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Cloning of *rpoZ*-dependent Transcriptional Activator Genes of *kasT*, a Pathway-specific Regulator of Kasugamycin Biosynthesis

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Introduction

The core enzyme of bacterial RNA polymerase (RNAP) consists of four subunits with a stoichiometry of $\alpha_2\beta\beta'\omega$ and requires an additional σ subunit to form the holoenzyme for promoter-specific initiation of transcription. Of the five subunits, little has been reported on the biological importance of the subunit ω except that a defect in the subunit results in a slow-growth phenotype in *Escherichia coli* and that the ω subunit of *Thermus aquaticus* facilitates the assembly of $\alpha_2\beta$ and β' for formation of the core enzyme.

We have clarified that *rpoZ*, the gene encoding the subunit ω is required for the production of kasugamycin (KSM), an aminoglycoside antibiotic effective against rice blast disease caused by *Pyricularia oryzae*, as well as for the formation of aerial mycelia, an early stage of morphological differentiation, in *Streptomyces kasugaensis*.[1] Indeed, the *rpoZ*-disrupted mutant produces neither KSM nor aerial mycelia. Furthermore, transcriptional analysis of KSM biosynthetic genes revealed that the KSM non-producing phenotype of the *rpoZ*-defective mutant is due to the lack of transcription of *kasT*, which encodes KasT, a KSM-biosynthesis specific transcriptional activator. Of particular relevance is the observation that KasT shows 50% identity to StrR, a specific transcriptional activator in the streptomycin (SM) biosynthesis.[2]

As *strR* transcription is under the control of AdpA, the specific transcriptional activator, in SM-producing *Streptomyces griseus*,[3] we assumed that *kasT* might also require an activator, designated as KasZ, for its transcription and that *rpoZ* might be crucial for the *kasZ* expression as illustrated in Fig. 1. To examine the mechanisms involved in the *rpoZ*-dependent KSM biosynthesis, we have established a system for cloning *kasZ* and genes associated with *kasT* transcription. We present here this cloning system together with several genes of interest subsequently isolated by this system.

Results and Discussion

Cloning system for *kasZ* and genes relevant to *kasT* expression in *S. kasugaensis*. As shown in Fig. 1, there is the possibility that regulators in addition to KasZ are required for *kasT* transcription. To clone these regulatory genes, we constructed the cloning vector pAK557, which harbors a reporter gene that can monitor transcription from the *kasT* promoter. The reporter gene was generated by fusion of the *kasT* promoter region (P_{kasT}) to a promoterless *dbfB* structural gene that encodes an extradiol dioxygenase of *Terrabacter* sp. strain DBF63.[4] Subsequently, the gene cassette was ligated into pSK2162, a vector derived from an *S. kasugaensis* plasmid, for the formation of pAK557 (Fig. 2). As *dbfB* is under the control of P_{kasT}, any gene encoding a P_{kasT}-specific transcriptional activator, which is cloned into the vector, can be identified by the induced DbfB activity that degrades the colorless substance, 2,3-dihydroxybiphenyl (DHB), into a yellow oxidation product (Fig. 3).

To enhance the expression of the genes cloned into the *Bam*HI, *Hind*III or *Pst*I cloning sites, these sites are located downstream of P_{ermE}, the strong promoter of an erythromycin resistance gene.

The utility of the pAK557 system was demonstrated by

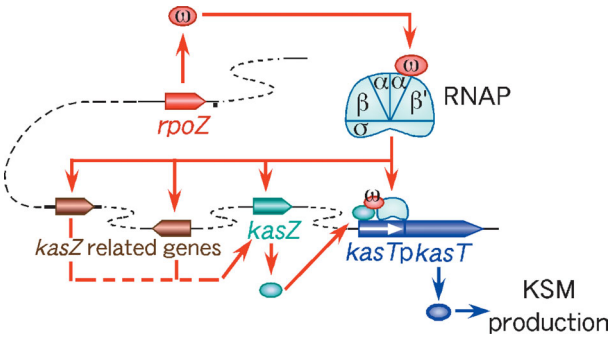

Fig. 1. Transcriptional activation of *kasT* by KasZ and related genes with reference to *rpoZ*.

