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Characterization of negative regulatory genes for the biosynthesis of rapamycin in *Streptomyces rapamycinicus* and its application for improved production

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Abstract Sequence analysis of the rapamycin biosynthetic gene cluster in *Streptomyces rapamycinicus* ATCC 29253 identified several putative regulatory genes. The deduced product of *rapY*, *rapR*, and *rapS* showed high sequence similarity to the TetR family transcription regulators, response regulators and histidine kinases of two-component systems, respectively. Overexpression of each of the three genes resulted in a significant reduction in rapamycin production, while in-frame deletion of *rapS* and *rapY* from the *S. rapamycinicus* chromosome improved the levels of rapamycin production by approximately 4.6-fold (33.9 mg l^{-1}) and 3.7-fold (26.7 mg l^{-1}), respectively, compared to that of the wild-type strain. Gene expression analysis by semi-quantitative reverse transcription-PCR (RT-PCR) in the wild-type and mutant strains indicated that most of the rapamycin biosynthetic genes are regulated negatively by *rapS* (probably through its partner response regulator RapR) and *rapY*. Interestingly, RapS negatively regulates the expression of the *rapY* gene, and in turn, *rapX* encoding an ABC-transporter is negatively controlled by RapY. Finally, overexpression of *rapX* in the *rapS* deletion mutant resulted in a 6.7-fold (49 mg l^{-1}) increase in rapamycin production compared to that of wild-type strain. These results demonstrate the role of RapS/R and RapY as negative regulators of rapamycin biosynthesis and provide valuable information to both understand the complex regulatory mechanism in *S. rapamycinicus* and exploit the regulatory genes to increase the level of rapamycin production in industrial strains.

Keywords Rapamycin · Negative regulator · *Streptomyces rapamycinicus* · ABC-transporter

Introduction

Rapamycin is a 31-membered macrocyclic polyketide produced by *Streptomyces rapamycinicus* (previously classified as *Streptomyces hygroscopicus* ATCC 29253) [23] possessing various biological and pharmacological activities including antifungal [38], immunosuppressive [4], antitumor [8], neuroprotective [43], and anti-aging activities [13] (Fig. 1a). Furthermore, rapamycin has been approved in the US by the FDA for the clinical prevention of organ transplant rejection [19]. Because of its pharmacological importance and broad application, intense effort to understand its biosynthetic routes and to enhance its yield has been made during the past decades [35]. In brief, the polyketide chain of rapamycin is biosynthesized by type I modular polyketide synthase (PKS) systems (RapA, B, and C) using 4,5-dihydrocyclohex-1-ene-carboxylic acid (DHCHC) as a starter unit. The DHCHC is derived from chorismate by the action of chorismatase RapK [1]. The linear polyketide chain is condensed with pipecolate, synthesized by a lysine cyclodeaminase RapL [9], through a peptide synthetase RapP [22], followed by cyclization to generate pre-rapamycin. Pre-rapamycin is further modified by a series of post-PKS tailoring steps [11] (Fig. 1a). Nevertheless, the regulatory

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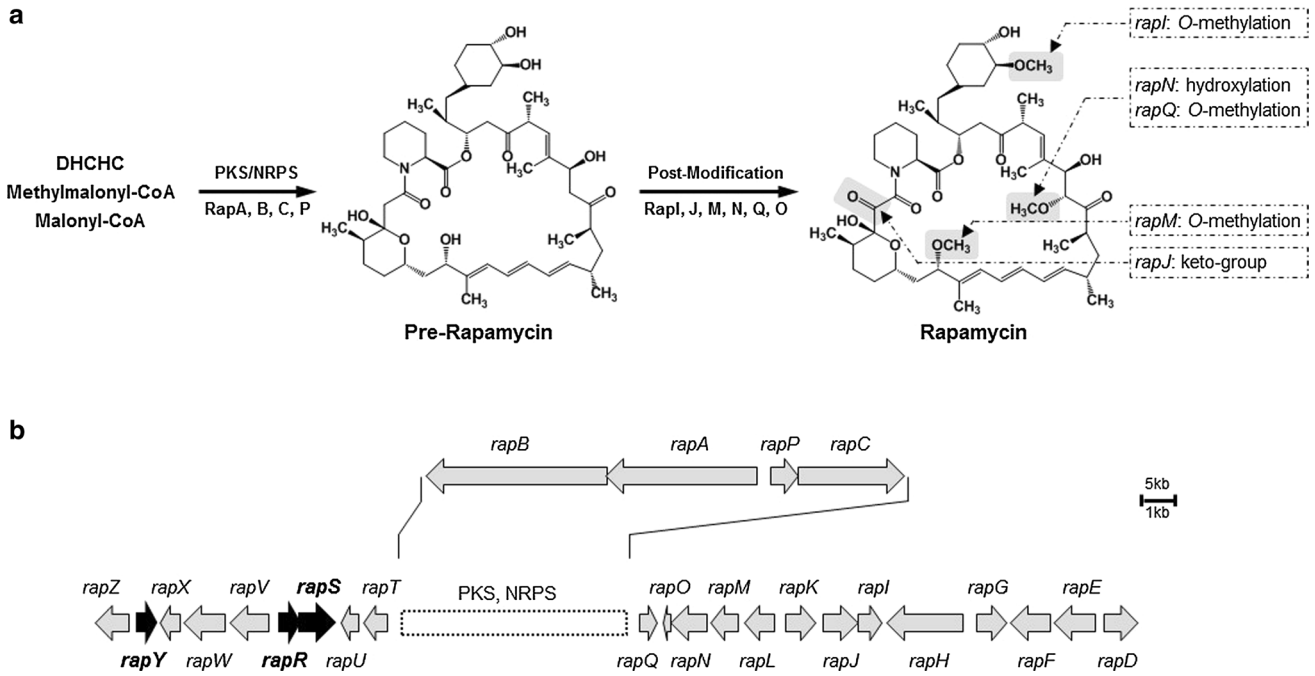

Fig. 1 The biosynthesis of pre-rapamycin, rapamycin, and its biosynthetic gene cluster. **a** RapA, B, and C use DHCHC (4,5-dihydrocyclohex-1-ene-carboxylic acid) as a starter unit, and (2S)-malonyl-CoA (2S)-methylmalonyl-CoA as extender units. RapP then incorporates an L-pipecolic acid unit, and the macrolide ring is closed generating pre-rapamycin. Post-modification genes (*rapI*, *rapJ*, *rapM*, *rapN*, *rapQ*, and *rapO*) produce rapamycin. **b** Rapamycin biosynthetic gene cluster of *S. rapamycinicus*. The putative negative regulatory genes (*rapY*, *rapR*, and *rapS*) are represented by *black*

mechanism of the pathway-specific regulators for rapamycin biosynthesis has only been partially revealed.

