Sco-pRA_{lon} strain at the 16th h, 48th h and 72nd h, respectively. Similarly, in the Sco-pRA strain, the *bldN* gene expression was downregulated by 1.6 and 1.7-fold at the 16th h and 48th h, respectively. However, there was no statistically significant change in gene expression at the 72nd h (Figure 7.31).

The expression of the *bldM* gene decreased by 3.4-fold at the 16th h and increased by 2-fold at the 48th h in the Sco-pRA_{lon} strain compared to the Sco strain. At 72nd h, no significant change in *bldM* expression was observed for this strain. In the Sco-pRA strain, the level of *bldM* gene expression was reduced by 1.2-fold at the 16th h, with no statistically significant change at either the 48th or 72nd hours (Figure 7.32).

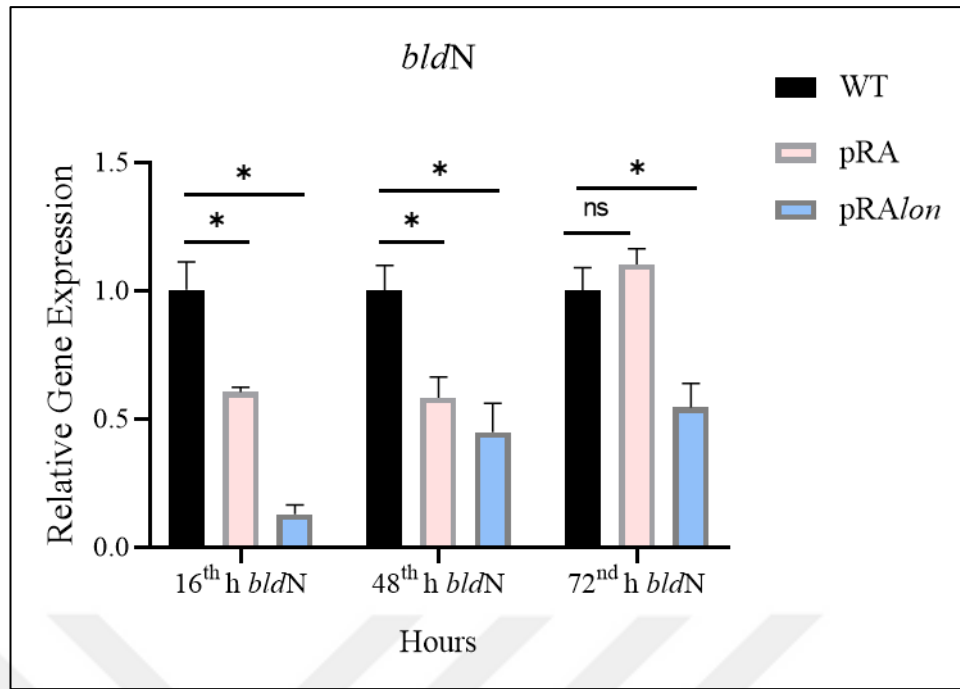


Figure 7.31: The differences in *bldN* gene expression in recombinant strains at 16th, 48th and 72nd hours. The "*" sign indicates the statistically significant results ($p < 0.05$).

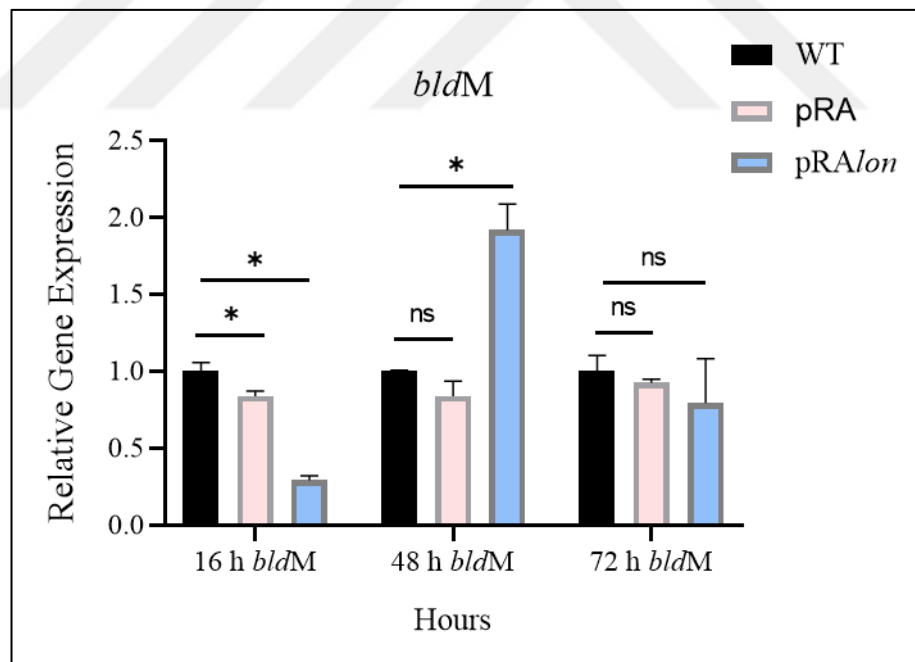


Figure 7.32: The differences in *bldM* gene expression in recombinant strains at 16th, 48th and 72nd hours. The "*" sign indicates the statistically significant results ($p < 0.05$).

The expression of *whiA*, which belonged in the ‘early’ *whi* regulatory genes, was found to be increased by 1.5-fold at 16th h. Gene expression decreased by 2.5-fold, and 2.3-fold at 48th h and 72nd h, respectively, in the Sco-pRA_{lon} strain compared to Sco. In the Sco-pRA strain, the level of *whiA* gene expression was reduced by 1.2-fold at 16th h, with no statistically significant change at either the 48th h or 72nd h (Figure 7.33).