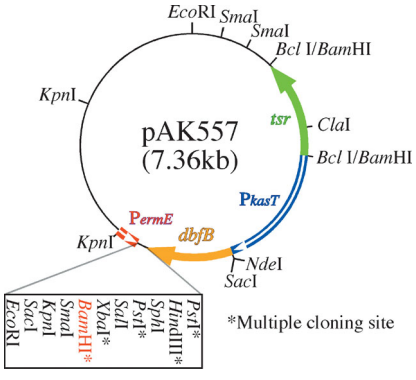

Fig. 2. Cloning vector pAK557.

transformation of the wild-type *S. kasugaensis* strain A1R6, the *rpoZ*-disrupted *S. kasugaensis* mutant R6D4 and *Streptomyces lividans* TK21. Of these transformants, only A1R6 transformants showed the expected yellow coloration, as shown in Table 1. This suggested that transcriptional activators of P_{kasT} are present only in wild-type *S. kasugaensis* and that their expression requires the presence of *rpoZ*. On the other hand, despite the presence of *rpoZ*, the *S. lividans* TK21 transformants failed to degrade DHB, probably because the streptomycete lacks the transcriptional activator genes specific to P_{kasT}. We consequently selected *S. lividans*TK21 as the host for cloning these activator genes.

We performed a shotgun cloning experiment as follows. Genomic DNA of the *rpoZ*-disrupted mutant R6D4 was partially digested with *Sau*3AI and the DNA fragments, with an average size of 4 kb, size-fractionated by agarose gel electrophoresis were ligated into *Bam*HI-digested pAK557. After transformation of *S. lividans* with the ligation mixture, the resulting 32,000 transformed colonies were screened with the DHB assay and 37 positive clones were selected. These cloned DNA fragments were PCR-amplified using primers with *Xba*I tails and subcloned into the *Xba*I site of pUC18/19 for nucleotide sequence determination and subsequent BLAST homology analysis.

Cloning of *bphC* in *S. kasugaensis*. Of the 37 clones isolated, transformants of KW6 and KW13, which overlapped in a 3.5-kb region, showed the highest intensity of yellow coloration after DHB application. As illustrated in Fig. 4, three full-length open reading frames (*orf1–orf3*) were present in the region. The *orf3* was identified as a homolog of *bphC*, a gene encoding biphenyl-2,3-diol 1,2-dioxygenase,[5] which also degrades DHB in a manner similar to DbfB (Table 2). Deletion analysis with a *dbfB*-less plasmid demonstrated that *orf3* was responsible for the observed DHB degradation and that its expression requires the presence of the promoter region of *orf2*. We concluded therefore that *orf3* is the *bphC* ortholog of *S. kasugaensis* and is co-transcribed from the *orf2* promoter. As wild-type *S. kasugaensis* alone fails to degrade DHB, the degradation activity of these transformants was probably due to a *bphC* gene dosage effect. After the remaining positive clones were PCR amplified with a *bphC*-specific set of primers, additional 11 clones were found to contain the *bphC* gene and were therefore eliminated from further analysis.

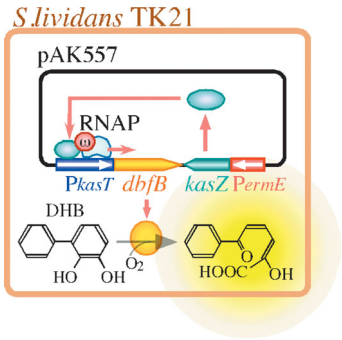

Fig. 3. Principle for cloning of kasZ and related genes using pAK557.

Table 1. DHB degradation and KSM production by pAK557-harboring *S. kasugaensis* and *S. lividans*.

Transformant	*rpoZ*	DHB degradation	KSM production
*S. kasugaensis*AIR6/pAK557	+	+	+
*S. kasugaensis*R6D4/pAK557	−	−	−
*S. lividans*TK21/pAK557	+	−	−

Classification of positive clones. Based on the yellow intensity elicited by DHB application in the clone-harboring transformants of wild-type *S. lividans* TK21 and *rpoZ*-disrupted *S. kasugaensis* mutant R6D4, the clones were classified into three groups, Groups A, B and C, as shown in Table 3. Briefly, clones in Group A developed a dark yellow coloration (+++ or ++++) in *S. lividans* but a faint yellow coloration (+) in mutant R6D4. Similarly, but of lower intensity, clones in Group C caused a relatively-dark yellow (++ or +++) in *S. lividans* but no or a faint yellow coloration (− or +) in mutant R6D4. Meanwhile, clones of Group B gave rise to similar levels of yellow coloration in the both streptomycetes. It is most likely that the color intensity correlates with the amount of DbfB produced through an activation of $P_{kas}T$ by protein products of the cloned genes and that the far less intensity developed in the *rpoZ*-disrupted mutant is due to the need of *rpoZ* or ω-containing RNAP for transcription of the genes. From this viewpoint, the genes classified into Group A, in marked contrast to those of Group B, are thought to require *rpoZ* for their expression. Despite the variation in the DHB degradation, however, all the clones listed in Table 3 failed to recover KSM production in mutant R6D4.