From a sequence analysis of the rapamycin biosynthetic gene cluster, five open reading frames (ORFs) have been identified, which may have potential regulatory functions [30] (Fig. 1b). RapG contains a helix-turn-helix (HTH) motif for DNA binding and has been identified to be a potential positive regulatory protein showing similarity to the SoxS and Rob proteins from *Escherichia coli* [30]. RapH also contains a DNA-binding motif and an ATP-binding site at the C-terminus and the N-terminus, respectively [30], and it also shows high sequence similarity (72 %) to the recently characterized positive regulatory protein FkbN from FK506 biosynthetic gene cluster [29]. Likewise, RapY has a HTH motif near the N-terminus and exhibits sequence similarity to repressors of antibiotic export in the actinorhodin and tetracenomycin biosynthetic gene clusters [30]. The RapR and RapS were suggested to be a two-component signal transduction system [30] such as CutR–CutS from *Streptomyces lividans* [5] and AfsQ1–AfsQ2 from *Streptomyces coelicolor* [42]. Therefore, it has been suggested that *rapH* and *rapG* function as positive regulatory genes, whereas *rapY* and *rapR–rapS* may be putative negative regulatory genes. Indeed, the positive regulatory roles of *rapH* and *rapG* in rapamycin biosynthesis have been demonstrated by overexpression, gene deletion, and complementation [24]. However, the functions of the putative negative regulators RapR, RapS, and RapY, and their interaction with biosynthetic enzymes and other regulatory proteins still remain unknown.

Here, we characterize the negative regulatory roles of RapY and the RapR–RapS two-component system in the rapamycin biosynthesis of *S. rapamycinicus* through overexpression, in-frame deletion, complementation, and transcriptional analysis of the rapamycin biosynthetic genes in the wild-type and mutant strains. In addition, comparative transcriptional analysis of wild-type and deletion mutants revealed that RapY represses the expression of the ABC-transporter gene RapX, which plays a critical role in enhanced rapamycin production. Furthermore, we have partially established the complex regulatory network involving five pathway-specific regulatory genes, which will form the foundation for a detailed understanding of the regulatory network that controls rapamycin biosynthesis.

Materials and methods

Bacterial strains, culture conditions, and genetic manipulation

All strains and plasmids used in this study are described in Table 1. *S. rapamycinicus* ATCC 29253 and its derivative recombinant strains were propagated on Medium I

Table 1 Strains and plasmids used in this study

Strain/plasmid	Characteristics[a]	Reference
Bacterial strains		
Escherichia coli		
DH5α	Plasmids construction and general subcloning, F$^-$ *recA lacZ*ΔM15	New England Biolabs
ET12567/pUZ8002	Non-methylating ET12567 containing non-transmissible RP4 derivative plasmid pUZ8002, Cml^r, Tet^r, Kan^r	[11]
Streptomyces		
S. rapamycinicus ATCC 29253	Wild-type rapamycin producing strain	[23]
WT/pSET152 (*ermEp**)	Wild-type stain with integrative plasmid pSET152 (*ermEp**)	This study
WT/pRAPY	Wild type with integrative plasmid pRAPY, Apra^r	This study
WT/pRAPR	Wild type with integrative plasmid pRAPR, Apra^r	This study
WT/pRAPS	Wild type with integrative plasmid pRAPS, Apra^r	This study
WT/pRAPX	Wild type with integrative plasmid pRAPX, Apra^r	This study
ΔrapY	Mutant of *S. rapamycinicus* ATCC29253 with an in-frame deletion of internal to *rapY*	This study
ΔrapS	Mutant of *S. rapamycinicus* ATCC29253 with an in-frame deletion of internal to *rapS*	This study
ΔrapY/pRAPY	ΔrapY with integrative plasmid pRAPY, Apra^r	This study
ΔrapS/pRAPS	ΔrapS with integrative plasmid pRAPS, Apra^r	This study
ΔrapS/pRAPX	ΔrapS with integrative plasmid pRAPX, Apra^r	This study
Plasmids		
pGEM T-Easy	*E. coli* vector for cloning PCR products, Amp^r	Promega
pSET152 (*ermEp**)	Integrative plasmid containing *ermEp**, *oriT*, *attP*, *ΦC31 int* and *aac(3)IV*	[3]
pKC1139	Temperature-sensitive *E. coli–Streptomyces* shuttle vector containing *oriT* and *aac(3)IV* for gene disruption	[3]
pRAPY	pSET152 (*ermEp**)-based integrative plasmid containing single copy of *rapY*, Apar^r	This study
pRAPR	pSET152 (*ermEp**)-based integrative plasmid containing single copy of *rapR*, Apar^r	This study
pRAPS	pSET152 (*ermEp**)-based integrative plasmid containing single copy of *rapS*, Apar^r	This study
pRAPX	pSET152 (*ermEp**)-based integrative plasmid containing single copy of *rapX*, Apar^r	This study
pΔRAPY	pKC1139 based deletion plasmid with in-frame deletion of 87 bp internal to *rapY*	This study
pΔRAPS	pKC1139 based deletion plasmid with in-frame deletion of 678 bp internal to *rapS*	This study

