

Dual Positive Feedback Regulation of Protein Degradation of an Extra-cytoplasmic Function σ Factor for Cell Differentiation in *Streptomyces coelicolor**

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Background: The appropriate protein levels of ECF (extra-cytoplasmic function) σ factors are essential for bacteria functions.

Results: SigT degradation is dependent on ClpP protease and accelerated by secondary metabolites.

Conclusion: SigT degradation is regulated in a dual positive feedback manner.

Significance: This novel mechanism expands our understanding of ingenious cooperation of intracellular molecules for proper physiological functions of bacteria.

Here we report that in *Streptomyces coelicolor*, the protein stability of an ECF σ factor SigT, which is involved in the negative regulation of cell differentiation, was completely dependent on its cognate anti- σ factor RstA. The degradation of RstA caused a ClpP/SsrA-dependent degradation of SigT during cell differentiation. This was consistent with the delayed morphological development or secondary metabolism in the $\Delta clpP$ background after *rstA* deletion or *sigT* overexpression. Meanwhile, SigT negatively regulated *clpP/ssrA* expression by directly binding to the *clpP* promoter (*clpPp*). The SigT-*clpPp* interaction could be disrupted by secondary metabolites, giving rise to the stabilized SigT protein and retarded morphological development in a non-antibiotic-producing mutant. Thus a novel regulatory mechanism was revealed that the protein degradation of the ECF σ factor was initiated by the degradation of its anti- σ factor, and was accelerated in a dual positive feedback manner, through regulation by secondary metabolites, to promote rapid and irreversible development of the secondary metabolism. This ingenious cooperation of intracellular components can ensure economical and exquisite control of the ECF σ factor protein level for the proper cell differentiation in *Streptomyces*.

Throughout evolutionary history, organisms have adopted various regulatory measures for rapid accommodation to diverse environments. Extra-cytoplasmic function (ECF)² σ

factors, the largest group of σ^{70} family in bacteria, have been extensively studied. They are seen to alternatively recruit RNA polymerase core enzyme to modulate the gene expression of their regulons to facilitate a rapid response and adaptation to environmental stresses (1–3).

Meanwhile, to meet the physiological requirements of bacteria, the intracellular protein levels and transcriptional regulatory activities of ECF σ factors are also elaborately modulated at multiple levels. These include transcription, translation, antagonism from cognate anti- σ factors, and protein degradation. The gene expression of most ECF σ factor genes is rapidly induced by extracellular environmental signals and is frequently autoregulated to confer a prompt resistance to damages (4–8). BldN, an ECF σ factor from *Streptomyces coelicolor*, can be initially translated as a pro-protein, and becomes matured during cell differentiation (9). Anti- σ factors traditionally function as antagonists to inhibit the transcriptional activities of cognate ECF σ factors, and ECF σ factors are activated after conformation changes (10) or sequential proteolysis of anti- σ factors by intra-membrane and intracellular proteases (11–16). The intracellular ECF σ factors are also maintained at an appropriate level by protein degradation. In bacteria, protein degradation is executed mainly by the ATP-dependent proteases ClpP, Lon, HslUV, and FstH (17, 18). Under conditions of oxidative stress, the degradation of SigR', an isoform of the ECF σ factor SigR in *S. coelicolor*, is known to be partially dependent on the ClpP protease (19).

We previously described that loss of the ECF σ factor SigT or its anti- σ factor RstA in *S. coelicolor* resulted in retarded production of the blue pigment actinorhodin (Act) on nitrogen-limited media. We also found that RstA had a positive role on autoregulation of *sigT* expression (20). Meanwhile, although the locations of *sigT* and *rstA* are close to each other (21), they are not co-transcribed (20). This is another distinctive characteristic of SigT from other classical ECF σ factors. Moreover, on rich media, both $\Delta sigT$ and $\Delta rstA$ mutants show accelerated morphological development and secondary metabolism. This

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² The abbreviations used are: ECF, extra-cytoplasmic function; Red, undecylprolinine; Act, actinorhodin; RNAP, RNA polymerase.

Dual Positive Feedback Regulation of SigT Degradation

TABLE 1
Plasmids and cosmids

Plasmid or cosmid	Description	References
pLM29	<i>sigT</i> in pPROEX-HTb	21
pET28a	Expression vector in <i>E. coli</i>	Novagen
pL85	BamHI/HindIII <i>sigT</i> fragment from pLM29 into pET28a	This study
pET32a	Expression vector in <i>E. coli</i>	Novagen
pLM26	Kanamycin resistant gene and <i>ermEp*</i> in pIJ8630	21
pL86	NdeI/NotI <i>3flag-sigT</i> into pLM26	This study
pLM36	NdeI/NotI <i>rstA-3flag</i> into pLM26	21
pTA2	T vector, self-ligation	Toyobo, Japan
pL87	<i>clpP1/P2</i> promoter (<i>clpPp</i>) in pTA2	This study
pL88	<i>sigT</i> promoter (<i>sigTp</i>) in pTA2	This study
pL89	200 bp of <i>clpP1</i> in pTA2	This study
pL90	300 bp of <i>sigT</i> in pTA2	This study
Cosmid StC49	Cosmid containing <i>clpP1/P2</i>	John Innes Centre
pL92	$\Delta clpP1/P2::oriT-aac(3)IV$ in cosmid StC49	This study
Cosmid N6–68	Cosmid containing <i>ssrA</i>	This study
pL94	$\Delta ssrA::aadA$ in cosmid N6–68	This study
pL95	$\Delta ssrA::FRT$ in cosmid N6–68	This study
pIJ779	Plasmid with <i>FRT-aadA-FRT</i> for disruption cassette amplification	25
pIJ773	Plasmid with <i>FRT-oriT-aac(3)IV-FRT</i> for disruption cassette amplification	25
pIJ8660	Promoter-probing plasmid	24
pL146	<i>aadA</i> in pTA2	This study
pL147	<i>aadA</i> into EcoRI site of pIJ8660	This study
pL148	<i>rstA-3flag</i> in NdeI/NotI site of pL147	This study
pL149	<i>sigTp</i> replaces <i>ermEp*</i> in pLM26	This study
pL150	<i>sigT-linker</i> fused to <i>egfp</i> in pL149	This study

unusual phenomena triggered us to discover that the SigT protein had disappeared in the $\Delta rstA$ mutant (21). This had not previously been reported and suggested a potentially unique regulatory mechanism of RstA on SigT, beyond the putative traditional antagonistic effects.

Here we present further evidence that RstA is required for protein stability of SigT, and that degradation of RstA causes a rapid ClpP/SsrA pathway-dependent degradation of SigT in a dual positive feedback manner. We also suggest that secondary metabolites could act as regulators on SigT degradation for the timely and irreversible progress of cell differentiation. This novel mechanism enriches our understanding of how cellular components work cooperatively to keep the appropriate protein levels of transcriptional regulatory factors for proper cell development and environmental adaptation.

EXPERIMENTAL PROCEDURES

Media—*Escherichia coli* strains were routinely cultured in a LB medium. Liquid Tryptic Soy Broth (TSB) plus 5% PEG6000 was used for mycelium preparation in the primary metabolism. Solid R2YE and liquid R5 media were used for cell differentiation of *S. coelicolor*, and MS medium for spore preparation (22, 23).

