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Mini-Review

Extracellular production of recombinant enzymes by *Streptomyces lividans*

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ABSTRACT

We constructed the *Streptomyces* hyperexpression vector pTONA5a. It includes a metallo-endopeptidase (SCMP) promoter isolated from *Streptomyces cinnamoneus* TH-2 and a metalloendopeptidase terminator isolated from *S. aureofaciens* TH-3. The pTONA5a plasmid, which also contains *oriT*/*mob*, can be transferred into streptomycetes through conjugation with *Escherichia coli*. The resulting transformants can be selected with kanamycin and/or thiostrepton. The SCMP promoter functions constitutively in the presence of a rich inorganic phosphate source and glucose. Using pTONA5a and *S. lividans*, we constructed a series of plasmids carrying *Streptomyces* genes encoding aminopeptidases, asparaginases, and proteases. It is particularly interesting that despite the lack of N-terminal Sec and Tat secretion signals, some of these enzymes were secreted into the medium in high amounts. A useful method for the industrial production of these enzymes was constructed until the unusual extracellular expression remained unknown.

KEYWORDS: extracellular production, *Streptomyces*, hyperexpression

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ABBREVIATIONS

SCMP	:	a metalloendopeptidase from *Streptomyces cinnamoneus* TH-2
LB	:	Luria-Bertani broth
Km	:	kanamycin
Nal	:	nalidixic acid
DDBJ	:	the DNA Data Bank of Japan
kibilysin	:	a metalloendopeptidase from *Streptomyces aureofaciens* TH-3
SOT	:	a trypsin from *Streptomyces omiyaensis*
LAP	:	leucine aminopeptidase
PAP	:	proline aminopeptidase
APP	:	aminopeptidase P
14270ASNase	:	*Streptomyces thermoluteus* subsp. *fuscus* NBRC 14270
SGR ASNase	:	*Streptomyces griseus* NBRC 13350
14270 X-PDAP	:	an X-prolyl aminopeptidase from *Streptomyces thermoluteus* subsp. *fuscus* NBRC 14270
14271 X-PDAP	:	an X-prolyl aminopeptidase from *Streptomyces thermocyaneoviolaceus* NBRC 14271
14273 X-PDAP	:	an X-prolyl aminopeptidase from *Streptomyces thermocoerulescens* NBRC 14273

SCO X-PDAP : an X-prolyl aminopeptidase from *Streptomyces coelicolor* A3(2)
Sgma : a metalloendopeptidase from *Streptomyces griseus* NBRC 13350
AdpA : A-factor-dependent transcriptional activator

1. INTRODUCTION

The *Escherichia coli* expression system is a first choice for recombinant production because of its convenience. However, it is often unsuccessful in yielding to be caused by the formation of insoluble inclusion bodies. The *Pichia* expression system is also used for recombinant production. This system is superior in high expression because fermentation is performed at high cell concentrations. However, this system is unsuitable for recombinant production from *Streptomyces* enzymes because the codon usage of *Streptomyces*, which is high in GC-content, differs from that of *Pichia*.

Streptomyces lividans has been studied intensively during the last 10 years as an alternative expression system [1]. For *Streptomyces* sp. to survive in a continuously changing environment, it must secrete numerous enzymes to degrade various substances [2]. Some of these enzymes have industrial applications. Consequently, *Streptomyces* sp. has excellent secretion capacity. In addition, secreted recombinant proteins are released in the culture medium, resulting in easy downstream processing. Based on these facts, *Streptomyces* sp. makes an attractive host.

Recently, we developed a hyperexpression vector for streptomycetes named pTONA5a (Fig. 1) [3]. Herein, we describe our further attempt at overexpression and extracellular production of various useful *Streptomyces* enzymes in *S. lividans* using pTONA5a.

