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One Extra Copy of *lon* Gene Causes a Dramatic Increase in Actinorhodin Production by *Streptomyces coelicolor* A3(2)

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Abstract

ATP-dependent Lon protease plays important roles in different physiological processes, including cellular differentiation of the bacteria and is a part of an important stress response regulon (HspR/HAIR). In *Streptomyces*, biosynthesis of secondary metabolites starts with cellular differentiation and stress is one of the factor that affect metabolite production. To clarify the effect of Lon protease on secondary metabolite production, we constructed a recombinant strain of *Streptomyces coelicolor* A3(2) that has one extra copy of *lon* gene with its own promoter and transcriptional terminator in its genome. Expression of *lon* gene in the recombinant strain was determined by quantitative real time (RT-qPCR). Actinorhodin and undecylprodigiosin production of the recombinant cell was measured in liquid R2YE and it was found to produce about 34 times more actinorhodin and 9 times more undecylprodigiosin than the wild-type at 168 h of growth. Development of stable *Streptomyces* strains capable of producing high amounts of secondary metabolites is valuable for biotechnology industry. One extra copy of *lon* gene is enough to boost antibiotic production by *S. coelicolor* A3(2) and this change do not cause any metabolic burden in the cell.

Introduction

ATP-dependent Lon protease, which is present in all species from bacteria to human, is responsible for the degradation of unstable natural proteins and improperly folded proteins [1, 2]. Lon and two other proteases (ClpAP and ClpXP) are responsible for 70–80% of energy-dependent protein degradation in *Escherichia coli* [3]. This enzyme plays important roles in the control of physiological processes, such as cell division [4], DNA replication [5], adaptation to stress conditions [6] by degrading naturally folded regulator proteins.

Lon protease is also known to play a critical role in bacterial differentiation. For example, in *Bacillus subtilis*, Lon affects sporulation by degrading sigma factor σ^H [7]. The *lon* gene (*lon*D) has also been shown to be necessary for the developmental cycle of *Myxococcus xanthus* [8]. Similarly, cell differentiation in *Vibrio parahaemolyticus* is shown to be regulated by Lon protein [9]. In a recent study by Cerletti et al. [10], LonB protease was demonstrated to regulate carotenogenesis in the extremophilic archaeon *Haloferax volcanii*.

Lon protease also controls the expression of virulence genes in *Brucella abortus* and *Salmonella enterica* by regulating the degradation of transcription factor HilA [11]. In *Pseudomonas aeruginosa*, Lon mutants were shown to have reduced pathogenicity and also exhibit severe deficiency in swarming motility [12]. Recently, Lee et al. [13] determined that Lon protease modulates virulence traits in enterobacterial pathogen of apple, *Erwinia amylovora*.

Lon is not a vital enzyme for most bacterial species under normal growth conditions, but its absence results in a number of dysfunctions in the cells. Mutations in *E. coli lon* gene result in pleiotropic phenotypes, such as increased sensitivity to UV irradiation [14] and SOS-inducing agents [15], filament formation, mucoidy, and reduced degradation of various abnormal and normal proteins [16]. *E. coli*

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mutants deficient in either Lon or Clp proteases also show an extended growth lag after the downshift, which was more severe in the double mutant (*lon, clp*) [17]. In contrast to *E. coli,* one of the *lon* genes (*lon*V) in *M. xanthus*, is shown to be essential for viability, as attempts to construct a deletion mutant have failed [8].

Streptomycetes are gram positive, filamentous bacterium which produce a wide variety of bioactive compounds such as antibiotics, anticancer agents, immunosuppressant drugs, herbicides etc. They have a complex life-cycle with morphological differentiation [18]. In the liquid medium, the germinating spore cells form young vegetative mycelium. These young mycelium, also called "primary mycelium", initiate pellet formation. Over time, the cell density increases and the cells in the pellet center begin to die (programmed cell death) and growth stops. The new mycelium with multi nucleoid (secondary mycelium) develops in and around the pellet and passes to the second growth phase. Secondary metabolites are produced by these new mycelium [19]. The expression of secondary metabolite biosynthesis genes are generally coordinated depending on the growth phase [20].

