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GENOMICS, TRANSCRIPTOMICS, PROTEOMICS

# The ECF sigma factor SigT regulates actinorhodin production in response to nitrogen stress in *Streptomyces coelicolor*

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**Abstract** Sigma factors of the extracytoplasmic function (ECF) subfamily are important regulators of stress responses in bacteria. This work described the characterization of ECF sigma factor SigT in *Streptomyces coelicolor*. We found the absence of *sigT* almost abolished the production of the antibiotics actinorhodin (Act) under nitrogen stress. Under nitrogen-limited conditions, significantly reduced Act production and linked *act*II-ORF4 transcription with respect to wild type were observed in the *sigT*-null mutant. Using reporter (*xylE*) fusion to *sigT* promoter, we demonstrated that *sigT* was induced by nitrogen limitation in a SigT-dependent manner. Transcriptional analyses showed that SigT controlled the expression of *relA*, the ppGpp synthetase gene, and consequently affected the Act production upon nitrogen starvation. Co-transcription analysis revealed that *sigT* was co-transcribed with *rstB* (gene upstream of *sigT*) but not with *rstA* (gene downstream of *sigT*). Phenotypic and transcriptional results suggested RstA may modulate the activity of SigT positively.

**Keywords** Sigma factor · Nitrogen stress · Secondary metabolism · *Streptomyces coelicolor*

## Introduction

During the complex life span, the soil-dwelling bacterial genus *Streptomyces* is challenged with diverse nutritional and environmental stresses, including high osmolarity, heat and cold shock, pH variation, and nutrient starvation, etc. (Chater 2001). Stresses lead to the reduction or cessation of growth, and also to a major switch in gene expression through the use of alternative sigma factors for altering the recognition specificity and binding strength of RNA polymerase to the target promoters (Ishihama 2000).

Among $\sigma^{70}$ family, the extracytoplasmic function (ECF) sigma factors present the largest subfamily. They are involved in responses to a wide range of environmental stresses, such as metal homeostasis, starvation, high temperature, reactive oxygen species, and antibiotics (Helmann 2002; Anthony et al. 2005; Alvarez-Martinez et al. 2006, 2007; Bang et al. 2005). In most cases, genes encoding for ECF sigma factors are co-transcribed with a gene encoding their cognate regulator, an inner membrane anti-sigma factor (Helmann 2002).

The best-understood archetypes of ECF sigma factors include *Streptomyces coelicolor* SigR, SigE, and SigB, etc. SigE was characterized to control cell envelope stress response (Paget et al. 1999a, b), SigR controls disulphide stress response (Paget et al. 1998), and SigB is responsible for osmotic stress response (Kormanec et al. 2000). Nevertheless, up to date, the ECF sigma factor responding to nutrient starvation has not been identified in *S. coelicolor*. SigB of *S. coelicolor* may be a candidate as its structural homologue in *Bacillus subtilis* could respond to nutrient stress (Hecker and Völker 1998).

A very important aspect of *Streptomyces* biology is the ability of these bacteria, during stationary phase in liquid culture or at the onset of differentiation on solid medium, to synthesize a wide variety of secondary metabolites, including many antibiotics (Bibb 2005). Nutrition plays an important role in the onset and intensity of secondary metabolism. Control of antibiotics biosynthesis was demonstrated to be a multifunctional process in which limiting

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nutrients such as nitrogen and phosphate play different roles (Doull and Vining 1990). Two sigma factors in *S. coelicolor* had been reported to affect the production of the pH-reactive antibiotic actinorhodin (Act; red at pH below 8.5, blue at pH above) and of the red-pigmented tripyrrole antibiotic undecylprodigiosin (Red). One is principal and essential sigma factor HrdB, mutation of *hrdB* could abolish Act and Red production by reducing the ppGpp pool size (Wang et al. 2010). The other is SigB; the *sigB* mutant produces dramatically higher levels of Act precociously, while producing very low amounts of Red. This phenomenon results primarily from the absence of catalase B which is critically required for osmoprotection and proper differentiation of *S. coelicolor* cells (Cho et al. 2001). However, no ECF sigma factor in *S. coelicolor* has been characterized that could connect nutrient stress with antibiotics production.

In our previous work, we described that both ECF sigma factor SigT and its putative anti-sigma factor RstA negatively regulate morphological differentiation and secondary metabolism in *S. coelicolor* (Mao et al. 2009). Here, we show that SigT regulates the Act production of *S. coelicolor* upon nitrogen starvation, and this regulation role might result from the control over the transcription of *relA* by SigT. Promoter activity and real-time PCR analysis showed *sigT* gene expression was induced by nitrogen stress in SigT-dependent manner. Our findings suggested that SigT may function as a link between nitrogen stress and antibiotics production. Our results also revealed that the gene *rstA* encoding putative anti-sigma factor of SigT was not co-transcribed with *sigT*. The phenotypic and transcriptional analysis of *rstA*-null mutant coincided well with the proposal that RstA acts as a positive regulator of SigT.

## Materials and methods

### Bacterial strains and culture conditions

The *S. coelicolor* strains used in this study are listed in Table 1. *Escherichia coli* TG1 was used for plasmid construction. *E. coli* ET12567/pUZ8002 was used for DNA introduction into *S. coelicolor* by conjugation (MacNeil et al. 1992). *E. coli* BW25113/pIJ790 and DH5α/BT340 were used in λRED-mediated PCR-targeted mutagenesis of *rstB* (Gust et al. 2003).

*S. coelicolor* strains were grown and sporulated on solid R2YE and mannitol soya flour agar medium at 30°C. Transformations were performed as previously described (Kieser et al. 2000). SMMS (a solidified version of supplemented minimal medium, SMM) (Floriano and Bibb 1996; Takano et al. 1992), R5 and R2 (Kieser et al. 2000) were used to assess antibiotic production. RNA was isolated from liquid cultures grown in SMM (Takano et al. 1992; Hobbs et al. 1989) or tryptic soya broth (TSB). For the nitrogen-limited condition, a modification of R2 solid medium was used. The composition of this modified medium, per liter, was: sucrose 103 g; $K_2SO_4$ 0.25 g; $MgCl_2{\cdot}6H_2O$ 10.12 g; glucose 10 g; $KH_2PO_4$ 7.2 mM; $CaCl_2{\cdot}2H_2O$ 2.94 g; L-proline 1.3 mM; TES buffer 5.73 g; trace element solution 0.2 ml; total nitrogen: 1.3 mM; pH 7.2. For the nitrogen-rich condition, nitrogen-limited R2 medium was supplemented with Difco casamino acids of 2 g (BD, NJ, USA). For growth and antibiotics production in liquid, SMM was used. The composition of this medium, per liter, was: PEG6000 49.95 g; $MgSO_4{\cdot}7H_2O$ 0.6 g; TES buffer 5.73 g; glucose 10 g; trace element solution 0.1 ml; $NaH_2PO_4$+$K_2HPO_4$ (50 mM each) 50 ml; Difco casamino acids 2 g; total phosphate 5 mM; total nitrogen 2 g; pH 7.2. For the analysis under nitrogen-limited or phosphate-limited condition, the *S. coelicolor* strains were grown in SMM medium until mid-exponential phase ($OD_{450nm}$≈0.5), and subjected to nitrogen or phosphate depletion by rapidly transferring the cultures to nitrogen-limited SMM (without Difco casamino acids) or phosphate-limited SMM (total phosphate concentration was reduced to 0.05 mM).