Plasmid Construction—The plasmids and primers used in this study are listed in Tables 1 and 2, respectively. *E. coli* TG1 was used for routine plasmid construction. Plasmid pLM29 (21) was digested with BamHI/HindIII, and the purified *sigT* fragment was inserted into the BamHI/HindIII site of pET28a to create pL85. The *3flag-sigT* fragment was amplified with primers 1 and 2, ligated to pTA2 after dA addition, and digested with NdeI/NotI. After purification, the *3flag-sigT* fragment was inserted into the NdeI/NotI site of pLM26 (21) to generate plas-

TABLE 2
Oligonucleotides used in this study

Primer no.	Sequence
1	CATATGGACTACAAGGACCACGACGGCGATTACAAGGAC CAGCACATCGACTACAAGGACGACGACGACAAGGCGGGC GGCGCGAGTCACTAC
2	TCACGCTCGTCCACCTCCGCCCTTC
3	GGATCCCCCTCGCGCCGTCCCCGCAC
4	GGATCCCCGTCTCACGTTTCCCTGCCG
5	GGATCCGCACAACCATCGTCCGCGCGCTG
6	GGATCCCCGTCTTCTGCCTCCCCATTC
7	CGTCGTAAGACGAGCAGGTGG
8	CGGGTCGGAGGCAAGGAGCAG
9	CGCTGGGGGACCGTGAGGAG
10	TCGCTGGTCCGCGGGCAAGG
11	GAGTCCCGGAAGCGGCTCCAGCCCCGAGGAAACGTGAT TCCGGGGATCCGTCGACC
12	CCGTGCCAAGGAGCGGCAGCAGTACAGCCTCTTGCCCTATG TAGGCTGGAGCTGCTTC
13	GCGAGCCCCCTCTTCGGAGGACTTGAAAAATCAACATGGGAT TCCGGGGATCCGTCGACC
14	TCGCGCTTACGCGAGCACACGCCCTTCGACCTCGTGGTG TAGGCTGGAGCTGCTTC
15	TACAAGCTTATCAAGCGCAAGCAGCGGGC
16	ATCGAATTCGGGGTTCGGGTGGCAGAGC
17	GCCAGGTTTCCAGTCAACA
18	Bi o t i n -GCCAGGTTTCCAGTCAACA
19	GAGCGGATAACAATTCACACAGG
20	6FAM-GTTGTAATAACGACGGC
21	CTGCAGTTCGAAGTTCCTATTC
22	GCTTGATGTGATGGCTGGAG

TABLE 3
S. coelicolor strains used in this study

Strain	Genotype or description	References
M145	Wild type	47
LM21	$\Delta sigT$	21
LM22	$\Delta rstA$	21
L43	$\Delta rstA\Delta clpP1/P2::oriT-aac(3)IV$	This study
L44	$\Delta rstA\Delta ssrA::aadA$	This study
L45	$\Delta clpP1/P2::oriT-aac(3)IV$	19
L46	$\Delta ssrA::FRT$	This study
M1141	Δact	42
M1146	$\Delta act\Delta red\Delta cpk\Delta cda$	42

mid pL86. Primers 3, 4, and primers 5, 6 were used to amplify *clpPp* and *sigTp*, respectively, and each was ligated into pTA2 for plasmids pL87 and pL88, respectively. About 200 bp of the *clpP1* coding region and 300 bp of the *sigT* coding region were obtained with primers 7, 8 and primers 9, 10, respectively, and each was inserted into pTA2 for plasmids pL89 and pL90, respectively. *sigTp* was digested with BamHI from pL88 and replaced *ermEp** in pLM26 (21) to produce plasmid pL149. The *sigT-linker* was digested with NdeI from pLM34 (21) and fused to *egfp* in pL149 to create plasmid pL150. The streptomycin-resistant gene *aadA* was amplified from pIJ779 with primers 21 and 22, and ligated to the pTA2 vector to generate plasmid pL146. Then the EcoRI fragment *aadA* was ligated to EcoRI site of pIJ8660 (24) to create pL147. The *rstA-3flag* fragment was digested from pLM36 (21) with NdeI/NotI, and inserted into the NdeI/NotI site of pL147 for plasmid pL148.

Construction of *S. coelicolor* Strains—*S. coelicolor* strains used in this study are listed in Table 3. The *clpP1/P2* and *ssrA* genes were disrupted by the PCR-targeting strategy (25). *clpP1/P2::FRT-oriT-aac(3)IV-FRT* disruption cassette was amplified with primers 11, 12 from EcoRI/HindIII fragments of pIJ773, and electrotransformed into *E. coli* BW25113/pIJ790 containing cosmid StC49 to generate disruption cosmid pL92. The *ssrA::FRT-aadA-FRT* disruption cassette was amplified

with primers 13, 14 from EcoRI/HindIII fragments of pIJ779, and electrotransformed into *E. coli* BW25113/pIJ790 containing cosmid N6–68 to generate disruption cosmid pL94. The in-frame deletion cosmid pL95 was created by passage of cosmid pL94 through *E. coli* BT340 at 42 °C. Disruption cosmids pL92 and pL94 were conjugated through *E. coli* ET12567/pUZ8002 into LM22 (Δ rstA) to knock out *clpP1/P2* and *ssrA*, respectively, to give rise to strains L43 and L44, respectively. Cosmid pL95 was introduced into M145 for strain L46 by in-frame deletion of *ssrA* (25).

Protein Techniques—*E. coli* BL21(DE3) containing pL85 was induced with 0.1 mM isopropyl 1-thio- β -D-galactopyranoside at 18 °C to express the soluble His-SigT protein. The protein was purified with Ni²⁺-NTA according to the manufacturer's instructions (Qiagen). *S. coelicolor* mycelia were collected after inoculation of spores in liquid medium or on cellophane for the time indicated, and lysed for protein preparation as previously described (21). About 20 μ g of total protein was subject to Coomassie Blue staining or Western blot with mouse α -FLAG antibody (M2, Sigma) or rabbit α -GFP antibody (Proteintech Group). Purified *E. coli* RNA polymerase core enzyme (Epicenter), purified SigT, and *E. coli* cell lysate with expressed SigT were separated on a 6% SDS-PAGE for Western blot with antibodies against *E. coli* RNA polymerase subunit β and β' (Santa Cruz Biotechnology), or on 12% SDS-PAGE for Coomassie Blue staining.

RNA Analysis—Total RNA was extracted from mycelia cultured in TSB or on cellophane overlaid on R2YE medium and treated with RNase-free DNase I as described (21). About 10 μ g of RNA was subject to formaldehyde gel-based Northern blot analysis (26). The RNA on the nylon membrane was hybridized with an internal biotin-labeled *ssrA* DNA probe, and detected with streptavidin-HRP and BeyoECL plus, as described by the manufacturer (Beyotime, China). Ribonuclease protection assays were demonstrated to analyze *sigT* and *clpP1* mRNA in a low abundance. Briefly, about 10 μ g of total RNA was co-precipitated with 0.1 ng of biotin-labeled antisense RNA probe. Protected RNA fragments were prepared as described by the manufacturer (Ambion), and separated on a 7 M urea, 5% polyacrylamide gel after heat-denaturing for *clpP1*, or subject to Northern blot for *sigT*. After electrophoresis, the RNA fragments were blotted to a positive charged nylon membrane, UV-fixed, and then detected with streptavidin-HRP and North2South Chemiluminescent Substrate (Thermo Scientific).

Electrophoretic Mobility Shift Assay (EMSA)—About 1 ng of the probe was incubated with 0, 0.4, 1.0, 2.0, and 4.0 μ M purified His-SigT, respectively, at 25 °C in buffer (10 mM Tris, 100 mM Na₂HPO₄, pH 8.0, 50 μ g/ml of sheared sperm DNA) for 30 min and loaded on a 5% native polyacrylamide gel for separation in 0.5 \times TBE running buffer. The probe was then electroblotted to the nylon membrane, UV-fixed, and detected with streptavidin-HRP and BeyoECL plus (Beyotime, China). For inhibitory assays by secondary metabolites, the crude extract of secondary metabolites, HPLC-purified γ -actinorhodin or undecylprodigiosin with a decreased concentration as indicated, were incubated with His-SigT at 25 °C for 30 min, and 1 ng of 5'-labeled *clpPp* probe was then added for a further 30-min incubation. For the *E. coli* RNAP core enzyme binding assay, an increased

amount of purified SigT protein was incubated without or with 0.4 μ g of core enzyme for about 30 min at room temperature, and then 1 ng of biotin-labeled *clpPp* probe was added for a further 30-min incubation.

Preparation of Secondary Metabolites—Preparation of crude extract of undecylprodigiosin (Red), Act, and their intermediates has been described previously (27, 28). Briefly, Red and its intermediates were extracted with acidified methanol (0.5 M HCl) from wild type mycelia cultured in a R5-medium for 1 day. The supernatant was collected by a brief centrifugation, filtered, vacuum evaporated, and solubilized in methanol as 100 μ g/ μ l. For the preparation of extracellular Act and its intermediates, the 6-day wild type culture supernatant in the R5-medium was acidified to pH 3.0, extracted with 2 volumes of ethyl acetate, and vacuum evaporated. The 6-day wild type cells in the R5-medium were lysed with ultrasonication, and the supernatant was extracted with 2 volumes of ethyl acetate and vacuum evaporated for preparation of intracellular Act and its intermediates. The residues were resuspended in DMSO as 100 μ g/ μ l.

