

# Spatial and temporal regulation of protein expression by *bldA* within a *Streptomyces lividans* colony

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**Abstract** The *bldA* gene encodes the only tRNA for the UUA codon that, although dispensable in genes important for primary vegetative growth of *Streptomyces* spp., is important in genes that serve a regulatory purpose in the differentiation. To investigate this role further, the spatial and temporal expression profiles of the *bldA*-regulated and unregulated genes within a *Streptomyces* colony were examined using modified genes for the green fluorescent protein (*gfp*) as an expression-tag. A comparative study, based on computer-assisted quantitative analysis of the GFP fluorescence, revealed that the presence of TTA codons in *gfp* results in a temporal delay of translation and, consequently, changed the spatial pattern of the GFP expression within a colony, especially during early differentiation. The delay of GFP expression was undetectable at 60 h post-inoculation. These results provide the first extensive evidence that the *bldA* does indeed play a significant regulatory role during colony differentiation.

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**Key words:** Visualization; *bldA* regulation; Green fluorescent protein; *Streptomyces*

## 1. Introduction

A bacterial colony on solid medium is a regulated population consisting of cells in different growth stages. Thus, it can be regarded as a closed regulatory circuit. A change in gene regulation within a colony over time alters the cellular physiology and is regarded as a mimicked definition of differentiation. *Streptomyces* colonies represent a good model to investigate bacterial differentiation because of their complex life cycle including spore germination, substrate mycelium growth into the medium, aerial mycelium extension into the air and sporulation taking place in the aerial hyphae tips. Many mutants that are defective in differentiation have been described and the corresponding genes have been isolated [1]. One of these genes, *bldA*, encodes a leucyl-tRNA that recognizes the UUA codon [2,3]. *bldA* is needed in several species of *Streptomyces* for the expression of many genes which are not necessary for growth but are indispensable for secondary metabolism, morphological differentiation, carbon catabolite regulation and cell-cell signaling [4–10]. This suggests that *bldA* functions as a regulator of mRNA translation in early stages of differentiation. To clarify *bldA* regulation, it is im-

portant to understand the spatial and temporal profiles of *bldA*-regulated gene expression during differentiation. Studies using either phenotypic analysis or the immunoblot detection of reporter gene expression have provided significant temporal information about the *bldA* regulation [11,12]. However, spatial information of *bldA* regulation remains obscure because of technical limitations. In this study, using laser scanning confocal microscopy (LSCM) and a specifically engineered green fluorescent protein (GFP) as an expression-tag to overcome the limitations of existing techniques [13–15], we demonstrate the regulation profile of *bldA*, resolved in time and space during a colony differentiation and not summed over the whole bacterial population.

## 2. Materials and methods

### 2.1. General technique

*Streptomyces lividans* TK21 and *Escherichia coli* DH5a were used as host strains. The cultivation and transformation of *E. coli* and *S. lividans* and DNA handling were according to the standard protocols [16,17]. *S. lividans* transformants were selected with thiostrepton (5 mg/ml). Enzymes for genetic manipulation were purchased from TAKARA. All chemicals used in this study were obtained from Sigma. For monitoring GFP expression in *S. lividans* single colonies, thin R2YE medium (up to 3 mm in depth) was used.

### 2.2. Modifications of GFP gene and plasmid constructions

The *gfp*(S65T) gene for a red shift mutant of GFP [18,19] was kindly provided from H. Takahashi (Mitsubishi-kasei Institute of Life Sciences). The gene was modified by site-directed mutagenesis using a polymerase chain reaction (PCR). Two modified *gfp* genes, *gfp*(TTA) and *gfp*(TTG), of which expressions are *bldA*-dependent and independent, respectively, were constructed with the following relevant features (Fig. 1). The original *gfp*(S65T) gene contains three TTA codons, corresponding to Leu-18, Leu-83 and Leu-153. The first TTA codon for Leu-18 was changed to the synonymous TTG codon in *gfp*(TTA) and all the three codons were changed to TTG in *gfp*(TTG). To ensure efficient translation, the initial six codons were changed to codons suitable for the codon usage in *Streptomyces* and a typical ribosomal binding site (RBS: 5'-GGAGG-3') was added 6 bp upstream of the initiation codon in both genes. The modified genes were confirmed by sequencing. Both genes were designed to be transcribed from a strong promoter, *p-tra*, of the *Streptomyces nigricans* plasmid pSN22 [20]. The *tra* promoter has a sequence that is likely recognized by the vegetative sigma factor of *Streptomyces* and is considered to be a constitutive promoter [21,22]. The promoter-modified gene fragments were inserted between *Eco*RI and *Hind*III sites of pIJ486 [23] and the resultant plasmids were introduced into *S. lividans* TK21 by transformation. The thiostrepton-resistant transformants containing the plasmids were named TK21GFT (*gfp*(TTG) inserted) and TK21GFS (*gfp*(TTA) inserted), respectively.

