# FEBS*Letters*

# Involvement of SigT and RstA in the differentiation of *Streptomyces coelicolor*

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## A R T I C L E   I N F O

## A B S T R A C T

**SigT is an ECF sigma factor in *Streptomyces coelicolor*. *sigT* and its putative anti-sigma factor gene *rstA* are located in one putative operon, and SigT could physically interact with RstA. Deletion of *sigT* or *rstA* caused accelerated morphological development and enhanced production of antibiotics, concomitant with over-expression of *chpE*, *chpH*, *actII-orf4* and *redD*. Furthermore, SigT was undetectable after loss of *rstA*. These data suggested that SigT has a negative role on differentiation and that RstA negatively regulates the SigT activity through a putative antagonistic mechanism and at the post-transcriptional level.**

*Structured summary:*
MINT-7262599, MINT-7262614: *RstA* (uniprotkb:Q9S6U2) *physically interacts* (MI:0915) with *sigT* (uniprotkb:O86856) by *pull down* (MI:0096)
MINT-7262539: *rpoC* (uniprotkb:Q8CJT1) *physically interacts* (MI:0915) with *sigT* (uniprotkb:O86856) by *pull down* (MI:0096)
MINT-7262574: *RstA* (uniprotkb:Q9S6U2) *physically interacts* (MI:0915) with *sigT* (uniprotkb:O86856) by *anti tag coimmunoprecipitation* (MI:0007)

## 1. Introduction

Extracytoplasmic functions (ECF) sigma factors are the alternative sigma factors of the bacterium $\sigma^{70}$ family controlling genes responding to various environmental stimuli [1]. Whole genome sequencing project of the soil-dwelling model actinomyces *Streptomyces coelicolor* has revealed remarkable 51 ECF sigma factors, the highest number in bacteria so far identified, while another gram-positive model bacterium *Bacillus subtilis* has seven and the gram-negative model bacterium *Escherichia coli* contains only one. The abundance of ECF sigma factors in *S. coelicolor* implies that they might play major roles in elaborate transcriptional regulation on stress-responsive genes for the adaptation to the complex environments [2,3].

In *S. coelicolor*, only four ECF sigma factors, SigR, SigE, BldN and SigU, have been extensively studied to date in modulation of stress responses and morphological development. SigR and its anti-σ factor RsrA are responsible for the accommodation to the disulphide stresses by maintaining the intracellular reductive environments through the selective transcriptional activation of the *sigR* operon and other target genes [1,3–5]. Recently, it is reported that the decreased synthesis of mycothiol triggers the expression of *sigR* oper-on, and SigR in turn initiates the transcription of genes for mycothiol synthesis and detoxification [6]. SigE is involved in the maintenance of cell wall integrity by transcriptional modulation of operons for cell wall glycan synthesis [7–9]. However, *sigE* itself is auto-regulated by its own four-gene operon including two-component system of CseB/CseC, which senses the outside stimuli and mediates the signal transduction to up-regulate the *sigE* expression, and CseA, a lipoprotein negatively regulating the *sigE* expression [1,3,9,10]. In addition, SigE is also required for the appropriate sporulation and actinorhodin production at a low concentration of magnesium [8]. BldN is indispensable for the aerial formation and translated primarily as a pro-protein at the vegetative phase but proteolytically cleaved at its N-terminus into a mature form after cells develop into aerial hyphae [11,12]. Over-expression of SigU or removal of its anti-σ factor RsuA results in the delayed development into aerial hyphae [13]. Extracellular proteomic analysis reveals that the exalted SigU activity enhances the secretion of some secreted proteins, lipoproteins and proteases, which provides implications for SigU in morphological differentiation and possibly in cell envelope stress responses [14].

Another ECF sigma factor SigT was previously mentioned in the possible modulation of oxidative stress responses to diamide [1]. Here we presented evidence of SigT and its putative anti-σ factor RstA in regulation of morphological differentiation and secondary metabolism of *S. coelicolor*.

