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# Spatio-temporal expression of the pathway−specific regulatory gene *redD* in *S. coelicolor*[*]

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**Abstract:** Confocal laser scanning microscopy was used to observe the spatio-temporal expression of the pathway-specific gene *redD* during *S. coelicolor* cell cultivation. The corresponding mutant *S. coelicolor* lyqRY1522 carrying *redD::eyfp* in the chromosome was constructed. The temporal expression results of the fusion protein during submerged cultivation demonstrated that expression of *redD* began in the transition phase, continuing through the exponential growth phase to the stationary phase, and reached maximum in the stationary phase. On the other hand, *redD* was expressed only in substrate mycelia during solid-state culture, while aerial mycelia remained essentially non-fluorescent throughout culture. Results demonstrated that the expression pattern of *redD* coincides with that of the biosynthesis of the antibiotics during culture, revealing a direct correlation between the spatio-temporal distribution of regulatory gene expression and second metabolism.

**Key words:** *Streptomyces coelicolor*, EYFP, Temporal and spatial expression, Pathway-specific gene *redD*
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## INTRODUCTION

*Streptomycetes* are gram-positive soil bacteria renowned for their ability to produce a large number of different secondary metabolites. Their life cycle involves complex morphological differentiation, which is believed to be closely co-ordinated with gene expression that promotes a variety of physiological and structural changes including the onset of antibiotic biosynthesis. *S. coelicolor* has been extensively studied as a model system with the aid of genetic and molecular techniques (Hopwood *et al*., 1995). Its ability to produce four chemically distinct antibiotics, actinorhodin (Act), undecylprodigiosin (Red), Calcium-Dependent Antibiotic (CDA) and methylenomycin (Mmy), enables studies of pathway-specific and pleiotropic regulation of antibiotic production, in which pathway-specific regulator genes, *actII-orf4* (Fernandez-Moreno *et al.*, 1994) and *redD* (Takano *et al.*, 1992), controlled productions of two dramatic pigmented antibiotics, Red and Act, respectively. Many researches revealed a direct correlation between the regulatory gene expression and second metabolism (Huang *et al.*, 2001).

Green fluorescence protein (GFP) had been established as an important gene reporter and used as a protein tag for analysis of gene expression since its discovery a decade ago (Prasher *et al.*, 1992). Early attempts to use wild-type GFP as a reporter for gene expression in *S. coelicolor* failed to reveal fluorescence, reflecting the relatively AT-rich nature of the wild-type GFP gene (74 mol% G+C of the *S. coelicolor* chromosome). In addition, the wild-type GFP gene contains three TTA codons, which may also have hindered synthesis of GFP. Recently, GFP variants such as EGFP (enhanced green fluorescence

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protein), ECFP (enhanced cyan fluorescence protein) and EYFP (enhanced yellow fluorescence protein) with enhanced fluorescence intensity, stability and removal of all three TTA codons, had been developed (Brendan *et al.*, 1996). These variants exhibit brighter fluorescence and high levels of gene expression in *Streptomyces* (Beavis and Kalejta, 1999; Yang *et al.*, 1998).

In this study, the corresponding mutant *S. coelicolor* lyqRY1522 carrying *redD::eyfp* in the chromosome was constructed. Then, confocal laser scanning microscopy was used to observe the spatio-temporal expression of *redD* during cell cultivation. This research is focused at obtaining more information on the relationship between gene expression and physiological differentiation, and better understanding the regulatory hierarchy of secondary metabolism in *S. coelicolor*.