If necessary, 25 mg $ml^{-1}$ kanamycin, 25 mg $ml^{-1}$ thiostrepton or 25 mg $ml^{-1}$ apramycin was added to the growth medium.

*E. coli* was grown in Luria broth at 37°C, supplemented with 100 μg ampicillin $ml^{-1}$, 50 μg apramycin $ml^{-1}$, 25 μg chloramphenicol $ml^{-1}$, 50 μg kanamycin $ml^{-1}$, and 50 μg streptomycin $ml^{-1}$ if necessary.

**Table 1** List of *Streptomyces* strains used in this study

| Strains | Description | Source or reference |
|---|---|---|
| M145 | Wild-type SCP1-,SCP2- | Kieser et al. (2000) |
| LM21 | *sigT* in-frame deletion mutant | Mao et al. (2009) |
| LM22 | *rstA* in-frame deletion mutant | Mao et al. (2009) |
| MRB1 | M145/pMRB2,*rstB* in-frame deletion mutant | This work |
| LM23 | *sigT*-complemented strain | Mao et al. (2009) |
| LM24 | *rstA*-complemented strain | Mao et al. (2009) |
| MRB2 | M145/pMRB6,*rstB*-complemented strain | This work |
| MRB3 | M145/pMRD278 | This work |
| MRB4 | M145/pMRB3 | This work |
| MRB5 | LM21/pMRB3 | This work |
| MRB6 | M145/pMRD279 | This work |

### Plasmid construction

The plasmids and cosmids used in this work are listed in Table 2. The primers used in this study are listed in Table 3.

The *sigT* promoter was cloned by PCR amplification with primers F3 and R3, and cloned into pTA2 to yield pMRB4,

**Table 2** List of plasmids and cosmids used in this study

| Plasmid | Description | Source or reference |
|---|---|---|
| PIJ8630 | Promoter-probe plasmid using *egfp* as reporter | Sun et al. (1999) |
| PTA2 | Vector system for the cloning of PCR products | Toyobo, Osaka, Japan |
| PIJ773 | pBluescript KS(+) derivative. Template plasmid containing the apramycin resistance gene *aac(3)IV* and the *ori*T of plasmid RP4 | Gust et al. (2003) |
| PIJ778 | pBluescript KS(+) derivative. Template plasmid containing the streptomycin/spectinomycin resistance gene *aadA* and the *ori*T of plasmid RP4 | Gust et al. (2003) |
| Cosmid N03-16 | 33-kb DNA fragment from *S. coelicolor* cloned into Supercos-1 carrying the *rstB* gene | Gift from Prof. Zhong-Jun Qin, Shanghai |
| PMRB1 | *rstB* gene in cosmid N03-16 was replaced by *ori*T + *aadA* cassette from pIJ778 | This work |
| PMRB2 | *ori*T + *aadA* cassette in cosmid pMRB1 was replaced by "scar" | This work |
| PLM1 | PIJ8630 derivative. *ermE*p* controls the expression of *egfp* gene | Mao et al. (2009) |
| PMRD278 | PLM1 derivative. *ermE*p* controls the expression of *xylE* gene | Unpublished, our lab |
| PMRD279 | PIJ8630 derivative. Promoter-probe plasmid using *xylE* as reporter instead of *egfp* | Unpublished, our lab |
| PMRB3 | *Bgl*II-*Xba*I fragment carrying *sigT* promoter cloned into pMRD279 | This work |
| PMRB4 | PCR product carrying the *sigT* promoter cloned into pTA2 vector | This work |
| PMRB5 | PCR product carrying the *S. coelicolor rstB* gene with *Nde*I site cloned into pTA2 vector | This work |
| PMRB6 | *Nde*I fragment carrying the *rstB-linker* gene cloned into pLM1 | This work |

which was verified by sequencing. pMRB4 was digested with *Bgl*II and *Xba*I, and the resulting 140 bp DNA fragment was cloned into the vector pMRD279 containing the *xylE* reporter gene (encoding catechol 2,3-dioxygenase, which converts colorless catechol into the yellow-colored 2-hydroxymuconic semialdehyde), yielding pMRB3. pMRB3 was introduced into the *S. coelicolor* strains M145 and Δ*sigT* by conjugation, yielding *S. coelicolor* strains MRB4 and MRB5. As a positive control, plasmid pMRD278, containing the *ermE*p* (the strong constitutive promoter drives the expression of *ermE* gene, which is 23S rRNA dimethylase gene from *Saccharopolyspora erythraea*), was also conjugated into the *S. coelicolor* strain M145, yielding the *S. coelicolor* strain MRB3. As a negative control, pMRD279 was introduced into *S. coelicolor* strain M145 yielding MRB6.

To construct the complement *rstB* mutant, the *rstB* gene was amplified by PCR using primers F6 and R6 with *Nde*I sites and cloned into pTA2 to generate pMRB5. pMRB5 was digested using *Nde*I, and the resulting DNA fragment was subcloned into pLM1, yielding pMRB6.

## Construction and complementation of the in-frame *rstB* deletion mutant

The disruption of *rstB* gene was achieved using the λRED-mediated PCR targeting system developed by Gust et al. (2003). Two primers, F1 and R1, were used to amplify the cassette *aadA*/*oriT* from plasmid pIJ778 to replace the *rstB* gene in the cosmid N03-16, giving the plasmid pRMB1. The cassette in pMRB1 was further removed by FLP recombinase, leaving an 81-bp "scar" sequence. The resulting plasmid, named pMRB2, was then conjugated into M145. The double-crossover replacements in *S. coelicolor* were verified by Southern blotting and PCR analysis (Fig. S1). One positive strain was designated MRB1.

To complement the mutant, pMRB6, a derivative of pIJ8630 containing the functional *rstB* gene was transferred into *S. coelicolor* strain MRB1 by conjugation. Insertion of complementation constructs was confirmed by PCR.

## Fermentation and antibiotic quantification

For production of secondary metabolites, fermentation of *S. coelicolor* strains was carried out as described previously (Kieser et al. 2000). Antibiotic assays were performed as previously described (Kieser et al. 2000).

## Isolation and manipulation of DNA

*Streptomyces* genomic DNA was isolated as described previously (Kieser et al. 2000). DNA restriction and modifying enzymes were used as recommended by the manufacturers (Sangon and Takara). Standard recombinant DNA techniques were used as previously described (Sambrook et al. 2000). DNA fragments were purified from agarose gels with the Gel Purification Kit (Axygen). Southern blotting was carried out as previously described (Sambrook et al. 2000).

