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NOTES

Thiopeptide Non-producing *Streptomyces* Species Carry the *tipA* Gene: A Clue to Its Function

BONG-SIK YUN[a,b], TOMOMI HIDAKA[a], TOMOHISA KUZUYAMA[a] and HARUO SETO[a,†,*]

[a] Institute of Molecular and Cellular Biosciences, The University of Tokyo, Bunkyo-ku, Tokyo 113-0032, Japan
[b] Korea Research Institute of Bioscience and Biotechnology, P.O. Box 115, Yusong, Taejon 305-600, Korea

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Thiopeptide antibiotics are distinguished by structural features containing thiazole(s) and/or oxazole(s) with highly modified amino acids in mono-, di- or tri-cyclic peptide ring(s). Several thiopeptides are known to induce the biosynthesis of many proteins of unknown function in *Streptomyces lividans*[1]. Two of these proteins, TipAL and TipAS, were characterized and their corresponding genes containing the promoter region (*ptipA*) were cloned and sequenced[1]. TipAL is a regulatory protein which autogenously activates transcription of its own promoter after forming an irreversible complex with thiostrepton or other related thiopeptide antibiotics[2], and this transcriptional activation of *ptipA* by TipAL results in the overexpression of TipAS, an in-frame translation product of the *tipAL* gene[2,3]. TipAS also forms a complex with thiopeptide compounds but does not activate transcription of its promoter *ptipA*. These proteins require the cyclic core and dehydroalanine side chains in the structures of thiopeptides for recognition and transcriptional activation[3]. Although disruption of the *tipA* gene weakly increased antibiotic susceptibility in *S. lividans*, its functions are still under investigation.

The inducible promoter *ptipA* has been cloned into a series of vectors to allow regulated expression of genes in *Streptomyces* and those vectors have been employed to give a very sensitive and specific microbiological disc assay to screen for compounds inducing their transcription. Our screening to find *tipA* promoter-inducing compounds of microbial origin has resulted in the isolation of only the thiopeptide class of antibiotics[4~8]. This finding led us to hypothesize that the *tipA* gene may be related to the production of thiopeptide antibiotic or the resistance specific against thiopeptides in *Streptomyces*. In this paper, we investigated the distribution of the *tipA* gene in several different *Streptomyces* species to study the relationship between thiopeptide production and the *tipA* gene as a preliminary step to understand the function of the *tipA* gene.

Nine thiopeptide producing strains were selected as producers of *tipA* promoter-inducing principles, and nine thiopeptide nonproducing *Streptomyces* were randomly selected for this experiment (Table 1). Thiopeptide production by these strains was confirmed by resistance to thiostrepton, a representative thiopeptide antibiotic, and Southern blot analysis of the chromosomal DNAs isolated from these *Streptomyces* species with the thiostrepton resistant gene (*tsr*)[9] as a probe. All the thiopeptide producing strains were resistant at concentrations up to 500 μg/ml thiostrepton, while nonproducing strains were sensitive with different MIC values of 0.16 to 4 μg/ml, as shown in Table 1. This result showed that all the thiopeptide producing strains possess the *tsr* gene. This implication was supported by the Southern blot analysis using the *tsr* gene. The cultures of *Streptomyces* species were grown at 30°C for one day in 10 ml of GPY medium (glucose 1.0%, polypepton 0.5%, yeast extract 0.4%, $MgSO_4 \cdot 7H_2O$ 0.05%, K_2HPO_4 0.1%). The mycelia were washed with 25% sucrose and then disrupted by lysozyme. The chromosomal DNAs from each culture were obtained through the procedures of phenol-chloroform extraction and ethanol precipitation and were digested by *Bam*HI for the Southern blot analysis. Two primers for the *tsr* gene corresponding to the nucleotides 391~402 (12-mer, CCCGCGGTGCAG) and 1117~1131 (15-mer, ATCGCGCTGCACGAG) were synthesized and used to prepare a *tsr* probe by PCR. All thiopeptide producing strains hybridized with this DNA probe, while the nonproducing strains did not as shown in Fig. 1. This result was confirmed by repeated hybridization.

We then investigated the distribution of the *tipA* gene

† Present address: Faculty of Applied Bio-Science, Tokyo University of Agriculture, 1–1–1 Sakuragaoka, Setagaya-ku, Tokyo, Japan.
* Corresponding: haseto@nodai.ac.jp

Table 1. Thiopeptide producing and nonproducing *Streptomyces* species used in this experiment.