The regulation of secondary metabolite production is a highly complex process and involves many different regulatory protein families, extracellular and intracellular signaling molecules [21]. Internal signals such as ions [22], alarmones [23], or reactive oxygen species [24] are directing the regulation of secondary metabolites by influencing the transcription of the secondary metabolite gene cluster or by inducing a transcriptional activator of the target gene cluster [23, 25–27]. Stress conditions (shortage of nitrogen, phosphorus and carbon sources, amino acid starvation, lack of energy etc.) are also known to induce secondary metabolite biosynthesis in bacteria [21, 28–31]. In stress conditions protein degradation increase so that amino acids are provided in order to be used in the synthesis of proteins required for adaptation to new environmental conditions and the role of Lon protease in this process is critical [32]. Being an important part of stress response and regulating cellular differentiation, Lon seems to be a good candidate that will affect secondary metabolite production, however, there is no enough study showing the effect of Lon protease on secondary metabolism. To the best of our knowledge there is only one study that relate Lon with antibiotic production. Whistler et al. [33] demonstrated that Lon is a global regulator that negatively influences antibiotic pyoluteorin production in *Pseudomonas fluorescens* Pf-5. They proposed that Lon may represses antibiotic production by degrading a sigma factor required for pyoluteorin biosynthetic gene transcription or by degrading a positive regulator of antibiotic production.

Sobczyk et al. [34] cloned the *lon* gene of *Streptomyces lividans* and disrupted its expression by inserting the hygromycin resistance gene. Although the cell cycle has not changed drastically, it has been shown that the Lon mutant grows more slowly than the wild-type and that spore germination is affected. *lon* gene has been found to be a part of HspR/HAIR stress response regulon in the same study. Another study reported that *S. lividans* Lon protease could cause toxic effect when expressed in high copy [35].

In the present study, we determined the effect of one extra copy of *lon* gene on antibiotic production by *Streptomyces coelicolor* A3(2).

Materials and Methods

Bacterial Strains, Plasmids and Culture Conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* strains were grown in LB liquid and solid medium at 37 °C. *E. coli* ET12567 containing the RP4 derivative pUZ8002 was used as the nonmethylating plasmid donor for intergeneric conjugation with *S. coelicolor* A3(2). *E. coli* DH5α was used as a host strain for cloning of *lon* gene into pRA (5769 bp) vector. pRA is derived from pSET152 and has the ability to integrate into *att*B site of the chromosomes. *S. coelicolor* strains were grown in TBO, TSB, YEME, MS and R2YE medium at 30 °C. Apramycin (50 µg/ml), chloramphenicol (25 µg/ml), kanamycin (50 µg/ml) and nalidixic acid (25 µg/ml) were added into growth media as required.

DNA Procedures

Isolation of plasmids, restriction enzyme digestions, agarose gel electrophoresis, Southern hybridization, competent cell preparation and transformation of *E. coli* cells were performed according to standard molecular biology techniques [36]. Isolation of *Streptomyces* total DNA were as described by Keiser [20]. Recombinant plasmid was transferred to *S. coelicolor* by intergeneric conjugation [37].

Southern Blot Hybridization

The Southern blot method was performed according to Sambrook et al. [36]. First, chromosomal DNA samples were digested with *EcoR*I and *Sph*I restriction endonucleases. The transfer of DNA fragments from the gel to the membrane was achieved by the blotting system (Amersham) under vacuum with 40–50 mbar. The membrane was incubated first in prehibridization solution at 42 °C for 5 h, then in the hybridization solution containing *lon* gene probe overnight at 42 °C.

The *lon* gene fragment (1140 bp) to be used as a probe was obtained by PCR using Q5 polymerase enzyme and specific primers, *lon* probe F and *lon* probe R (Table 2). This

Table 1 Bacterial strains and plasmids used in this study

Bacterial strains/plasmids	Genotype	Reference
Plasmid		
pUZ8002	*tra, neo, RP4*	Paget et al. [53]
pRA	Integrative and conjugative vector derived from pSET152	Perez-Redondo et al. [54]
E. coli strains		
E. coli DH5α	F– *rec*A1, *end*A1, *gyr*A96, *thi*-1, *hsd*R17 (rK–, mK+), *sup*44, *rel*A1λ, (σ80 d*lac*ZAM15), D (*lac*ZYA-*arg*F) U169	Hanahan [55]
E. coli DH5α +pRA	Strain, carring pRA plasmid	This study
E. coli DH5α +pRA*lon*	Strain, carring pRA*lon* recombinant plasmid	This study
E. coli ET12567/pUZ8002	*dam, dcm, hsdS, cat, tet*	MacNeil et al. [56]
ET12567/pUZ8002 + pRA	Strain, carring pRA plasmid	This study
ET12567/pUZ8002 + pRA*lon*	Strain, carring pRA*lon* recombinant plasmid	This study
Streptomyces coelicolor strains		
S. coelicolor A3(2)	Wild-type prototrophic	Hopwood [57]
S. coelicolor A3(2) + pRA	Strain, carring pRA plasmid	This study
S. coelicolor A3(2) + pRA*lon*	Strain, carring pRA*lon* recombinant plasmid	This study