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## 2. Materials and methods

### 2.1. Bacterial strains and growth media

The *S. coelicolor* strains in this study were listed in Table 1. *E. coli* ET12567 [15] with the helper plasmid pUZ8002 was for plasmid introduction into *S. coelicolor* by conjugation [16]. *S. coelicolor* strains were grown at 30 °C on MS and R2YE solid media for morphological and secondary metabolism analysis or in YEME for in vivo protein interaction assay [16].

### 2.2. Plasmid construction

All the plasmids used in this paper were in Table 2 and all primers in this study were listed in Supplementary data S1. The detailed procedure for plasmid construction was in Supplementary data S2.

### 2.3. In-frame deletion of sigT or rstA and complementation

Plasmid pLM27 or pLM28 was introduced into M145, respectively, to delete *sigT* or *rstA* by in-frame deletion strategy via double cross-over homologous recombination [16]. Details of construction of *sigT* or *rstA* mutant were described in Supplementary data S3, and the genotypes were verified by PCR and Southern blot (Supplementary data S4). Plasmid pLM34 or pLM36 was transformed into *sigT* or *rstA* mutant, respectively, for complementation.

### 2.4. Protein extraction and Western blot

For assays of protein expression levels, spores were spread on cellophane overlaid on MS plates [16], and cells were collected at different developmental stages and resuspended in lysis buffer (10 mM Tris, pH 8.0, 1 mM EDTA, pH 8.0, 100 mM NaCl, 10% glycerol, 0.5% NP-40, 1 mM PMSF). Total protein was extracted by ultra-sonication. About 20 μg of total protein was subjected to Western blot [17] with an α-GFP antibody (Proteintech Group, USA) or α-FLAG M2 antibody (Sigma), and coomassie brilliant blue R250 staining of total protein for the loading control (Beyotime, China).

### 2.5. In vitro GST pull-down assay

The over-night cultured *E. coli* BL21 (DE3) containing pLM29, pLM30, pLM31, pLM32 or pGEX-6P-1, respectively, were re-inoculated in liquid LB at 37 °C to $OD_{600} = 0.4$, and induced with 0.1 mM IPTG for additional 5 h. Cells were collected, resuspended in PBS and total protein in supernatant was recovered by ultra-sonication and subsequent centrifugation at 13 000×g for 10 min. The GST pull-down assay was demonstrated as described previously [18], and eventually the eluted complex was subjected to Western blot

**Table 1**
*Streptomyces coelicolor* strains.

| Name | Description or genotype | Reference |
|---|---|---|
| M145 | Wild type, SCP1−, SCP2− | [16] |
| J1981 | *rpoC::6×His* of M145 | [21] |
| LM21 | *sigT* in-frame deletion mutant | This work |
| LM22 | *rstA* in-frame deletion mutant | This work |
| LM23 | LM21/pLM34 | This work |
| LM24 | LM22/pLM36 | This work |
| LM25 | M145/pLM34 | This work |
| LM26 | J1981/pLM34 | This work |
| LM27 | LM22/pLM34 | This work |
| LM28 | M145/pLM34/pLM36 | This work |
| LM29 | M145/pLM1 | This work |
| LM30 | LM22/pLM1 | This work |