## RNA isolation

Total RNA was isolated from mycelia harvested from R2YE plates over cellophane or SMM and TSB liquid

**Table 3** Lists of primers used in this study

| Name | Sequence | Use |
|---|---|---|
| F1 | GGCGATGAGCCGCTGACCGCACAGGCGGACCAG TCCACGATTCCGGGGATCCGTCGACC | |
| R1 | CCGTGCCAAGGAGCGGCAGACGATACAGCCTCT | |
| | TGCCTATGTAGGCTGGAGCTGCTTC | Amplification of disruption cassette for *rstB* deletion |
| F2 | GCACAACCATCGTCCGCCGCTG | Identifying knock out of *rstB* |
| R2 | TCGAATTCTCGCTCACGGCTTCATTC | |
| F3 | GAGATCTGCGCACAACCATCGTCCG | Amplification of *sigT* promoter |
| R3 | GCTCTAGACGTCGTTCCTGCCTCCCC | |
| F4 | CCGCGTCGCCGACATCAAGGTG | Co-transcription of *rstB* and *sigT* |
| R4 | CTCGTCCACCTCCGCCCTTCACTG | |
| F5 | GCTAGGATCCCGGGCATGAAGGTGACGAGCAT | Amplification of *rstB* probe |
| R5 | ACTAGGATCCCGTCGTTCCTGCCTCCCCATTC | |
| F6 | ACATATGGCGGAACGGAGCACAGC | Amplification of *rstB–linker* gene |
| R6 | ACATATGCTCCGGGCCCGGCAGCTCCGGG CCCGGCAGGCTGGTCACCTTGATGTCGG | |
| F7 | GCGATTCAACCGCAGTGAAGGG | Co-transcription of *rstA* and *sigT* |
| R7 | TGTCATTGGTGGCGTCGGGCAGTA | |
| F8 | ACATGATATCCTTCCGCTGCTCACCCAC | Co-transcription of *rstA* and *sigT* |
| R8 | TGTCATTGGTGGCGTCGGGCAGTA | |
| F9 | GACCACGCGTATCGATGTCGACTTTTTTTTT TTTTTTV | Oligo dT-anchor primer |
| F10 | GACCACGCGTATCGATGTCGAC | PCR anchor primer |
| R9 | GAGCTTGTGCCCGCTGTGCAGT | Specific primer 1 for *rstB* in 5′RACE |
| R10 | GCCGGGTTCCTCTCGTGGG | Specific primer 2 for *rstB* in 5′RACE |
| R11 | CCCCTGTGCCTCGTCGCTGTC | Specific primer 3 for *rstB* in 5′RACE |
| R12 | GCGATCCCCAGCGGTGCTC | Specific primer 1 for *rstA* in 5′RACE |
| R13 | CCGAGCAGTCCTTGGATCTC | Specific primer 2 for *rstA* in 5′RACE |
| R14 | CAGCTTTCCAGGTGCTGTCG | Specific primer 3 for *rstA* in 5′RACE |
| F11 | ATACTGTCCGACGAACTG | Gene expression of *act*II-*ORF4* for real-time RT PCR |
| R15 | CTACACGAGCACCTTCTC | |
| F12 | GAGAAGCAGGAGAAGAAG | Gene expression of *relA* for real-time RT PCR |
| R16 | CCACTTGATGGTGTTCAT | |
| F13 | TCGACATGCAGGGCTACC | Gene expression of *sigT* for real-time RT PCR |
| R17 | CAGCGGCTCTTGATCGTTC | |
| F14 | CTCTTCCTGGACCTCATC | Gene expression of *hrdB* for real-time RT PCR |
| R18 | TTGTAGCCCTTGGTGTAG | |
| F15 | CGGAAAGTGCTCAACGAG | Gene expression of *redD* for real-time RT PCR |
| R19 | GAGTCTCAGGAAGCGGTT | |

cultures. Total RNA was prepared as described by Mao et al. (2009). RNA concentrations were determined by absorbance at 260 nm. The integrity of the RNA was indicated by visualization of sharp rRNA bands after electrophoresis in agarose containing 2.2 M formaldehyde.

## RT-PCR and quantitative real-time PCR

DNase I-treated RNA (1 μg) was used as a template for reverse transcription (RT) at 42°C with the Primer-Script RT-PCR kit (Takara) and random hexamer primers. The

resulting cDNA was used for PCR amplification with sequence-specific primers under the following conditions: 95°C for 3 min, followed by 35 cycles of 95°C for 15 s, 55°C for 30 s, and 72°C for 30 s. DNase I-treated RNA samples that had not been subjected to RT were used as negative controls. Genomic DNA samples were used as positive controls.

Real-time PCRs were carried out on an iCycler MyiQ2 real-time PCR detection system (Bio-Rad Laboratories), and the data were analyzed using the software provided by the supplier. Assays were performed using 1 μl of template cDNA, 5 pmol of each primer, 0.5× SYBR Green (Takara), and 1 U of Taq polymerase (Takara) in a final volume of 25 μl. Thermal cycle conditions were as follows: 95°C for 3 min, followed by 40 cycles of 95°C for 15 s, 52°C for 20 s, and 72°C for 20 s. A final dissociation was run to generate a melting curve and verify the specificity of the amplification product. All samples were run in triplicate. The threshold cycle (CT) value was calculated for each sample. A ΔCT value was calculated by subtracting the mean CT value of the target gene from the mean CT value of the *hrdB* reference gene (Kelemen et al. 1996). Relative quantification of gene expression was determined using the $2^{-\Delta CT}$ method (Livak and Schmittgen 2001).

### 5′ RACE experiment

Transcription start sites (TSS) were determined using the 3′/5′ RACE kit (Roche) according to manufacturer's instructions. After treatment with DNase I (Takara), RNA was reverse-transcribed using a gene-specific primer, purified, and poly (dA) tailed at 3′ ends. The resulting cDNA was amplified by PCR using a poly(dT) primer and a gene-specific primer complementary to a region upstream of the original cDNA primer. Amplicons from the first PCR were submitted to a second PCR using the poly(dT) primer and a distinct gene-specific nested primer. PCR products were ligated into the pTA2 vector (Toyobo). Ten distinct clones were sequenced, generally resulting in the same TSS.

### Promoter activity assay using the reporter *xylE* gene

The catechol-2,3-dioxygenase activity of the *xylE* reporter gene was measured as previously described to quantify promoter activity (Kieser et al. 2000).

## Results

### Transcriptional analysis of *sigT*, *rstB*, and *rstA*

In the *S. coelicolor* chromosome, *rstB* (SCO3893), *sigT* (SCO3892), and *rstA* (SCO3891) are physically linked and have the same transcriptional direction (Fig. 1a). Amino acid homology analysis suggested that SigT is a structural homologue of SigM in other actinobacteria (data not shown). Domain prediction with COG (Tatusov et al. 2000) and Pfam databases revealed that *rstB* probably encode a serine/threonine protein kinase (STPK) (COG0515, pfam NO PF00069.18). Proteins of the STPK family are believed to be implicated in signal sensing and transduction (Barthe et al. 2010). RstA, being a member of zinc-associated anti-sigma factor (ZAS) family (Zdanowski et al. 2006; Dona et al. 2008), has been demonstrated to interact with SigT in vivo and in vitro, suggesting it being a putative anti-sigma factor against SigT (Mao et al. 2009).

Although *rstB*, *sigT*, and *rstA* are closely linked, whether these three genes are co-transcribed as an operon has not been documented. To this end, RT-PCR analysis was performed with total RNA obtained from transition-phase cultures of wild-type strain. A 742-bp cDNA product covering the *sigT-rstB* intergenic region was obtained (Fig. 1b). Co-transcription analysis of *rstA* and *sigT* using primer pairs F8 and R7 failed to produce the expected 932 bp product. Nevertheless, the expected product was detected when using a forward primer F7, around 40 bp upstream of the *sigT* translation stop codon (Fig. 1b). These results suggested that *sigT* is likely not co-transcribed with *rstA* but with *rstB*, and one or more transcription start sites of *rstA* may lies within the coding region of *sigT*.

In most cases, genes in the vicinity of the genes coding for sigma factors of *S. coelicolor* were found functionally related to sigma factors (Helmann 2002). So, to obtain an overall picture of transcription of the genes probably related to *sigT*, we determined transcription of *rstB*, *sigT* and *rstA* in different genetic backgrounds such as the parental strain M145, and the deletion mutants, Δ*sigT*, Δ*rstA*, and Δ*rstB*. The expression of *rstB* was not detected in the Δ*sigT* mutant (Fig. 1c). Considering that *rstB* is cotranscribed with *sigT*, one reasonable explanation for the absence of transcript of *rstB* was that SigT might direct its own transcription. The *rstA* mutation as well as *rstB* mutation showed almost no effect on the expression of *sigT* under this normal condition (Fig. 1c).