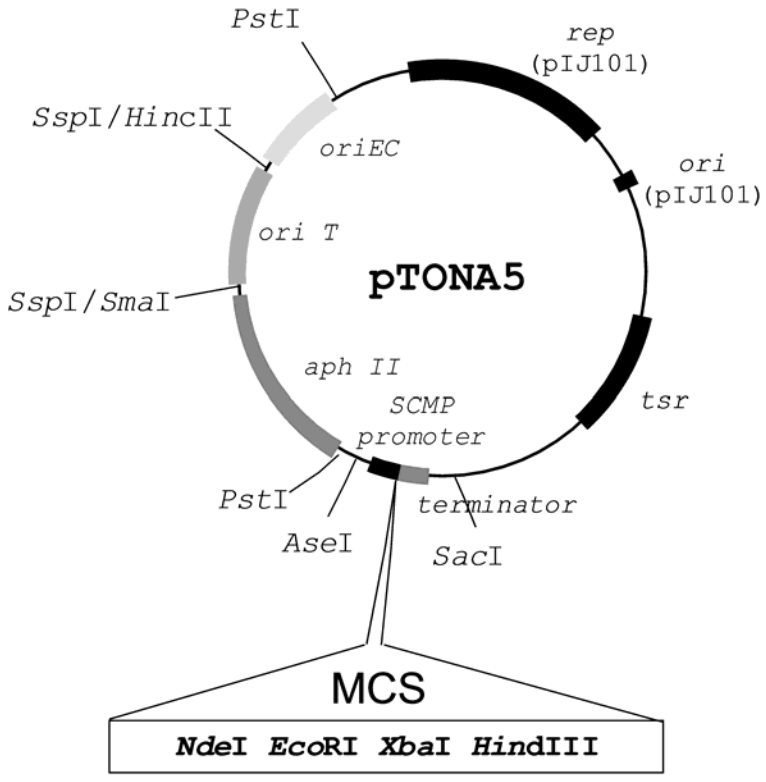

Fig. 1. Map of the expression vector pTONA5. *aphII*, kanamycin-resistance gene; *tsr*, thiostrepton-resistance gene; MCS, multicloning site; *ori*, origin for streptomycetes; *rep*, replication gene in streptomycetes; *oriEC*, origin for *E. coli*; *oriT*, origin for transfer.

2. Expression of recombinants

The pTONA5 derivatives were transformed in *E. coli* S17-1. A single colony of the transformant was cultured using Luria–Bertani broth (LB) containing 50 μg/ml kanamycin (Km) at 37°C for 7 h. Cells were harvested and washed with LB broth by pelleting the cells three times to remove Km. The cells were suspended in 500 μl of LB broth and then mixed with spores of *S. lividans* 1326. The mixture was spread on an ISP No. 4 agar plate and incubated at 30°C overnight. A 3 ml aliquot of soft-agar nutrient broth containing Km (50 μg/ml) and nalidixic acid (Nal, 67 μg/ml) was dispensed as upper layers on the plate, which was then incubated at 30°C for 3-5 days. A single colony was streaked on an agar plate with a medium containing 2.0% soybean meal, 2.0% mannitol, Km (20 μg/ml), and Nal (5 μg/ml) in tap water, which was autoclaved twice. The plate was incubated at 30°C for 5-7 days. The resultant *S. lividans* 1326 transformants were inoculated and grown in 50 ml of a culture medium containing 0.8% K_2HPO_4, 2.0% glucose that was sterilized separately, 0.05% $MgSO_4 \cdot 7H_2O$, 0.5% polypeptone, and 0.5% yeast extract (termed PG medium) in a 500-ml baffled flask at 30°C for 4 days with rotary shaking at 200 rpm.