Table 2 Primer pairs used in this study

Primer	Sequence 5′→3′	Use
lon F	CGATGAGCAGGTGCGGAGCG	PCR
lon R	CTCCTACAACGTCGGCATGAT	PCR
lon probe F	ACAACTCCGGCTACTCG	PCR
lon probe R	GGTGACGGTGAACGGCA	PCR
gyrA F	GGTACACCGAGTGCAAGAT	RT-qPCR
gyrA R	GTCGTAGTTGTCCGTGAAGT	RT-qPCR
lon F	AGCTGGTGAAGGAGTACA	RT-qPCR
lon R	TCAGGAACGGCGAGTAG	RT-qPCR
actIIORF4 F	GAGCTGCGGCTTTTTGGAAT	RT-qPCR
actIIORF4 R	TGCGAAATTACCAGGGACCG	RT-qPCR
sigB F	GGAGTTGTCGAAGCTGTTCTT	RT-qPCR
sigB R	GTTCATCTCGATCAGCGTGTT	RT-qPCR
redK F	GCAAGGACTTCGTGATCGAG	RT-qPCR
redK R	GCGTTGGAGACGTAGAGCAG	RT-qPCR
hrdB F	TGCTCTTCCTGGACCTCAT	RT-qPCR
hrdB R	GAACTTGTAGCCCTTGGTGTAG	RT-qPCR

DNA fragment was labelled with the DIG DNA labelling and detection kit (Roche). The same fragment was also used as a positive control in Southern Blot hybridization.

Plasmid Construction and Transformation

The 3397 bp genomic DNA region of *S. coelicolor* A3(2), which contains the *lon* coding sequence with its own putative promoter and transcriptional terminator, was amplified using forward (*lon* F) and reverse (*lon* R) *lon* primers (Table 2) and High Fidelity Q5 DNA polymerase (New England Biolabs). Blunt end PCR fragments were ligated into pRA vector, predigested with *Eco*RV, to obtain the recombinant plasmid pRA*lon*.

Sequencing of DNA fragment containing *lon* coding region in pRA*lon* was carried out at Iontek Company (Istanbul, Turkey) by the dideoxy chain-termination method using an ABI Prism 310 instrument (Perkin–Elmer) and DYEnamic ET Terminator Cycle Sequencing Kit (Amersham).

Actinorhodin and Undecylprodigiosin Determination

Actinorhodin and undecylprodigiosin production were determined spectrophotometrically as described by Keiser [20]. 10^8 spores from each strain were inoculated into R2YE medium and incubated at 30 °C, 200 rpm for 48 h. Pellets with equal wet weight from this preculture were used to inoculate fresh R2YE medium and grown at 30 °C, 200 rpm. For antibiotic and dry weight measurements, samples were taken at every 24 h of fermentation. 1 ml of culture was treated overnight with 250 µL of KOH (5 M) at 4 °C. Then the sample was centrifuged at 13000 rpm for 5 min and the A_{640} value of the supernatant was measured to determine actinorhodin concentration. Molarity was quantified by the formula: Absorbance = ε × [M], where ε640 = 25230 for the pure compound. To determine undecylprodigiosin concentration (a mixture of at least four prodiginines), the mycelium (pellet from actinorhodin measurement) was washed two times with 0.5 M HCl. Then, the pellet was dissolved in 1 ml of methanol-HCl (0.5 M) mixture and incubated at room temperature for 2 h. Then, the A_{530} of the supernatant was determined (ε530 = 100500).

For the dry weight measurement, the pellets from 24, 48, 72, 96, 120, 144, and 168 h samples were washed twice with dH_2O, dried at 80 °C for 5 days and then the weights were measured.

Gene Expression Quantification

For RNA extraction, the cell pellets were resuspended in TE solution with lysozyme and were incubated at 37 °C for 60 min. Total RNA isolation was performed with RNA Isolation Kit (Macherey–Nagel, Germany) according to manufacturer's instructions with small modifications. Possible DNA contamination was eliminated using Deoxyribonuclease I (Thermo Scientific, USA). The absence of contaminated DNA in the RNA samples was confirmed by PCR reactions without reverse transcriptase. The RNA concentration and purity was determined by spectrophotometric measurement. RNA samples were aliquoted then stored at − 80 °C for future use.

cDNA was synthesized by the Iscript Advanced cDNA Synthesis KIT (Bio-rad Laboratories, USA), using 300 ng of total RNA. Reaction mixtures, prepared according to manufacturer's recommendations, were first incubated at 70 °C for 5 min, followed by 2–3 min of incubation in ice to eliminate hairpin formation and primer dimer formation. For the first-strand cDNA synthesis conditions were as follows: incubation at 25 °C for 5 min, 46 °C for 60 min, followed by 85 °C for 5 min. For quantitative RT-PCR StepOnePlus™ Real-Time PCR System (Applied Biosystems) and the SYBR Green qPCR master mix (Bio-rad Laboratories, USA), including 500 nM primers (each) and 25 ng cDNA in a total volume of 20 µl were used. The thermal profile consisted of 10 min initial denaturation at 98 °C followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Each cDNA sample was quantified three times in independent experiments. The primers used for transcriptional analysis were designed via a web-based tool, primer 3. Sequences of primer pairs were given in Table 2. The target gene expression level was normalized internally to the level of the *gyr*A and *hrd*B genes. Relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ formula [38] and the error bars represent the standard deviations obtained from three independent biological replicates.