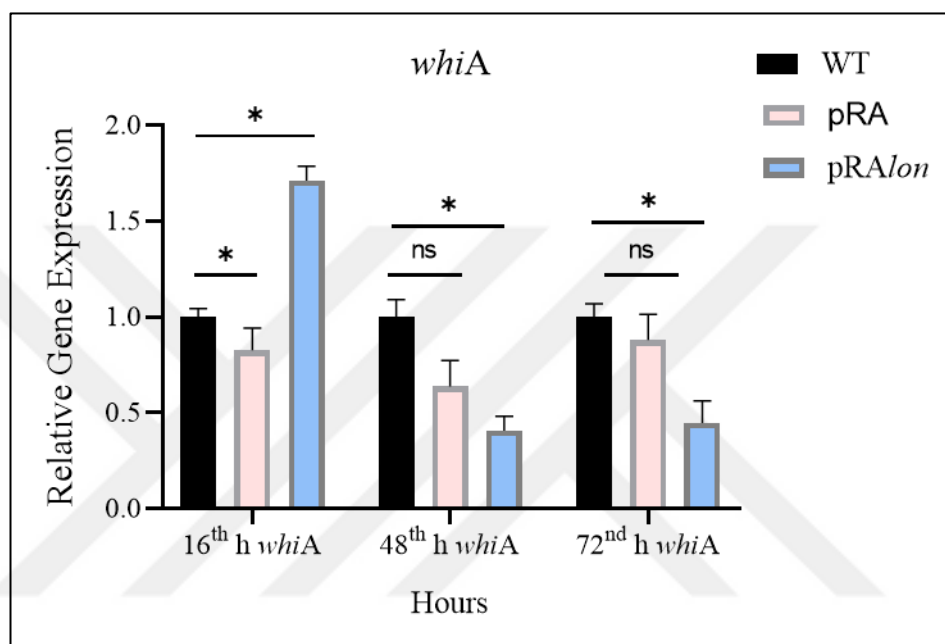


Figure 7.33: The differences in *whiA* gene expression in recombinant strains at 16th, 48th and 72nd hours. The "*" sign indicates the statistically significant results ($p < 0.05$).

whiG gene expression, which is involved in the early stage of sporulation and is responsible for the expression of an RNA polymerase sigma factor for other *whi* genes, was increased 11-fold and 1.6-fold at 16th h and 48th h, respectively, and gene expression decreased 2.5-fold at 72nd h in the Sco-pRA_{lon} strain compared to Sco. In the Sco-pRA strain, *whiG* gene expression increased 2.9-fold at the 16th h and decreased 1.8-fold at the 72nd h. No statistically significant change was observed in *whiG* expression at 48th h in the Sco-pRA strain (Figure 7. 34)

The *whiH* gene is essential for septation and for the expression of some late sporulation genes. In the Sco-pRA_{lon} strain, *whiH* gene expression was 3 and 1.6 times higher than in Sco at 16th h and 48th h, respectively. However, the *whiH* gene showed a 2.8-fold decrease in expression at 72nd h. In the Sco-pRA strain, *whiH* gene expression

increased 1.9-fold at 48th h and decreased 1.7-fold at 72nd h. No statistically significant change in *whiH* expression was observed at 16th h in the Sco-pRA strain (Figure 7.35).

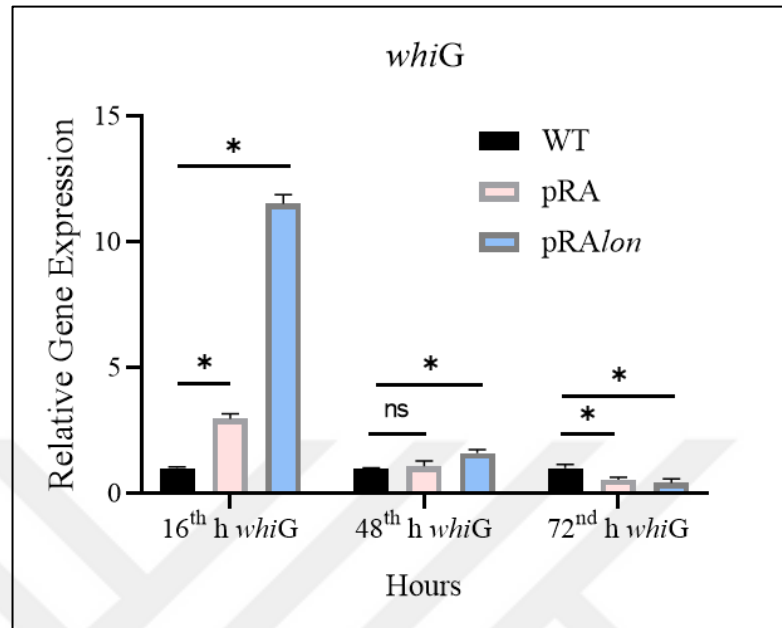


Figure 7.34: The differences in *whiG* gene expression in recombinant strains at 16th, 48th and 72nd hours. The "*" sign indicates the statistically significant results ($p < 0.05$).

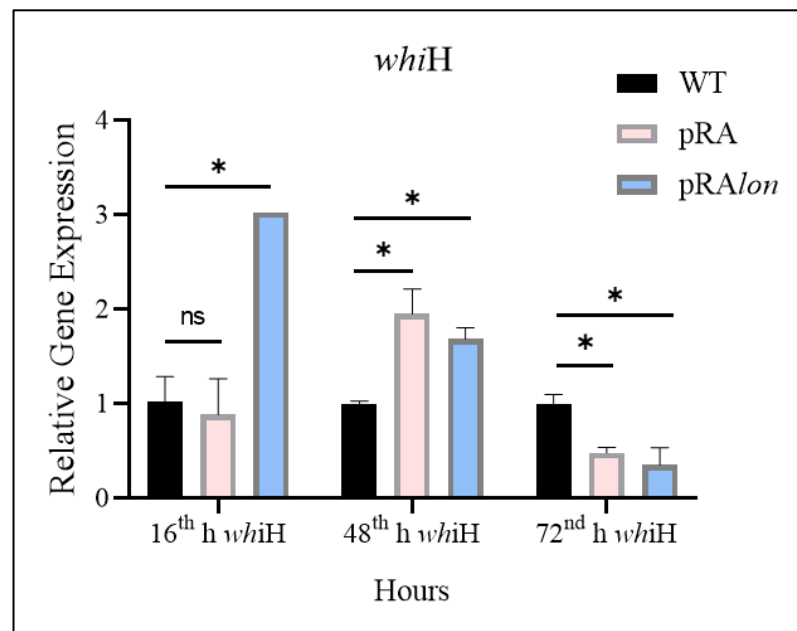


Figure 7.35: The differences in *whiH* gene expression in recombinant strains at 16th, 48th and 72nd hours. The "*" sign indicates the statistically significant results ($p < 0.05$).

The results of the analysis of *chpA* gene expression levels, which are involved in hydrophobic sheath formation, revealed no statistically significant differences between the Sco-pRA_{Lon} and Sco strains at 16th h. At 48th h, there was a 5.5-fold increase in *chpA* gene expression, and at 72nd h, there was a 1.6-fold decrease. A comparison of the *chpA* gene expression levels of the Sco-pRA strain with the Sco strain revealed no statistically significant differences at the 16th h, 48th h, or 72nd h (Figure 7.36).