### 2.3. Fluorescence microscopy

Single colonies of transformants were observed from the agar side using an OLYMPUS IX70 microscope (OLYMPUS) with a xenon

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lamp and a filter set for observation of fluorescence from fluorescein isothiocyanate. The fluorescence and bright images were captured by a chilled CCD camera system controlled by IP Lab Spectrum (Signal Analytics Corporation) on a Macintosh 8500. For quantitative analysis, the same exposure time was used for samples at the same time point. To detect the GFP fluorescence within a colony, LSCM was used. To prepare samples supplied for LSCM, the colony on the thin R2YE plate was sliced vertically through the center of the colony by a razor blade. The slice was put on a 0.1 mm thick glass slide on which 2 ml of water was dropped. To observe very young (10 h) cultures where colonies could not be identified, spores were streaked onto the thin R2YE plate and the slice was made using the streaked trace as a landmark. The distribution of fluorescence signal within a colony was monitored by a GB-200 confocal laser scanning microscope (OLYMPUS). The argon laser beam (wavelength 488 nm) was used for excitation and the fluorescence signals were split with a dichroic mirror (550 nm) and a 500–530 nm bandpass filter for monitoring. To gain high reliability in quantitative analysis of captured images, the same operation parameters (i.e. laser strength and confocal aperture) were used for samples at the same time point. The confocal images were saved as TIF files and image analysis of the files was performed by IP Lab Spectrum. At each time point, colonies of approximately the same diameter were used for analyses.

### 3. Results and discussion

#### 3.1. Differences between genes in temporal expression

To elucidate the effect of *bldA* regulation on GFP expression, we first analyzed the fluorescence emitted from single colonies of TK21GFS and TK21GFT by fluorescence microscopy at 24 and 48 h post-inoculation. Single colonies within 48 h post-inoculation were captured as single images in our system. The fluorescence intensity of a TK21GFT colony was stronger than that of a TK21GFS colony at 24 h post-inoculation (Fig. 2A). This difference was conserved among 10 colonies tested. To present the difference quantitatively, the fluorescence intensity was calculated as an average intensity value per pixel of a selected region across the colony. As shown in Fig. 2B, the maximum fluorescence intensity value measured from a TK21GFT colony was approximately twice that from a TK21GFS colony. At 48 h post-inoculation, the difference in the fluorescence intensities between the strains was reduced, whereas the fluorescence distributions remained distinct between the strains (Fig. 2A,B). The fluorescence distribution pattern emanating from the TK21GFT colony was triangular (Fig. 2B). Since the expression of *gfp(TTG)* is constitutive, the fluorescence distribution should reflect the cell density at each location in a TK21GFT colony. On the other hand, the fluorescence distribution pattern in TK21GFS differed from the triangular pattern. The difference in the fluorescence distribution patterns between colonies is thought to

result from the time delay of GFP expression in TK21GFS and/or a different spatial distribution of GFP expression by *bldA* regulation through UUA codons. Differences in the fluorescence between the strains, particularly early in colony growth, and the reduction in this difference over time suggest that the *bldA* regulation is more effective early in colony differentiation.