Results

Cloning and Expression of *Streptomyces coelicolor* A3(2) *lon* gene

A 3397 bp genomic DNA region of *S. coelicolor*, that contain ATP-dependent Lon protease gene (SCO5285, GeneBank accession number: AL939123.1) was amplified by PCR with its own putative promoter and transcriptional terminator. HAIR motif with a "ATTGAGT-cgatgta-ACT CAAC" sequence at the upstream of the *lon* gene [34] was included in the promoter region (Fig. 1). *lon* gene was cloned into *Eco*RV site of pRA (derived from pSET152) and after verification by DNA sequencing the recombinant plasmid (pRA*lon*) was transferred to *S. coelicolor* cells by conjugation. Possible recombinant strains were first selected according to apramycin resistance, followed by Southern hybridization. Hybridization sites of *lon* probe on pRA*lon* plasmid and in the genome of *S. coelicolor* was shown in Fig. 2. The presence of second *lon* gene in the genome of recombinant strain (Sco-pRA*lon*) was confirmed by Southern hybridization (Fig. 3a and b). *S. coelicolor* with empty pRA (Sco-pRA) was used as the control strain. A hybridization band of 4195 bp was obtained for the wild-type, Sco-pRA and Sco-pRA*lon* with a *lon* gene probe. Second *lon* copy (a hybridization band of 6455 bp) was present only in Sco-pRA*lon* strain (Fig. 3b). The relative expression of *lon* gene in the Sco-pRA*lon* strain was found to be about 16, 24 and 12-fold higher compared to the wild-type at 60th, 72th and 96th hour of growth, respectively (Fig. 3c).

Growth of Recombinant Strain and Antibiotic Production

Growth of Sco-pRA*lon*, Sco-pRA and wild-type strains were compared on R2YE liquid culture and quite similar growth profile were obtained for all strains until 96 h of growth, then the growth of Sco-pRA*lon* was impaired

Fig. 1 The diagram of the 494 base region before the start codon of the *lon* gene containing the promoter region. Black arrow indicates forward primer binding site, red frames show HAIR sequence of *lon* gene and blue frame shows start codon of *lon* gene (Color figure online)

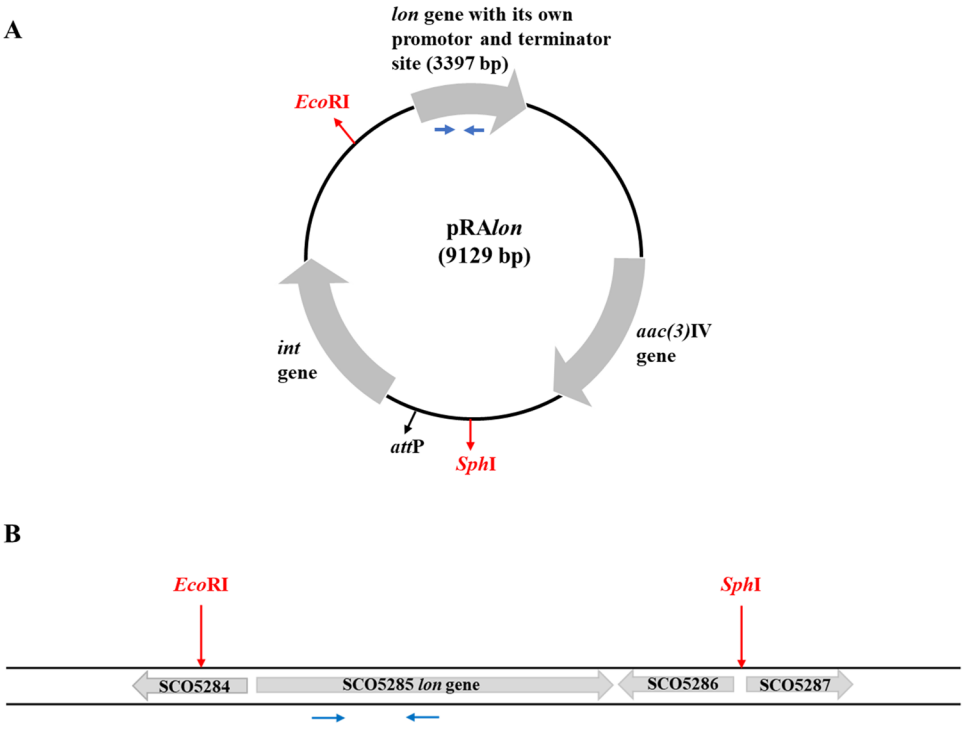

Fig. 2 Hybridization sites of the *lon* probe in **a** pRA*lon* plasmid and **b** the genome of *S. coelicolor*A3(2) are illustrated with blue arrows. Red arrows indicate *Sph*I-*Eco*RI restriction enzyme cutting sites (Color figure online)