A set of genes encoding a putative two-component system in Clone A23. Clone A23 was found to contain two full-length genes encoding a putative two-component system, consisting of a sensor kinase and a response regula-

Table 2. Analysis of overlapped region of clone KW6 and KW13.

ORF	Putative function
orf1	NDP-sugar epimerase
orf2	HpcE homolog (decarboxylase/isomerase)
orf3	BphC homolog (extradiol dioxygenase)
Δorf4	HppA homolog (phenol hydroxylase)

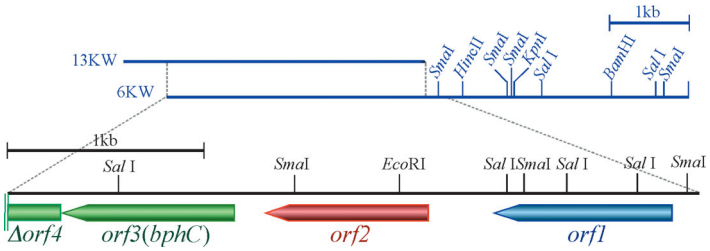

Fig. 4. Physical maps of Clone KW6 and KW13.

Table 3. Summary of cloned fragments in this study.

Group	Clone	Size of cloned fragment (kb)	Intensity of yellow induced by:		Putative function of gene(s) encoded
			S. lividans TK21	*S. kasugaensis*R6D4	
A	A23	4.5	++++	−	two-component regulatory system
	A17	4.5	+++	+	transposase
	KW22	5.2	+++	+	
	K8	4.3	++++	+	*bldA* (tRNA^{UUA}-Leu)
	K9	3.7	++++	+	
	K33	3.6	++++	+	function unknown
	KW19	4.2	+++	+	acyl-CoA synthetase
	KW24	4.5	++++	+	function unknown
	KW36	5.4	+++	+	asparagine synthetase, methyltransferase
	AS6	5.0	+++	+	function unknown
B	A12	3.7	++	++	1-deoxy-D-xyllulose-5-phosphate synthase
	M7	5.0	++++	+++	LacI-family trancriptional regulator
	K13	5.2	++++	+++	glycogen debranching enzyme, trypsin-like protease
	K14	5.0	+++	++	regulatory protein
	K17	4.5	+	+	enolase, cytochrome P-450
	K23	3.7	+++	++	*rrnF* (16SrRNA, 5SrRNA, 23SrRNA)
	KW37	4.0	+++	+++	translocase SecA subunit
	KW40	3.4	+++	++	ABC transporter
C	A18	3.6	++	−	zinc protease
	A22	4.5	++	−	ABC transporter
	A24	5.0	++	+	function unknown
	A26	3.0	+	−	DNA polymerase III β subunit
	KW16	3.3	++	+	function unknown
	KW17	4.5	++	+	leucyl-tRNA synthetase

tor, as a signal transduction system prevalent in prokaryotes. Such systems as *absA1*/*absA2*, *cutR*/*cutS* and *afsQ1*/*afsQ2* are known to be involved in antibiotic regulation in *S. coelicolor*.[6]

bldA in Clones K8 and 9. The bldA gene, encoding a leucyl tRNA for a rarely used UUA codon in streptomycetes, was present in a 0.7-kb overlapping region of clones K8 and K9. In fact, a subclone of pAK557, harboring a PCR-amplified *bldA*, induced an intense color reaction in *S. lividans*. In *S. coelicolor* and *S. griseus*, *bldA* is associated with antibiotic production by translationally regulating antibiotic-specific activator genes.[7,8] It is therefore most likely that extra copies of *bldA* enhanced the production of the regulators in *S. lividans* and that, because of their low specificity to $P_{kas}T$, these regulators could only activate the promoter when present in high concentrations. It should be mentioned that clone KW17 in Group C encodes a putative leucyl-tRNA synthase, an enzyme that adds leucine to the product of *bldA* (Table 3).

Concluding Remarks

Initially, we assumed that KasZ, an AdpA homolog, might be required for *kasT* transcription in *S. kasugaensis* and that *kasZ* expression may require *rpoZ*, or more specifically, ω-containing RNAP. However, no *adpA* homologs were identified among the selected clones. We are now therefore in the process of cloning an *adpA* homolog of *S. kasugaensis* by PCR amplification with primers specific to *adpA* and its homologs in *S. coelicolor* and *Streptomyces avermitilis*.

The isolation of a set of genes encoding a putative two-component system and also of *bldA*, whose transcription is highly dependent on *rpoZ* presence is of great interest. To study their roles in KSM biosynthesis, we are planning to examine the expression of these genes in the *rpoZ*-disrupted mutant R6D4 and to construct gene-disrupted mutants of *S. kasugaensis*.

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