[a] *F*$^-$ does not carry the F plasmid, *recA1* for reduced occurrence of unwanted recombination in cloned DNA, *lacZΔM15* partial deletion of the *lacZ* gene that allows α complementation of the β-galactosidase gene, *Cml*^{r} chloramphenicol resistance, *Tet*^{r} tetracycline resistance, *Kan*^{r} kanamycin resistance, *Apra*^{r} apramycin resistance, *Amp*^{r} ampicillin resistance, *oriT* origin of transfer, *attP* plasmid *ΦC31*attachment site, *ΦC31 int* integrase gene, *aac(3)IV* apramycin resistance gene, *ermEp** mutated constitutive promoter

agar plates [11] at 28 °C. The rapamycin strains were cultured in tryptic soy broth (TSB; Difco with 1.5 % glucose and 0.01 % $FeSO_4$ $7H_2O$ adjusted pH 6.0) liquid medium with shaking at 28 °C, 250 rpm [10]. *E. coli* DH5α was used for the propagation of plasmids using standard protocol [21]. pGEM T-Easy vector (Promega) and Litmus28 (New England Biolabs) were used for subcloning. Antibiotics were selectively used at the following concentrations: for *S. rapamycinicus*, 25 μg ml^{-1} apramycin; for *E. coli*, 25 μg ml^{-1} apramycin, 50 μg ml^{-1} chloramphenicol, and 50 μg ml^{-1} kanamycin. To avoid the methyl-specific restriction, conjugation of plasmids into *S. rapamycinicus* was performed using *E. coli* ET12567 carrying the conjugation-facilitating plasmid pUZ8002 [21].

Construction of mutant strains overexpressing *rapY*, *rapR*, and *rapS*

The integrative *E. coli–Streptomyces* vector pSET152 [3] with the strong constitutive *ermE** promoter (*ermEp**) [39] was used to overexpress genes in *S. rapamycinicus*. For the overexpression of putative negative regulatory genes, *rapY*, *rapR*, and *rapS* genes were amplified from the genomic DNA of *S. rapamycinicus* by PCR using the following primers: *rapY* (Forward, 5′-**TTAATTAA**ACTAGTCACCGTTCCGCCTTACCTTCGAAG-3′; reverse, 5′-TCTAGAGGTCAGCGGACGGCCGCTC-3′), *rapR* (Forward, 5′-**TTAATTAA**ACTAGTGGGCCGCCACATCCTTATCCG-3′; reverse, 5′-**TCTAGA**GGGTTCAGTCGCACAGCCGGTAAC-3′) and *rapS*

(Forward, 5′-**TTAATTAA**ACTAGTCGGCTGCGCGGCAAACTC-3′; reverse, 5′-**TCTAGA**CTTCAACGGCGGCAAGGGAAG-3′), the *Pac*I and *Xba*I restriction sites are in bold. The PCR product of the three genes (*rapY*, *rapR*, and *rapS*) were separately cloned into the pGEM T-Easy vector and sequenced. They were then digested by *Pac*I/*Xba*I and ligated into the pSET152 derivative containing *ermEp**, generating pRAPY, pRAPR, and pRAPS, respectively. Each of these plasmids was then introduced into *S. rapamycinicus* by conjugation from *E. coli* ET12567/pUZ8002, as described elsewhere [18], yielding the mutant strains WT/pRAPY, WT/pRAPR, and WT/pRAPS, respectively. Their genotypes were verified by Southern blot hybridization. These pSET152-derived vectors were also used for complementation experiments.

Construction of the *rapY* and *rapS* deletion mutant strains

The mutants were constructed by in-frame deletion of the conserved regions of the target genes using the temperature-sensitive vector pKC1139 [3]. The deletion cassette was designed based on the original rapamycin cluster sequence (GenBank X86780). The *rapY* deletion plasmid, pΔRAPY, was constructed by cloning two 1-kb fragments homologous to the *rapY*-flanking region and generated using the following primers: a *HindIII*–*Xba*I fragment containing the 3′-region of *rapZ* and 5′-region of *rapY*, forward, 5′-**AAGCTT**TACAGCACGCACGCGGCTACG-3′ and reverse, 5′-**TCTAGA**CGACATCCGCGCGTACCCGG-3′ (Nucleotides in bold show the restriction sites); an *Xba*I–*EcoR*I fragment containing the 3′-region of *rapY* and 5′-region of *rapX*, forward, **TCTAGA**ATCCGCGGCAAGGTCACCGACA-3′ and reverse 5′-**GAATTC**GCTCTCCCTCGCCAGCGGTCT-3′ (Nucleotides in bold show the restriction sites). The PCR-generated DNA fragments were cloned separately in a pGEM T-Easy vector and then sequenced. After digestion with the appropriate restriction enzymes, the fragments were cloned into pKC1139 yielding pΔRAPY.

The *rapS* deletion plasmid, pΔRAPS, was obtained by combining two 1-kb *rapS*-flanking DNA fragments. These fragments were prepared by PCR using the following primers: a *HindIII*–*Xba*I fragment containing the 3′-region of *rapR* and 5′-region of *rapS*, forward, 5′-**AAGCTT**GACCTGCTCGCACCGTGCC-3′ and reverse 5′-**TCTAGA**TGTGAGATTCGGCCGGGTGGG-3′; a *Xba*I–*EcoR*I fragment containing the 3′-region of *rapS* and 5′-region of *rapU*, forward 5′-**TCTAGA**CGCAATGCCATCCAGTACAACCACCC-3′ and reverse 5′-**GAATTC**CGCGGTCGCAACACGGTGG-3′ (Nucleotides in bold show the restriction sites). The PCR-generated DNA fragments were cloned separately in the pGEM T-Easy vector and then sequenced. After digestion with the appropriate restriction enzymes, the fragments were cloned into pKC1139 yielding pΔRAPS.

These deletion plasmids were then introduced into *S. rapamycinicus* as described above. The desired double-crossover mutants, namely ΔrapY and ΔrapS, were selected on the basis of their phenotype of apramycin sensitivity as described previously [29] and confirmed selectively by Southern blot analysis.