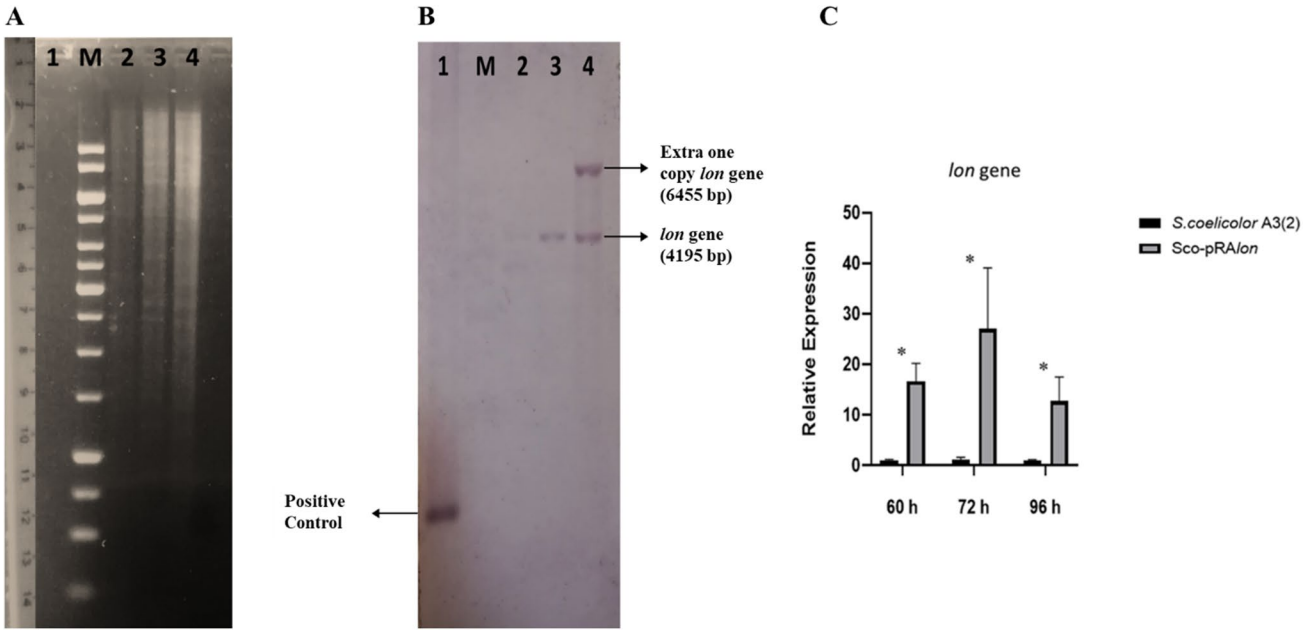

Fig. 3 Determination of the presence of an extra copy of *lon* gene in *S. coelicolor* genome by Southern Blot. **a** Red safe-stained agarose gel and **b** Southern blots results where *lon* probe was used in hybridization. 1140 bp *lon* gene fragment is positive control (lane 1), hybridization pattern of the *Sph*I-*Eco*RI-digested chromosomal DNA of the wild-type *S. coelicolor* (lane 2), *Sph*I-*Eco*RI digested chromosomal DNA of the Sco-pRA (lanes 3), *Sph*I-*Eco*RI digested chromosomal DNA of the Sco-pRA*lon* (lane 4) probed with the *lon* gene fragment. M: 1 kb DNA marker (Fermentas). **c** Relative gene expression of the *lon* gene in the Sco-pRA*lon* strain compared with the wild-type (WT) grown in R2YE medium for 60th, 72 th and 96th hour. Fold changes were calculated using the $2^{-\Delta\Delta Ct}$ method. The statistical analyses performed with GraphPad Prism 8.0.2 software. *P* values were calculated using the Mann–Whitney test. $P < 0.05$ was considered as statistically significant. Asterisks (*) represents $P < 0.05$. Vertical bars indicate standard deviation from the mean value

compared to others (Fig. 4a). Recombinant strains were shown to sporulate efficiently on TBO medium (Fig. 4b).