**Table 2**
Plasmids in this study.

| Plasmid | Description | Reference |
|---|---|---|
| pKC1139 | Bifunctional *oriT* RK2 plasmid for gene deletion | [34] |
| pGH113 | Conjugal plasmid with *ermEp** | [20] |
| pIJ8630 | Promoter-probing plasmid with *egfp* | [35] |
| pPROEX-HTb | *E. coli* expression vector | Invitrogen |
| pGEX-6P-1 | *E. coli* expression vector | GE |
| pET32a | *E. coli* expression vector | Navagen |
| pLM1 | *ermEp** in pIJ8630 | This work |
| pLM15 | *hrdB* in pTA2 | This work |
| pLM16 | *redD* in pTA2 | This work |
| pLM17 | *actII-orf4* in pTA2 | This work |
| pLM18 | *chpE* in pTA2 | This work |
| pLM19 | *chpH* in pTA2 | This work |
| pLM26 | Kanamycin resistant gene in pLM1 | This work |
| pLM27 | *sigT* in-frame knock-out plasmid | This work |
| pLM28 | *rstA* in-frame knock-out plasmid | This work |
| pLM29 | *sigT* in pPROEX-HTb | This work |
| pLM30 | *sigT* in pGEX-6P-1 | This work |
| pLM31 | *rstA* in pET32a | This work |
| pLM32 | *rstA* in pGEX-6P-1 | This work |
| pLM33 | *sigT*-linker in pTA2 | This work |
| pLM34 | *sigT*-linker in pLM1 | This work |
| pLM35 | *3flag-rstA* in pTA2 | This work |
| pLM36 | *3flag-rstA* in pLM26 | This work |

with a α-His antibody (Tiangen, China) or α-GST antibody (Tiangen, China).

### 2.6. In vivo His pull-down assay

For in vivo His pull-down assay, *S. coelicolor* spores were inoculated in YEME (10.3% sucrose). Mycelia were collected, resuspended in lysis buffer (100 mM $NaH_2PO_4$, 10 mM Tris, pH 8.0, 20 mM imidazole, 10% glycerol, 1 mM PMSF) and destroyed by ultra-sonication. His pull-down assay was demonstrated as described by the manufacture (Qiagen). The $Ni^{2+}$-NTA binding complex was eluted with 500 mM imidazole and loaded for Western blot [17] with the α-GFP antibody or α-6×His antibody (Tiangen, China).

### 2.7. Co-immuno-precipitation

Crude extract of *S. coelicolor* mycelia from YEME was prepared in IP buffer (50 mM Tris, pH 7.6, 150 mM NaCl, 0.5% NP-40, 10% glycerol, 1 mM PMSF). About 1 ml of lysate was pre-cleared at 4 °C for 3 h with 20 μl of protein G-Sepharose beads (Roche). 1.0 μg of α-FLAG M2 antibody was added to the lysate at 4 °C for 3 h and subsequently 20 μl of protein G-Sepharose beads were added for over-night incubation. After wash with IP buffer for 5 times, the immuno-complex was eluted with loading buffer by boiling for 5 min and subjected to Western blot with the α-GFP antibody or α-FLAG antibody.

### 2.8. Antibiotic assay

Quantitative measurements of the actinorhodin (Act) or undecylprodigiosin (Red) were described previously [16]. Briefly, to analyze the total actinorhodin, about 10 mg of cells at various developmental stages on R2YE plates overlaid with cellophane were gathered, extracted with KOH (1 M final concentration), centrifuged at 5000×g for 5 min and the supernatant was determined by the spectrophotometric assay at wavelength 640 nm. For undecylprodigiosin measurement, the pellet recovered from above assay was vacuum freeze-dried, extracted with HCl-acidified methanol over-night and the $A_{530}$ of the supernatants was determined after centrifugation at 5000×g for 5 min.

*2.9. RNA preparation*

About 50 mg of mycelia collected from cellophane overlaid on R2YE plates were resuspended in 500 μl of buffer (1% SDS, 4% β-mercaptoethanol, 5 mM EDTA in DEPC-treated H$_2$O) and homogenized by ultra-sonication. The lysate was immediately extracted with 500 μl of phenol (pH 5.3) twice and once with 500 μl of chloroform and precipitated with isopropanol. The genomic DNA was removed by RNase-free DNase I (Takara). Total RNA was precipitated after extract once with phenol/chloroform/isoamyl alcohol (pH 5.3), precipitated with isopropanol and resuspended in DEPC-treated H$_2$O. The concentration of RNA was determined by spectrometry.