Construction of the mutant strain overexpressing *rapX*

A 707-bp PCR fragment carrying the native ribosome-binding site of *rapX* was amplified with primers *rapX*-forward: 5′-**TTAATTAA**ACTAGTCCGACCCCATCGACAAGATC-3′ and *rapX*-reverse: 5′-**TCTAGA**GCGACGATGAGTATCTGGAGAC-3′ (the restriction sites are in bold) by PCR using genomic DNA of *S. rapamycinicus* as the template, cloned into a pGEM T-Easy vector, and were then sequenced. The sequence-verified fragments were digested with *Pac*I/*Xba*I, and then placed into the same sites of pSET152 yielding pRAPX. This plasmid was then introduced into the wild-type *S. rapamycinicus* and ΔrapS strains, and verified by Southern blot hybridization, thus generating the WT/pRAPX and ΔrapS/pRAPX mutant strains, respectively.

Analysis of cell growth and rapamycin production

Cell growth was measured by collecting and weighing mycelia as described previously [29]. *S. rapamycinicus* and the mutant strains were cultivated on a TSB medium. At first, seed cultures were cultivated at 28 °C in 50-ml tubes containing 5 ml of TSB medium for 2 days, and then transferred to 10 μl of fresh TSB medium and shaken at 300 rpm for 2–6 days at 28 °C, as described previously [18]. To analyze the rapamycin in the culture broth, the cultures were separated from the mycelia, extracted with the same volume of ethyl acetate, and filtered. A portion of this solution was then subjected to HPLC analysis as described previously [18]. Authentic rapamycin (MP Biomedicals, CA, USA) standards were used to generate a calibration curve. Five separate cultivations and extractions were carried out to obtain an average production yield of rapamycin.

RNA preparation and gene expression analysis by RT-PCR

Total RNAs were isolated from wild-type and mutant strains using an RNeasy Mini spin column (Qiagen) as described previously [18]. RT-PCR was carried out using a Qiagen OneStep RT-PCR kit according to the method of Jung et al. (2011) with some modifications. A total of 100 ng DNase I-treated RNA was used as a template. The RT-PCR conditions were as follows: cDNA synthesis, 50 °C for 30 min followed by 95 °C for 15 min;

amplification, 33–40 cycles of 94 °C for 1 min (*rapB*, *rapP*, *rapC*: 33 cycles; *rapX*, *rapW*, *rapN*, *rapM*, *rapL*, *rapI*, *rapH*, *rapG*: 35 cycles; *rapA*, *rapQ*: 36 cycles; *rapK*, *rapJ*: 37 cycles; *rapY*, *rapV*, *rapR*, *rapS*, *rapU*, *rapT*: 40 cycles), 55 °C for 1 min, and 72 °C for 45 s. The cycle number for each gene was optimized to obtain enough visibility of the RT-PCR band and ensure that amplification was in the linear range and the results were semi-quantitative. Primers (Tm value 50–60 °C; 19–21 mer) were designed by software Primer3 [37] to generate PCR products approximately 500 bp except for the *rapY* gene (440 bp) (Table S1 in the supplemental material). With each set of primers, negative controls were carried out with *Taq* DNA polymerase (New England Biolabs) in the absence of RT reaction to confirm that the amplified products were not derived from chromosomal DNA. The 16 s rRNA was used as a positive control, as it is expressed at a constant level (50 °C for 30 min followed by 95 °C for 15 min; amplification, 26 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 45 s.). The RT-PCR experiments were done in duplicate using RNA samples from two independent cultures.

Results

Sequence analysis of the putative negative regulatory genes in the rapamycin gene cluster

In the rapamycin biosynthetic gene cluster, three putative negative regulatory genes *rapY*, *rapS*, and *rapR* were previously identified by sequence analysis [30, 40] (Fig. 1b). BLAST analysis of the deduced product of *rapY* (204 amino acid residues) revealed its identity/similarity to the known TetR family proteins: 34/52 % with TetR from *E. coli* [2], 25/49 % with AcrR from *E. coli* [25], 27/45 % with PhlF from *Pseudomonas fluorescens* F113 [7], 28/48 % with ArpA from *Streptomyces griseus* [12], 24/38 % with TcmR from *Streptomyces glaucescens* [15]. Members of this TetR family are characterized by an HTH motif for DNA binding at the N-terminal region, and many of them have been found in the polyketide biosynthetic gene clusters of *Streptomyces* [36]. For instance, TetR binds to the intergenic region between *tetR* and *tetA* containing two identical operators and thus prevents transcription of the tetracycline resistance determinant antiporter TetA [14]. Likewise, PhlF, which binds to the intergenic region between *phlA* and *phlF* genes, was characterized as a repressor specific for the biosynthesis of phloroglucinol [7]. In addition, AcrR, another known TetR family member, was characterized as a specific secondary modulator to downregulate *acrAB* encoding a multidrug efflux pump in *E. coli* [25]. The deduced products of *rapR* (220 amino acid residues) and *rapS* (399 amino acid residues) showed sequence similarity to two-component signal transduction systems which are composed of a cytoplasmic response regulator (RapR) and a membrane-integrated histidine kinase (RapS). BLAST analysis of the response regulator RapR shows 53/69 and 35/52 % sequence identity/similarity with CutR from *S. lividans* [5] and AfsQ1 from *S. coelicolor* [42], respectively, whose genes are members of the PhoB/OmpR subfamily of response regulators [44]. The histidine kinase sensor protein RapS has an ATPase domain at the C-terminal region and kinase domain in the middle, it shows 28/40 and 32/47 % sequence identity/similarity with CutS from *S. lividans* [5] and AfsQ2 from *S. coelicolor* [42], respectively. Although a number of two-component systems exist in *Streptomyces* sp., only a few systems which affect antibiotic production have been characterized, namely AbsA1–AbsA2 in *S. coelicolor* [26, 41] and CutR–CutS in *S. lividans* [5]. AbsA1 (sensor kinase) controls the phosphorylation state of AbsA2 (response regulator), and the phosphorylated AbsA2 interacts with the pathway-specific activator genes of each of the three antibiotic biosynthetic gene cluster (*actII-ORF4*, *cdaR*, and *redZ*) to reduce their expression in *S. coelicolor* [26]. This detailed sequence analysis suggests that RapY, RapR, and RapS act as negative regulators in rapamycin biosynthesis.