The extracellular γ -actinorhodin was purified by HPLC (29) and resuspended in DMSO as 10 μ M. For purification of undecylprodigiosin, the total prodigiosin was prepared from the mycelia of a *S. coelicolor* Δ act mutant M1141 cultured in R5-medium for 6 days as described above. The concentrated extract was resolved by HPLC in a C18 column with solution A (CH₃OH:CH₃CN:H₂O = 40%:10%:50%) and solution B (CH₃OH in 0.1% trifluoroacetic acid) and with detection at 530 nm. Undecylprodigiosin was eluted in a linear A:B gradient from 100:0% to 20:80% in 10 min, followed by 20:80% to 0:100% in 20 min, and collected at about 26.5 min. The purity and molecular weight were determined by HPLC and mass spectrometry.

DNase I Footprinting Assay—The FAM-labeled non-radioactive DNase I footprinting assay was carried out as previously described with minor modifications (30–32). Briefly, the purified SigT protein was ultrafiltered with YM-10 (Millipore) for a 10-kDa cut-off and eluted in the 20 mM Tris buffer, pH 7.5. A 5'-FAM-labeled *clpPp* probe was amplified with universal primers 19 and 20 from plasmid pL87 and then gel purified. About 50 ng of *clpPp* probe was incubated with SigT protein at room temperature for 30 min, and 0.03 units of DNase I (Promega) was added with 10 mM MgCl₂ and 1 mM CaCl₂. After exact incubation at room temperature for 1 min, reactions were stopped with an equal volume of 100 mM EDTA, pH 8.0, immediately followed by phenol/chloroform extraction and precipitation with 0.75 M NH₄Ac, 40 μ g of glycogen and ethanol. DNA mixed with Liz-500 DNA marker (MCLAB) was loaded on ABI 3130 DNA sequencer and electropherograms were analyzed with Genemapper version 4.0 software (Applied Biosystems) to align and determine the protected region. A DNA sequencing ladder was prepared with the 5'-FAM-labeled primer 20 according to Thermo Sequenase Dye Primer Manual Cycle Sequencing Kit (U.S. Biochemical Corp.).

Non-radioactive Labeling of DNA and RNA Probes—The *ssrA* DNA probe for Northern blot was internally labeled by PCR. Primers 15 and 16 were used to amplify *ssrA* from the M145 genome with *Taq* polymerase. Then the gel-purified *ssrA*

Dual Positive Feedback Regulation of SigT Degradation

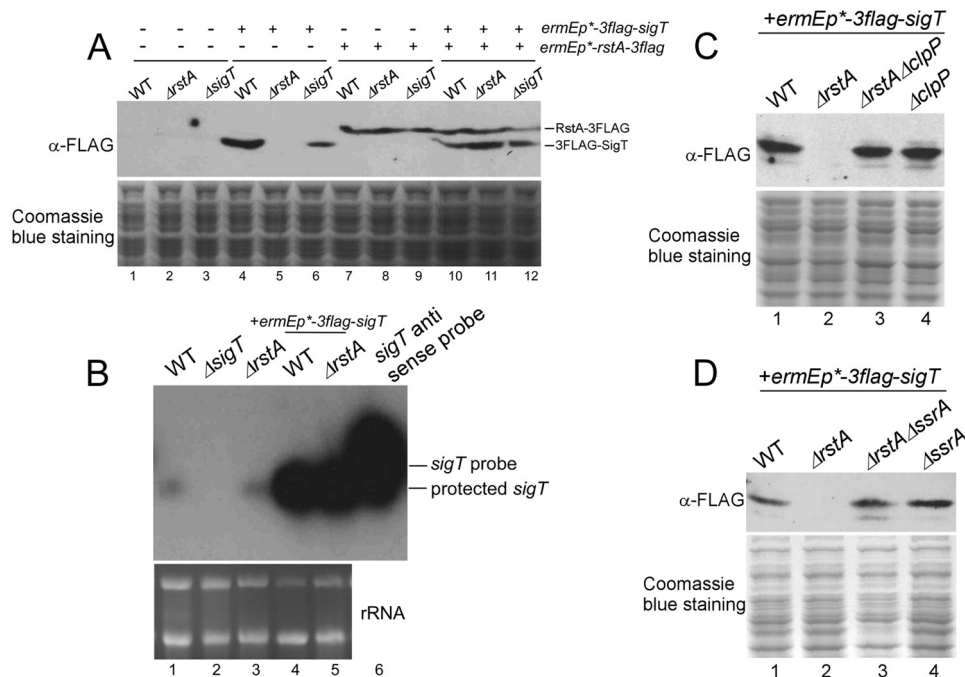


FIGURE 1. ClpP/SsrA is involved in degradation of SigT without RstA. *A*, complementation of RstA for SigT protein stability. Total protein was prepared from strains M145 (wild type, WT), LM22 (Δ *rstA*), LM21 (Δ *sigT*) containing either plasmid pL86 (*3flag-sigT*) or plasmid pL148 (*rstA-3flag*) or both, in TSB liquid medium for 2 days. SigT and RstA proteins were analyzed by Western blot with α -FLAG antibody. Coomassie Blue staining of total protein served as a loading control. *B*, *sigT* expression profile in wild type M145 (WT), LM21 (Δ *sigT*), LM22 (Δ *rstA*), M145/pL86 (WT + *3flag-sigT*), and LM22/pL86 (Δ *rstA* + *3flag-sigT*) as demonstrated by a ribonuclease protection assay. rRNA was shown as a loading control in a formaldehyde gel. *C* and *D*, ClpP/SsrA is required for SigT degradation without RstA. Total protein was prepared from strains M145/pL86 (wild type + *3flag-sigT*), LM22/pL86 (Δ *rstA* + *3flag-sigT*), L43/pL86 (Δ *rstA* Δ *clpP* + *3flag-sigT*), and L45/pL86 (Δ *clpP* + *3flag-sigT*) (*C*), strains M145/pL86 (wild type + *3flag-sigT*), LM22/pL86 (Δ *rstA* + *3flag-sigT*), L44/pL86 (Δ *rstA* Δ *ssrA* + *3flag-sigT*), and L46/pL86 (Δ *ssrA* + *3flag-sigT*) (*D*) in TSB liquid medium for 2 days and analyzed as in *A*.

fragment was used to amplify the biotin-labeled probe with biotin-11-dUTP (Fermentas) with universal primers 17 and 19.

clpP1 and *sigT* antisense RNA were internally labeled with biotin by *in vitro* transcription. The template for *in vitro* transcription was amplified with KOD plus from plasmids pL89 and pL90, respectively, with universal primers 17 and 19, then gel purified and eluted in diethyl pyrocarbonate-treated water. The *in vitro* transcription was carried out with T3 RNA polymerase (Fermentas) and the Biotin RNA Labeling Mix (Roche) at 37 °C for at least 3 h. The DNA template was removed by digestion with RNase-free DNase I (Takara) and the RNA probe was purified with the RNeasy Mini Kit (Qiagen) and eluted in diethyl pyrocarbonate-treated water.

For preparation of EMSA probes, the 5'-biotin-labeled universal primers 18 and 19 were used to amplify the 5'-biotin *clpPp* and *sigTp* probes from plasmid pL87 and pL88, respectively. The unlabeled universal primers 17 and 19 were used to amplify the unlabeled *clpPp* probe for competition assays.

RESULTS

ClpP/SsrA Is Required for Degradation of SigT without RstA—Traditionally, the antagonistic effects of anti- σ factors often result in opposite phenotypes between σ factor and anti- σ factor mutants (7, 33, 34). We have previously reported an exception that deletion of the ECF σ factor SigT or its cognate anti- σ factor RstA in *S. coelicolor* causes accelerated morphological development and increased antibiotic production. This prompted us to discover the disappearance of SigT protein in the Δ *rstA* mutant at the post-transcription level (21). Here we

further demonstrate that complementation of *rstA* in the Δ *rstA* mutant could restore the SigT protein (Fig. 1*A*, lane 11). In contrast, deletion of *sigT* did not affect the RstA protein (Fig. 1*A*, lane 9). These results further suggest the requirement of RstA for the presence of SigT. We then checked whether the absence of the SigT protein in the Δ *rstA* mutant resulted from a repressed expression of *sigT* or a decay of *sigT* mRNA. It was observed that *sigT* mRNA in the Δ *rstA* mutant was stable and present in comparable levels to those of the wild type, both under *sigT* operon promoter (Fig. 1*B*, lanes 1 and 3) and under the strong constitutive promoter *ermEp** (Fig. 1*B*, lanes 4 and 5). *sigT* mRNA expressed from *ermEp** was about 30 times higher than that from *sigT* native promoter. This suggested that absence of the SigT protein in the Δ *rstA* mutant could not have resulted from the possible disappearance of *sigT* mRNA.