*2.10. Low resolution S1 nuclease protection assay*

About 30 μg of total RNA was subjected to S1 nuclease mapping as described [18] with some modifications. Briefly, RNA was hybridized to about 10 ng of single strand DNA (ssDNA) probe(s) in aqueous buffer (40 mM PIPES, pH 6.4, 0.4 M NaCl, 1 mM EDTA) at 65 °C for 16 h after denaturation at 90 °C for 10 min. The single stranded nucleic acids were removed with 100 U of S1 nuclease (Promega) at 37 °C for 1 h, and the hybrids were precipitated and subjected to Southern blot [18]. The biotin-labeled probes for Southern blot hybridization were prepared by PCR with the universal primer 30 and 31 from pTA2 vector with cloned fragments in the presence of biotin-11-dUTP (Fermentas) [19], and the signals were visualized by Chemiluminescent Biotin-labeled Nucleic Acid Detection Kit (Beyotime, China) as described by the manufacture.

The ssDNA probes for RNA hybridization were prepared with λ exonuclease (Fermentas). The 5′ phosphorylated primer 29 and un-phosphorylated primer 31 were used to amplify the dsDNA from pTA2 vector with cloned fragments. The PCR products were digested with λ exonuclease to remove the 5′ phosphorylated sense strand DNA. The anti-sense ssDNA was extracted once with phenol/chloroform/isoamyl alcohol (pH 8.0) and precipitated with isopropanol.

# 3. Results and discussion

*3.1. SigT is an ECF sigma factor in S. coelicolor*

SCO3892 was designated an ECF sigma factor SigT in *S. coelicolor* genome annotation. It contained two typical independent domains, σ2 and σ4, essential for promoter recognition and RNA polymerase recruitment [1,3]. Furthermore, the in vivo His pull-down assay showed that SigT could interact with RpoC, the β′ subunit of RNA polymerase core enzyme of *S. coelicolor* (Fig. 1). SigT was C-terminally tagged with GFP and expressed under the up-mutant *ermE* promoter (*ermEp*\*) [20], while RpoC was tagged with 6×His in an engineered strain J1981 [21]. SigT could be co-pelleted with RpoC but not in the control strain M145 expressing SigT-GFP (Fig. 1). As a control, GFP, instead of SigT-GFP, was not detected in the same His
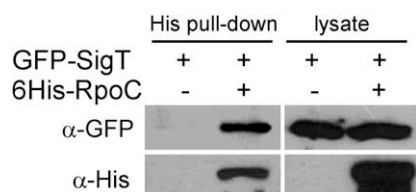


**Fig. 1.** Interaction between SigT and RNA polymerase. In vivo His pull-down assay. M145 or J1981 with plasmid pLM34, respectively, was cultured in YEME, lysed by sonication and subjected to His pull-down assay and Western blot.

pull-down assay as demonstrated for SigT-GFP (data not shown). These data suggested SigT was an ECF sigma factor in *S. coelicolor*.

*3.2. RstA, a putative anti-σ factor, interacts with SigT*

Genome sequencing revealed that SCO3891 resided with *sigT* in the same putative operon (Fig. 2A), and encoded a putative anti-σ factor of SigT. It was thus designated *rstA*. The RstA contained a conserved motif HXXXCXXC at its N-terminus [13]. This motif has been demonstrated required for the activity of RsrA, the anti-σ factor of SigR, in the maintenance of intracellular thiol-disulfide redox balance [22–24]. Interestingly, *trxAB* operon just located downstream of the putative *sigT* operon (Fig. 2A). It was hence supposed that SigT/RstA was also involved in the regulation of redox equilibration and *sigT* mutant has already been mentioned to be sensitive to diamide [1]. However, we did not observe the sensitivity of *sigT* mutant to H$_2$O$_2$. Another characteristic of RstA as an anti-σ factor was a putative trans-membrane domain (data not shown), since many ECF sigma factors responded to external signals through its membrane associated anti-σ factor [25].