Based on the co-transcription profile, we decided to map the TSS of *sigT* and *rstA* by 5′ RACE experiments. Two TSSs upstream of *rstB*, named *sigT*p1 and *sigT*p2, were identified (Fig. 2a, Fig. S3a and b). *sigT*p1 is more distal and *sigT*p2 is more proximal to the predicted translation initiation codon of *rstB* with relative positions of −28 and −74, respectively. The deduced −35 regions of these promoters lack a conserved AAC motif of most ECF sigma factor promoters (Helmann 2002).

Three TSSs, named *rstA*p1, *rstA*p2, and *rstA*p3, were identified for *rstA* (Fig. 2b, Fig. S3c, d and e). *rstA*p1, *rstA*p2 and *rstA*p3 are located at nucleotides −8, −79, and −165, relative to the translation start site of *rstA*, respectively.

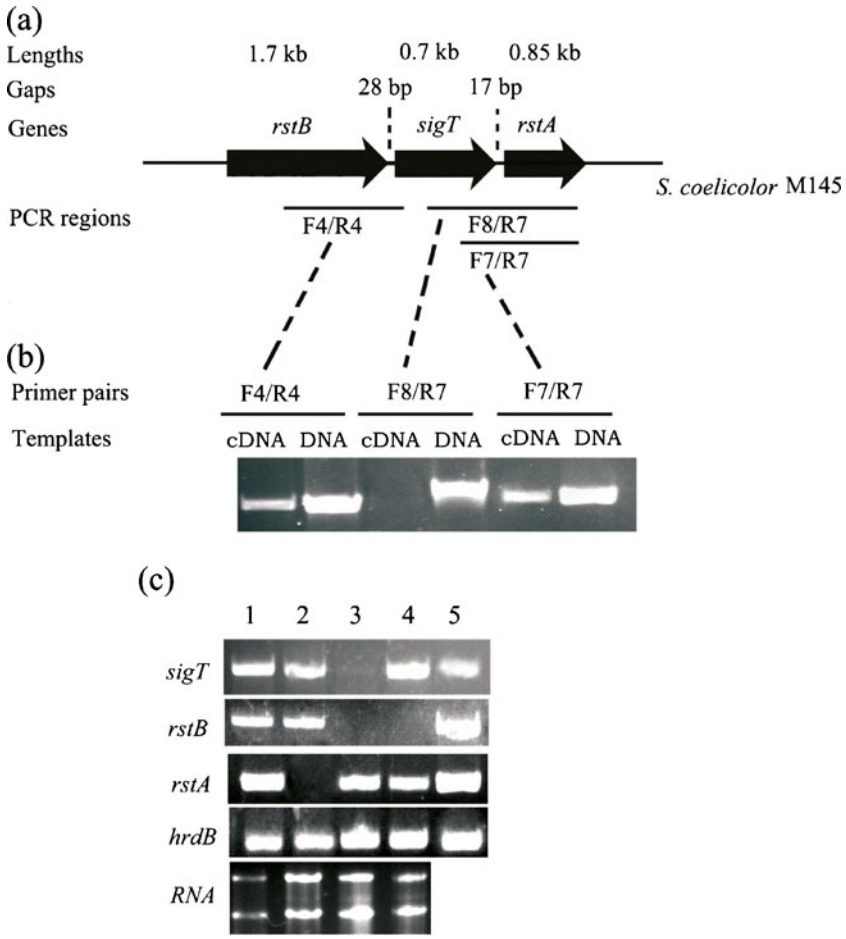


**Fig. 1** Organization and transcription of three genes in *sigT* cluster of *S. coelicolor*. Physical map of the *sigT* cluster (*sigT, rstA*, and *rstB*) in *S. coelicolor*. The transcription directions of these three genes were indicated by *filled arrowheads*. **b** RT-PCR of transcripts overlapping the consecutive adjacent genes in the *sigT* cluster. RNA of strain M145 was isolated from transition phase cultures and reverse-transcribed into cDNA. The cDNAs prepared from M145, Δ*rstA*, Δ*sigT* and Δ*rstB* strains and M145 chromosomal DNA were used as templates. Three paired primers (F4/R4, F8/R7, and F7/R7) were used for amplification of segments as indicated in (**a**). **c** Expression of *sigT*, *rstA*, and *rstB* in different *S. coelicolor* strains. *S. coelicolor* strains Δ*sigT*, Δ*rstA*, Δ*rstB*, and M145 were grown in TSB liquid medium, and total RNA was isolated from the transition phase cultures and transcribed into cDNA. *Lanes 1, 2, 3,* and *4* indicated the M145, Δ*rstA*, Δ*sigT*, and Δ*rstB* genetic backgrounds, respectively. *Lane 5* is a positive control with chromosomal DNA from M145 as a template. The *hrdB* gene was used as an internal control

These results corroborated that *rstA* is not co-transcribed with *sigT* in a single operon. No distinct band was detected when using a reverse primer internal to the *sigT* ORF.

## Phenotypic analysis of the *S. coelicolor rstB*, *sigT*, and *rstA* mutant upon nitrogen stresses

Mao et al. (2009) reported the over-production of antibiotics (Act and Red) and accelerated morphological development in Δ*sigT* and Δ*rstA* mutants on R2YE medium suggesting SigT and RstA were both involved in negative regulation on differentiation and secondary metabolism in *S. coelicolor* and these features appeared to be medium-dependent.

The fact that ECF sigma factor could respond to environmental stresses (Helmann 2002) led us to evaluate the potential role of SigT under various stress conditions. It was reported that a *sigT* mutant was sensitive to diamide, suggesting that SigT like SigR was also involved in the response to oxidative stress (Helmann 2002). However, no increased sensitivity with respect to wild type was observed when the Δ*sigT* mutant was exposed to diamide and hydrogen peroxide (data not shown) (Paget et al. 1998). To investigate if SigT was a functional homologue of SigM in *B. subtilis*, the Δ*sigT* mutant was exposed to high concentration salt, the strains was not impaired in growth compared to wild type (data not shown) (Horsburgh and Moir 1999; Thackray and Moir 2003).

Given that nitrogen stress plays an important role on production of antibiotics (Chakraburtty and Bibb 1997), we tested the Δ*sigT* mutant on nitrogen-limited and nitrogen-