Given the direct protein interaction between SigT and RstA (21), our results suggest that RstA is probably required for SigT protein stability. In bacteria, ClpP/SsrA is one of the major ATP-dependent protein degradation systems that includes ClpP1/P2 (ClpP) protease encoded by *clpP1/P2* (*clpP*) and the tmRNA-mediated recognition tag encoded by *ssrA* (35). After further deletion of *clpP1/P2* in a Δ *rstA* mutant (Δ *rstA* Δ *clpP*) or *ssrA* in a Δ *rstA* mutant (Δ *rstA* Δ *ssrA*), reappearance of the SigT protein was observed, with protein levels that were comparable with those in the wild type, Δ *clpP1/P2* mutant (Δ *clpP*) and Δ *ssrA* mutant, respectively (Fig. 1, *C* and *D*). Thus, our results strongly suggest that RstA can stabilize and protect SigT from

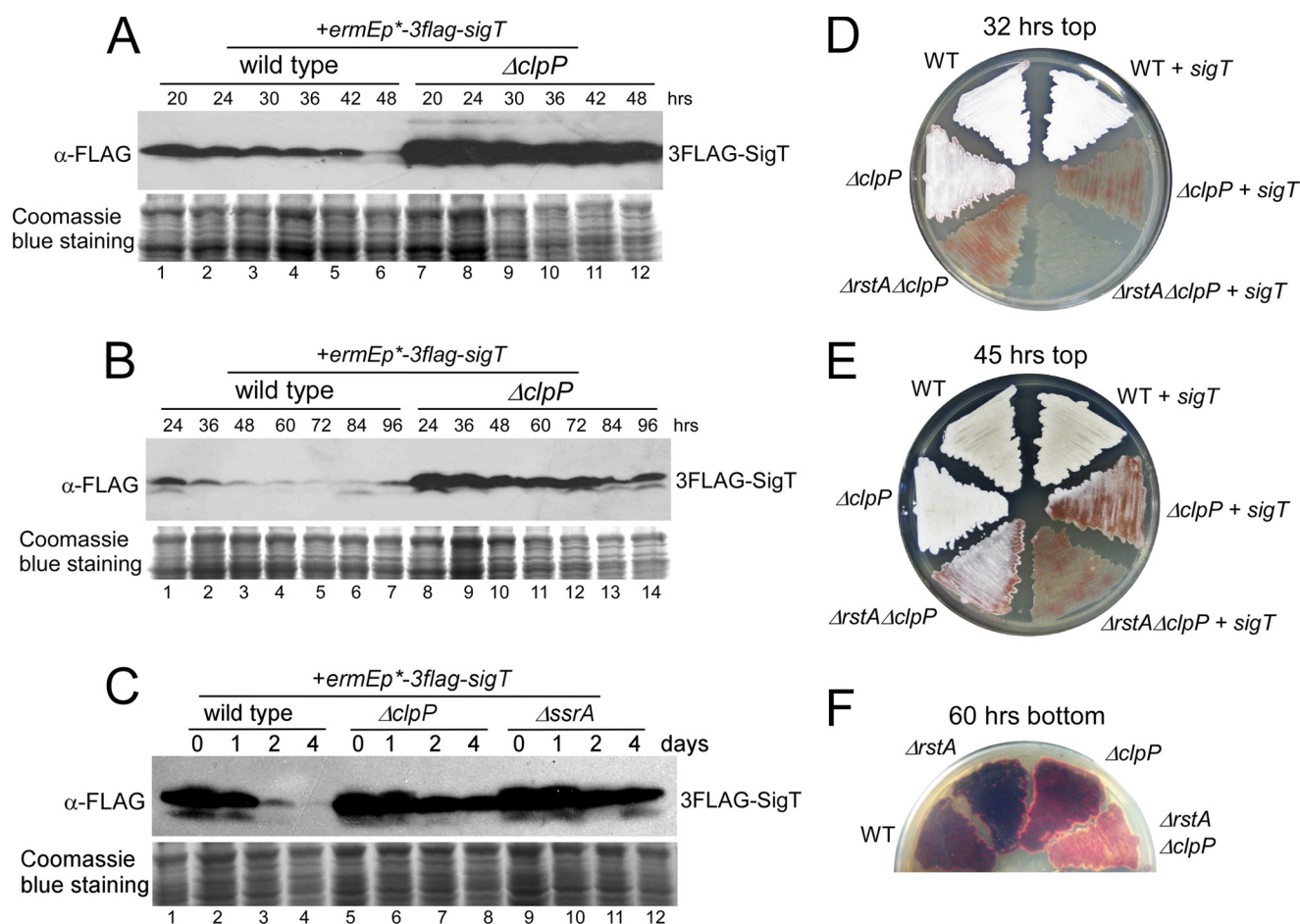


FIGURE 2. **SigT is degraded dependent on ClpP/SsrA during cell differentiation.** A–C, ClpP protease is required for SigT degradation during cell differentiation. SigT protein dynamics of strains M145/pL86 (wild type + 3flag-sigT) and L45/pL86 ($\Delta clpP$ + 3flag-sigT) on R2YE medium covered with cellophane (A and B) or in a liquid R5-medium (C) for the times indicated as demonstrated by Western blot. Coomassie Blue staining of total protein served as a loading control. Spores of strains M145 (wild type, WT), M145/pL86 (WT + sigT), L45 ($\Delta clpP$), L43 ($\Delta rstA \Delta clpP$), L45/pL86 ($\Delta clpP$ + sigT), and L43/pL86 ($\Delta rstA \Delta clpP$ + sigT) were streaked on R2YE medium for 32 (D) or 45 h (E), respectively, and photographed. F, spores of strains M145 (wild type, WT), LM22 ($\Delta rstA$), L45 ($\Delta clpP$), and L43 ($\Delta rstA \Delta clpP$) were streaked on R2YE medium for 60 h and photographed.

degradation, and that ClpP/SsrA is required for SigT degradation after *rstA* deletion.

SigT Degradation Is Dependent on ClpP Protease during Cell Differentiation—We then checked the physiological conditions under which SigT was degraded. On a solid R2YE medium, the SigT protein in the wild type was degraded gradually when cells were developing from substrate mycelia to aerial mycelia (Fig. 2A, lanes 1–5), and the protein level dropped sharply after sporulation (Fig. 2A, lane 6). The constitutively low level of SigT protein in the prolonged spore phase (48 h later) was repeatedly observed (Fig. 2B, lanes 1–7). However, the SigT protein was much more abundant in the $\Delta clpP$ mutant under all developmental phases than that in the wild type, especially after sporulation (Fig. 2, A and B). It should be noted that the prominent morphological development and secondary metabolism of *S. coelicolor* concurred on the solid R2YE medium. SigT protein levels were then checked in the liquid R5-medium, where *S. coelicolor* cells grow only as mycelia but produce abundant red and blue secondary metabolites, undecylprodiginine (Red) and actinorhodin (Act), respectively (22). When the cells cultured in the TSB medium at the logarithmic phase were transferred to R5-medium, SigT protein levels in the wild type decreased

slightly in the first day (Fig. 2C, lanes 1 and 2). This corresponded to the time when cells mainly produce Red (22). Beyond this, however, the protein level fell dramatically after 2 days, and was almost undetectable 4 days later (Fig. 2C, lanes 3 and 4). This corresponded to the time when Act is also produced (22). These phenomena were consistent with the observations on the R2YE medium, where cells mainly produce Red in the aerial phase (36 h) and then both antibiotics in the spore phase (48 h and later). In contrast, almost no degradation of the SigT protein was observed in the $\Delta clpP$ mutant even after 4 days in R5-medium (Fig. 2C, lanes 5–8). This suggests, on a genetic level, that ClpP protease is required for developmental phase-dependent SigT degradation.

In the wild type, loss of the ECF σ factor SigT or anti- σ factor RstA results in accelerated morphological development and secondary metabolism (21). This could be at least partially explained by the observations that SigT was unstable in the $\Delta rstA$ mutant, whereas RstA remained stable in the $\Delta sigT$ mutant, as shown above. Meanwhile, overexpression of *sigT* in the wild type did not have a significant impact on morphological development (Fig. 2, D and E). This is possibly because SigT would be degraded in the wild type during cell differentiation.