In vitro and in vivo protein interaction assays were carried out to confirm the interaction between SigT and RstA. GST, GST-SigT, GST-RstA, His-SigT and His-RstA, were expressed in *E. coli*, respectively. The reciprocal in vitro GST pull-down assays showed that SigT could specifically interact with RstA (Fig. 2B and C). Furthermore, when SigT-GFP and RstA-3FLAG were co-expressed in *S. coelicolor* under *ermEp*\*, the SigT-GFP could be specifically co-immuno-precipitated with the α-FLAG antibody in the strain co-expressing these two fusion proteins, but not in the strain only expressing SigT-GFP (Fig. 2D), indicating the specific interaction between SigT and RstA.

*3.3. Functional analysis of sigT and rstA in morphological development*

These two genes were knocked out from M145 genome based on the in-frame deletion strategy, which removed the σ2 and σ4 domains of SigT and motifs of RstA, respectively (Supplementary data S3 and S4) [16].

On R2YE medium after growth for 40 h, both *sigT* and *rstA* mutants have exhibited apparent white lawn surface, indicating the flourishing aerial mycelium formation, while the wild type cells has just begun to differentiate into aerial hyphae. Reintroduction of *sigT* or *rstA* into *sigT* or *rstA* mutant, respectively, could restore the accelerated morphological development to the level of wild type (Fig. 3A). After incubation for a longer time, spores were observed in *sigT* and *rstA* mutants earlier than wild type (data not shown). In addition, *sigT* mutant differentiated more rapidly than *rstA* mutant (Fig. 3A). Similar phenotypes were also observed on MS and MM media (data not shown). The emergence of aerial mycelia is facilitated by a group of small cell wall proteins, such as chaplins, SapB and rodlins to overcome the surface tension of the mycelium–air interface [26–28]. We observed two chaplin genes, *chpE* and *chpH*, were expressed higher in *sigT* and *rstA* mutants than the wild type, especially at the aerial hypha stage, and even much stronger expression was observed in *sigT* mutant (Fig. 3B). These phenomena suggested both *sigT* and *rstA* had negative roles in morphological development at least by negatively regulating the expression of *chp* genes.