Actinorhodin and undecylprodigiosin production of the cells were measured in liquid R2YE for 168 h. Sco-pRA*lon* strain produced about 20–34 times more actinorhodin (Fig. 5a) and 5–9 times more undecylprodigiosin (Fig. 5b) than the wild-type and control strain Sco-pRA at 168 h of growth depending on the amount and age of the inoculum. These results were consistent in repeated experiments.

Expression of sigma factor *sigB*, which is responsible from general stress response in the cell and that of *actII-IORF4* and *red*K, which encodes regulatory protein for actinorhodin biyosynthetic gene cluster and one of gene in undecylprodigiosin biyosynthetic gene cluster, respectively, were measured by RT-qPCR. *sigB*, *actIIORF4* and *red*K expressions in Sco-pRA*lon* strain were found to be increased significantly compared to the wild-type (Fig. 6).

Discussion

From bacteria to human, ATP-dependent Lon protease is one of the important enzyme in protein quality control [39, 40]. In this study, the effect of Lon on antibiotic production by *S. coelicolor* A3(2) was determined. For this, a recombinant strain (Sco-pRA*lon*), which have one extra copy of *lon* gene (with its own putative promoter and transcriptional terminator) was prepared. Actinorhodin concentration was dramatically increased in recombinant strain and it was found to be around 34 times more than the wild-type strain.

The elicitation mechanism of secondary metabolism is a complex process and many different factors play role in this process. No significant increase was observed in antibiotic production in the control strain obtained by transferring the empty pRA vector into *S. coelicolor* compared to the wild-type. It seems that the excessive increase in antibiotic production by Sco-pRA*lon* recombinant strain is due to the expression of extra *lon* gene. pRA and other

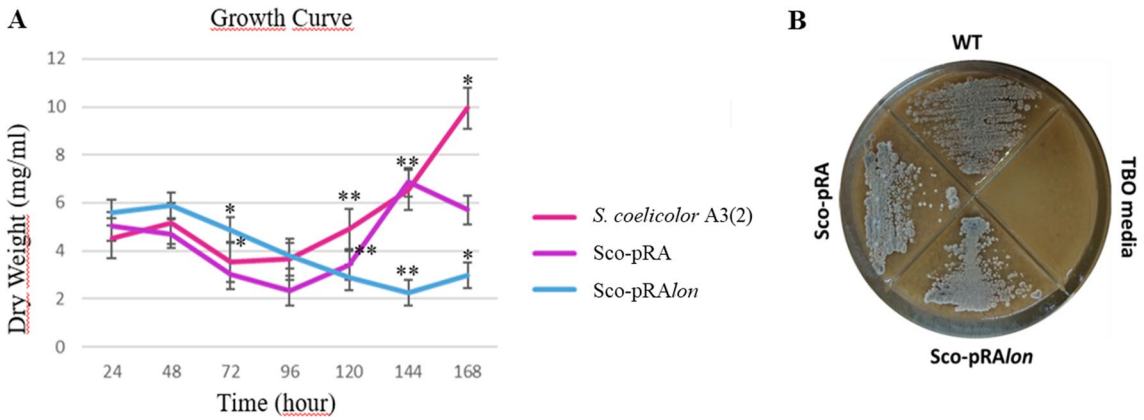

Fig. 4 Comparison of **a** growth and **b** sporulation of bacterial strains in R2YE liquid and TBO solid media, respectively

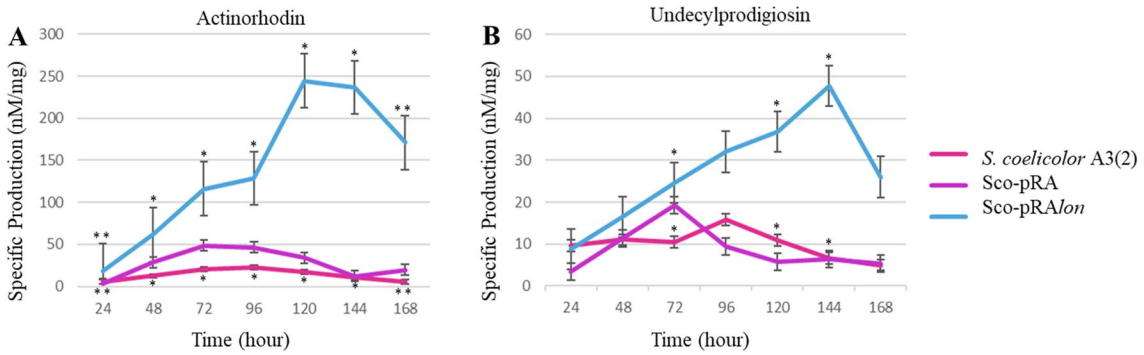

Fig. 5 Comparison of **a** specific actinorhodin and **b** undecylprodigiosin production (nmol/mg dry weight) by wild-type, Sco-pRA and Sco-pRA*lon* strains grown in R2YE. Vertical bars indicate standard deviation from the mean value. *P* values < 0.05 were considered to be statistically significant. One asterisks (*) represents $P < 0.05$ and double asterisks represents (**) $P < 0.005$ according to the *t* test (Microsoft Office Excel). Data are the average of six independent experiments