Rapamycin biosynthetic gene clusters were also reported in *Streptomyces iranensis* [16] and *Actinoplanes* sp. N902–109 [17] as well as *S. rapamycinicus*. Comparative sequence analysis revealed that the five regulatory genes (RapH, RapG, RapR, RapS, and RapY) are well conserved in *S. iranensis* and share >90 % similarity with those from *S. rapamycinicus* (Fig. S1 and Table S2 in the supplemental material). *Actinoplanes* sp. N902–109 only contains a RapH homologue showing relatively low similarity (70 %) in its rapamycin gene cluster (Fig. S1 and Table S2 in the supplemental material). This suggests that *S. rapamycinicus* and *S. iranensis* would share similar regulatory mechanism for rapamycin biosynthesis, while *Actinoplanes* sp. N902–109 may use relatively simpler regulatory strategy than *Streptomyces*.

Overexpression of *rapY*, *rapR*, and *rapS* genes in *S. rapamycinicus*

To investigate the functions of *rapY*, *rapR*, and *rapS* genes in the regulation of rapamycin production in *S. rapamycinicus*, the genes were introduced and overexpressed in the *S. rapamycinicus* wild-type strain. The ΦC31-based integrating plasmids pRAPY, pRAPR, and pRAPS which individually contain *rapY*, *rapR,* and *rapS* regulatory genes under the control of a strong constitutive *ermE** promoter were introduced into the wild-type *S. rapamycinicus* strain generating WT/pRAPY, WT/pRAPR, and WT/pRAPS, respectively. All overexpression mutants were confirmed

Table 2 Rapamycin titers from *S. rapamycinicus* ATCC 29253 wild-type and mutant strain

Strain	WT (wild type)	WT/pRAPY	WT/pRAPR	WT/pRAPS	ΔrapY/pRAPY	ΔrapS/pRAPS	ΔrapY/pRAPS	ΔrapS/pRAPY
Rapamycin (mg/l) ± standard deviation	7.35 ± 0.91	2.52 ± 0.48	1.95 ± 0.15	2.08 ± 0.42	7.24 ± 0.39	7.56 ± 0.63	12.06 ± 0.87	17.28 ± 0.93

by Southern blot hybridization (Fig. S2 in the supplemental material). As a control, pSET152 (*ermEp**) was introduced into the wild-type strain. There were no differences in rapamycin production and growth or morphology between the exconjugants with pSET152 and the wild-type strain when grown on TSB medium (data not shown).

HPLC analysis of rapamycin production of all overexpression mutants grown on TSB medium for 5 days showed that the rapamycin titer decreased significantly compared to the wild-type strain (Table 2). Overexpression of *rapY* reduced rapamycin production by approximately 66 % to 2.5 mg l^{-1}. In the case of the WT/pRAPS strain, the production level of rapamycin (2.08 mg l^{-1}) was reduced by 71.5 %. Likewise, when *rapR* was overexpressed in the wild-type strain, rapamycin production decreased a similar amount. These results indicate that RapY and the RapR–RapS two-component system play significant roles as negative regulators in rapamycin biosynthesis.

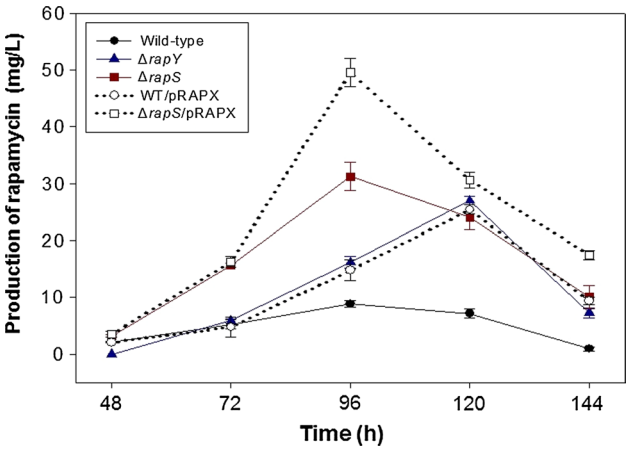

Fig. 2 Time course of rapamycin production for wild-type (*filled circle*), ΔrapY (*filled triangle*), ΔrapS (*filled square*), WT/pRAPX (*open circle*), and ΔrapS/pRAPX (*open square*) strains

Inactivation of *rapY* and *rapS* by in-frame deletion

To confirm the negative regulatory roles of RapY and the RapR–RapS two-component system in rapamycin biosynthesis, the *rapY* gene and one of the two-component system genes, *rapS*, were inactivated by an in-frame deletion to avoid any polar effect. Because it can be expected that inactivation of *rapR* or *rapR–rapS* would have similar effects on the rapamycin biosynthesis as *rapS* inactivation as observed in DraR–DraK two-component system in *S. coelicolor* [48] and the efficiency of homologous recombination in the *S. rapamycinicus* is very low, we aimed to inactivate only one gene of the two-component system. The *rapY* deletion plasmid (pΔRAPY) was designed to delete a 29-amino acid region which contains an HTH motif, and the *rapS* (pΔRAPS) deletion plasmid was prepared to delete 226 amino acids including the kinase domain and a part of ATPase domain (Table S3 in the supplemental material). All in-frame deletion mutants that were generated using pKC1139 were confirmed by Southern blot hybridization (Figs. S3 and S4 in the supplemental material). Compared with the wild-type strain, no change in the sporulation and growth behavior was observed in all deletion mutants (data not shown).

Rapamycin production in wild-type, ΔrapY, and ΔrapS strains was monitored throughout a 144-h (6-day) incubation period. The rapamycin production time courses in the wild-type and ΔrapS strains were similar. Maximal levels of rapamycin were observed at 96 h and then decreased in both strains. In the wild-type strain, rapamycin was detectable at 72 h of cultivation, gradually increased until 96 h, and then decreased. However, the rapamycin production level of the ΔrapS strain increased notably between 48 and 96 h by approximately 4.6-fold (33.9 mg l^{-1}) compared to the wild-type strain. On the other hand, in the ΔrapY strain, rapamycin production increased steadily over 120 h up to 26.7 mg l^{-1}, which is approximately 3.7 times higher than the maximum titer of the wild-type strain (7.3 mg l^{-1}) (Fig. 2). These results confirmed that *rapY* and *rapS* (and/or *rapR*) genes play key roles as negative regulators of rapamycin biosynthesis.