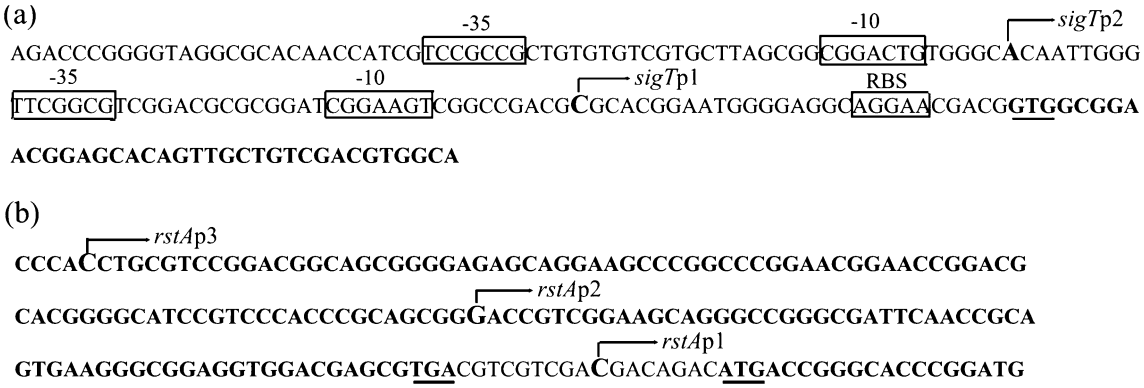


**Fig. 2** Mapping of the 5′end of the *sigT* and *rstA* mRNA by 5′RACE analysis. RNA was isolated from transition phase cultures of strain M145 grown in TSB medium and equal amount (5 μg) of total RNA were used for 5′RACE analysis. **a** Nucleotide sequence of the promoter region of *sigT* and *rstB*. Transcription start sites of *sigT* are denoted as *sigT*p1 and *sigT*p2. The putative −10 and −35 regions and ribosome binding site are indicated with *boxes*. The translation initiation codon of *rstB* is *underlined*. **b** Nucleotide sequence of the promoter region of *rstA*. Transcription start sites of *rstA* are denoted by *rstA*p1, *rstA*p2, and *rstA*p3. The translation initiation codon of *rstA* and translation stop codon of *sigT* are *underlined*

rich R2 medium. To exclude the effects of phosphate, excess phosphate (7.2 mM) was added into R2 medium as mentioned in methods. Under the nitrogen-limited condition, the Δ*sigT* mutant produced less Act than the parental strain M145 (Fig. 3c). While under the nitrogen-rich condition, the Δ*sigT* mutant exhibited the same phenotype as wild type (Fig. 3b). Notably, the Δ*rstA* mutant produced markedly less Act than the Δ*sigT* mutant (Fig. 3c).

The production of the blue Act pigments (Bystrykh et al. 1996) or the development of aerial mycelium in the Δ*sigT* mutant could be restored to wild-type level by the introduction of the *sigT* gene into the Δ*sigT* mutant which gave rise to *S. coelicolor strain sigT*$^+$, confirming that these phenotype was caused by mutation of the gene.

Growth and antibiotic production kinetics of Δ*sigT*, Δ*rstA*, and Δ*rstB* mutants upon nutritional downshift

To further assess the effect of the *sigT* mutation on antibiotic production under phosphate- and nitrogen-limited conditions, M145 and Δ*sigT* mutant were grown in SMM liquid medium until mid-exponential phase

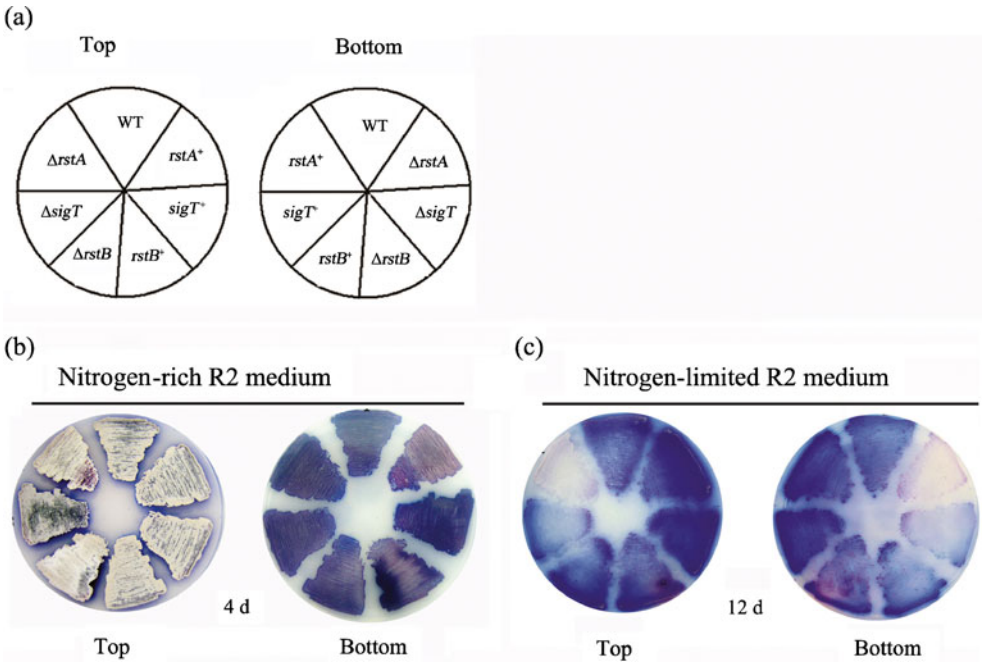


**Fig. 3** Phenotype of *S. coelicolor* M145, Δ*rstB*, Δ*sigT*, and Δ*rstA* mutants and complemented *rstA*$^+$, *sigT*$^+$, and *rstB*$^+$ transformants on modified versions of R2 solid medium. **a** Plate legend, **b** growth of different *S. coelicolor* strains on nitrogen-rich R2 medium (Difco casamino acids: 2 g) for 4 days. **c** Growth of different *S. coelicolor* strains on nitrogen-limited R2 medium (total nitrogen: 1.3 mM, $KH_2PO_4$ 7.2 mM) for 12 days

(optical density at 450 nm [$OD_{450}$], 0.5) and subjected to amino acid shift-down (detail in methods) (Chakraburtty and Bibb 1997; Ryu et al. 2007). The level of antibiotics Act and Red was measured after nutritional depletion. Under nitrogen deprivation, the *sigT* deletion resulted in abolishment of Act production (Fig. 4a, b). This lowered Act production was also observed for the Δ*rstA* mutant but not for the Δ*rstB* mutant (Fig. 4c, d). The production of Red was barely detectable either in the wild type or in the Δ*sigT* mutant upon nitrogen deprivation.

Following nitrogen depletion, growth retardation was observed for the Δ*sigT* mutant throughout the course of experiment (Fig. 4c).

To summarize, these data obtained from batch culture was in agreement with what observed in surface-grown culture, suggesting SigT was responsive to nitrogen starvation.

## *sigT* gene expression is induced by nitrogen stress in auto-regulated manner

The roles of *sigT* on *S. coelicolor* development led us to investigate its expression during cell differentiation. The transcription of *sigT* was monitored by RT-PCR during differentiation of *S. coelicolor* M145 on solid R5 medium. This transcription was increased along with the development of aerial mycelium and peaked at 48 h, around the time when antibiotic production is most abundant (Bibb 1996). Then it decreased rapidly (Fig. S2), suggesting that SigT may participate in the regulation of antibiotic synthesis.