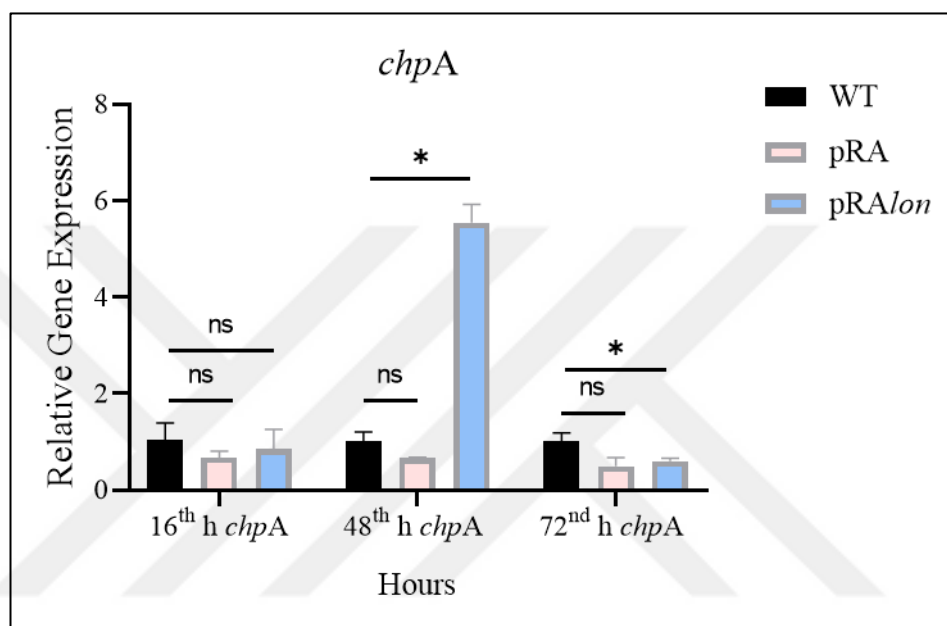


Figure 7.36: The differences in *chpA* gene expression in recombinant strains at 16th, 48th and 72nd hours. The "*" sign indicates the statistically significant results ($p < 0.05$).

The *rdlA* gene is involved in rodlet layer formation. The *rdlA* gene expression level increased 2.8-fold and 8-fold at the 16th and 48th h, respectively, in the Sco-pRA_{Lon} strain compared to the Sco strain. Conversely, at the 72nd h, the *rdlA* gene expression was downregulated by 2.3-fold in the Sco-pRA_{Lon}. A comparison of the *rdlA* gene expression levels of the Sco-pRA strain with the Sco strain revealed no statistically significant differences at the 16th and 72nd hours. However, at the 48th h, *rdlA* gene expression was upregulated by 4.9-fold in the Sco-pRA strain (Figure 7.37).

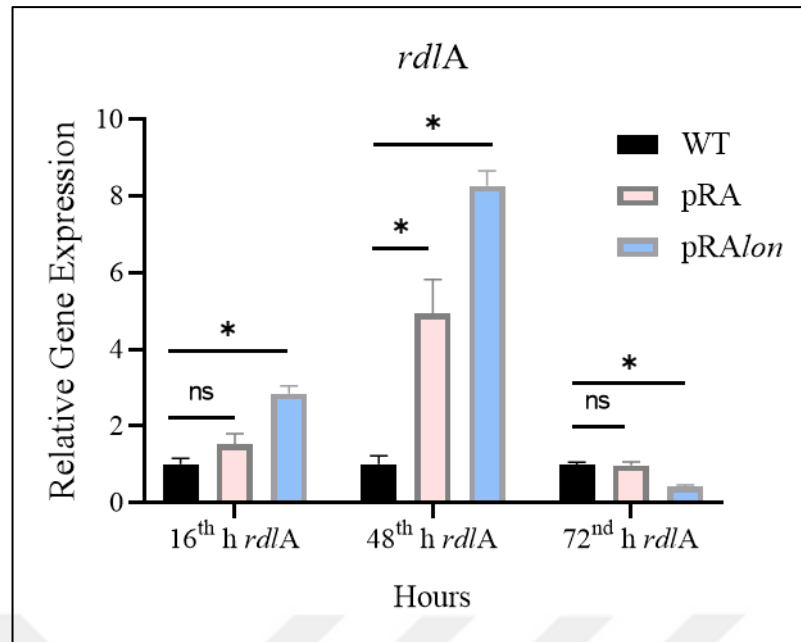


Figure 7.37: The differences in *rdlA* gene expression in recombinant strains at 16th, 48th and 72nd hours. The "*" sign indicates the statistically significant results (p<0.05).

The expression of the *ftsZ* gene, which is involved in Z-ring formation, was found to have been upregulated by 1.6-fold and 1.7-fold, respectively, in the Sco-pRA_{lon} strain at 16th and 48th hours compared to the wild-type Sco strain. Conversely, *ftsZ* expression was observed to decrease by 2-fold in the Sco-pRA_{lon} strain at 72nd h. The expression of the *ftsZ* gene in the Sco-pRA strain did not exhibit a significant change at either the 16th or 48th h in comparison to the wild-type Sco strain. Nevertheless, *ftsZ* expression exhibited a 2-fold decrease in the Sco-pRA strain at 72nd h (Figure 7.38).

The *ssgB* gene expression, which is responsible for the production of the protein that localizes FtsZ proteins during the sporulation phase, exhibited a 2-fold decrease at 16th h and a 2.3-fold increase at 48th h in the Sco-pRA_{lon} strain. No statistically significant change in *ssgB* expression was observed in the strain at 72nd h. In the Sco-pRA strain, *ssgB* gene expression increased 1.6-fold at the 16th h and decreased 1.6-fold at the 48th h. No statistically significant change was observed in *ssgB* expression at 72nd h in the Sco-pRA strain. The change in gene expression of both recombinant strains was compared to that of the Sco wild type strain (Figure 7.39).