To confirm that the increased production of rapamycin was solely due to the disruption of *rapY* gene, complementation experiments in ΔrapY were carried out by reintroducing *rapY*. Plasmid pRAPY was transferred from *E. coli* ET12567/pUZ8002 to the ΔrapY, generating ΔrapY/pRAPY (Fig. S2 in the supplemental material). This self-complementation lowered rapamycin production to levels comparable to those observed in the wild-type strain (Table 2), demonstrating that the absence of *rapY* is the sole reason for increased production of rapamycin in the ΔrapY strain. Similarly, self-complementation

of ΔrapS by pRAPS lowered the rapamycin production to wild-type strain levels (Table 2), thus showing that the deletion of *rapS* is responsible for the enhanced production of rapamycin.

Interestingly, in the cross-complementation experiments, ΔrapS/pRAPY and ΔrapY/pRAPS produced approximately 17.3 and 12.1 mg l^{-1} of rapamycin, respectively, suggesting that the RapR–RapS two-component system may occupy a higher hierarchical position than RapY in the regulatory network and may negatively regulate rapamycin biosynthesis through another independent regulator(s) from RapY.

Transcription analysis of the rapamycin biosynthetic gene cluster in the wild-type, ΔrapY, and ΔrapS strains

To validate the negative regulatory role of *rapY* and *rapS*, transcription analysis of the rapamycin biosynthetic gene cluster was conducted by semi-quantitative RT-PCR in the wild-type, ΔrapY, and ΔrapS strains but omitting five genes (Fig. 3). The nucleotide sequence of *rapO* encoding a putative ferredoxin is too short to perform RT-PCR, and it is not likely that the *rapF*, *rapE*, and *rapD* genes located at the right end of the cluster and *rapZ* gene at the left end of the cluster are involved in rapamycin biosynthesis because no obvious function can be suggested for them [11, 40]. Total RNA was isolated from the wild-type, ΔrapY, and ΔrapS strains after 24, 48, and 60 h of cultivation, and used as the template for gene expression analysis by RT-PCR.

For the PKS, NRPS genes (*rapB*, *rapA*, *rapC*, and *rapP*) and 27-*O*-methyltransferase gene (*rapQ*) [22, 30, 40], transcripts were detected exclusively in the ΔrapS strain at 24 h. Whereas, in the wild-type and ΔrapY strains the transcripts of those genes were detected after 48 h. The levels of transcription of those genes were evidently higher in the ΔrapS and ΔrapY stains compared to the wild-type strain at 48 and 60 h. This observation is consistent with the time course rapamycin production (Fig. 2). While rapamycin biosynthesis increased relatively slowly during the first 96 h of cultivation in the wild-type and ΔrapY strains, rapamycin production in the ΔrapS strain increased more rapidly during the same culture period. Interestingly, the expression level of *rapB* was similar in the ΔrapY and ΔrapS strains at 48 and 60 h, whereas the transcription level of *rapA* was observably higher in the ΔrapY than that in the ΔrapS strain at 60 h. This observation is consistent with the observed prolonged rapamycin production times of up to 120 h in the ΔrapY strain. In the ΔrapS strain, rapamycin production increased relatively rapidly up to 96 h but began to decrease after 96 h (Fig. 2). In the wild-type strain, relatively low expression of the *rapA* gene compared to *rapB* was also observed throughout the incubation period, which is in agreement with the previous report [18] and suggests the possibility that *rapA* and *rapB* genes are transcribed independently although the transcriptional direction of those genes is the same. In the cases of *rapK*, *rapJ*, and *rapI* transcribed in the same direction, their transcripts were only present in the ΔrapS strain at 24 h, which is also consistent with

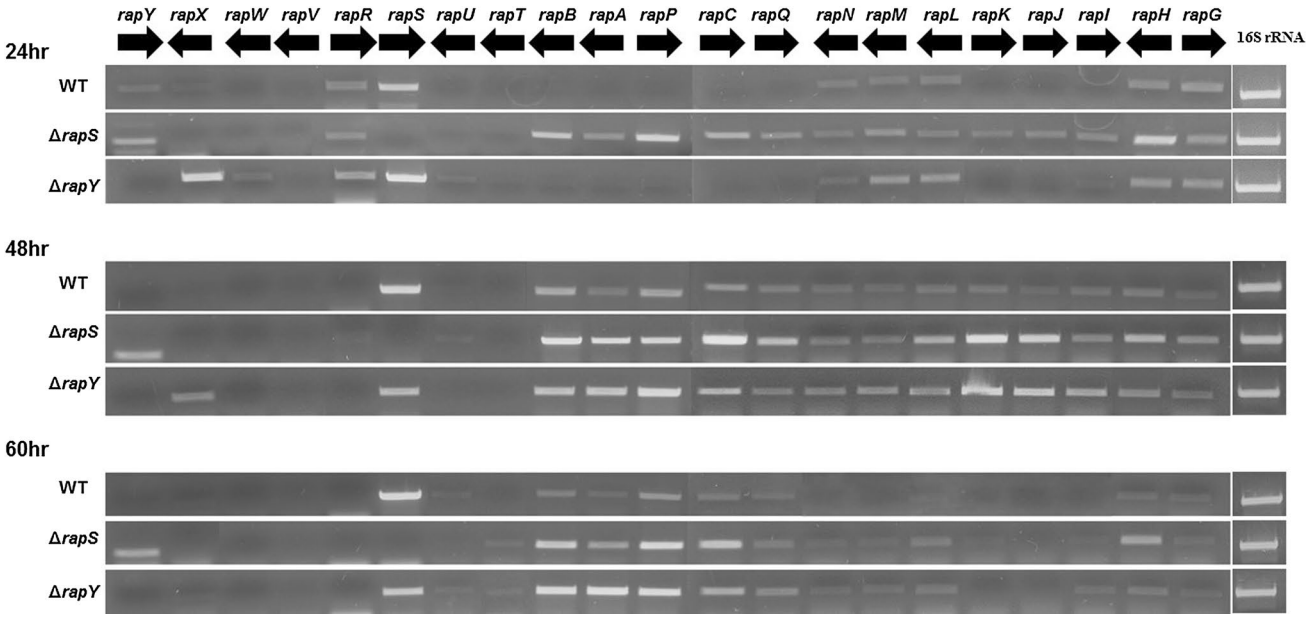

Fig. 3 Gene expression analysis of the rapamycin cluster by RT-PCR. Total RNAs were isolated from a culture of wild-type, ΔrapY, and ΔrapS strains at the time indicated. 16S rRNA gene was used as a control