Using the reporter *xylE* gene coupled to the *sigT* promoter (Sola-Landa et al. 2005), the regulatory effects of phosphate or nitrogen on expression of *sigT* genes were studied in *S. coelicolor* M145 and the Δ*sigT* mutant in

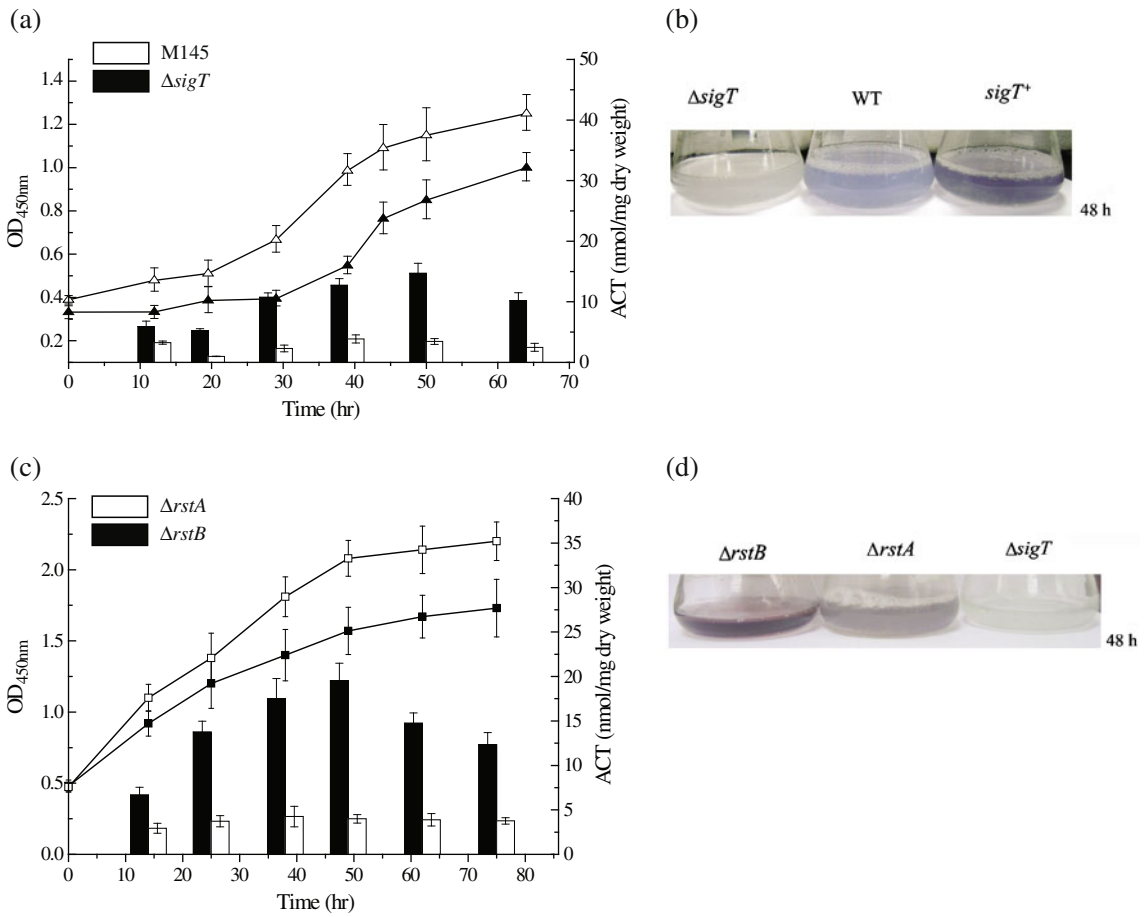


**Fig. 4** Growth and antibiotic synthesis kinetics of *S. coelicolor* strain Δ*sigT* and M145 grown in SMM after nitrogen or phosphate shiftdown. The *S. coelicolor* strains were grown in SMM until mid-exponential phase ($OD_{450nm}$≈0.5), and subjected to nitrogen shiftdown by rapidly transferring the cultures to nitrogen-limited SMM (without Difco casamino acids), **a** growth and actinorhodin level of *S. coelicolor* M145 and Δ*sigT* under nitrogen limitation. **b** Photograph showing the difference in antibiotic synthesis between the M145 and Δ*sigT* under nitrogen limitation. $sigT^+$ indicates the complementation of the Δ*sigT* mutant. **c** Growth and actinorhodin level of *S. coelicolor* mutants Δ*rstA* and Δ*rstB* under nitrogen limitation. **d** Photograph showing the difference in antibiotic synthesis between Δ*rstA* and Δ*rstB* under nitrogen limitation. *Filled triangles*, *S. coelicolor* mutant Δ*sigT*; open triangles, *S. coelicolor* M145; *filled squares*, *S. coelicolor* mutant Δ*rstB*; *open squares*, *S. coelicolor* mutant Δ*rstA*. *Vertical error bars* correspond to the standard error of the mean of four replicated cultures

SMM. Nutrient deprivation was performed the same as described in the Materials and methods section. Results showed that nitrogen limitation caused a fivefold induction of the *sigT* promoter activity with respect to that under the nitrogen-rich condition during the transition stage (Fig. 5a). This *xylE* expression level paralleled that in the positive control strain MRB3 (M145/*ermE*p*::*xylE*) (data not shown). In contrast, no induction of *sigT* promoter activity was observed for phosphate stress (Fig. 5a). The SigT dependence of the nitrogen stress response at *sigT* promoter was tested by introducing the *sigT*p-*xylE* construct into the Δ*sigT* mutant, and induction was carried out under nitrogen starvation. The level of *xylE* expression in the *sigT* null mutant was not increased by nitrogen stress (Fig. 5a). The same induction of *sigT* upon nitrogen depletion was also observed in RT-PCR experiments using exponential-phase cultures of Δ*sigT* strain (Fig. 5b). Interestingly, the induction of *sigT* was also absent in the Δ*rstA* mutant