3. DNA accession numbers

The accession numbers assigned to the sequences in the DNA Data Bank of Japan (DDBJ) database were the following: a metalloendopeptidase (SCMP) from *S. cinnamoneus* TH-2, AB189036; a metalloendopeptidase (kibilysin) from *S. aureofaciens* TH-3, AB281185; a trypsin (SOT) from *S. omiyaensis*, AB362837; a leucine aminopeptidase from *S. griseus* NBRC 12875 (LAP), AB125217; a proline aminopeptidase (PAP) from *S. aureofaciens* TH-3, AB248820; an aminopeptidase P (APP) from *S. costaricanus* TH-4, AB284164, an asparaginase from *S. thermoluteus* subsp. *fuscus* NBRC 14270 (14270 ASNase), AB469678. The gene ID of an asparaginase from *S. griseus* NBRC 13350 (SGR ASNase) was assigned to SGR3975 in the *Streptomyces griseus* genome project (http://streptomyces.nih.go.jp/griseus/). All sequences of X-prolyl aminopeptidases from *S. thermoluteus* subsp. *fuscus* NBRC 14270 (14270 X-PDAP), *S. thermocyaneoviolaceus* NBRC 14271 (14271 X-PDAP), and *S. thermocoerulescens* NBRC 14273 (14273 X-PDAP) were assigned accession numbers AB510493, AB510494, and AB510495 in the DDBJ database, respectively. The gene ID of an X-PDAP from *S. coelicolor* A3(2) (SCO X-PDAP) was assigned to SCO5122 in *Streptomyces coelicolor* genome project (http://www.ncbi.nlm.nih.gov/genome?term=streptomyces%20coelicolor).

4. Extracellular production of secreted proteins

First, to verify our expression system, we examined the yield of targets and N-terminal sequence of secreted proteins using three genes of known secreted enzyme and a gene annotated as a secretory protein. The result is shown in Table 1. SCMP [4], kibilysin [5], and SOT [6] have pre-structure and pro-structure in their primary sequences. Moreover, an inactive mutant SOT that substituted the active Ser with Ala was also processed and secreted correctly [7]. The N-terminal sequences of the secreted recombinants

Table 1. Extracellular expression of secretory enzymes from *Streptomyces*.

Protein	Source strain	Composition	Yield (mg/ml)	N-terminal sequence of the secreted protein	Ref.
LAP	*S. griseus* NBRC 12875	Monomer	0.8	AAPDIPL	3
SCMP	*S. cinnamoneus* TH-2	Monomer	0.6	GTG	4
Kibilysin	*S. aureofaciens* TH-3	Monomer	0.6	AAGTG	5
SOT	*S. omiyaensis*	Monomer	1.0-1.6	VVGGTRA	6
SOT mutant*	*S. omiyaensis*	Monomer	1.0-1.6	VVGGTRA	7

*The active Ser of this mutant was substituted with Ala.

Table 2. Extracellular expression of non-secretory enzymes from *Streptomyces*.

Protein	Source strain	Composition	Yield (mg/ml)	Ratio of activity in supernatant/cel	N-terminal sequence of the secreted protein*	Ref.
PAP	*S. aureofaciens* TH-3	Hexamer	1.9	10.5	MSTVSRLP	8
APP	*S. costaricanus* TH-4	Dimer	0.2	1.9	Start Met	9
14271 X-PDAP	*S. thermocyaneoviolaceus* NBRC 14271	Dimer	1.2	11.0	MTTEPLSFPR	10
14270 ASNase	*S. thermoluteus* subsp. *fuscus* NBRC 14270	Dimer	0.1	4.5	MHSSSPADAPVVRE	11
SGR ASNase	*S. griseus* NBRC 13350	Dimer	0.2	3.2	MTSTDAPSAISSVPA PAPPV	11

*N-terminal sequences of actually secreted proteins are underlined.

described above were the same as those of the wild-types. The N-terminal sequence of LAP, which has a signal peptide for secretion in its primary sequence, showed additional Ala compared to the known sequence. Type I signal peptidases of gram-positive bacteria acted by recognition of the "Ala-Xaa-Ala" sequence: six consecutive Ala around the digested sequence of LAP. Therefore, the N-terminal sequence of the recombinant was secreted with additional Ala. Based on these results, it is suggested that the secretion function is active in our system.