#### 3.2. Imaging of GFP expression within a colony

To provide more detailed information on the spatial and temporal profiles of *bldA* regulation, LSCM analysis was performed. Five–seven independent TK21GFS and TK21GFT colonies at 10, 24, 48 and 60 h post-inoculation were examined. The images of the cross section along the vertical axis of a colony and the result of quantitative analysis of each image are shown in Fig. 3. At 10 h post-inoculation, when spores germinate and substrate mycelium grows, limited detectable fluorescence was present on the medium surface in TK21GFS cultures. However, GFP expression of substrate mycelium in the medium was clearly detectable in TK21GFT cultures. Thus, *bldA* strictly regulates the expression of genes containing TTA codons just after spore germination. The GFP fluorescence, detected in TK21GFS at the medium surface, implies that *bldA* is expressed within 10 h after spore germination. At 24 h post-inoculation, the distribution patterns of GFP expression in both strains were similar, however, the GFP expression level in TK21GFS remained lower than that in TK21GFT. This difference was consistent with the result of fluorescence microscopic analysis at the same time point. At 48 h post-inoculation, possibly the last stage of substrate mycelium growth, the difference of the maximum fluorescence intensity was further reduced, but the GFP distribution was very different between the two strains. The fluorescence intensity in mycelium at the medium surface was higher than that at the basal area in all TK21GFS images, whereas fluorescence was almost equally distributed in both areas in most images (six of seven) of TK21GFT. The difference clearly indicates the suppression of GFP expression in recently divided substrate mycelium in TK21GFS cultures. Therefore, the timing delay of gene expression introduced by *bldA* results in a large spatial difference of gene expression within a colony. The differences demonstrated by LSCM analyses were more drastic than that shown by fluorescence microscopic analyses. This inconsistency may be due to differences in spatial resolution between both techniques. At 60 h post-inoculation, when aerial mycelium may begin to grow into the air, the specific differences in both intensity and distribution

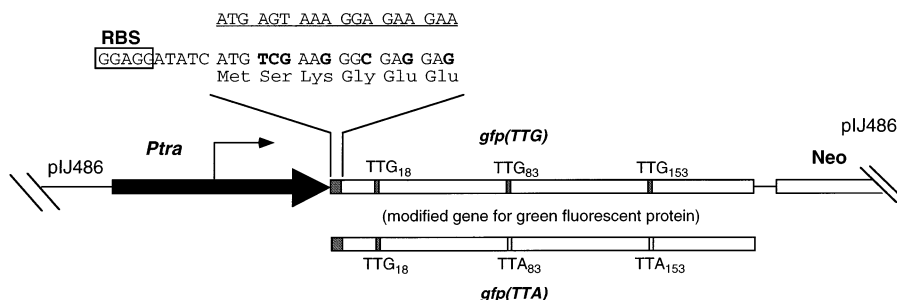


Fig. 1. The structures of the modified *gfp* genes used in this study. The modified location, the TTA codons for Leu-18, Leu-83 and Leu-153 and the N-terminal portion are indicated by shadowed boxes. N-terminal sequences of the wild-type S65T and the modified genes are presented. Altered bases are shown in bold letters. The RBS sequence is indicated by boxed characters.