the early rapamycin production in the ΔrapS strain; *rapK* encodes the chorismatase for the starter unit biosynthesis [1]; *rapJ* encodes a cytochrome P450 monooxygenase for the introduction of a keto group at C9 [11]; and *rapI* encodes the methyltransferase for *O*-methylation at C39 [11]. At 48 h, in the ΔrapY and ΔrapS strains, the expression levels of *rapK*, *rapJ*, and *rapI* genes were observably higher than those in the wild-type strain. Then, transcripts of *rapK*, *rapJ*, and *rapI* genes disappeared in all three strains at 60 h. In contrast, there was no significant difference in the expression patterns of *rapN* (cytochrome P450 monooxygenase gene for hydroxylation at C27; [11]), *rapM* (methyltransferase gene for *O*-methylation at C16; [11]), and *rapL* (lysine cyclodeaminase gene; [11, 20]), which are transcribed in the opposite directions from *rapK–rapJ–rapI* genes, observed among three strains at 24 and 46 h. Transcripts of these genes were slightly visible at 24 h, maintained up to 46 h, and then diminished at 60 h in all strains, although faint bands were observed in the ΔrapY and ΔrapS strains. There was no significant difference in the transcription patterns of the *rapR* gene encoding a response regulator of the two-component system in all three strains throughout the incubation period, suggesting that the presence or absence of other negative regulatory genes *rapY* and *rapS* did not observably affect the expression of *rapR*. Similarly, the expression levels of *rapS* in the wild-type and ΔrapY strains were similar during the observed period, implying that the expression of *rapS* was not affected by *rapY*. However, the transcription of *rapY* in the absence of *rapS* was dramatically increased compared to the wild-type strain at all times, suggesting that RapS (and/or its partner response regulator RapR) negatively controls the expression of *rapY*. The transcripts of the positive regulatory gene *rapH* were clearly observed at 24 h and maintained at 48 h, but almost disappeared at 60 h in the wild-type and the ΔrapY strains. However, in the ΔrapS strain compared with the other strains, a relatively strong band was observed at 24 and 48 h and still maintained at 60 h, showing that RapS (and/or RapR) affects the expression of *rapH* negatively. The expression of another positive regulator gene *rapG* did not seem to be affected by the absence of the *rapS* and *rapY* genes. The transcripts of genes with unknown functions (*rapU*, *rapT*, *rapV*, and *rapW*) [30] were hardly visible in all three strains at all times. However, although the transcriptional direction of *rapV*, *rapW*, and *rapX* genes was the same, the *rapX* gene which encodes a putative ABC-transporter was expressed uniquely at 24 h, maintained up to 48 h, and disappeared at 60 h in the ΔrapY strain, suggesting that the expression of *rapX* is negatively controlled by RapY. For detection of the transcripts of *rapU*, *rapT*, *rapV*, and *rapW* genes, we tested several primer pairs and changed PCR cycles, but did not obtain visible RT-PCR products.

Overexpression of *rapX* gene in the ΔrapS strain

Interestingly, the improved productivity of rapamycin in the ΔrapY strain seems to be closely related to the high expression levels of the *rapX* gene, which encodes a putative ABC-transporter as well as other genes in the rapamycin cluster (Fig. 3). To investigate the direct effect of overexpression of *rapX* on rapamycin production, pRAPX, which contains *rapX* genes under the control of a strong constitutive *ermE** promoter, was introduced into the *S. rapamycinicus* wild-type strain, generating WT/pRAPX (Fig. S2 in the supplemental material). HPLC analysis of the culture broth extract of the WT/pRAPX strain grown for 120 h revealed a considerable increase in the rapamycin titer, approximately 3.5-fold (25.8 mg l^{-1}) higher than the level present in the wild-type strain (Fig. 2). This suggests that the increased expression of *rapX* gene in the ΔrapY strain is a major factor contributing to the enhanced rapamycin production.

Inactivation of both negative regulatory mechanisms, the RapS–RapR two-component system and RapY, could have synergistic effects to increase rapamycin production. However, the rates of homologous recombination in the *S. rapamycinicus* are extraordinarily low compared to other *Streptomyces* species even when longer flanking sequences are used (data not shown). Therefore, instead of double inactivation, we tried to exploit the positive role of RapX, the expression of which is negatively controlled by RapY but does not seem to be influenced by the two-component system RapS–RapR directly, in the absence of *rapS*, one of the two-component system genes. The *rapX* gene was overexpressed in the ΔrapS strain (Fig. S2 in the supplemental material), and as a result, the production of rapamycin by the ΔrapS/pRAPX strain was further increased up to 49 mg l^{-1} after 96 h (4 days) cultivation, which is approximately a 6.7- and 1.4-fold enhancement compared to the wild-type and ΔrapS strains, respectively (Fig. 2).

Discussion

Many secondary metabolites are produced by polyketide biosynthetic pathways that are regulated by one or more regulatory genes [31]. These complex regulatory systems can have more than one regulatory gene, such as the tylosin cluster from *Streptomyces fradiae* [6] and the daunorubicin cluster from *Streptomyces peucetius* [32, 33, 46]. The rapamycin biosynthesis of *S. rapamycinicus* is one of the most complicatedly regulated pathways involving five regulatory genes (*rapH*, *rapG*, *rapY*, *rapR*, and *rapS*). It has been previously shown that *rapH* and *rapG* genes are positive regulatory genes, and the products of the two genes were shown to interdependently regulate the rapamycin biosynthetic

gene cluster [24]. While complementation of *S. rapamycinicus* ΔHG (the *rapH* and *rapG* deletion mutant) with *rapH* alone did not restore rapamycin production, complementation with *rapG* alone partially restored a low level of production, indicating that *rapG* may directly regulate the initiation of rapamycin biosynthesis. Only when both *rapG* and *rapH* were expressed with the native promoters was wild-type yield restored [24]. However, our transcriptional analysis suggests the possibility that these proteins independently regulate the rapamycin gene cluster. As described above, there was no observable difference in the expression level of *rapG* in the ΔrapS and ΔrapY strains, whereas a higher and prolonged transcription of *rapH* was observed in the ΔrapS strain compared to the wild-type and ΔrapY strains (Fig. 3). This result suggests the possibility that the RapS (and/or RapR) negatively regulate the expression of *rapH* gene, and in turn, the rapamycin biosynthetic gene cluster is positively controlled by RapH independently of RapG.