*3.4. Negative roles of sigT and rstA on secondary metabolism*

We then examined the roles of *sigT* and *rstA* on secondary metabolism. After grown on the R2YE plate for 1 day, the red antibiotic undecylprodigiosin (Red) were readily observed in *sigT* and *rstA* mutants, and *sigT* mutant was much redder than *rstA* mutant, but the red appearance was not observed in the wild type, *sigT* or
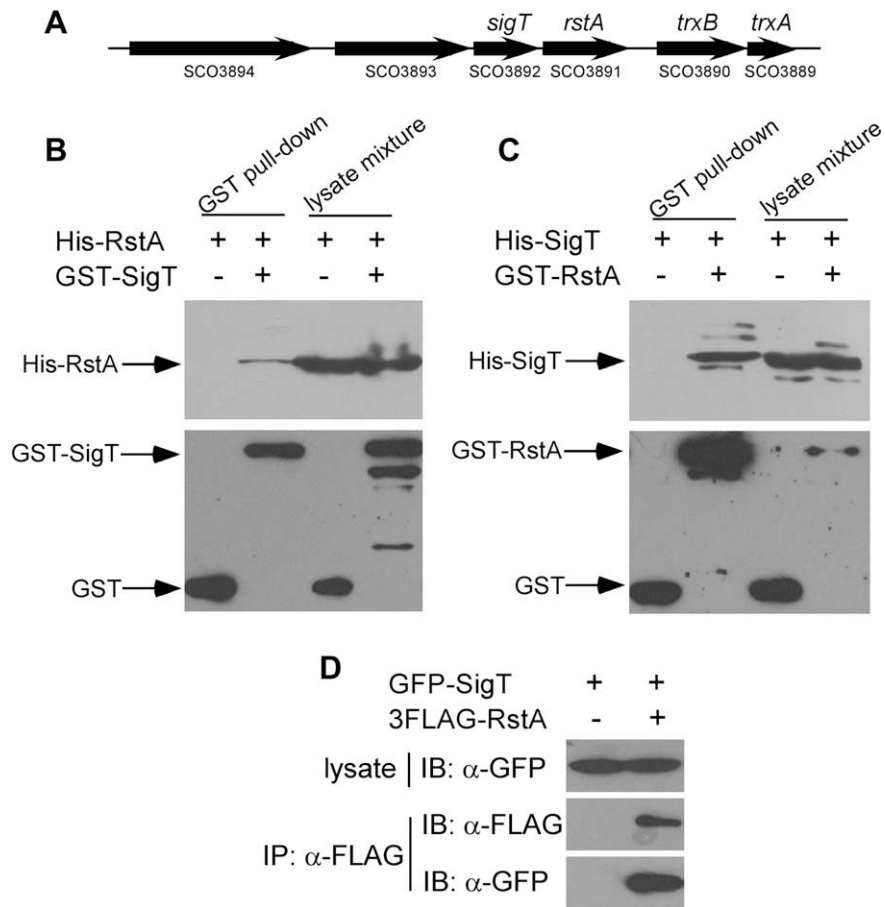
**Fig. 2.** Interaction between RstA and SigT. (A) Genomic organization of *sigT* and *rstA*. (B) GST, His-RstA, GST-SigT, (C) GST, His-SigT, GST-RstA were expressed in *E. coli*, respectively, lysed by sonication and subjected to GST pull-down assay and Western blot. (D) Co-immuno-precipitation assay. GFP-SigT was expressed in M145 alone or M145 with 3FLAG-RstA. Strains were cultured in YEME, lysed by sonication and subjected to co-immuno-precipitation assay and Western blot.
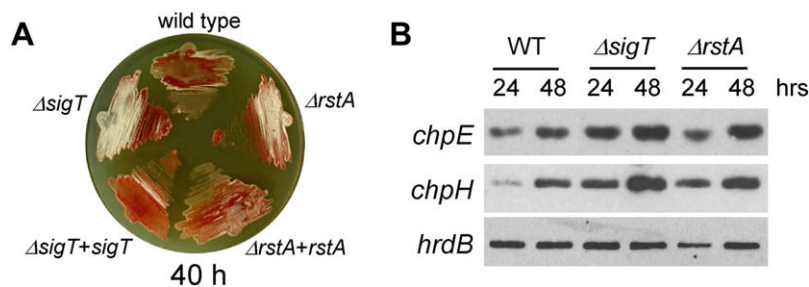


**Fig. 3.** Phenotypic analysis on morphogenesis after *sigT* or *rstA* deletion. (A) Wild type strain M145 (WT), *sigT* mutant (*ΔsigT*), *rstA* mutant (*ΔrstA*) and the complemented strains were streaked on the R2YE plate simultaneously, incubated at 30 °C for the indicated hours and photographed from the top of the plate. (B) RNA was extracted from M145 (WT), *sigT* mutant (*ΔsigT*) and *rstA* mutant (*ΔrstA*) after grown on R2YE medium overlaid with cellophane for 24 and 48 h, respectively. S1 nuclease protection assay was demonstrated with ssDNA probe of *chpE*, *chpH* and *hrdB*, respectively.

*rstA* complementation strain (Fig. 4A). After further incubation for 1 day, we found the similar production pattern of the blue antibiotic actinorhodin (Act) (Fig. 4A), indicating advanced production of Red and Act after deletion of *sigT* or *rstA*. Quantitative measurement of Red and Act production also revealed that *sigT* and *rstA* mutants produced antibiotics much earlier and higher than wild type, and *sigT* mutant showed more prominent antibiotics production than *rstA* mutant (Fig. 4B and C). Consistent with over-production of antibiotics, the *actII-orf4* and *redD*, which encode the positive transcription activators of gene clusters for Red and Act production, respectively [29,30], were also expressed higher in *sigT* and *rstA* mutant than in wild type, and even much more remark-

able over-expression was observed in *sigT* mutant (Fig. 4D). These results suggested both *sigT* and *rstA* also have negative roles on secondary metabolism.