## MATERIALS AND METHODS

### Strains and culture conditions

*Escherichia coli* DH5α was grown at 37 °C in Luria-Bertin (LB) medium supplemented with apramycin (50 μl/ml), and *E. coli* ET12567 (*dam*$^-$) was cultured at 37 °C in LB medium supplemented with chloramphenicol (30 μl/ml) or apramycin when necessary. *S. coelicolor* strain M145 (wt) used throughout these studies was grown at 30 °C and maintained using standard procedures (Kieser *et al.*, 2000). For broth culture, the spores were pre-germinated as described (Kieser *et al.*, 2000), and then inoculated into modified liquid medium ($R_5^-$), pH 7.2, without additional $KH_2PO_4$, $CaCl_2$ and L-proline, but supplemented with kanamycin (20 μl/ml). Six percent PEG8000 was added to enhance cell dispersal, and antifoam289 was added (0.05%, v/v) before use, $R_5^-$ medium was used for regeneration of the *S. coelicolor* protoplast.

### DNA manipulation

Standard molecular biological techniques were used for all DNA manipulations (Sambrook *et al.*, 1989). All of the PCR reactions were carried out using *pfu* polymerase with hot start, and PCR products were purified using QIA Quick Kit (QIAGEN Companies, Valencia, CA, USA). Preparation of genomic DNA and protoplasts of *S. coelicolor*, protoplast transformation, selection and screening were performed as described by Kieser *et al.*(2000).

### Construction of plasmid pSET1522 carrying *redD::eyfp*

To fuse with *redD* and *eyfp*, pSET152, a plasmid clone vector containing a *Φ*C31 *int* chromosomal attachment site and oriT for transformation of DNA from *E. coli* to *Streptomyces*, was digested with EcoRI and XbaI, and used as a backbone for constructing the gene-integrated plasmid pSET1522 (Fig.1), which could directly integrate into the chromosome of *S. coelicolor* to yield mutant lyqRY1522 (Bierman *et al*., 1992). The 1.37 kb DNA fragment containing the entire *redD* coding region was amplified by PCR using wild-type *S. coelicolor* M145's genomic DNA as a template with the forward primer 1 (5' GCCCACTCTAGAGCAGCCCATGA TGACAATGTG 3') and the reverse primer 1 (5' GACCAAGCTTGGGTCGTCTGGCGCTGAG 3'). The forward primer was designed to contain an XbaI site (underlined) and the reverse primer was prepared with introduction of a HindIII site (underlined), with the stop codon (TGA) removed and changed. The 0.75 kb DNA fragment containing the *eyfp* gene was from plasmid pEYF (Clontech, Palo Alto, CA, USA) digested directly with EcoRI/HindIII. All DNA fragments were digested with the corresponding restriction endonucleases and cloned into the EcoRI/XbaI-digested pSET152 by four-way ligation to create the gene-integrated plasmid pSET1522.

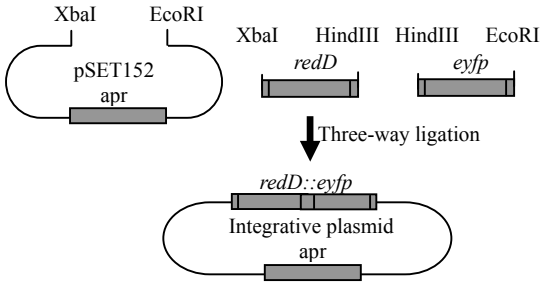


**Fig.1 Construction of the gene-integrated vector pSET1522**

pSET152 digested with XbaI and EcoRI as backbone was ligated with the two DNA fragments *redD* and *eyfp*. The sizes of DNA fragments *eyfp* and *redD* were 0.75 kb and 1.37 kb, respectively

**Western blot analysis**

Method for Western blot analysis was performed as described by Kyung *et al.*(2001), in which 30 μg of the total protein extract was separated by SDS-PAGE (10%) and the purified EYFP (27 kDa) was used as a positive control.

**Confocal microscopy and image processing**

A confocal laser-scanning microscope (CLSM 510, Zeiss, Germany) was equipped with a Zeiss inverted microscope and an argon laser (excitation at 458 nm, 488 nm and 514 nm). A 514 nm laser was used to excite and detect the fluorescence of EYFP in *S. coelicolor* lyqRY1522, and transmission images were obtained simultaneously. All images were stored in a workstation and further image processing was carried out using Adobe Photoshop after conversion of images to JPEG format. All samples were from $R_5^-$ medium, and cross-sections of thin agar slices or liquid samples were placed sideways on the microscope.