Strain	Produced compound	MIC (μg/ml)[a]	Lane No.[b]	*Tsr* probing[c]	*TipA* probing[c]	*TipA* PCR[d]
Streptomyces sp. DW76	Promothiocins	> 500	1	+	-	nt
Streptomyces sp. SF2741	Promothiocins	> 500	2	+	-	nt
S. morookaensis	Thioxamycin, thioactin	> 500	3	+	±	-
S. rochei	Thiotipin	> 500	4	+	-	nt
Streptomyces sp. CL10	Thiotipin	> 500	5	+	-	nt
Streptomyces sp. CR63	Berninamycin	> 500	6	+	-	nt
Streptomyces sp. DX49	Promothiocins	> 500	7	+	-	nt
Streptomyces sp.	Nosiheptide	> 500	8	+	-	nt
S. azureus	Thiostrepton	> 500	9	+	-	nt
S. fradiae	-	0.16	10	-	-	nt
S. roseoflavus	-	0.16	11	-	+	+
S. antibioticus IFO3126	-	0.16	12	-	+	+
S. aureofasciculus	-	4	13	-	+	±
S. lavendulae	-	0.8	14	-	±	-
S. citricolor	-	0.16	15	-	+	+
S. moderatus	-	4	16	-	-	nt
S. hygroscopicus	-	4	17	-	-	nt
S. lividans	-	0.8	18	-	+	+
Probe			19			

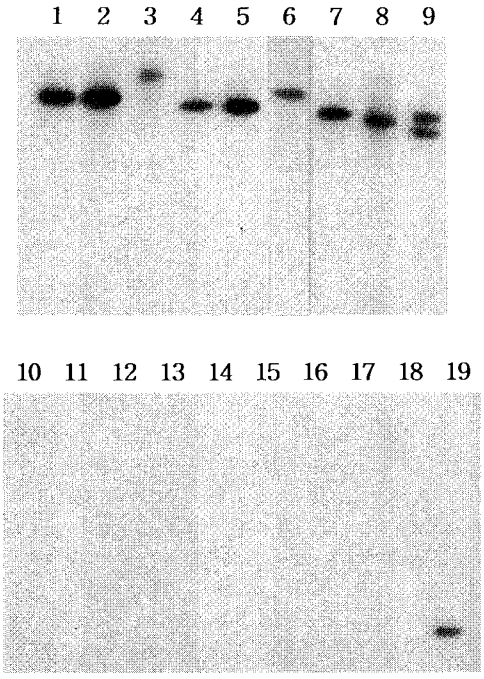
[a]Antimicrobial activity of thiostrepton against the thiopeptide producing and nonproducing strains. MIC indicates the minimum inhibitory activity. [b]Lane numbers for Figs. 1-3. [c]Southern blot analyses using thiostrepton resistance gene (*tsr*) and *tipA* gene probes, +: hybridized, -: not hybridized, ±: not clear. [d]PCR amplification of the *tipA* gene, +: amplified, -: not amplified, ±: amplified with a different size, nt: not tested.

among thiopeptide producing and nonproducing *Streptomyces* species by hybridization of their chromosomal DNAs with the *tipA* gene. The probes for hybridization (nucleotides 253~879) were prepared by PCR using two synthetic primers [nucleotides 253~283 (31-mer, ACCTCGACCGGCTGCAGCAGATCCTGTTCTA) and 849~879 (31-mer, ATCCTCGCCAACGCCGTCCGGCACACCCCCT)][1]. While thiopeptide producing strains did not show hybridization with the *tipA* probe at all, five of nine thiopeptide nonproducing strains (lanes 11, 12, 13, 15, and 18) showed positive results and lanes 3 and 14 were ambiguous, as shown in Fig. 2. This result was confirmed by repeated hybridization. Namely, the hybridized bands were found in 5 of 9 thiopeptide nonproducing strains examined.

Confirmation of these bands was carried out by PCR analysis (Fig. 3). The chromosomal DNAs of the strains *S. morookaensis* (lane 3), *S. roseoflavus* (lane 11), *S. antibioticus* (lane 12), *S. aureofasciculus* (lane 13), *S. lavendulae* (lane 14), *S. citricolor* (lane 15), and *S. lividans* (lane 18) were amplified by PCR with the primer sets indicated above. *S. roseoflavus*, *S. antibioticus*, and *S. citricolor* yielded PCR products of the same size as that of *S. lividans* (lane 18) while *S. morookaensis*, a thiopeptide producing strain, produced some nonspecific bands and *S. aureofasciculus* produced a PCR product with a different size, and *S. lavendulae* did not produce any product. These results imply that at least four species, *S. roseoflavus*, *S. antibioticus*, *S. citricolor*, and *S. lividans* have the *tipA* gene in their chromosomal DNA. Thus the *tipA* gene was found in 4, possibly 5, of the 6 thiopeptide nonproducing *Streptomyces* species examined.