### 3.5. RstA is required for the presence of SigT protein

We have shown that both *sigT* and *rstA* had negative roles in regulation of morphological development and secondary metabolism, but these phenotypes were not consistent with the hypotheses that RstA acts as the anti-σ factor of SigT, since traditional anti-σ factor mutant displays the opposite phenotypes to the corresponding sigma factor mutant, thus implying additional
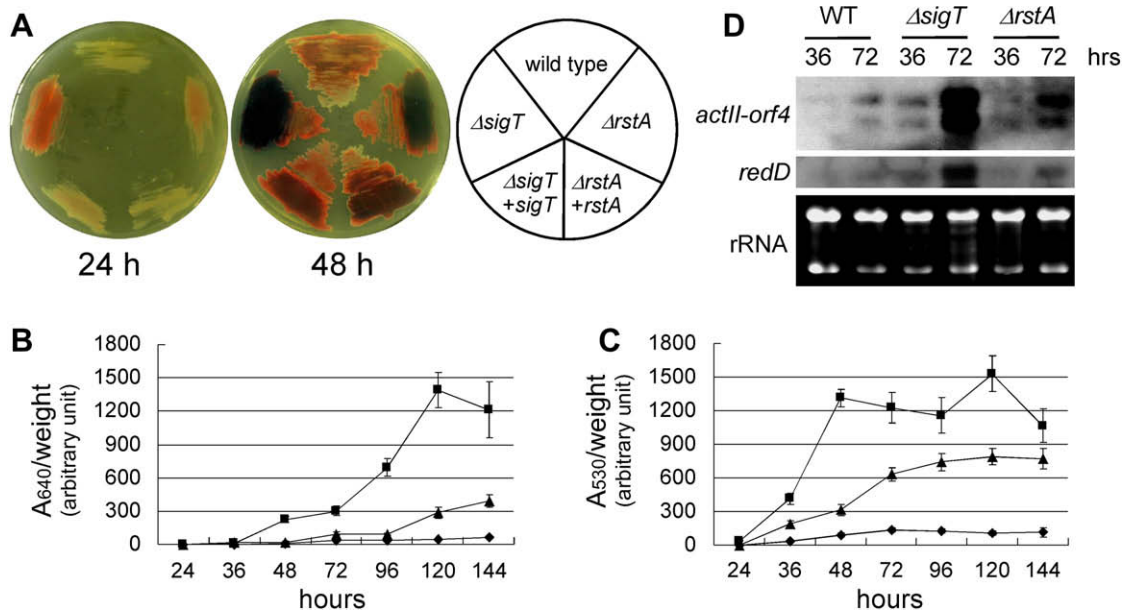
**Fig. 4.** Involvement of SigT and RstA on secondary metabolism. (A) M145 (WT), sigT mutant (ΔsigT), rstA mutant (ΔrstA) and the complemented strains were streaked on the R2YE plate, incubated at 30 °C for the indicated hours and photographed from the bottom of the plate. (B) and (C) M145 (WT) (♦), sigT mutant (ΔsigT) (■) and rstA mutant (ΔrstA) (▲) cells were collected from R2YE covered with cellophane and analyzed for actinorhodin (B) and undecylprodigiosin (C) production assay, respectively. The ratios of absorbance to mycelium dry weight were calculated and numbers in the graphs were the mean values of three independent experiments. S.D. (standard deviation) bars were shown on graphs. (D) RNA was extracted from M145 (WT), sigT mutant (ΔsigT) and rstA mutant (ΔrstA) after grown on R2YE medium overlaid with cellophane for the indicated hours, respectively. S1 nuclease protection assay was demonstrated with ssDNA probes of actII-orf4 and redD, respectively. For a loading control, about 1 μg of total RNA were loaded on 1% TBE agarose gel and photographed.

mechanisms of RstA in regulating the SigT activities. SigT-GFP was over-expressed under the ermEp* promoter in wild type, rstA mutant and wild type with rstA over-expression also under ermEp*. SigT-GFP fusion protein was almost invariable in wild type and rstA over-expression strains during the developmental course (Fig. 5A, lanes 1–4 and 9–12). But interestingly and surprisingly, this fusion protein was not detected when rstA was deleted (Fig. 5A, lanes 5–8), which suggested RstA was essential for the existence of SigT protein in S. coelicolor.