The other negative regulator, RapY, affects the expression of most of the rapamycin biosynthetic genes (except for *rapL*, *rapM*, and *rapN*), including the *rapX* gene, which encodes the putative ABC-transporter and can prevent self-poisoning by exporting the secondary metabolite from inside cells [27]. Likewise, in the case of AcrR of *E. coli*, which is a TetR family regulator, it has been reported that AcrR can repress the *acrAB* gene which encodes a multidrug efflux pump, and thus, prevent the unwanted overexpression of *acrAB* [25]. Elevated yields of the secondary metabolite by overexpression of an ABC-transporter gene has also been reported: overexpression of *avtAB* in *Streptomyces avermitilis* NRRL 8165 strain resulted in a twofold increase in avermectin production [35] and the amplification of *cefT* increased yield of cephalosporin in *Acremonium chrysogenum* C10 strain [47]. As expected, the introduction of an additional copy of the *rapX* gene into the wild-type strain led to enhanced rapamycin production, suggesting that RapX is an ABC-transporter system which can be exploited for the improved production of rapamycin biosynthesis. Presence of the RapX homologue in *S. iranensis* (94 % identity) (Fig. S1 and Table S2 in the supplemental material) also suggests that this simple but effective strategy can be applied in other *Streptomyces* to increase rapamycin production.

It is also noteworthy that the relative orientation of *rapY* and its proposed target *rapX* is unusual. Generally, TetR-like regulators are positioned in a divergent orientation relative to their targets such that they share an intergenic region [14]. However, in the rapamycin cluster they appear to have a convergent orientation.

There are several points which currently cannot be clearly explained. First, it is at present unclear how the rapamycin production level of the ΔrapS strain was 4.6-fold higher than the wild-type strain although transcription of the negative regulatory gene *rapY* in the absence of *rapS* was dramatically increased compared to the wild-type strain at all times. These observations could possibly be explained if the repressive effects of RapY can be overcome by the presence of rapamycin similar to how tetracycline binds TetR resulting in its release from the binding site [14]. Second, discrepancies in timing between changes in gene expression pattern and rapamycin production have been observed. For example, maximal rapamycin production was observed at 120 h in both the ΔrapY mutant and WT/pRapX strain, and the maximal yield was similar between the two strains (Fig. 2), which are consistent with the notion that RapY is the negative regulator of *rapX*. Therefore, *rapX* is expected to be overexpressed in the ΔrapY mutant at a later stage of time. Contrary to this speculation, *rapX* transcription was only detected at earlier stages in the ΔrapY mutant and disappeared at 60 h. Although we cannot currently explain these observations clearly, it has often been observed that the distinct changes in the transcription patterns precede the changes in the biosynthesis of polyketides such as tylosin and FK506 [28, 45]. Thirdly, although it seems that *rapS* and *rapR* constitute an operon, the transcription rates of *rapS* are higher than that of *rapR* in the wild-type and ΔrapY strains. Lastly, although RapS seems to negatively affect *rapH* transcription through RapR, the wild-type strain showed low expression of *rapH* at 48 and 60 h where *rapR* was not apparently transcribed. These observations were probably, at least in part, due to the poor primer efficiency in RT-PCR of *rapR* even though we tried to optimize the primer sequences and condition for RT-PCR. The apparently low expression of *rapA* compared to *rapB* may also be due to the poor primer efficiency and it would be possible that *rapA* and *rapB* are transcribed together.

In conclusion, we have characterized the roles of *rapY* and *rapS* in the negative regulation of rapamycin biosynthesis in *S. rapamycinicus*. Clearly, the two-component system RapR–RapS and RapY negatively regulate most of the rapamycin biosynthetic genes, but probably through different mechanisms. Interestingly, RapS (and/or RapR) and RapY negatively control *rapH* and *rapX*, respectively, and exert a major influence on rapamycin biosynthesis. The cross-complementation and transcription analysis together suggest that the two-component system occupies a higher hierarchical position than RapY in the regulatory network and negatively controls RapH and RapY in an independent manner. Therefore, in ΔrapS/pRAPY strain, overexpression of *rapY* decreases the expression of *rapX*, which is involved in rapamycin export, and the biosynthetic genes. However, the absence of *rapS* increases the expression of the positive regulatory gene *rapH*, resulting in the improved production of rapamycin up to approximately 17 mg l^{-1}. Moreover, it

seems that the repressive effects of RapY can be overcome by the presence of rapamycin as discussed above. On the other hand, in ΔrapY/pRAPS strain, although the absence of *rapY* increases the expression of *rapX* and the biosynthetic genes, *rapS* overexpression decreases the expression of *rapH,* resulting in the moderately improved production (12 mg l^{-1}) compared to the wild type. Taken together, the results presented herein provide a valuable initial understanding of the complex regulatory network involving five regulators for the biosynthesis of rapamycin. However, the regulatory mechanisms need to be examined in more detail with additional experiments, such as electrophoretic mobility shift assay analysis or quantitative real-time RT-PCR. In addition, the significantly improved production of rapamycin in the ΔrapS/pRAPX strain demonstrates the potential of manipulating regulatory/transport genes for strain development. Overexpression of the transporter gene *rapX*, which is upregulated by the absence of one negative regulatory gene *rapY*, in the background of another regulatory gene *rapS* deletion enhanced rapamycin production by 6.7- and 1.4-fold compared to the wild-type and ΔrapS strains, respectively. Application of this strategy to industrial strains may allow for the development of more improved strains.

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