5. Unusual extracellular production of proteins

None of the five enzymes [8-11] has any signal peptide for secretion (Table 2). Especially, it is reported that SGR ASNase activity was undetected in the medium. The activities distribution of the enzymes was higher in the culture supernatants than those in cell lysates (Table 2). Analysis of the N-terminal sequence shows that no common role exists in the pattern of the sequences (Table 2). Despite the lack of N-terminal Sec and Tat secretion signals, the enzymes above were secreted into the medium in high amounts by some unclarified mechanism.

Using four X-PDAPs, efficiencies of extracellular expression were compared (Fig. 2). Although each clone showed over 80% identity with each other, differences exist in contents of secreted activities [10]. Similarly, using homologues of SGR ASNase and 14270 ASNase from *S. coelicolor* (gene ID: SCO4085) and *S. avermitilis* (gene ID: SAV1316 and SAV4025), their extracellular expression was tried. However, no activity exists in their supernatants and cell lysates (data not shown) [11].

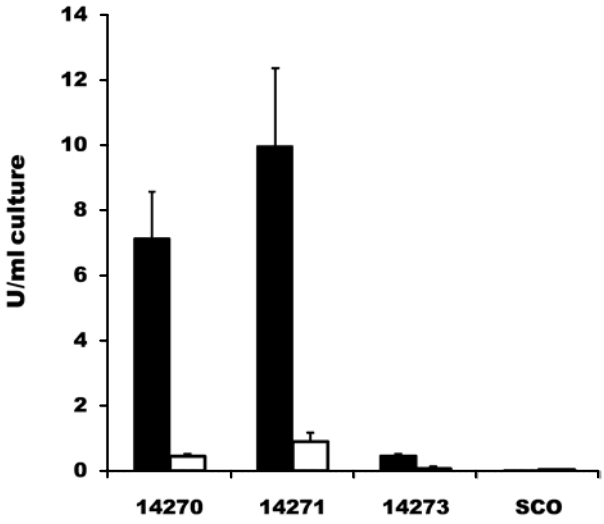

Fig. 2. Comparison of X-PDAP activities. Culture supernatants and cells were harvested after 5 d cultivation with 5 ml PG medium in test tubes at 30°C with 180 strokes. The activity was determined using 2 mM Ala-Pro-*p*NA in 100 mM Tris–HCl buffer (pH 7.5) at 37°C. Black columns show activities of the supernatants, white columns show those of the cell-free extracts. Cell-free extracts were prepared as follows: harvested cells were frozen, thawed, and suspended in yeast protein extraction reagent (Y-PER; Pierce Biotechnology Inc.). Cells were disrupted by sonication; their debris was removed by centrifugation. The resultant supernatants were used as cell-free extracts.