Dual Positive Feedback Regulation of SigT Degradation

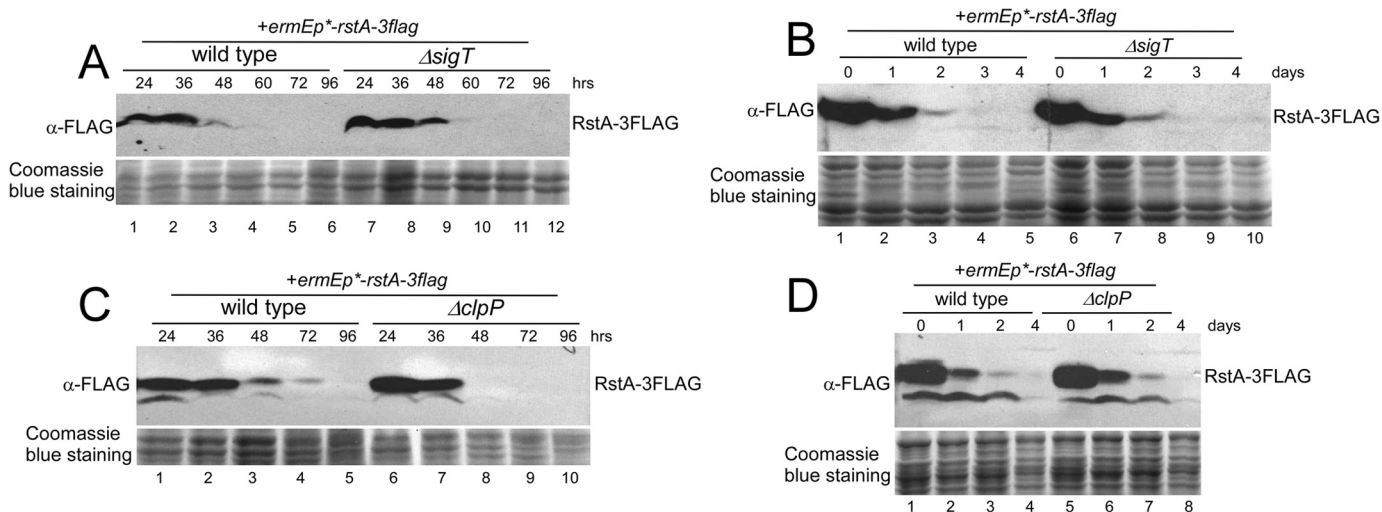


FIGURE 3. *A* and *B*, RstA degradation independent of SigT during cell differentiation. RstA protein dynamics in strains M145/pLM36 (wild type + *rstA-3flag*) and LM21/pLM36 (Δ *sigT* + *rstA-3flag*) on R2YE medium (*A*) or in liquid R5-medium (*B*) for the times indicated as demonstrated by Western blot with α -FLAG antibody. *C* and *D*, RstA degradation independent of ClpP protease. RstA protein dynamics in strains M145/pL36 (wild type + *rstA-3flag*) and L45/pL36 (Δ *clpP* + *rstA-3flag*) on R2YE medium (*C*) or in liquid R5-medium (*D*) for the times indicated as demonstrated by Western blot. Coomassie Blue staining of total protein served as a loading control.

However, when the SigT protein was stabilized in the Δ *clpP* mutant (Fig. 2, *A–C*), further deletion of *rstA* caused a delay in aerial hyphae formation (compare Δ *clpP* and Δ *rstA Δ *clpP* in Fig. 2, *D* and *E*). Meanwhile, overexpression of *sigT* in the Δ *clpP* or Δ *clpP Δ *rstA* mutant also led to a retarded aerial hyphae development, as compared with their parental strains, respectively (Fig. 2, *D* and *E*). *S. coelicolor* can produce a red antibiotic undecylprodiginine (Red) and a blue antibiotic actinorhodin (Act) and mixture of both makes the lawn appear purple. The wild type and Δ *clpP* mutant was more purple than the Δ *clpP Δ *rstA* mutant on a R2YE medium, and the Δ *clpP Δ *rstA* mutant appeared much redder (Fig. 2*F*). This suggests Act production was dramatically lessened after *rstA* deletion in the Δ *clpP* mutant, a situation that contrasted to that of the Δ *rstA* mutant in the wild type background (Fig. 2*F*) (21). This suggests a positive role of RstA on antibiotic production if SigT is stabilized. All of these phenotypes were consistent with the hypothesis that SigT negatively regulates cell differentiation and RstA was an anti- σ factor not only protecting SigT from degradation but also antagonizing SigT activity when the SigT protein was stabilized.****

SigT-independent Degradation of RstA during Cell Differentiation—Next we examined how RstA regulated SigT stability. It should be reasonable that RstA conformation alteration or protein degradation should result in dissociation of the SigT-RstA complex to mimic *rstA* deletion and leave SigT liable to degradation. RstA has an HXXXCXXC (where *X* is any amino acid) motif for possible disulfide formation (21), as does another anti- σ factor RsrA, which can release the ECF σ factor SigR under oxidation after conformation changes in *S. coelicolor* (10). However, we did not observe RstA mobility shift or SigT degradation under oxidative stresses from diamide or H₂O₂ (data not shown). It was then speculated that RstA should also be degraded during cell differentiation. Indeed, we found RstA degradation in wild type cells both on the solid R2YE medium (Fig. 3*A*, lanes 1–6) and in the liquid R5-medium (Fig.

3*B*, lanes 1–5) as observed for the SigT protein. Furthermore, we observed that deletion of *sigT* did not significantly affect RstA protein kinetics either on the R2YE medium (Fig. 3*A*, lanes 7–12) or in liquid R5-medium (Fig. 3*B*, lanes 6–10). This suggests that SigT is not essential for RstA stability. These data also strongly suggest that RstA has protective effects on SigT, and that RstA must be degraded before SigT degradation to leave SigT isolated and susceptible to ClpP/SsrA pathway-dependent degradation. Furthermore, we found RstA was not stabilized in the Δ *clpP* mutant either on R2YE or in R5-liquid medium (Fig. 3, *C* and *D*), suggesting that the RstA and SigT degradations are dependent upon different pathways, and that stabilized SigT in the Δ *clpP* mutant during cell differentiation does not result from a stabilized RstA, also supporting ClpP-dependent SigT degradation.

Endogenous Kinetics of the SigT Protein—We have shown that SigT expressed from a strong constitutive promoter *ermEp** is protected by RstA and its degradation is dependent on ClpP. Next we checked the endogenous SigT protein level during cell differentiation by monitoring the SigT-GFP fusion protein expressed under the *sigT* promoter (*sigTp*). It was observed that the SigT-GFP protein was rapidly degraded after the cells developed into its spore phase in wild type, and the Δ *clpP* mutant once again showed more abundant SigT-GFP fusion protein at all developmental phases (Fig. 4*A*), although a slightly higher SigT-GFP protein level was observed at the aerial hyphae phase (Fig. 4*A*, lanes 2 and 7). With the GFP protein as a reporter for the *sigTp* activity assay during cell differentiation, both wild type and the Δ *clpP* mutant exhibited a similar kinetics of the GFP protein, which gradually increased and then decreased when cells were developing into the aerial mycelium and spore phases, respectively (Fig. 4*B*). This expression pattern excluded the possibility that the SigT protein difference in wild type and in the Δ *clpP* mutant might result from different *sigTp* activities. We suspected that increased *sigTp* activity, occurring before the aerial hyphae, could buffer the fast degra-

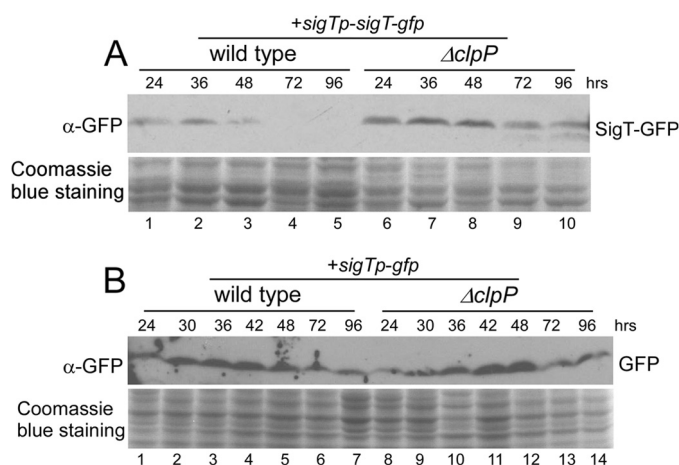


FIGURE 4. **Endogenous SigT protein dynamics.** Protein dynamics of SigT-GFP fusion protein (A) and GFP expressed under *sigTp* (B) in strains M145/pL97 (wild type + *sigTp-sigT-gfp*) and L45/pL97 ($\Delta clpP$ + *sigTp-sigT-gfp*) (A), in strains M145/pL96 (wild type + *sigTp-gfp*) and L45/pL96 ($\Delta clpP$ + *sigTp-gfp*) (B). Cells were collected on R2YE medium overlaid with cellophane after the indicated times and lysed for Western blot with α -GFP antibody. Coomassie Blue staining of total protein was used as a loading control.