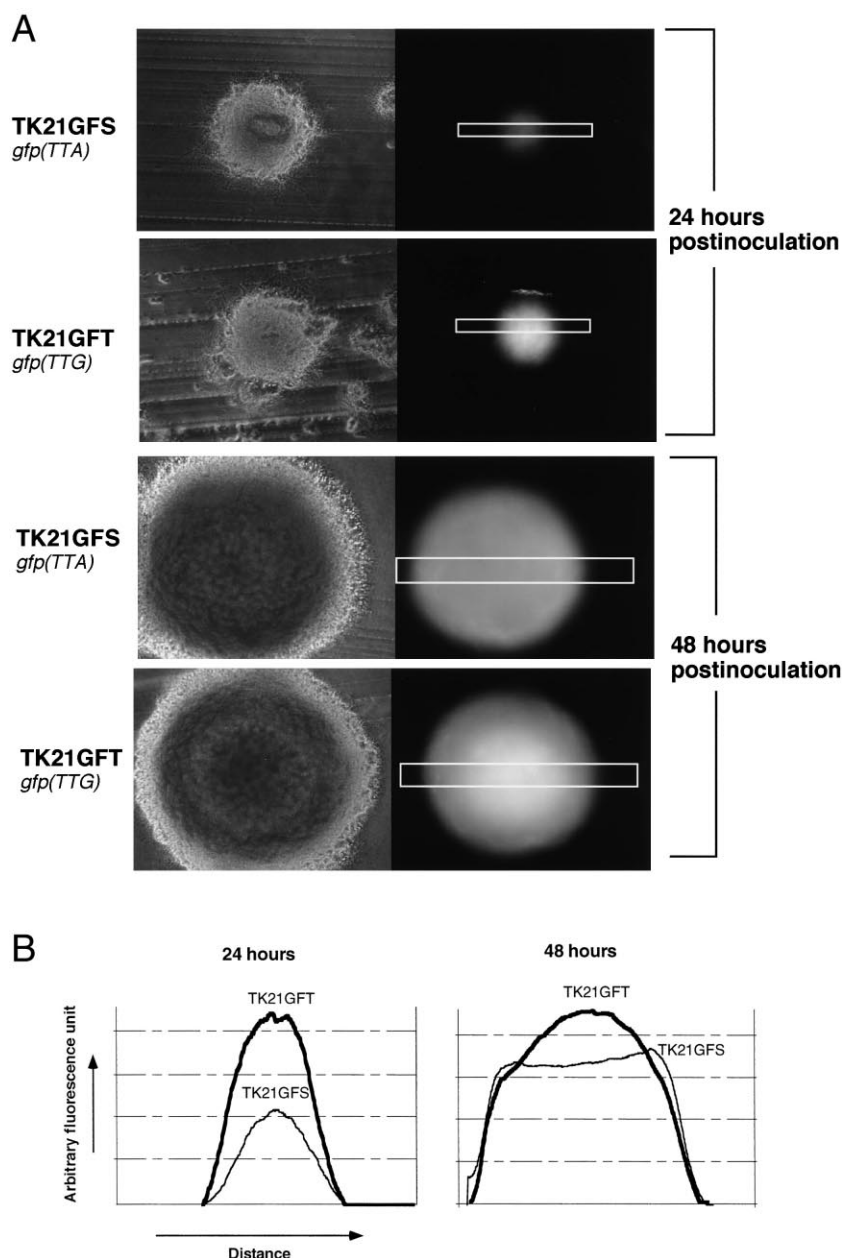


Fig. 2. (A) Bright-field (left) and fluorescent (right) microscopic images of the colonies at 24 and 48 h post-inoculation. Each image consists of  $550 \times 650$  pixels. White boxes indicate regions for quantitative analysis. (B) The results of quantitative analysis. A region was selected as a rectangle through the middle of the colonies. The shorter side consists of 20 (24 h) or 60 (48 h) pixels of the image. The average fluorescent intensity of 20 (60) pixels at any point along the  $X$ -axis was calculated and the values were plotted against the  $X$ -distance. Bold and thin lines indicate the fluorescence from TK21GFT and TK21GFS, respectively.

of fluorescence could not be observed in all images, indicating that the GFP expression in TK21GFS is equivalent to that in TK21GFT by this timepoint. These results suggest that the effect of *bldA* regulation on GFP expression within a colony may be ineffective at 60 h after spore germination. Therefore, *bldA*-dependent translation is fully active in any region of a colony at this time.

The levels of *gfp* mRNA should be equivalent throughout growth in TK21GFT and TK21GFS because both modified *gfp* genes are transcribed from the same constitutive promoter. The only difference in GFP expression between the two strains is in mRNA translation. Thus, in conjunction with previous studies, we speculate that GFP expression in

TK21GFS may be delayed in relation to GFP expression in TK21GFT [1]. The differences in GFP expression between the strains during early differentiation are consistent with this supposition. It has been demonstrated previously that a *bldA* mutant cannot differentiate from substrate mycelium to aerial mycelium [1,3]. This suggests that *bldA* regulation is dispensable for the growth of substrate mycelium. Our result, that *bldA*-dependent translation was suppressed in substrate mycelium, is in good agreement with these reports. Temporally, *bldA*-dependent translation begins in cells approximately 10 h after their division and *bldA* becomes fully active in a whole colony at 60 h after spore germination. Spatially, the alteration in the timing of gene expression results in a change