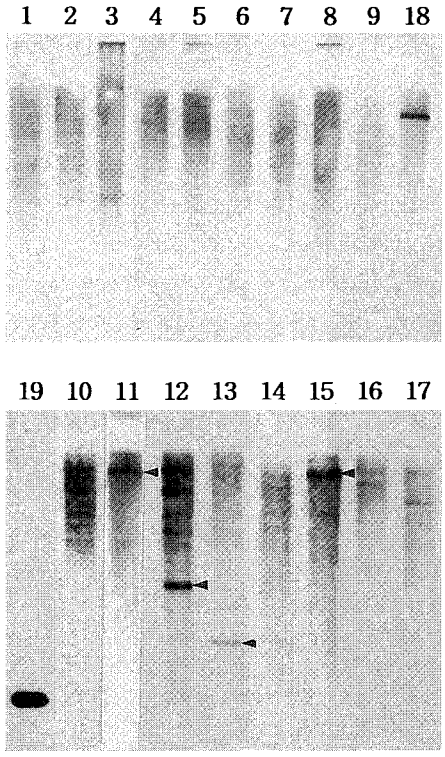
The *tipA* gene does not seem to be directly involved in thiopeptide production, because the gene exists only in some thiopeptide nonproducing species of *Streptomyces*. It was reported that the disruption of the *tipA* gene in *S. lividans* increased sensitivity to thiopeptide antibiotics up to about 5 times[3]. Thus, the *tipA* gene is suggested as serving as an autogeneously controlled resistance system specific to thiopeptides. We notice that the *tipA* gene does not exist in thiopeptide producing strains with the *tsr* gene, high-level thiopeptide resistance system, and the expression of *tipA* gene is dependent on the intracellular level of free thiopeptide compounds. In response to thiopeptides, TipAL activates transcription of its own promoter and thus results in the expression of TipAS, an alternate in-frame translation

Fig. 1. Southern blot analysis of *Streptomyces* species DNA using thiostrepton resistance gene (*tsr*) probe.



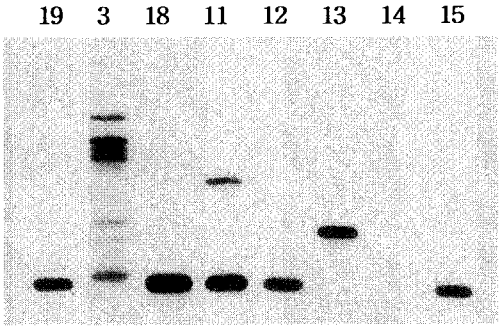
*Bam*HI-digested DNA from the thiopeptide producing and nonproducing *Streptomyces* species listed in Table 1 was analyzed by a Southern blot probed with the *tsr* probe. Lanes 1~9 contain DNA from thiopeptide producing *Streptomyces* strains and lanes 10~18 contain DNA from thiopeptide nonproducing strains. Lane 19 is the hybridization probe (nucleotides 391~1131) of the *tsr* gene.

Fig. 2. Southern blot analysis of *Streptomyces* species DNA using the *tipA* gene probe.



*Bam*HI-digested DNA from the thiopeptide producing and nonproducing *Streptomyces* species listed in Table 1 was analyzed by a Southern blot probed with the *tipA* gene. Lanes 1~9 contain DNA from thiopeptide producing *Streptomyces* strains and lanes 10~17 contain DNA from thiopeptide nonproducing strains. Lane 18 contains DNA from *S. lividans* which is used as a positive control and lane 19 is the hybridization probe (nucleotides 253~879) of the *tipA* gene.

Fig. 3. PCR amplification of the *tipA* gene using DNA from various *Streptomyces* species.



PCR amplification was carried out using two synthetic primers [nucleotides 253~283 (31-mer, ACCTCGACCGGCTGCAGCAGATCCTGTTCTA) and 849~879 (31-mer, ATCCTCGCCAACGCCGTC-CGGCACACCCCCT)] under the same conditions. DNAs used for PCR amplification are as described in Table 1.

product of the same gene. The expression of TipAS may control activation of the TipAL-dependent promoter by decreasing the intracellular concentration of free thiopeptides. The modulation of intracellular free thiopeptide level by the binding of thiopeptides with *tipA* gene products (TipAL and TipAS, antibiotic-inactivating proteins) would provide a self-contained antibiotic resistance system to diminish inhibition by transient exposure to thiopeptides. However, since *S. moderatus* and *S. hygroscopicus* without the *tipA* gene showed higher resistance to thiostrepton than those of *S. roseoflavus*, *S. antibioticus*, *S. citricolor* and *S. lividans* with the *tipA* gene, as shown in Table 1, this mechanism seems to be extremely low-level resistance or transient stress response system for

Streptomyces very sensitive to thiopeptide compounds. Thus, thiopeptide producing *Streptomyces* with high-level antibiotic resistance system would not require the *tipA* gene, a low-level thiopeptide resistance system.

Through this preliminary study on the function of *tipA* gene, we found that the *tipA* gene often existed in thiopeptide nonproducing *Streptomyces* but not in thiopeptide producing strains. Although one of major roles of the *tipA* gene is suggested to be antibiotic resistance specific to thiopeptides, we can not eliminate other possible physiological functions. It also should be investigated whether *S. roseoflavus*, *S. antibioticus*, and *S. citricolor* as *S. lividans* will be more sensitive strains to thiopeptides when the *tipA* gene is knocked out. Other roles of the *tipA* gene remain to be investigated.

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