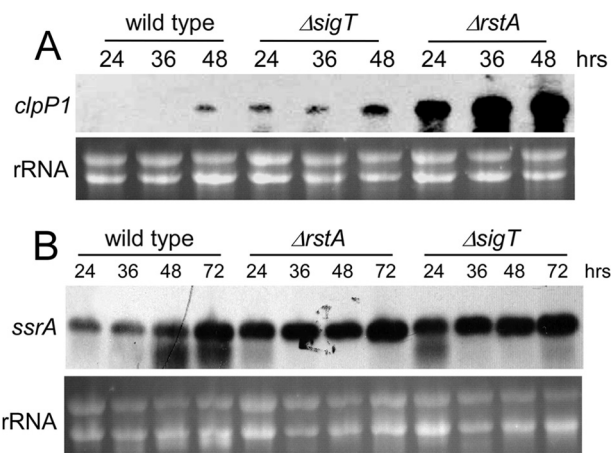


FIGURE 5. A and B, SigT negatively regulates *clpP1/P2* and *ssrA* expression. Ribonuclease protection assay of *clpP1* expression (A) and Northern blot analysis of *ssrA* expression (B) in M145 (wild type), LM21 ($\Delta sigT$) and LM22 ($\Delta rstA$) grown on R2YE medium overlaid with cellophane at various time points. About 10 μ g of total RNA run in a formaldehyde gel was for a loading control.

dation of the endogenous SigT protein, although decreased *sigTp* activity in the spore phase might speed up the disappearance of the SigT protein, thus leading to an appropriate intracellular SigT protein level and timely cell differentiation. Therefore, our results suggest that developmental phase-dependent SigT degradation requires ClpP protease during cell differentiation.

Negative Regulation of *clpP* and *ssrA* Expression by SigT and RstA—*clpP1*, *clpP2* are co-transcribed in one operon, and ClpP1 and ClpP2 are the subunits of the ClpP protease (19). Our gene expression analysis showed that *clpP1* expression was undetectable before 36 h, but increased after 48 h, when cells developed into spores (Fig. 5A). Wild type cells showed similar expression levels of *ssrA* at 24- and 36-h time points, when cells were in substrate mycelia and aerial mycelia, respectively, but 2 times higher expression was observed after sporulation, or 48 h later (Fig. 5B). This was consistent with the sharp drop of the SigT protein level after sporulation (Fig. 2, A and B). Deletion of

sigT or *rstA* could enhance the expression of *clpP1* at all developmental phases and a more dramatic expression augment was observed in the $\Delta rstA$ mutant (Fig. 5A), which might also be responsible for degradation of the SigT protein in the $\Delta rstA$ mutant. Meanwhile, $\Delta sigT$ and $\Delta rstA$ mutants showed about 5 times higher *ssrA* expression than wild type at 24 and 36 h, and about 3 times higher at 48 h, but no significant difference after 72 h (Fig. 5B). Furthermore, the *clpP1* expression profile was verified by a GFP reporter assay, in which GFP was expressed under the control of the *clpP* promoter (*clpPp*). It was found that the GFP protein level gradually increased with cell differentiation in the wild type on a R2YE medium, and we also observed more GFP protein in both the $\Delta sigT$ mutant and in the $\Delta rstA$ mutant at all developmental phases (data not shown). Thus, our results indicate that both SigT and RstA negatively regulated the expression of *clpP1/P2* and *ssrA*.

Interaction between SigT and the *clpP* Promoter—We then investigated whether SigT could directly regulate the expression of *clpP1/P2* and/or *ssrA*. The SigT protein was expressed and purified from *E. coli*. EMSA showed that SigT could bind to the *clpP1/P2* promoter (*clpPp*) (Fig. 6A, lanes 6-10) but not to the *sigT* promoter (*sigTp*) (Fig. 6A, lanes 1-5). Another σ factor, SigN (36) from *S. coelicolor*, was also purified from *E. coli*, but no binding activity of SigN to *clpPp* was observed (data not shown). Furthermore, the addition of an excessive unlabeled *clpPp* probe could restore the shifted band to the free probe position (data not shown). These results suggest a specific interaction between SigT and *clpPp*. Traditionally, the promoter recognition and DNA-binding activity of σ factors is exerted after formation of holoenzyme together with the core enzyme of RNA polymerase (RNAP) (37). We then checked whether SigT bound to *clpPp* with the *E. coli* RNAP core enzyme. About 2.1 μ g of purified *E. coli* core enzyme was co-separated with about 1 μ g of purified SigT and 20 μ g of *E. coli* lysate with expressed SigT. Subunit β and β' co-migrated almost to the same position in a 12% polyacrylamide gel, and showed about 2 times the signal strength of purified SigT by Coomassie Blue staining. This was consistent with loading of about 1 μ g of β , β' subunit and SigT, respectively (Fig. 6B, lanes 1 and 9). Immuno-blots with anti- β and β' subunits showed that a weak signal (about 0.02 μ g) was also observed in the purified SigT (Fig. 6B, lane 9), which suggested SigT was present to a level of about a 200-fold molar excess of RNAP. Meanwhile, about 2 μ g of SigT and 1 μ g of β , β' subunit were observed in total lysate (Fig. 6B, lanes 1, 9, and 12). This suggested that most of the RNAP was not co-purified with SigT, and that RNAP might not be involved in formation of the SigT-core enzyme complex for SigT-*clpPp* binding. Moreover, supplementation of another additional RNAP core enzyme did not significantly increase the binding affinity of SigT to *clpPp* (Fig. 6C), further supporting the idea that SigT can solely bind to *clpPp*. The binding of σ factors to promoters without core enzymes has also been in evidence in studies of several other σ factors, such as CorE in *Myxococcus xanthus* (38), SigA from *Bacillus subtilis* (39), and Tt-RpoE1 from *Thermoanaerobacter tengcongensis* (40). Furthermore, two binding sites of SigT on *clpPp* were determined by a DNase I footprinting assay (Fig. 6D). These two sites comprised the -10 and -35 regions of