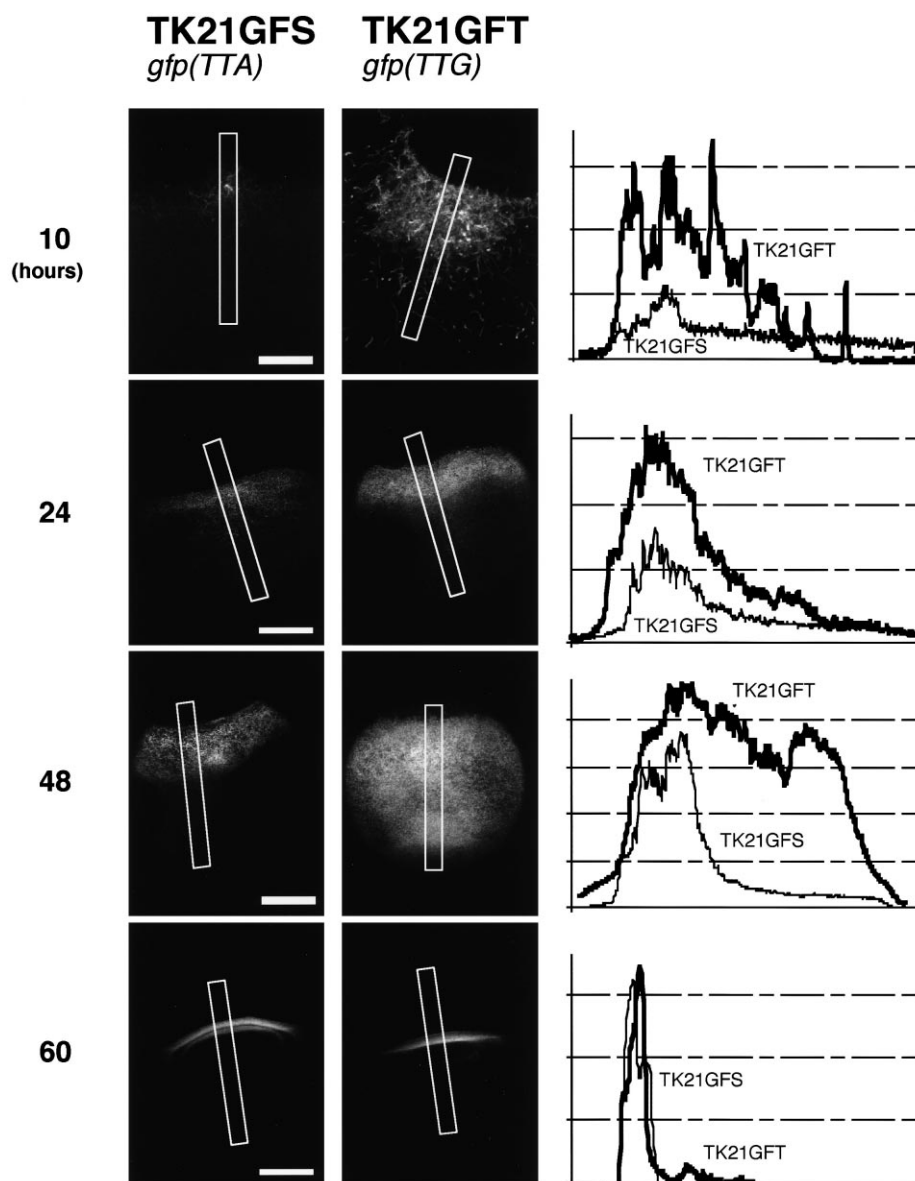


Fig. 3. Left panel: Confocal microscopic images of TK21GFS and TK21GFT colonies. Laser scanning is conducted 10 mm inside from the slide glass. Each image consists of  $1024 \times 768$  pixels. The white rectangles in the images indicate selected regions for quantitative analysis. Scale bars indicate 20 mm for the 10 h sample, 80 mm for the 24 and 48 h sample and 640 mm for the 60 h sample, respectively. Right panel: The results of quantitative analysis. A region was selected as a rectangle with the long side being perpendicular to the medium surface. The shorter side consists of 50 pixels of the image. The average fluorescent intensity of 50 pixels at any point along the X-axis was calculated and the values were plotted against the X-distance. Bold and thin lines indicate the fluorescence from TK21GFT and TK21GFS, respectively.

in the pattern of gene expression within *Streptomyces* colonies, especially in the late phase of substrate mycelium growth. This spatial inclination of gene expression within a colony, that is introduced by *bldA*, must be essential for the physiological differentiation which consists of harmonized genetic regulations. These results add to the basic knowledge needed to clarify developmental regulation within a *Streptomyces* colony.

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