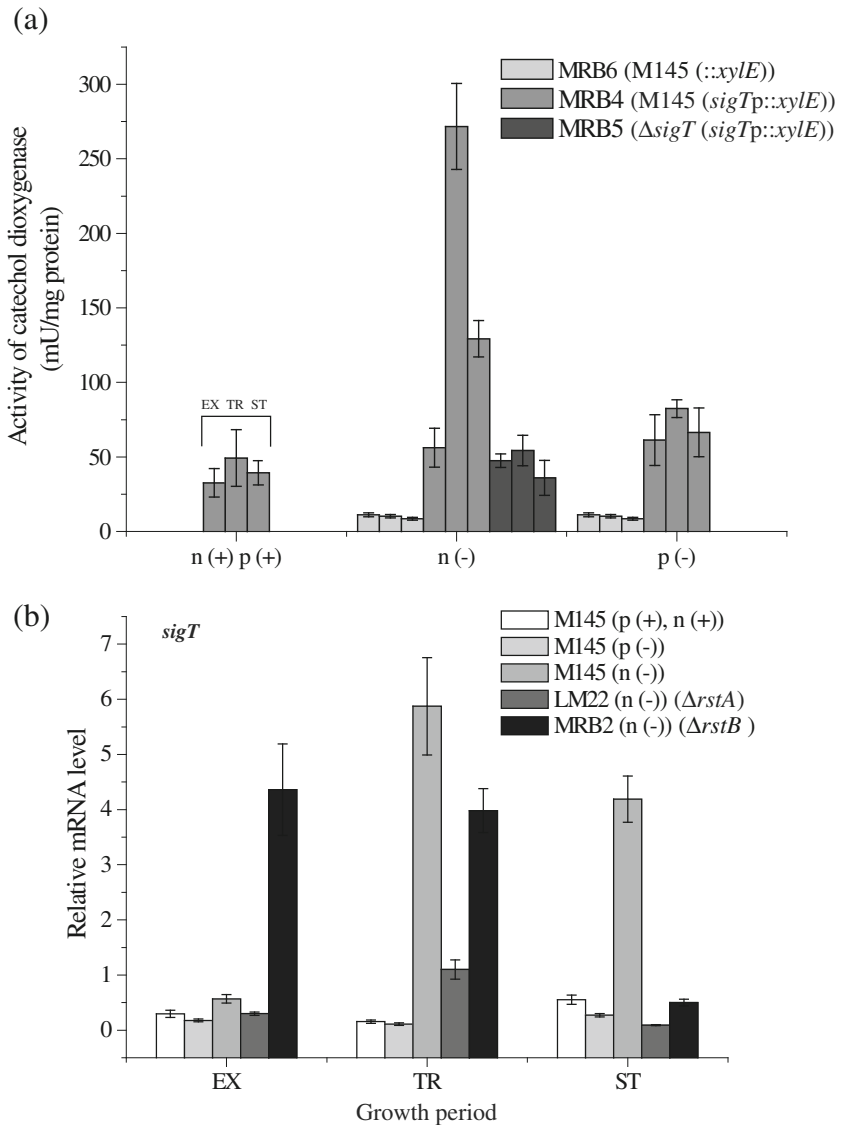


**Fig. 5** Expression of sigT was induced by growth in the nitrogen-limited SMM in a SigT-dependent manner. **a** Determination of *sigT* promoter activity using the *xylE* gene (encodes catechol dioxygenase) as reporter. For analysis of promoter activity in response to nitrogen or phosphate starvation, the *S. coelicolor* strains were grown in SMM until mid-exponential phase ($OD_{450nm}$≈0.5), and subjected to nitrogen or phosphate depletion as described in methods. The enzyme activity was measured at the indicated growth phases. Note that there is a drastic induction in *sigT* promoter activity under nitrogen limitation with respect to the negative control (*MRB6*) and that under nitrogen-rich condition. The promoter of the *sigT* gene was dependent on SigT as the above induction effect was abolished in the Δ*sigT* genetic background (*MRB5*). **b** qRT-PCR analysis of expression of *sigT* in *M145* and different mutant strains upon different nutritional stress. The results confirmed that *sigT* was responsive to nitrogen stress. *p (+)* phosphate-replete condition, *p (−)* phosphate-limited condition, *n (+)* nitrogen-replete condition, *n (−)* nitrogen-limited condition. *EX*, *TR*, and *ST* indicate exponential, transition, and stationary phases of growth, respectively. Results described in (**a**) and (**b**) represent the mean value of at least three independent experiments, each performed in duplicate, and the *vertical bars* indicate the standard error

upon nitrogen starvation as observed for the Δ*sigT* mutant in promoter activity assay (Figs. 5b and 6b). In contrast, the Δ*rstB* mutant displayed the similar induction of *sigT* with that in wild-type strain (Figs. 5b and 6b).

SigT regulates *relA* to response nitrogen stress

Several studies have documented that highly phosphorylated guanine nucleotides ppGpp play a central role in triggering

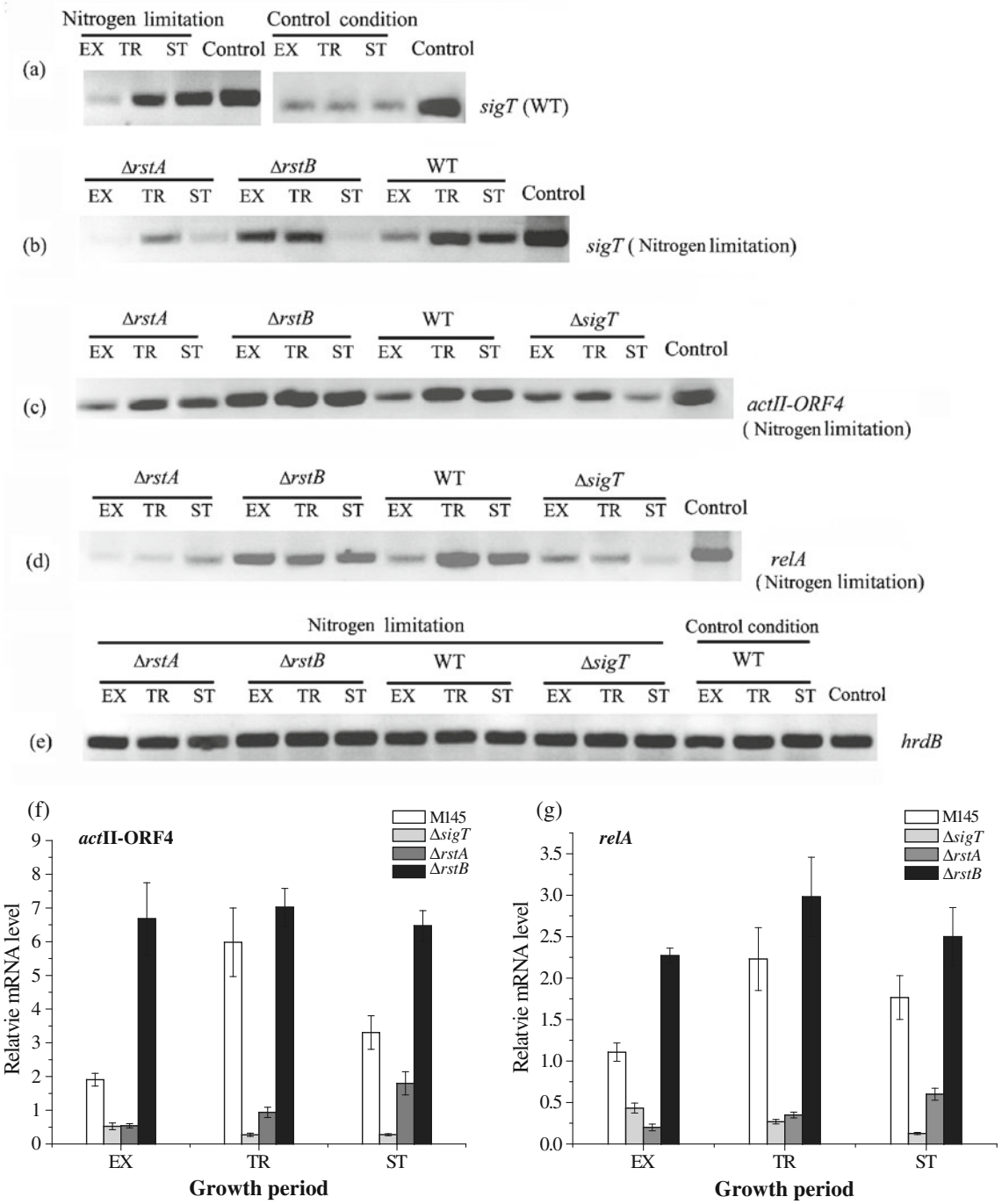


**Fig. 6** Relative mRNA levels measured by semi-quantitative and quantitative RT-PCR of differentially expressed genes upon nitrogen limitation in *S. coelicolor* M145, Δ*sigT*, Δ*rstA*, and Δ*rstB* strains. Total RNA was prepared from different *S. coelicolor* strains at exponential, transition, and stationary stages of growth in SMM after amino acid depletion then reverse-transcribed into cDNA. **a**–**b** show the expression of *sigT* under nitrogen-limited conditions and the control condition, respectively, **c**–**d** show the expression of *act*II-ORF4 and *relA*, **e** shows the expression of *hrdB* which was used as an internal control. **f**–**g** show the relative mRNA levels of the gene *act*II-ORF4 and *relA* in different *S. coelocolor* strains quantified by RT-PCR. *EX*, *TR*, *ST* indicate exponential, transition, and stationary phases of growth, respectively. *Error bars* correspond to the standard error of the mean of four culture replicates

antibiotic biosynthesis in *S. coelicolor* under stringent response (Ochi 1990; Strauch et al. 1991). The mutation in the ppGpp synthetase gene *relA* would impair both antibiotic production and the morphological differentiation of *S. coelocolor*. The former was ascribed to a marked decline transcription of genes *act*II-ORF4 and *redD* regulating production of Act and Red, respectively (Chakraburtty and Bibb 1997). The coincidence of antibiotic production in the Δ*sigT* mutant with that in the Δ*relA* mutant upon nitrogen starvation led us to probe a possible link between *sigT* and *relA* by RT-PCR analysis. Meanwhile, given that the Δ*rstA* mutant displayed a similar pattern of antibiotic production to the Δ*sigT* mutant upon nitrogen starvation, transcription analysis was also applied to the Δ*rstA* mutant and the Δ*rstB* mutant. The Δ*sigT* mutant displayed expression of *relA* and *act*II-ORF4 around eight times and 20 times less than the wild type as quantified by RT-PCR experiments (Fig. 6f, g). The Δ*rstA* mutant followed a similar transcription pattern to the Δ*sigT* mutant (around six times less than wild type both in expression of *relA* and *act*II-ORF4). Conversely, the transcription in the Δ*rstB* mutant was not affected by nitrogen starvation (Fig. 6f and g). We did not detect apparent difference on *redD* transcription between the Δ*sigT* mutant and wild-type strain (data not shown).