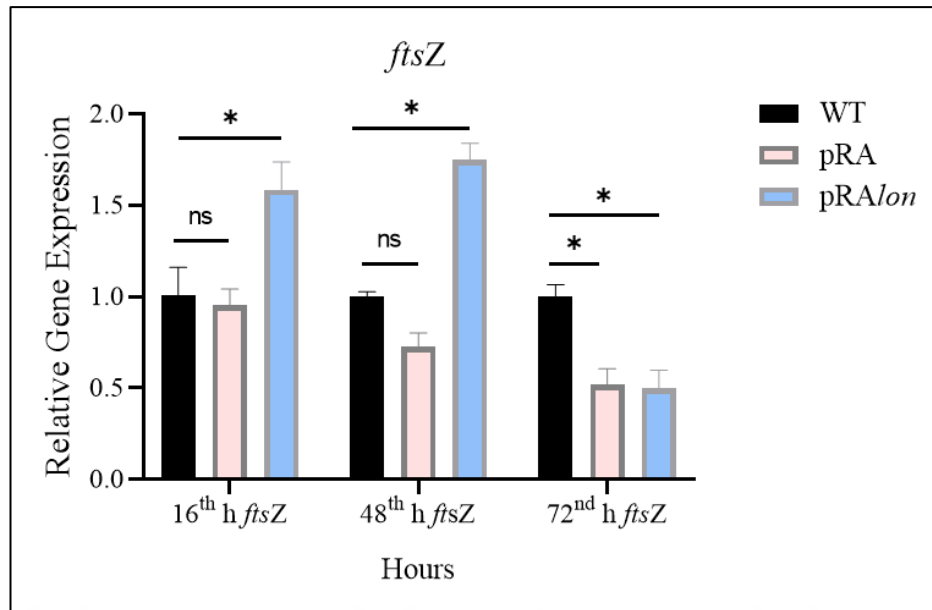


Figure 7.38: The differences in *ftsZ* gene expression in recombinant strains at 16th, 48th and 72nd hours. The "*" sign indicates the statistically significant results ($p < 0.05$).

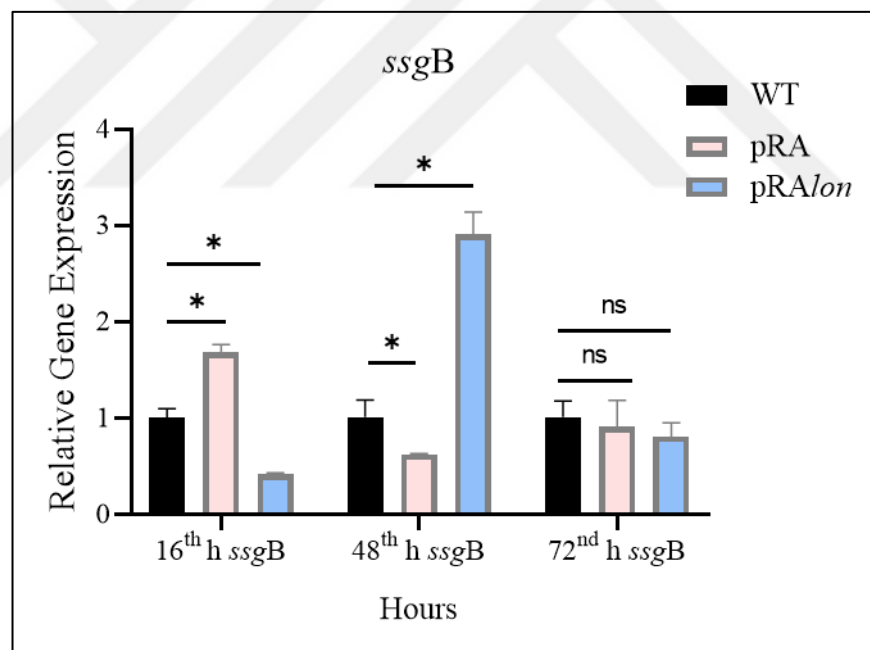


Figure 7.39: The differences in *ssgB* gene expression in recombinant strains at 16th, 48th and 72nd hours. The "*" sign indicates the statistically significant results ($p < 0.05$).

The *sigF* gene is responsible for the expression of sigma factor F, which activates the late sporulation gene. Expression of the *sigF* gene was found to be upregulated 4.7-fold and 1.8-fold in the Sco-pRA*lon* strain compared to the wild-type Sco strain at 16th h and 72nd h, respectively. At 48th h, no significant change in expression was detected for this strain. In the Sco-pRA strain, *sigF* gene expression was upregulated 1.2-fold at 16th h, downregulated 2-fold at 48th h, with no statistically significant change at 72nd h (Figure 7.40).

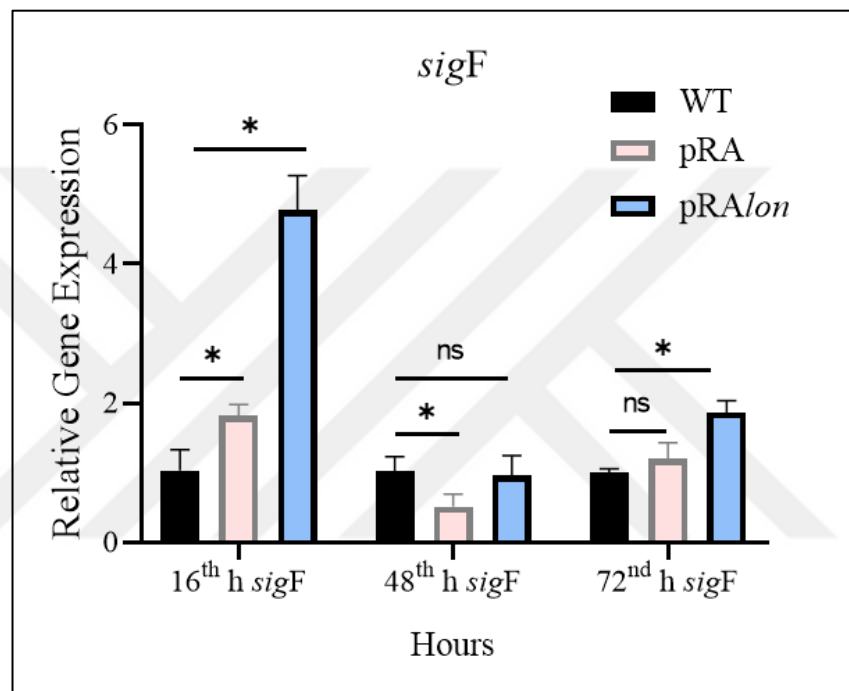


Figure 7.40: The differences in *sigF* gene expression in recombinant strains at 16th, 48th and 72nd hours. The "*" sign indicates the statistically significant results ($p < 0.05$).