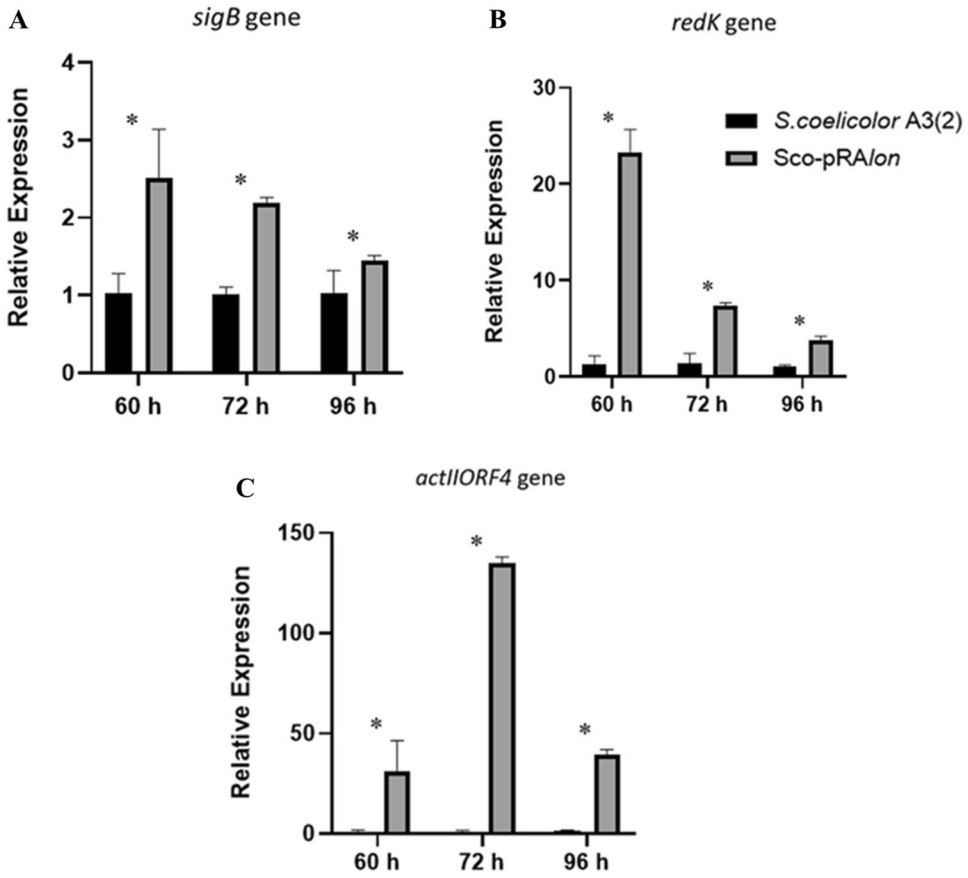

Fig. 6 Comparison of relative gene expression levels of **a** *sigB*, **b** *redK* and **c** *actIIORF4* in Sco-pRA*lon* and wild-type strains grown in R2YE medium. Fold changes were calculated using the $2^{-\Delta\Delta Ct}$ method. The statistical analyses performed with GraphPad Prism 8.0.2 software. *P* values were calculated using the Mann–Whitney test. $P < 0.05$ was considered as statistically significant. Asterisks (*) represents $P < 0.05$. Vertical bars indicate standard deviation from the mean value

integrative vectors enter to an *att*B site in a gene (SCAC2.o6c; SCO3798) encoding "putative chromosome condensation protein" in *S. coelicolor* A3(2) genome. This gene has been shown not to be essential for cell survival [41]. Although the vector integrate to this *att*B site with an efficiency of 300-fold, it is also known that there are other pseudo-*att*B sites in the genome that the vector might enter [41]. Three of these sites (pseB1: inside putative dihydropteroate synthase gene, pseB2: inside hypothetic protein gene and pseB3: inside aspartate aminotransferase gene) were shown to be active phage-integration sites. Neither *att*B nor pseudo-*att*B sites are inside the genes that may affect secondary metabolism.