Dual Positive Feedback Regulation of SigT Degradation

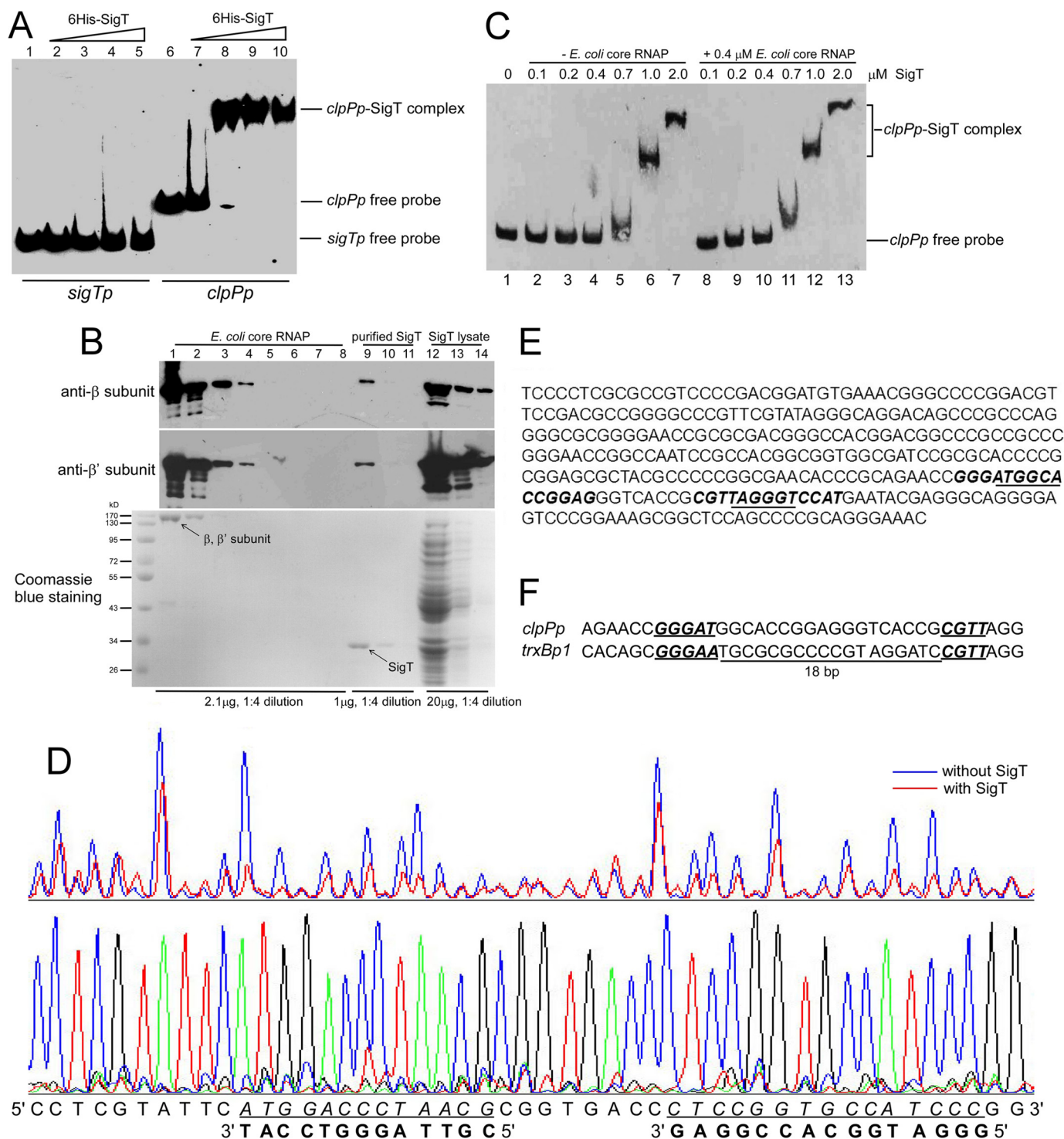


FIGURE 6. SigT recognizes *clpP* promoter. *A*, EMSA for SigT-*clpP* binding. About 1 ng of 5'-biotin-labeled *sigTp* and *clpPp* probes were incubated with an increasing amount of purified SigT protein, subjected to electrophoresis, and detected by ECL. *B*, evaluation of RNAP in purified SigT. About 2.1 μ g of purified *E. coli* RNAP core enzyme (lanes 1-8), 1.0 μ g of purified SigT (lanes 9-11), and 20 μ g of *E. coli* lysate containing SigT (lanes 12-14) were loaded on a 6% SDS-PAGE for Western blot with antibodies against β and β' subunits of *E. coli* RNAP, respectively, or on 12% SDS-PAGE for Coomassie Blue staining. All samples were diluted by 1:4. *C*, *E. coli* RNAP core enzyme was not required for SigT-*clpP* binding. EMSA of SigT-*clpP* binding with an increased amount of purified SigT as indicated without core enzyme (lanes 1-7) or with core enzyme (lanes 8-14). *D*, DNase I footprinting assay to determine the SigT recognition site on *clpPp*. 5'-FAM-labeled *clpPp* probe amplified from pL87 was used in the DNase I footprinting assay with purified SigT. The bottom strand of *clpPp* was analyzed. The protected region is *underlined* and in *italics*. The complementary strand is also shown. The sequencing ladder was generated with the same 5'-FAM-labeled primer. *E*, the promoter region of *clpPp*. The SigT binding site deduced from the DNase I footprinting assay is in *italics* and *bold*, and the -10 and -35 regions are *underlined*. *F*, alignment of recognition sites of SigT on *clpPp* with *trxBp1*. The conserved element is *bold italic* and *underlined*. The 18-bp interval region between two recognition sites is also shown.

clpPp, respectively (Fig. 6E) (41), consistent with SigT as an ECF σ factor. Recently, SigT was classified as an ECF27 with SigM from *Corynebacterium glutamicum*, and proposed to bind to a conserved element GGGAAN¹⁸-CGTT on *trxBp1* (3), which was also recognized by another ECF σ factor SigR from *S. coelicolor* (7, 10). The SigT recognition sites on *clpPp* had a GGGATN¹⁸-CGTT element, which matched that of *trxBp1* (Fig. 6F). Thus all our results indicate that SigT can regulate *clpP1/P2* expression by directly binding to its promoter.

Secondary Metabolites Prevent SigT-*clpPp* Binding—Next we examined whether a cell differentiation program could promote SigT degradation, probably by interfering with the SigT-*clpPp* interaction, to remove its negative effects. It was found that intracellular methanol (MeOH) extracts with undecylprodiginine (Red) and its intermediates (27), prepared from wild type cells cultured in R5-medium for 1 day, could inhibit SigT-*clpPp* binding (Fig. 7A). Meanwhile, γ -Act and its intermediates, extracted with ethyl acetate (31) from intracellular or extracellular parts of wild type cells cultured in R5-medium for 6 days, could also destroy SigT-*clpPp* binding (Fig. 7B). These results suggest that these are intracellular and secreted secondary metabolites that can damage the SigT-*clpPp* interaction. Furthermore, HPLC-purified undecylprodiginosin (data not shown) and γ -actinorhodin (29) from *S. coelicolor* could impede SigT-*clpPp* binding under a low concentration (10^{-7} M), respectively (Fig. 7, C and D). These results strongly suggest that during cell differentiation, a small amount of secondary metabolites can damage SigT-*clpPp* binding, and that continuously increased production of secondary metabolites can cause a gradually increased expression of *clpP1/P2* during cell differentiation as described in Fig. 5.

Stabilized SigT Protein in a Non-antibiotic-producing Mutant—Based on our observations, it was speculated that the removal of secondary metabolites could result in a tight binding of SigT to *clpPp* to suppress *clpP* expression, and lead to the stabilization of the SigT protein. Indeed, in R5-liquid medium, where cells grew as mycelia and underwent secondary metabolism, we did not observe SigT protein degradation in a mutant ($\Delta act\Delta red\Delta cpk\Delta cda$) (Fig. 7E), whose production of four secondary metabolites in *S. coelicolor* is abolished (42). Moreover, SigT protein was present at a higher and steadier level in the $\Delta act\Delta red\Delta cpk\Delta cda$ mutant than in the wild type on R2YE solid medium, where both morphological development and secondary metabolism were observed (Fig. 7F). Meanwhile, overexpression of *sigT* in $\Delta act\Delta red\Delta cpk\Delta cda$ caused delayed aerial hyphae development (Fig. 7G) and sporulation (Fig. 7H). These results further support our hypothesis that SigT, as a negative regulator for cell differentiation, is stabilized *in vivo* with reduced antibiotic production.

DISCUSSION

After fulfilling their mission to selectively regulate gene expression after stress responses, ECF σ factors are then rapidly degraded, as coordinated by the positive feedback autoregulation of gene expression (7, 18). In bacteria, this process remains essential for proper physiological adaptation and cell development in that it prevents the constitutive hyper-expression of

their regulons. However, exactly how the cellular components, including the ECF σ factors themselves, work collaboratively to promote degradation of ECF σ factors, thus enabling proper physiological adaptation, remains to be fully elucidated.

SigT negatively regulates cell differentiation in *S. coelicolor* (21). In this report, we show that ClpP protease is required for SigT protein degradation to remove its inhibitory effects on cell differentiation. This is consistent with the phenotypes of the $\Delta clpP$ and $\Delta ssaA$ mutants that display delayed morphological development and secondary metabolism (41, 43). Furthermore, SigT negatively regulates the *clpP1/P2* gene expression by binding to its promoter. So the initiation of SigT degradation results in up-regulation of *clpP1/P2* expression and enhancement of ClpP protease activity *in vivo*, which in turn causes more SigT to degrade. Furthermore, SigT negatively regulates the secondary metabolism, whereas antibiotics can disturb the SigT-*clpPp* binding. Thus initiation of SigT degradation also gives rise to increased production of antibiotics, whose interference with SigT-*clpPp* binding, in turn causes increased expression of *clpP1/P2* and accelerated degradation of SigT, for more rapid production of secondary metabolites (Fig. 8).