8. DISCUSSION

Streptomyces are a source of bioactive molecules used in various industrial, clinical, and agricultural applications [1]. Their life cycle, similar to that of fungi, distinguishes them from bacteria. The differentiation they undergo and the coexistence of unigenomic and multinucleated cells make them a good model for cell differentiation. Valuable secondary metabolites of *Streptomyces* are produced during their transient stage. The main goal of differentiation is the transformation of vegetative mycelia into aerial hyphae to form spores resistant to environmental conditions [3].

The differentiation process is initiated within the colony in response to various stress factors. This process is closely associated with the production of secondary metabolites, which are advantageous during differentiation. Additionally, PCD (Programmed Cell Death) causes the dispersion of cellular debris, enriching the colony environment with valuable nutrients. These situations also attract other bacterial species, intensifying the competition and prompting *Streptomyces* to the synthesis of antibiotics as a defense mechanism against rival bacterial strains. In *Streptomyces*, differentiation and secondary metabolite production are intertwined and co-regulated processes. Identifying genes and molecules whose mutations affect both secondary metabolite production and differentiation demonstrates that these two cellular processes can be controlled through the same control mechanisms. Stress in *Streptomyces* can trigger both differentiation and antibiotic production [3], [53], [54].

The ATP-dependent Lon protease, characterized by a conserved domain, is ubiquitously present across the evolutionary spectrum, from archaea to humans. This protease serves as a fundamental enzyme in ensuring protein quality control and actively regulates pivotal cellular processes, including cell division, sporulation, motility, pathogenicity, gene expression, and contributing to the stringent response in bacteria [9], [119]. Stress is known to induce the production of secondary metabolites in bacteria [120]. The fact that Lon protease plays an important role in the regulation of the stress response and its importance in bacterial differentiation processes positions this enzyme as a potential modulator of secondary metabolite biosynthesis. In a preceding investigation within our laboratory, two additional *lon* gene was integrated into the genome of the *S. coelicolor* (Sco) strain. The recombinant strain exhibited a marked increase in antibiotic production. Antibiotic measurements unveiled a 34-fold

increase in actinorhodin and a 9-fold increase in undecylprodigiosin production compared to the wild-type *S. coelicolor* strain [7]. By Lon's participation in stress response and cellular differentiation, as well as the documented impact of factors influencing antibiotic production in *Streptomyces*, it was postulated that developmental genes would be affected in the recombinant strain.

Considering the aforementioned information, the relationship between Lon protease, antibiotic production, and development in *S. coelicolor*, a microorganism of significant importance in both medical and biotechnological contexts, was investigated within the scope of this thesis study. The focus was on elucidating the relationship between Lon protease, high secondary metabolite production and developmental processes of *S. coelicolor*. The expression levels of developmental genes were quantified by RT-qPCR analysis. Transcriptional analysis was conducted using cDNA synthesized from RNA isolated from samples of Sco, Sco-pRA_{Lon}, and Sco-pRA strains grown on GYM solid medium at 16th h, 48th h, and 72nd h. While *gyrA* and *hrdB* genes were employed as reference genes, the cDNA of the Sco strain was designated as a positive calibrator. The rationale behind the transcriptional analysis of the Sco-pRA strain, which contains an empty plasmid, was to ascertain whether the observed changes in gene expression levels were attributable to the plasmid and to determine the specific effect of the plasmid by comparing the results with those of the control. The expression levels of 32 development-related genes were analyzed at the 16th h of growth. The genes analyzed included: *bld* genes (*bldD*, *bldG*, *bldA*, *bldH*, *bldN*, *bldM*) are responsible for the formation of aerial hyphae; *chpA*, *chpD*, *rdlA*, *rdlB*, *ramR*, and *ramC* are responsible for hydrophobic sheath formation; the *whi* genes (*whiA*, *whiG*, *whiH*, *whiE*) are responsible for controlling sporulation; the *ftsZ*, *ssgA*, *ssgB*, *parA*, *parB*, *divIVA*, *mreB*, and *pdtA* genes play a role in divisome, and spore wall forming; the *dasR*, *crgA*, *sigF*, and *sigH* genes are regulators; the *cdgE*, *cdgC*, *rmdA*, and *rmdB* genes are involved in c-di-GMP synthesis and degradation. Subsequently, our attention was directed towards 12 of these genes. The genes to be focused on were selected from 32 genes based on changes in gene expression levels and their roles in development. The expression levels of the 12 genes (*bldD*, *bldH*, *bldN*, *bldM*, *whiA*, *whiG*, *whiH*, *chpA*, *rdlA*, *ftsZ*, *ssgB*, *sigF*) were quantified at 48th and 72nd h of the life cycle. Following germination within the *Streptomyces* life cycle, substrate/vegetative mycelia (MI) are formed. These mycelia initially form unigenomic primary mycelia

with a compartmentalized structure. Subsequently, upon the induction of PCD, the primary micelles transform multinucleate secondary micelles (MII). Subsequently, MII are coated with a hydrophobic sheath, and aerial hyphae are produced. These mycelia in MII form serve as the foundation for the reproductive structure. Vegetative growth continues, with the BldD-c-di-GMP complex repressing the expression of other *bld* genes involved in the formation of aerial hyphae and sporulation-related *whi* genes until a signal is received [60]. In the initial stages of development, vegetative mycelia are the dominant form of the colony. As the concentration of c-di-GMP in the cell declines, the inhibitory effect of the BldD-c-di-GMP complex on target genes is alleviated, resulting in cellular differentiation [29], [62]. The formation of aerial hyphae is under the control of *bld* genes, while sporulation is regulated by *whi* genes. The majority of *bld* mutants are unable to produce antibiotics, and the mutant phenotype is characterized by bald colonies with defective or absent aerial hyphae. In contrast, *whi* mutants are unable to undergo septation and sporulation, resulting in white-colored colonies due to the absence of spore pigmentation [60].