"Duplicating a gene would double the gene's transcript" idea was not true for this study, since expression of *lon* gene in the wild-type and Sco-pRA*lon* strains was quite different. Understanding of the quantitative factors that govern gene expression is not very well understood. In the literature, there are different studies which show that transcription of a gene increase more than two times when it is integrated into the chromosome as an extra copy. For example, Schmid, Roth [42] have translocated the first four genes of the *his* operon (hisGDCB) of *Salmonella typhimurium*, along with the his promoter, to 16 chromosomal sites. They measured the level of *hisD* expression at these sites under different growth conditions and they have found that different chromosomal sites express the his genes at different levels. Other studies also demonstrates that expression of the genes varies depending on its position on the chromosome [43, 44]. Position effect on gene expression is also seen in eukaryotic organisms [45].

Stress is one of the important factor that triggers secondary metabolite biosynthesis in bacteria [21, 31]. In the case of amino acid starvation and energy shortage, bacteria produce stringent response alarmons (ppGpp and pppGpp), which are produced by "RelA" and "SpoT" proteins, cause cessation of tRNA and rRNA synthesis together with protein synthesis and increase protein degradation processes. By this way amino acids are used in the synthesis of proteins required for adaptation to new environmental conditions. The role of Lon protease in this process is critical [32]. Presence of one extra copy of *lon* in the genome of *S. coelicolor* could cause production of more precursors for antibiotic biosynthesis by direct protein degradation. Besides, ppGpp and pppGpp were also shown to be responsible for direct stimulation of transcription of several amino acid biosynthetic genes [46] which are precursors of some secondary metabolites including actinorhodin [47]. Traxler et al. [48] determined that starvation triggers induction of genes involved in the branched chain amino acid pathways as well

as genes in pathways which generate precursors for branched chain amino acid biosynthesis. Branched-chain amino acid catabolism, in turn, are known to provide precursors for the Type II polyketide antibiotic, actinorhodin, via pathways that are nutrient dependent [49]. Expression of sigma factor that is responsible from general stress response in bacteria, *sigB*, was shown to be increased in the recombinant strain. This result shows that with one extra copy of *lon* gene ScopRA*lon* is under stress. So it is possible that recombinant strain produce more branched chain amino acids which resulted in more actinorhodin production. Detailed experiments are underway to verify this possibility.

Recently, Bucca et al. [50] studied the genome-wide transcriptional and translational changes following heat stress exposure in *S. coelicolor* and find out little correlation between the "transcriptome" and 'translatome' data. Although key proteins like major molecular chaperones and proteases were found to be highly induced at both the transcriptional and translational level, many other transcripts were observed to be not induced at the transcriptional level but they were more highly polysome-associated following heat stress. Based on this study, a detailed transcriptomic and translatomic analysis of the recombinant strain would explain the reason why one extra copy of *lon* gene-boosted antibiotic production in *S. coelicolor*.

Although our attempts still continue, many trials to delete the *lon* gene by using REDIRECT method [37] were unsuccessful suggesting that this gene may be essential in *S. coelicolor*. To our knowledge there is no *Streptomyces* mutant strain with *lon* gene deletion. Sobczyk et al. [34] disrupted expression of *lon* gene in *S. lividans* and the mutant was shown to grow more slowly than the wild-type. However, it is known that disruption and deletion mutants may have different characteristics. Although Lon protease is not a vital enzyme for most bacterial species, there is at least one example where the *lon* gene is essential for viability [8]. Our preliminary results suggest that *lon* may be an essential gene for *S. coelicolor* A3(2). Ching et al. [51] observe a dramatic upregulation of surface antigen gene, surA1, in the *lon*$^{-}$ mutant, which could be caused by an energetic burden (phosphorylation sink) or by disturbance of the functions of the cell, thus having a role in inducing cell death. Although it remains to be elucidated, it is possible that SurA1 protein concentration increase to critical level in *S. coelicolor* so that we were not able to obtain *lon* deletion mutant of this strain.

The development of stable *Streptomyces* strains that produce high amount of secondary metabolites is highly attractive in biotechnology. Although Mak, Nodwell [52] concluded that actinorhodin is a pH-sensitive, strong bacteriostatic antibiotic, to our knowledge it is not used medically. However, it is known that actinorhodin and other colored metabolites produced by *Streptomyces* have been tested in some industrial areas as an alternative to toxic synthetic dyes and effective results have been obtained (https://www.asm.org/index.php/general-science-blog/item/6929bacterialdyesinfashion;https://www.ted.com/talks/natsai_audrey_chieza_fashion_has_a_pollution_problem_can_biology_fix_it). In this respect, our recombinant strain has the potential to be used in industry. But the subject we really wonder is "Does the presence of one extra copy of *lon* gene in the genome of other *Streptomyces*, which produce secondary metabolites with high medical and biotechnological value, will result the same increase in the production efficiency?" Our experiments are ongoing to find an answer to this question.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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