## Discussion

The evidences presented in this study suggest that the ECF sigma factor SigT is responding to the nitrogen stress and essential for normal Act production under nitrogen limitation. ECF sigma factors of *S. coelicolor* typically regulate a response to environmental stress (es) (Missiakas and Raina 1998; Helmann 2002). In consequence, in most cases, the absence of ECF sigma factor gene usually does not result in obvious phenotypes under normal conditions. To identify which stress is SigT in responding to, we exposed the Δ*sigT* mutant to various stress conditions such as oxidative stress, osmotic stress, and nutrient stress. The Δ*sigT* mutant only displayed a marked mutant phenotype upon nitrogen starvation. Our data showed that the *sigT* mutation did indeed impair Act production upon nitrogen limitation. While under a nitrogen-replete condition, the production of Act in the Δ*sigT* mutant was restored to the level of wild-type M145, indicating that this deficiency in Act production was nitrogen-dependent. qRT-PCR combined with promoter activity assay revealed that *sigT* was only induced after nitrogen limitation. These data confirmed that SigT could respond to nitrogen stress. To our knowledge, it is the first report of an ECF sigma factor involved in response to nitrogen starvation in *S. coelicolor*.

The absence of the transcript of *rstB* (*rstB* is co-transcribed with *sigT*) combined with the abolishment of induction of *sigT* promoters upon nitrogen deprivation in the Δ*sigT* mutant background was indicative of the *sigT* promoter to be SigT-dependent and demonstrated that SigT could control its own expression by positively auto-regulating transcription, a feature observed with most ECF sigma factors (Helmann 1999, 2002). In 5′RACE experiments, two promoters were identified for the *sigT* gene. Pioneering works had uncovered many ECF sigma factor genes such as *sigB* and *sigR* were also transcribed from two promoters, in which one promoter is responsible for constitutive transcription, and the other appears to be induced by a stress condition and to be auto-regulated (Paget et al. 1998; Cho et al. 2001). So, it is conceivable that only one promoter of *sigT* could be induced by stress condition and auto-regulated in analogy to the *sigR* or *sigB* promoters. Then, the key issue is to determine which promoter is SigT-dependent.

The stringent response plays a central role in responding to nutrient stress in bacteria and mediates its effect through the alarmone ppGpp (Toulokhonov et al. 2001; Braeken et al. 2006). ppGpp can bind to the β-subunit of RNA polymerase (Toulokhonov et al. 2001) and thereby triggers the initiation of antibiotic production (Bibb 2005). So the disruption of ppGpp synthetase gene *relA* resulted in deficient production of antibiotics (Act and Red) of *S. coelicolor* upon nitrogen limitation (Chakraburtty and Bibb 1997). This defect in antibiotic production was due to the markedly reduced transcriptions of pathway-specific regulatory genes *act*II-ORF4 and *redD*, for Act and Red production respectively, in the Δ*relA* mutant (Chakraburtty and Bibb 1997). In this study, the Δ*sigT* mutant showed a phenotypic similarity with the Δ*relA* mutant, in that both are deficient in Act production and both display delayed aerial mycelium development. In addition, *sigT* is also induced by nitrogen deprivation similar to *relA* (Chakraburtty et al. 1996). These evidences suggested a link between SigT and RelA. Transcription analysis confirmed this link in that the expression of *relA* was dramatically declined in the Δ*sigT* mutant compared to the wild type upon nitrogen starvation. These results suggest that the observed phenotypes of the *sigT* mutant upon nitrogen starvation may result primarily from the absence of *relA* and accordingly expression of *relA* is SigT-dependent.

The genes neighboring to an ECF sigma factor gene often encode the regulatory proteins of the sigma factor, such as an anti-sigma factor or an anti-anti-sigma factor. For instance, in the case of SigB, *rsbA* and *rsbB* upstream of the *sigB* gene encode an anti-sigma factor and an anti-anti-sigma factor for SigB, respectively (Lee et al. 2004). Previous work suggested that RstA encoded by the gene downstream of *sigT* may be a putative anti-sigma factor for SigT of *S. coelicolor* (Mao et al. 2009). To probe the function of RstB encoded by the gene upstream of *sigT*, the *rstB* gene was disrupted. Act production and *act*II-ORF4 as well as *relA* transcription of the Δ*rstA* mutant under

nitrogen limitation resembled well those of the Δ*sigT* mutant and were in contrast to those of Δ*rstB* mutant. In the transcriptional analysis under nitrogen starvation, we found the expression of *sigT* was reduced dramatically in the Δ*rstA* mutant in sharp contrast to that in the Δ*rstB* mutant. Giving that SigT is responsible for induction of its own structural gene as described above, the transcriptional profiles of *sigT* displayed in the Δ*rstA* mutant and the Δ*rstB* mutant may suggest that RstA regulates the activity of SigT positively and RstB is the negative regulator of SigT. Furthermore, co-transcriptional analysis and 5′RACE assay revealed that *sigT* was co-transcribed with *rstB* but not *rstA*. The data presented here were not in consistence with the notions that an anti-sigma factor gene is encoded with its cognate sigma factor gene in one operon and that an anti-sigma factor antagonizes the activity of sigma factor (Hughes and Mathee 1998). This raised the possibility that RstA may be just a positive regulator of SigT rather than an anti-sigma factor against SigT despite that it shares structural similarity with members of the ZAS family such as RsrA and that it could interact with SigT in vivo and in vitro (Kang et al. 1999; Mao et al. 2009). RstB was predicted to be a sensor STPK by bioinformatics analysis. We also found that RstA could interact with RstB in vitro (Mao et al. unpublished work). We propose from above evidences that RstA and RstB may constitute a partner pair in signal transduction for regulating expression of *sigT* transcriptionally in a similar manner with the CseB–CseC two-component signal transduction system in the SigE case, where CseC serve as a sensor histidine protein kinase and CseB serve as a response regulator (Hong et al. 2002; Paget et al. 1999b).

To conclude, our study demonstrated that *sigT* was induced by nitrogen stress in a SigT-dependent manner. SigT may activate the Act production under nitrogen limitation via activating the expression of *relA* gene directly or indirectly. Thus, SigT appears to link nutrient stress to antibiotic production by *Streptomyces*. Our results also suggested that the putative anti-sigma factor RstA may be a positive regulator of SigT at the transcriptional level. Although the detailed molecular mechanisms on regulation of Act production by SigT upon nitrogen stress remains unclear, our findings give a clue to understand the complex regulation system of antibiotic biosynthesis in *S. coelicolor* upon stringent response to nitrogen starvation. Further work will focus on defining the SigT regulon through in vitro transcription assays.

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