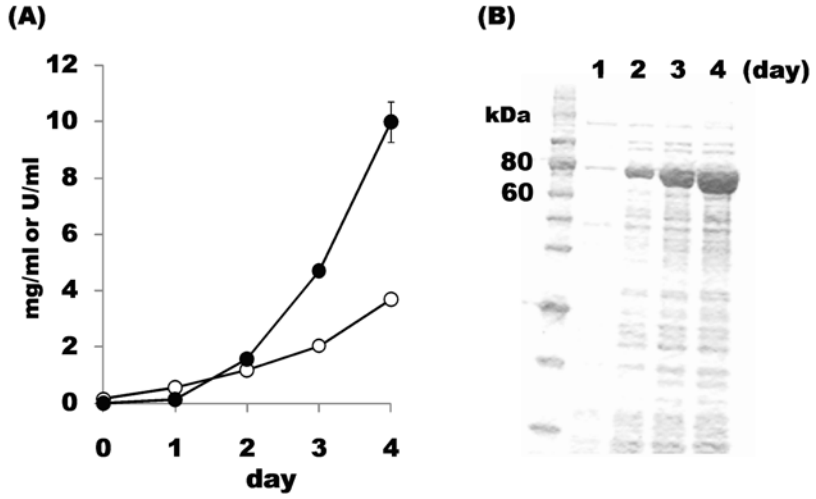

Fig. 3. (A) The time course of expression of 14271 X-PDAP. The transformant was subsequently used to conjugate *S. lividans* 1326, using a procedure similar to a method described in ref. 3. The resultant *S. lividans* transformant was inoculated and grown in culture PG medium at 30°C for 4 d at 200 rpm. **(B)** SDS-PAGE of the culture supernatants. All samples containing 10 µl of the supernatants were loaded onto a 12% polyacrylamide gel.

The time course of the expression was examined. The activity reached maximum at 4-day (Fig. 3A), and 14271 X-PDAP was the main band on SDS-PAGE (Fig. 3B). Based on this result, the extracellular expression under SCMP promoter is inferred to require long-term cultivation.

6. CONCLUSIONS AND FUTURE STUDY

This report described a hyperexpression system for *Streptomyces* enzymes. It is noteworthy that enzymes with no signal peptide were successful in unusual extracellular expression using pTONA5a and *Streptomyces lividans*.

This mechanism remains unclear, but we infer the following mechanism. SCMP belongs to a family of metalloendopeptidases that is classified as the family M4 in the MEROPS database (http://merops.sanger.ac.uk/index.shtml). Family M4 is known to be distributed in *Streptomyces* [4, 5]. The metalloendopeptidase from *S. griseus* (Sgma) also belongs to the family [12]. Reportedly, the enzyme transcription was controlled under an A-factor-dependent transcriptional activator (AdpA) that was reported to bind the region upstream of the Sgma promoter and activate the transcription. In *S. griseus*, the activation of AdpA is dependent on A-factor (2-isocapryl-3R-hydeoxymethyl-γ-butyrolactone). A-factor is a microbial hormone that triggers aerial mycelium formation and secondary metabolism in *S. griseus* [13]. Using extracellular proteome analysis, some proteins with no signal peptide were secreted, accompanied with various extracellular peptidases under the control of AdpA [14]. Akanuma *et al.* also described that the programmed cell death of *S. griseus* in liquid culture occurred and led to the resultant extracellular secretion of some cytoplasmic proteins [14]. The AdpA family was widely distributed in *Streptomyces*, similar to the family M4 peptidase. Kim *et al.* also proposed that a cascade of events links AdpA to mycelia autolysis in *S. coelicolor* [15].

Based on results of previous studies [12-15], it is possible that unusual extracellular production of cytoplasmic proteins autolysis results from autolysis linking of AdpA. Using sequence analysis to compare SCMP promoter and Sgma promoter revealed consensus AdpA-binding-like sequences (5'-TGGCSNGWWY-3') [13] in the SCMP promoter (Fig. 4), which suggests that the SCMP promoter is controlled under AdpA. In fact, AdpA induces a secretion of various proteases and peptidases and autolysis. Therefore, extracellular proteins should be attacked by such peptidases. Consequently, non-secretory proteins by secretion of unusual extracellular production