**Antibiotic assays for *S. coelicolor***

Actinorhodin (Act): Samples were treated with 1 mol/L KOH to obtain pH 12, and then the culture was filtered through a 0.2 μm filter unit. Absorbance of the filtrate was measured spectrophotometrically at 640 nm.

Undecylprodigiosin (Red): Samples were first extracted with 1 mol/L KOH to solubilize actinorhodin, and then centrifuged at 3000×g for 5 min. The mycelial pellet was washed and dried by vacuum, and then extracted with pH 2 methanol acidified with 1 mol/L HCl overnight at room temperature. The filtrate was detected spectrophotometrically at 540 nm after passage through a 0.2 μm filter unit.

All fermentation tests were performed using a completely randomized design. Every test was repeated three times, and results are the mean of values for five flasks. Statistical analysis was performed using a mean three repeat test based on Dunnett's LSD test.

## RESULTS AND ANALYSIS

**Construction of *S. coelicolor* lyqRY1522 carrying *redD::eyfp* in the chromosome**

Optimal application of EYFP as reporters for gene expression in *Streptomycetes* requires stable insertion of a gene at single copy in the chromosome. To achieve this, the gene-integrated plasmid pSET1522 was constructed, which is a derivative of the integrative pSET152, containing an origin of transfer from RK2, the pUC18 origin of replication and an apramycin-resistance gene, *aac* (3) IV, for maintenance and selection in *E. coli* and *Streptomyces*. It possesses a *Φ*C31 *int* gene and *attp* site, allowing insertion of the plasmid at the chromosome *Φ*C31 attachment site, and it uses apramycin resistance for selection in *S. coelicolor*. The constructed plasmid pSET1522 was transferred into *Escherichia coli* DH5α and subsequently introduced into the methylation-deficient *E. coli* ET12567. After protoplast transformation, selection and screening of *S. coelicolor* M145 (wt), the gene-fusional mutant *S. coelicolor* lyqRY1522 carrying *redD::eyfp* in the chromosome was acquired.

**Expression of the RedD-EYFP fusion proteins**

The RedD-EYFP fusion protein was detected by western blot, and results demonstrated that the *redD::eyfp* fusion gene was properly expressed (Fig.2). Fig.2 indicated that the peptide antibody recognized a single band at approximately 64.8 kDa region corresponding to the RedD-EYFP fusion protein (the sizes of EYFP and RedD are 27 and 37.8 kDa, respectively) in the *S. coelicolor* lyqRY1522 cell-free protein extracts, while no band showed in the *S. coeli-*

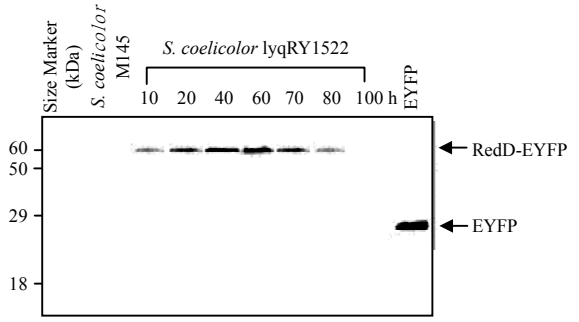


**Fig.2 Western blot analysis of *S. coelicolor* M145 (wild type) and *S. coelicolor* lyqRY1522 using anti-GFP peptide antibody**

Thirty micrograms portions of total protein extracts prepared from *S. coelicolor* M145 and lyqRY1522 were loaded into each lane. Samples of *S. coelicolor* lyqRY1522 were prepared at different culture time after inoculation of spores. Purified EYFP was used as a positive control

*color* wild type cell-free extract. Although the fusion protein was not expressed early in culture, it was observed at 10 h. As culture proceeded, the expression level of the fusion protein increased, reaching maximum at about 60 h after inoculation of the spores, and then subsequently decreased. These findings are consistent with the culture kinetics (see below).