Interpretation of 16th H Gene Expression Profile

The expression levels of developmental genes in the recombinant strain of Sco-pRA_{lon} were analyzed at 16th h in comparison with the Sco strain. The recombinant strain exhibited a 2.5-fold increase in *bldD* expression at 16th h. However, *bldA*, *bldG*, *bldM*, and *bldN*, which are known to be target genes of *bldD* to suppress, were found to be down-regulated by 2.6, 1.5, 3.4, and 8-fold, respectively. The observed decrease in the expression of these genes may be the result of the observed increase in the expression of *bldD*. In the recombinant strain, the expression of all *whi* genes analyzed at the 16th h were found to be upregulated. *whiG* expression is responsible for sigma factor synthesis which activates expression of important sporulation genes, *whiH* and *whiI*. *whiG* expression increased 11-fold and *whiH* gene expression increased 3-fold. The expression of the *whiA* gene, which is known to be involved in the termination of aerial hyphal growth and septation, was found to be upregulated by 1.5-fold, while the *whiE* gene, a late sporulation gene responsible for spore pigment [100], was upregulated by 2-fold. Furthermore, the expression of *sigF*, which is responsible for spore maturation, increased by 4.7-fold at the 16th h. It is noteworthy that the expression of sporulation-related genes increased at such an early stage. The early occurrence and increase in the expression of these genes indicated that they may cause

the precocious sporulation of the cell. Given that the recombinant strain is a prolific antibiotic producer, it is unlikely that precocious sporulation with the omission of aerial hyphae formation will occur. It is postulated that the observed increase in the expression of *bldD* and other factors act to prevent bypassing the formation of aerial hyphae.

BldD, a master regulator in *Streptomyces* differentiation, also affects primary and secondary metabolism. *bldD* null mutants exhibited a decrease in the expression of genes related to primary metabolism, including [60] the genes *leuB* and *leuC*, which are required to produce branched-chain amino acids [126]. The increase in *bldD* expression at 16th h in the recombinant strain with high antibiotic production suggested that these genes may also increase. Increased expression of branched amino acids during vegetative growth is one of the factors that may increase antibiotic production.

One of the key elements in the growth of *Streptomyces* hyphae is DivIVA, which accumulates at the hyphae tips and facilitates the recruitment of polarisome and other divisome components for apical growth. *divIVA* expression is essential for the cell and is known to be required for viability [44]. At 16th h, the expression of *divIVA* decreased by 3-fold in the Sco-pRA_{lon} strain. The observed decline in *divIVA* expression was an unanticipated outcome. A deficit in *divIVA* expression is known to give rise to the formation of non-branching hyphae. In the recombinant strain, which is presumed to develop in a healthy manner, the reduced expression of *divIVA* is not believed to exert any influence on the cell. The observed low expression may be insignificant at the protein level, or it may have increased expression at other stages of development. Nevertheless, it is known that a temporary cessation in cell growth occurs during the transition to the differentiation stage [51]. This indicates that Sco-pRA_{lon} may be entering the PCD at an early stage. To ascertain the most appropriate course of action, it would be beneficial to observe the transition from live to dead cells in the colony using confocal imaging.

The *ftsZ* gene, which is related to cell division, was found to be upregulated by 1.5-fold in the Sco-pRA_{lon} strain at 16th h. The *ftsZ* gene is involved in Z-ring formation and plays an important role in unigenomic spore formation at the septation stage. Furthermore, it has been observed that this gene has an increased expression during sporulation compared to other developmental stages. Also, FtsZ is involved in the cross-wall formation of the MII mycelia [51]. BldD acts as a repressor for *ftsZ*

expression [60], while WhiA protein acts as an activator for *ftsZ* expression. It can be postulated that the increase in *whiA* expression will increase *ftsZ* expression. The expression of *ssgA* and *ssgB*, which are responsible for the expression of proteins involved in the localization of FtsZ proteins in septation, was found to be downregulated by 1.4-fold and 2-fold, respectively, at the 16th h. Additionally, the formation of long aerial hyphae is observed in *ssgA* and *ssgB* mutants, suggesting that these proteins may play a role in regulating the growth of aerial hyphae, in conjunction with WhiA and WhiB. During vegetative growth, the expression of these genes is subject to BldD repression [60]. The observed decrease in expression may be related to increased *bldD* expression.

ParA and ParB are chromosome-packaging proteins that ensure the even distribution of genetic material during spore formation. There is no evidence to suggest that these proteins perform such a function in mycelia. In mutants lacking ParA and ParB, chromosomal defects were observed in the process of spore septation, whereas no abnormalities were detected in the growth of hyphae [100]. Nevertheless, given that ParA is known to be expressed at an early stage of development, it is postulated that it may play a role during hyphae growth, although it does not affect the phenotype. At 16th h, no significant change in *parB* expression was observed in Sco-pRA_{lon}, while *parA* expression increased by 1.4-fold.

The expression of the genes encoding the hydrophobic sheath-forming proteins Chaplin and Rodlin is activated by the σ^{BldN} . Upon the release of repression of BldD on *bldN* expression, BldN is expressed but retained by the anti-sigma factor until a signal is received [81], [84]. In the Sco-pRA_{lon} strain, no significant change was observed in the expression of *chpA* gene at 16th h. Interestingly, *chpD* gene expression increased by 1.6-fold. The observed increase in *chpD* expression at 16th h, concomitant with a reduction in *bldN* expression, is an unexpected finding.

The analysis of MreB expression, which is a shape-determining protein in other bacterial species, revealed no expression change in Sco-pRA_{lon} at the 16th h. Given that the MreB protein is a component of the *Streptomyces* spore wall complex and is involved in the final stages of sporulation [107], it is more probable that an expression change will be observed at an advanced stage of development. Another component of the *Streptomyces* spore wall synthesizing complex is PdtA involved in directing cell wall glycopolymer synthesis. Interestingly, its expression was upregulated 2-fold at

the 16th h in Sco-pRA_{lon}. PdtA is an essential enzyme for proper sporulation. In addition to this, Sigle et al. found that PdtA has a dual role. They observed that 34 percent of the spores of *pdtA* null mutants were nonviable, while apical growth and branching of vegetative mycelia were impaired under stress conditions [121].

This suggests that PdtA is involved in hyphae growth under stress conditions. The idea that the Sco-pRA_{lon} strain was stressed is supported by the profile shown by the *pdtA* gene, which was upregulated by 2-fold at the 16th h.

No significant change in the expression of *dasR*, which controls antibiotic production via N-acetylglucosamine in nutrient-poor conditions, was detected at the 16th h. *sigH*, which has a role in osmotic stress and differentiation, is among the targets repressed by *bldD* during vegetative growth [60]. At the 16th h, the expression of *sigH* was down-regulated 1.4-fold in the Sco-pRA_{lon} strain. The observed decrease in expression is thought to be related to the concurrent increase in *bldD* expression.