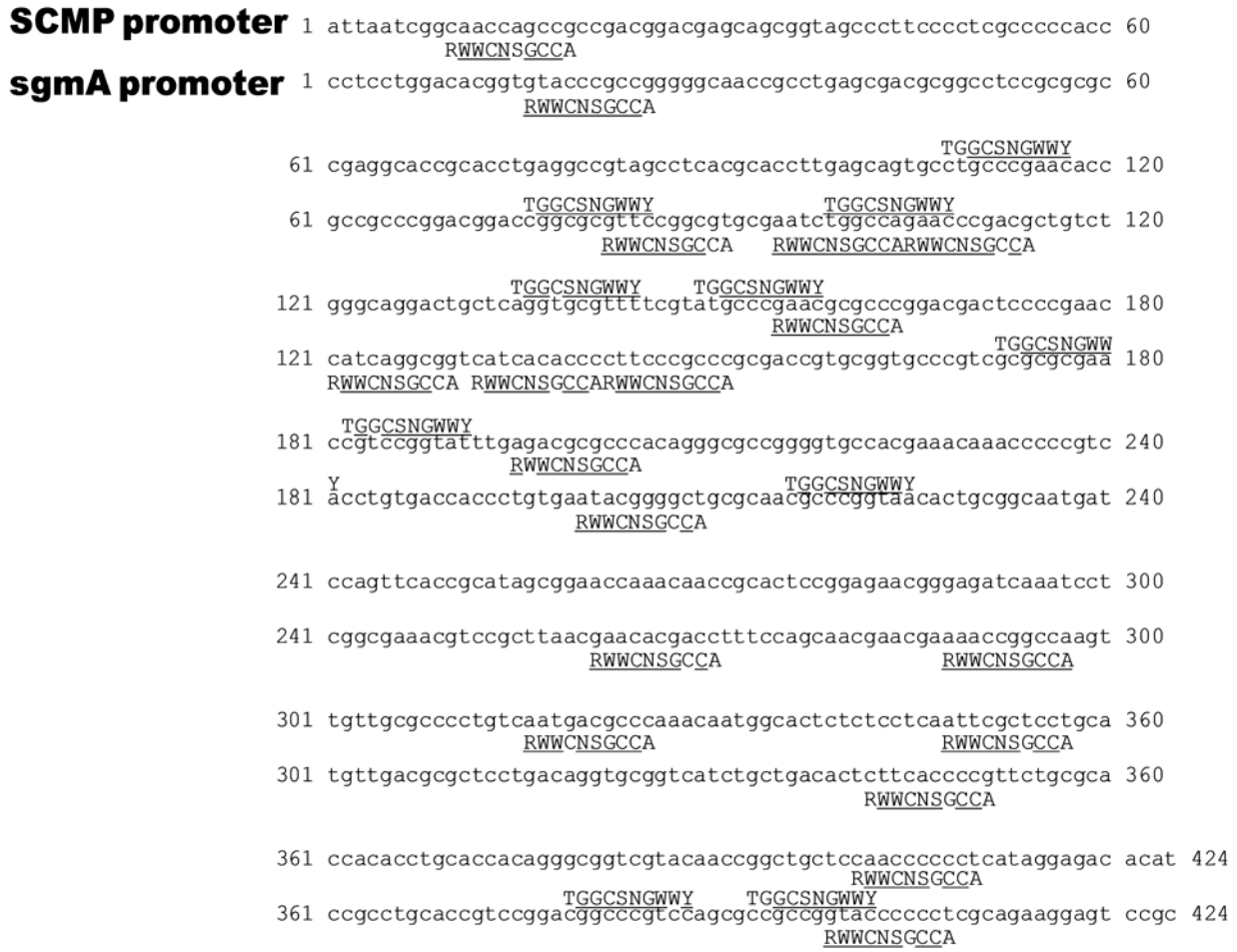

Fig. 4. Comparison of SCMP promoter with the Sgma promoter. The upper is SCMP promoter, the lower indicates the Sgma promoter. The consensus AdpA-binding sequence (5'-TGGCSNGWWY-3')-like sequences were indicated in both promoters.

might necessitate high stability. Among the four *Streptomyces* X-PDAPs used in this study, SCO X-PDAP showed very low stability [10]; the content of its extracellular production was the lowest among four X-PDAPs (Fig. 2). Moreover, the time of 14271 X-PDAP extracellular production was started late in the liquid culture (Fig. 3). Autolysis accompanied by unusual extracellular production of non-secretory proteins did not occur until the secondary metabolism was triggered by AdpA. These results support the notion that extracellular production by SCMP promoter is controlled under AdpA. Although the unusual extracellular production might be related with the autolysis triggered by AdpA, much more research is necessary to define the mechanism further.

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