Since integration of the plasmid containing ermEp*-sigT-egfp into the genome was verified by PCR (data not shown), GFP under ermEp* was expressed both in wild type and rstA mutant. We found steady expression of GFP during morphological development in both strains on MS medium (Fig. 5B) or on R2YE medium (data not shown), and no significant difference of the GFP protein levels between wild type and rstA mutant was observed (Fig. 5B). These results suggested ermEp* was still active in rstA mutant as in wild

type. Thus, these results indicated RstA was required for the post-transcriptional regulation of SigT protein.

Here we presented evidence that an ECF sigma factor SigT could directly interact with its putative anti-σ factor RstA. Phenotypical and gene expression analysis showed that both SigT and RstA were the negative regulators in morphological development and secondary metabolism. Anti-σ factors execute their roles by specifically antagonizing its cognate sigma factors in transcription initiations by interfering with the binding of sigma factors to the promoters or to the RNA polymerase core enzyme [31,32]. Thus, it is anticipated that loss of anti-σ factors should result in enhanced activity of sigma factors and thus causing phenotypes or gene expression patterns opposite to the mutant of sigma factors, such as rsuA to sigU, rsbA to sigB, etc., in S. coelicolor [13,14,33]. But it was an exception for SigT-RstA, since we repeatedly observed accelerated differentiation in sigT and rstA mutants. It could at least be explained by the disappearance of the SigT protein in rstA mutant,
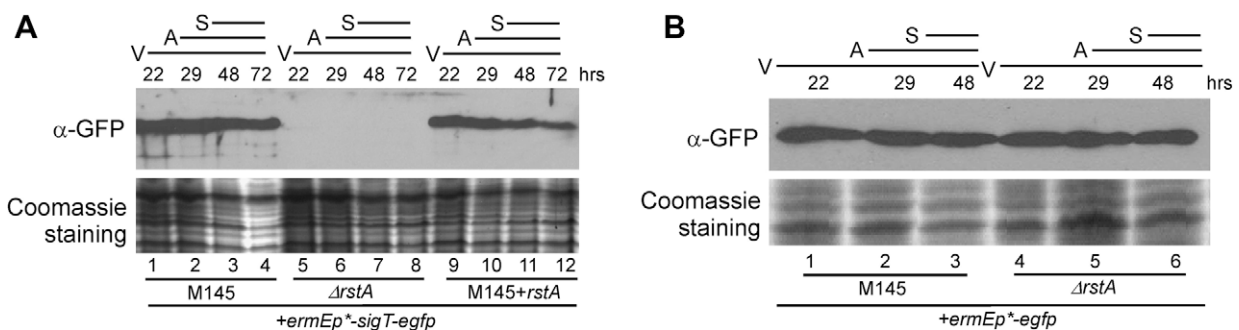


**Fig. 5.** Presence of SigT protein depends on RstA. (A) Cells of wild type (M145), rstA mutant (ΔrstA) and wild type with rstA over-expression (M145 + rstA) containing pLM34 were collected from MS plates overlaid with cellophane after incubation for the indicated hours, destroyed by ultra-sonication and about 20 μg of total protein were subjected for Western blot with an α-GFP antibody and coomassie blue staining. Vegetative mycelia (V), aerial hyphae (A) and spores (S) indicating different morphological phases were shown. (B) Cells of LM29 (M145 + ermEp*-gfp) and LM30 (ΔrstA + ermEp*-gfp) were gathered from MS plates overlaid with cellophane after incubation for the indicated hours and treated in the same way as in (A). Vegetative mycelia (V), aerial hyphae (B) and spores (S) indicating different morphological phases were shown.

which resulted in the decreased but not enhanced SigT activities. However, more experimental efforts, such as microarray, were still needed for the further proper elucidation of more prominent phenotypes observed in *sigT* mutant.

## Acknowledgements

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2009.09.025.

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