The secondary messenger c-di-GMP, which is found only in prokaryotes, plays a crucial role in the *Streptomyces*'s life cycle. BldD, a master regulator during vegetative growth [61], exerts its repressor effect on differentiation genes through a complex with c-di-GMP. Post-translationally controlled σ^{WhiG} is a sigma factor for important early sporulation genes. Until the time of its function in differentiation, σ^{WhiG} is retained by the anti-sigma factor RSIG [97]. The binding of the sigma factor and anti-sigma factor in the correct conformation is mediated by the c-di-GMP dimer. Changes in the level of c-di-GMP in the cell have been found to control differentiation. This secondary messenger is synthesized by diguanylate cyclases (DGCs) and degraded by phosphodiesterase. In general, overexpression of DGCs leads to impaired differentiation and antibiotic production, as well as inhibition of sporulation. Conversely, overexpression of *cdgC*, which is responsible for the synthesis of c-di-GMP, has been observed to increase actinorhodin production without affecting differentiation in *Streptomyces* [48]. RmdA and RmdB are phosphodiesterase enzymes that are responsible for the degradation of c-di-GMP. No spore pigmentation and delayed sporulation was observed in *rmdA* and *rmdB* mutants. When the *rmdA* and *rmdB* genes were deleted together, the resulting mutant strain was bald and failed to form aerial hyphae [66]. This phenotype is a similar profile to the strain overexpressing DGCs. The observation that the CdgC mutation exhibits a distinct mutant phenotype relative to other DGCs may suggest that these enzymes are involved in disparate

control pathways, despite performing analogous functions. At the 16th h, *cdgC* expression was found to be downregulated 3.3-fold in Sco-pRA_{lon}, while *cdgE*, which is functional for the synthesis of c-di-GMP, expression was observed to be increased 1.5-fold. It is noteworthy that the expression of one enzyme related to c-di-GMP synthesis decreased while the expression of the other increased. The observed increase in the expression of BldD target genes, which are known to exhibit a repressor effect, may be related to this situation. While *ftsZ* and *bldH* expression were among the BldD targets, their expression increased despite the increase in *bldD* expression. However, the observed decrease in the expression of other *bld* genes, especially *bldN* gene expression is reduced by 8-fold, which may be related to the observed increase in *cdgE* expression in addition to *bldD* expression. However, no phenotypic effect was observed in *cdgE* mutations in the studies. Therefore, the connection established may be unrelated. The 16th h *rmdA* and *rmdB* gene expressions were down-regulated 2.7 and 1.6-fold in Sco-pRA_{lon}, respectively. The reduction in the concentration of c-di-GMP prompts the cell to differentiate, and the decline in the production of RMD enzymes may have prevented the early surge in the expression of *whi* genes, thereby preventing the onset of early sporulation.

Interpretation of 48th h and 72nd h Gene Expressions

At the 16th h, the *bldD* gene exhibited a 2.5-fold increase in expression, followed by a decrease of approximately 2-fold at the 48th and 72nd h. This decrease may indicate that the cell is undergoing differentiation. BldD is also known to have a repressive effect on its own promoter. At the 16th h, the excess expression may result in a decrease in its expression later. However, at 48th h, *bldH* and *bldM* expression increased 5.8 and 2-fold, respectively, in the Sco-pRA_{lon} strain. This suggests that the expression of *bldD* is decreased and the repressor effect on its target genes is abolished. BldH (AdpA), which has a dual role between differentiation and secondary metabolite production, is involved in the regulation of secondary metabolite production in *Streptomyces*. Its mutation affects both antibiotic production and hyphae formation. BldH is known to activate actinorhodin expression in *S. coelicolor* [86], [124]. In the Sco-pRA_{lon} strain, which is a high actinorhodin producer, *bldH* expression increased 5.8-fold at 48th h. BldM plays a role in the formation of aerial hyphae as a homodimer. It is involved in sporulation as a heterodimer with WhiI. The observed increase in the expression of genes that are required for aerial hyphae formation at the 48th h indicates

that the cell prepare to form aerial hyphae. The lack of significant change in the expression of these genes at 72nd h may be related to the fact that they are not involved in the advanced stages of differentiation. However, the expression of *bldN*, which is responsible for the expression of chaplins and rodlets [81], and is also an activator for *bldM* expression, decreased by approximately 2-fold at 48th h. The decrease in *bldN* gene expression was unexpected. *chpA* and *rdlA* are target genes of the σ^{BldN} . Expression of *chpA* shows a 5-fold increase at 48th h and an 8-fold increase in the expression of *rdlA* in Sco-pRA_{lon}. The increase in *chpA* and *rdlA* expression suggests that *bldN* expression may have increased earlier than 48th h. In a study, it was observed that *chp* and *rdl* expression was absent in *bldN* mutants and the hydrophobic sheath was not formed [83]. The observed increase in the expression of target genes and the formation of a hydrophobic structure in the Sco-pRA_{lon} strain indicate that *bldN* expression may have been elevated outside of the examined time frame, or that the observed decrease in expression did not affect σ^{BldN} function.

whiG is one of the target genes of BldD during vegetative growth. In the *Streptomyces* life cycle, the *whiG* is expressed at earlier stages than sporulation and its activity is controlled post-transcriptionally. The σ^{WhiG} is sequestered by the anti-sigma factor RSIG via the c-di-GMP dimer. A reduction in c-di-GMP levels allows for the transcription of early sporulation genes. The precise manner by which BldD exerts its influence on post-transcriptionally controlled *whiG* expression remains uncertain [60]. In the Sco-pRA_{lon} strain, *whiG* expression increased by 11-fold at the 16th h. This early increase in expression suggests that there may be a difference in the timing of differentiation between the recombinant strain and the wild type strain. This increase in *whiG* expression resulted in a very early increase in the expression of its target gene *whiH*. At 48th h, expression of *whiG* and *whiH* increased 1.6-fold for both genes in the Sco-pRA_{lon} strain. The expression of these genes, which were identified as early sporulation genes, decreased approximately 2.5-fold at 72nd h. The observed reduction in expression may be attributed to the fact that genes involved in the initial stages of sporulation are no longer required.

SigF, known to be involved in spore maturation, was upregulated 4.7-fold at 16th h and 1.8-fold at 72nd h in the Sco-pRA_{lon} strain. The observed increase in the expression of *sigF* at the 16th h is unexpected, given that this gene functions in the final stages of development and is expected to be expressed late. A review of the literature revealed

that *sigF* expression is hierarchically related to *whiG* expression, with WhiG indirectly regulating *sigF* expression [71]. The increased *whiG* expression at 16th h may have triggered the subsequent increase in *sigF* expression at the same time. Interestingly, no detectable transcription of *sigF* was observed in mutants lacking WhiA, B, G, H, I, or J [71]. This profile illustrates the correlation between *sigF* expression and the expression of *whi* genes. At 48th h, *sigF* expression remained relatively unchanged in the Sco-pRA_{lon} strain. WhiE, responsible for spore pigmentation, is a late sporulation gene. The *whiE* promoter is activated by SigF [124]. At 16th h, the increase in *sigF* expression may have increased *whiE* expression.