### Growth kinetics and antibiotic titers of *S. coelicolor*

Measurement of both the growth kinetics and antibiotic titers of *S. coelicolor* lyqRY1522 and *S. coelicolor* M145 (wild-type) grown in $R_5^-$ liquid medium (Fig.3), revealed that both Red and Act synthesis began in the transition phase, continuing through the exponential growth phase to the stationary phase, when they reached maximum. The experiments indicated that there was little difference between the mutant and M145 in the Red production and cell growth. However, compared with the wild-type strain, the Red production of lyqRY1522 increased by about 50%.

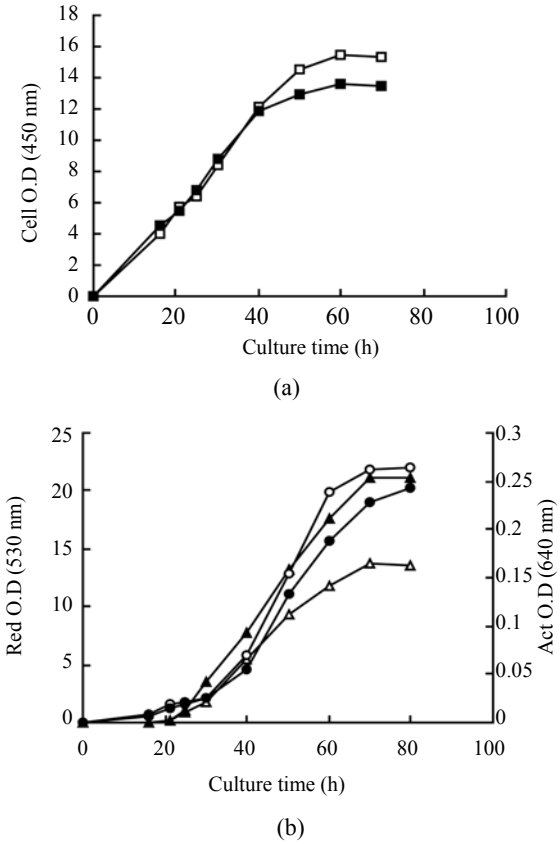


**Fig.3 (a) Growth kinetics and (b) antibiotic titers in cultures of *S. coelicolor* lyqRY1522 and wild type M145 growing in $R_5^-$ liquid medium**

Cells grown for 30 h after inoculation of spores were inoculated and incubated at 28 °C in 500 ml baffled shake flasks containing 125 ml of $R_5^-$. Growth kinetics were significantly different at $P<0.05$ based on Dunnett's LSD test. Values are means of three replications. (■, ●, ▲) represent *S. coelicolor* lyqRY1522 and (□, ○, Δ) represent wild type M145, in which (■, □) represent growth kinetics, with (▲, Δ) for Red and (●,○) for Act

### Detection of spatio-temporal distribution of RedD-EYFP

To examine the spatio-temporal expression distribution of *redD::eyfp*, the EYFP fluorescence protein of samples taken at different times of solid and liquid culture of mutant *S. coelicolor* lyqRY1522 were observed using confocal laser scanning microscopy. Samples of Fig.4 came from the mycelium of liquid culture, while samples of Fig.5 are cross-sectional views of solid culture.