Much evidence has supported the classical hypothesis that the transcription activity of a σ factor is antagonized by its cognate anti- σ factor. Consequently, the σ factor and its anti- σ factor mutants display opposite phenotypes (37). The antagonism of anti- σ factor on its cognate σ factor is a *trans*-regulation, which includes repression after binding but then derepression after disassociation, and during this whole process the matured σ factor is stable (19). However, as we have reported, the protein stability of the ECF σ factor SigT is entirely dependent on its anti- σ factor RstA, which protects SigT from degradation, and RstA degradation causes the instability of SigT. It should be noted that the wild type and the $\Delta clpP$ mutant have similar SigT protein levels at the logarithmic phase in the TSB medium (Figs. 1C and 2C). This suggests that SigT is stable during primary metabolism. However, when cells are entering the secondary metabolism, RstA degradation-triggered SigT degradation occurs, and the dual positive feedback regulation takes place for a rapid SigT degradation and accelerated cell differentiation (Fig. 8).

Our results also suggest that secondary metabolites, whose production is under the negative control of SigT, can act as regulators to promote SigT degradation, thus leading to rapid occurrence of the secondary metabolism as an irreversible biological process. Meanwhile, because γ -actinorhodin and undecylprodiginosin are structurally unrelated compounds, how they play a similar role on inhibiting SigT binding to *clpPp* remains for further investigations. They might interfere with the $\sigma 2$ and/or $\sigma 4$ regions of SigT for DNA binding. However, we observed that many antibiotics produced from other microbial cannot destroy SigT-*clpPp* interaction (data not shown), suggesting that only the antibiotics from *S. coelicolor* can have the specific inhibitory effects on SigT-*clpPp* binding. One of the approaches is to obtain the crystal structures of the SigT-compound complex or SigT-DNA complex to elucidate the interactive residues of SigT to DNA and antibiotics, just as actinorhodin and its pathway-specific regulator ActR in *S. coelicolor* (44).

Dual Positive Feedback Regulation of SigT Degradation

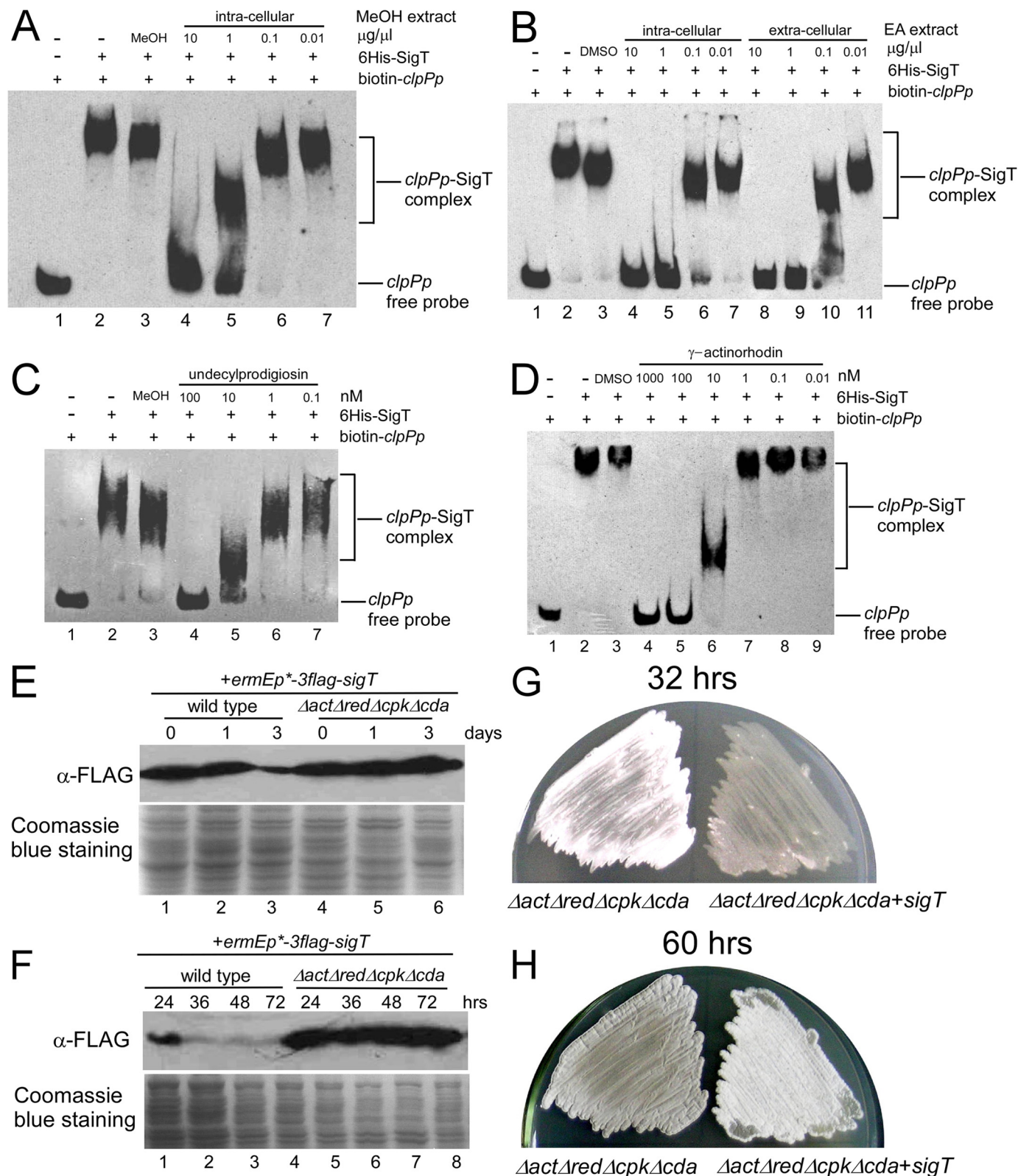


FIGURE 7. A–D, inhibition of SigT-*clpPp* binding by secondary metabolites. Intracellular crude extracts with methanol (*MeOH*) from wild type cells (A), intracellular or extracellular crude extracts with ethyl acetate (*EA*) from wild type cells (B), HPLC-purified undecylprodigiosin (C) or γ -actinorhodin (D) was incubated with SigT in a concentration gradient before addition of biotin-labeled *clpPp* probe, respectively, and subjected to EMSA. Methanol or DMSO were used as the solution control. E–H, SigT is stabilized in non-antibiotic-producing cells. SigT protein dynamics in strains M145/pLM86 (wild type + *3flag-sigT*) and M1146/pLM86 (Δ *act* Δ *red* Δ *cpk* Δ *cda* + *3flag-sigT*) grown in liquid R5- (E) or on R2YE (F) medium for the times indicated as demonstrated by Western blot with α -FLAG antibody. Coomassie Blue staining of total protein was for a loading control. Spores of strains LM1146 (Δ *act* Δ *red* Δ *cpk* Δ *cda*) and M1146/pLM86 (Δ *act* Δ *red* Δ *cpk* Δ *cda* + *sigT*) were streaked on R2YE medium for 32 (G) and 60 h (H), respectively, and photographed.

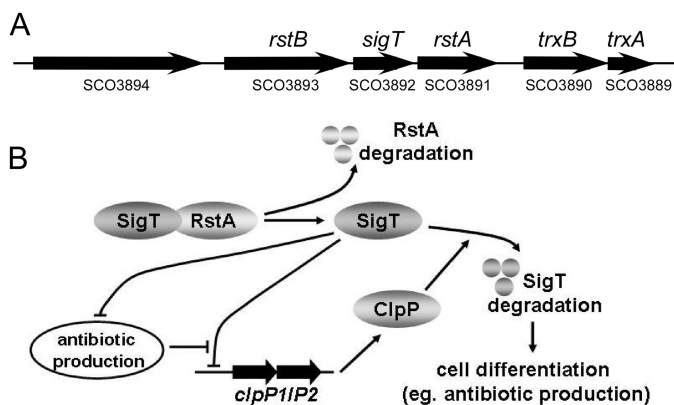


FIGURE 8. A proposed regulatory model for protein degradation of ECF σ factor SigT during cell differentiation. *A*, genomic organization of *sigT*, *rstA*, and their adjacent genes. *B*, a proposed model of dual positive feedback regulatory mechanism of SigT degradation dependent on ClpP protease and secondary metabolites.

Here we first report that an ECF σ factor is directly involved in a feedback regulation by secondary metabolites in *Streptomyces*. Given the common observations that the regulatory activities of “pseudo” γ -butyrolactone receptors (29), pathway-specific regulators (45, 46), and a LysR-type regulator (31) were also regulated by the secondary metabolites for antibiotic production, we believe that the secondary metabolite-mediated feedback regulation on the transcriptional regulator activities in *Streptomyces* is a general physiological strategy in bacteria, and that this evolutionary-based, economical, fine-tuned and cooperative regulation is essential for harmonic environmental adaptation and proper cell development.

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Dual Positive Feedback Regulation of SigT Degradation

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