The expression level of the *whiA* gene, which is responsible for the arrest of aerial hyphae growth and transcription of proteins involved in septation such as *ftsZ* [94], was found to be upregulated 1.5-fold at 16th h in the Sco-pRA_{lon} strain. This early increase in the expression of WhiA confirms that differentiation is triggered early in the recombinant strain. The correlation between the transcriptional activator *whiA* and the upregulation of *ftsZ* gene expression at 16th h is evident. The decrease in *whiA* gene expression at 48th h and 72nd h suggests that the cell may have progressed early on the differentiation pathway, septation may have started early, and the expression of *whiA* and *ftsZ* at 72nd h may not have been necessary.

Recent studies have indicated that the secondary messenger c-di-GMP plays a significant role in *Streptomyces* development. Vegetative growth is maintained by the repression of differentiation genes by the c-di-GMP/BldD complex. Concurrently, the sigma factor WhiG, which is responsible for the activation of essential sporulation genes, is retained by the anti-sigma factor via c-di-GMP until the point of activation. It has been demonstrated that alterations in c-di-GMP levels can influence *Streptomyces* differentiation [61]. The stringent response product ppGpp (alarmone) and c-di-GMP are synthesized in the cell via GTP. In *Streptomyces*, different phenotypes of differentiation and antibiotic production have been observed in response to changes in ppGpp levels and gene mutations responsible for ppGpp synthesis [111]. In the wild-type strain *S. clavuligerus*, ppGpp levels increase during differentiation, while c-di-GMP levels decrease. However, there was no decrease in c-di-GMP levels observed in *relA* mutants, which are responsible for ppGpp synthesis [125]. Furthermore, overexpression of the c-di-GMP synthesis enzyme CdgB in *S. coelicolor* demonstrated that differentiation and antibiotic production did not occur in this strain

[64], while *rmdA* and *rmdB* null mutants, responsible for c-di-GMP degradation, did not sporulate [65]. Given the role of c-di-GMP in the differentiation of *Streptomyces*, it can be concluded that purine balance is important for differentiation. The expression of *relA* in the Sco-pRA_{lon} strain is upregulated in R2YE, even though *relA* expression is typically triggered by starvation [8]. As a result, it is hypothesized that the level of ppGpp may be elevated in this recombinant strain. It is further hypothesized that an increase in ppGpp synthesis may have led to a reduction in the level of GTP, subsequently causing a decline in the level of c-di-GMP in the recombinant strain. If the predicted decrease in c-di-GMP levels in the recombinant strain does occur, it may be the cause of the early differentiation thought to occur in the recombinant strain.

The expression of developmental genes were observed to change in both the Sco-pRA and Sco-pRA_{lon} strains compared to the wild type. Sco-pRA strain was used as the control to examine the effect of empty plasmid on the gene profile. It is known that plasmids used in recombinant technologies may exert stress on the cell, which can in turn affect differentiation in the *Streptomyces* life cycle. By using the Sco-pRA strain as the control, the alteration in gene expression levels of developmental genes in the Sco-pRA_{lon} strain was attributable to *lon*. To date, there is no evidence that Lon protease plays a role in differentiation in *Streptomyces*. To our knowledge, this is the first study showing that the *lon* gene inserted into the genome may directly or indirectly trigger differentiation in *Streptomyces*.

Further experiments can be carried out to understand which pathways in the cell are affected by the two extra copies of *lon* gene in the genome. To elucidate the role of Lon in *S. coelicolor*, it is possible to compare proteome data obtained from different stages of development of the wild type and the recombinant strains. The study of protein-protein interactions may also help us to understand the effect of *lon* overexpression on the cellular processes in the recombinant strain. As c-di-GMP plays an important role in *Streptomyces* development, it is possible to determine the binding affinity of c-di-GMP to its developmental targets (BldD and WhiG). These and similar experiments may reveal the functions of the Lon protease in *Streptomyces* metabolism. These studies may contribute to the elucidation of the relationship between Lon, differentiation and secondary metabolite production in *Streptomyces*. It may also provide the necessary knowledge to induce secondary metabolite production in other *Streptomyces*.

This study has investigated the impact of the additional copies of the *lon* gene in the genome of the recombinant strain on the expression of genes involved in *S. coelicolor* development. Our results show that differentiation is triggered early in the recombinant strain, although developmental stages, including aerial hyphal formation, are not skipped. To our knowledge, the spore chains of the recombinant strain are also healthier than those of the wild-type strain (unpublished data). We hypothesize that the transient phase may be longer in the recombinant strain than in the wild type. This is because although differentiation and spore formation were triggered early in the Sco-pRA*lon* strain, the spores were healthy, antibiotic production was high and the aerial hyphal formation stage was not skipped. Further experiments are needed to confirm these hypotheses.

It is curious whether the production of other secondary metabolites is increased in the Sco-pRA*lon* strain. This possibility should be evaluated in another study. If this possibility is correct, the recombinant strain could be a valuable resource in the current era of urgent need for the discovery of new bioactive compounds for both clinical and industrial applications. In any case, the value of our recombinant strain is unquestionable, as the increasing prevalence of antibiotic resistance and the accelerated search for new antibiotics have led to a renewed interest in actinorhodin as an antimicrobial agent [127]. Given that 80% of currently used antibiotics originate from *Streptomyces*, elucidating the relationship between antibiotic production and development represents a crucial step in the production of antibiotics. The findings of our study indicate that Lon may exert a more significant and far-reaching influence on the life cycle and antibiotic production in *Streptomyces* than was previously acknowledged.

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PRESENTATIONS FROM THE THESIS

Kara S., Bayraktar A., Tunca S., (2023) “Transcriptional Analysis Of Developmental Genes Of Antibiotic Overproducer Recombinant *Streptomyces coelicolor*”,Gebze Technical University Graduate School of Natural and Applied Sciences 7th Graduate Research Symposium, Kocaeli, 31 May- 1 June.

Kara S., Bayraktar A., Tunca S., (2024), "The expression of the developmental genes of *Streptomyces coelicolor* is affected by the overexpression of the Lon protease gene", 5th International Eurasian Conference on Science, Engineering and Technology (EurasianSciEnTech 2024), Ankara, June 26-28.