During liquid culture, at the beginning, only a part of the mycelia showed weak EYFP. With culture

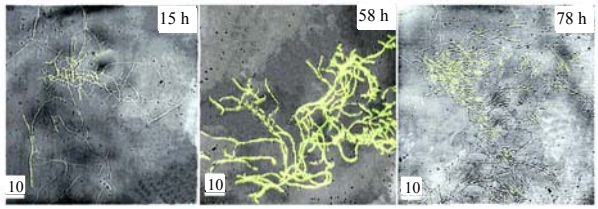


**Fig.4 Detection of gene expression of *redD::eyfp* in *S. coelicolor* lyqRY1522 in liquid culture**

EYFP images were acquired by the method of image processing described in the text and shown in each panel. Times in the fluorescence images represent the sampling points after inoculation. Scale bar=10 μm

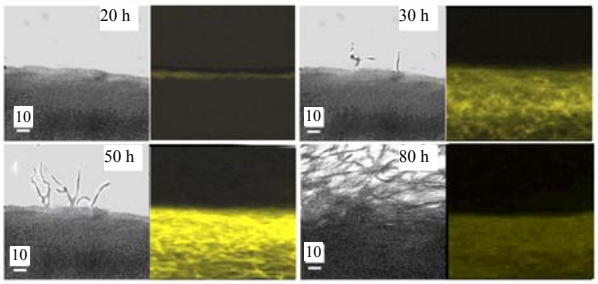


**Fig.5 Detection of spatio-temporal distribution of RedD in *S. coelicolor* lyqRY1522 on solid medium**

EYFP images and transmission images of confocal microscopy are shown on the right and left of each panel, respectively. Times of sampling points are shown in the gray photographs. Scale bar=10 μm

proceeding, the fluorescence level of EYFP and the fluorescence percentage of mycelia increased. Most of the mycelia at 58 h exhibited the highest intensity of EYFP. Then, the EYFP fluorescence decreased rapidly toward the end of the culture. During solid culture, at 20 h the fluorescence was initially detected near the surface of the agar. Then, the fluorescence level of EYFP and the fluorescence percentage of mycelia increased in the substrate mycelium embedded in agar, whereas aerial hyphae from the bacterial colonies exhibited no fluorescence. The fluorescence reached maximum at 50 h, but was mainly confined to the substrate mycelia. At 80 h, the aerial hyphae formed a complex network of filaments, but remained non-fluorescent, while the previously fully fluorescent substrate mycelia exhibited rapid decrease in fluorescence. The experiments also indicated that the concentration of mycelia or colony number per agar plate greatly affected the expression time of genes, which demonstrated that signaling between bacteria also affected gene expression (Gary and Stephen, 1999).

## DISCUSSION AND CONCLUSION

The temporal expression mode of the fusion protein during submerged cultivation indicated that the expression of *redD* began in the transition phase, continuing through the exponential growth phase to the stationary phase, when it reached maximum. During solid-state culture, *redD* expressed only in substrate mycelia, while the aerial mycelia remained essentially non-fluorescent throughout the cultivation.

Antibiotic production in *Streptomyces* species is generally dependent on the growth phase and involves expression of physically clustered regulatory and biosynthetic genes. In addition, when the fluorescence in substrate mycelia of lyqRY1522 emerged, the microorganism started to excrete Act; and the growth of *S. coelicolor* lyqRY1522 reached the climax at which the fluorescence in substrate mycelia was strongest too. The results above indicated that the *redD* plays a pivotal role in determining the onset of Act production, thus the expression of the regulative gene *redD* coincided with the biosynthesis of the antibiotics during batch fermentation, which revealed a direct correlation between the spatio-temporal distribution of regulatory gene expression and second metabolism.

Since the pathway-specific regulatory gene *redD* control Red production, accumulation of the *redD* transcripts increases remarkably upon entry into the stationary phase, followed by the emergence of transcripts for biosynthetic genes. Furthermore, addition copies of *redD* cause the overproduction of Red. In this research, the copy number of *redD* in *S. coelicolor* chromosome was increased by gene-integration, indicating the increase of the corresponding antibiotic. However, the Act production of the mutant was increased only 50%, which demonstrated that the pathway-specific regulation was not the sole factor in controlling antibiotic synthesis. This suggested that the regulatory network was very complicated. Analysis of the spatio-temporal expression of regulatory genes is essential for a complete understanding of gene regulatory patterns and morphology, including physiological development in *Streptomyces*. Ultimately, this understanding will provide new strategies for manipulating secondary metabolism.

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