J. Microbiol. Biotechnol. (2001), **11**(6), 1077–1086

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

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Overproduction of *Streptomyces griseus* Protease A and B Induces Morphological Changes in *Streptomyces lividans*

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Received: November 5, 2001
Accepted: December 5, 2001

Abstract The *sprA* and *sprB* genes encoding chymotrypsin-like proteases *Streptomyces griseus* protease A (SGPA) and *Streptomyces griseus* protease B (SGPB) and the *sprT* gene that encodes *Streptomyces griseus* trypsin (SGT) were cloned from *Streptomyces griseus* ATCC10137 and overexpressed in *Streptomyces lividans* TK24 as a heterologous host. The chymotrypsin activity of the culture broth measured with the artificial chromogenic substrate, N-succinyl-ala-ala-pro-phe-*p*-nitroanilide, was 10, 14 and 14 units/mg in the transformants haboring the *sprA*, *sprB* and *sprD* genes, respectively. The growth of *S. lividans* reached the maximum cell mass after 4 days of culture, yet SGPA and SGPD production started in the stationary phase of cell growth and kept increasing for up to 10 days of culture in an R2YE medium. The trypsin activity of the culture broth measured with the artificial chromogenic substrate, N-α-benzoyl-DL-arginine-*p*-nitroanilide, was 16 units/mg and SGT production started in the stationary phase of cell growth and kept increasing for up to 10 days of culture in an R2YE medium. The introduction of the *sprA* gene into *S. lividans* TK24 triggered the biosynthesis of pigmented antibiotics, actinorhodin and undecylprodigiosin, and induced significant morphological changes in the colonies in Benedict, R2YE, and R1R2 media. In addition, the introduction of the *sprT* gene also induced morphological changes in the colony shape without affecting the antibiotic production, thereby implying that certain proteases would appear to play very important and specific roles in secondary-metabolites formation and morphological differentiation in *Streptomyces*.

Key words: SGPA, SGPB, SGPD, SGT, *S. griseus*, *S. lividans*, morphogenesis

Streptomycetes is a group of soil eubacteria that are able to produce many valuable secondary metabolites and differentiate into aerial hyphae and then spores. *Streptomyces griseus* is one of the most intensively studied strains as regards its regulatory cascade concerning physiological and morphological differentiation. A-factor (2-isocapryloyl-3-R-hydroxy-methyl-γ-butyrolactone) is a microbial hormone that is known as a positive regulator for the production of streptomycin and sporulation in *Streptomyces griseus* [29]. Previous data suggest that certain proteases are produced in a different manner in *S. griseus* IFO13350 and its A-factor deficient strain, HH1. In addition, some serine protease and metalloprotease inhibitors induce the retardation of spore formation. Accodingly, it has been suggested that certain proteases may be involved in the differentiation process in *Streptomyces* [17].

S. griseus produces many kinds of secondary metabolites plus a mixture of proteases that is sold under the commercial name of Pronase with a variety of industrial use. Due to the importance of proteases, many proteases have already been purified from Pronase and their biochemical properties and three-dimensional structures have been studied [1, 25, 28, 31, 32]. Many genes such as *sprA*, *sprB*, *sprC*, *sprD*, *sprE* and *sprT* that encode *S. griseus* protease A(SGPA), *S. griseus* protease B(SGPB), *S. griseus* protease C(SGPC), *S. griseus* protease D(SGPD), and *S. griseus* protease E(SGPE), and *S. griseus* trypsin (SGT), respectively, have also been cloned and analyzed [7, 18]. All the proteases identified in *S. griseus* belong to the bacterial serine protease that catalyzes the hydrolysis of amides and esters through a common catalytic mechanism involving a triad of the residues serine, histidine and aspartic acid [30].

Four chymotrypsin-like serine proteases, SGPA, SGPB, SGPC and SGPD, are closely related to the mammalian Asp-Ser-Gly serine proteases and very stable in unusual condition such as 6 M guanidine [30]. The *sprA* and *sprB* genes (Genebank accession No. M17103 and M17104) encoding SGPA and SGPB have already been isolated from a *S. griseus* genomic library [7]. The amino acid

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sequence from its nucleotide sequence suggests that SGPA is produced as a precursor consisting of three portions; an amino-terminal pre sequence (38 amino acid residues), pro sequence (78 residues), and a mature chymotrypsin consisting of 181 amino acids with a molecular weight of 18 kD, while SGPB is produced as a precursor consisting of an amino-terminal pre sequence (38 amino acid residues), pro sequence (76 residues), and mature chymotrypsin consisting of 185 amino acids with a molecular weight of 18.4 kD [7].

The *sprD* gene encoding SGPD has also been isolated from *S. griseus* IFO13350 using the PCR method based on the nucleotide sequence (Genbank accession No. L29018) [3]. SGPD forms a very stable α_2 dimer and the amino acid sequence of the protein in the region of the specificity pocket is similar to that in SGPA, SGPB and SGPC [30]. The *sprD* from *S. griseus* IFO13350 encodes a protein consisting of an amino-terminal pre-peptide (64 amino acids), pro-pepetide (140 amino acids) and mature protease (187 amino acids) with a molecular weight of 17 kDa. [3].

SGT is a bacterial serine protease that is more similar to a mammalian protease than to *S. griseus* protease A and B [9, 22]. The *sprT* gene (Genebank accession No. M64471) encoding *S. griseus* trypsin has also been isolated from a *S. griseus* genomic library [18]. The amino acid sequence from its nucleotide sequence suggests that *S. griseus* trypsin is produced as a precursor consisting of an amino-terminal pre sequence (32 amino acid residues), pro sequence (4 residues), and mature trypsin consisting of 223 amino acids with a molecular weight of 23 kDa.

In a previous study, the current authors isolated the *sprT* gene from *S. griseus* ATCC10137 and optimized the overexpression system in *S. lividans* TK24 under various conditions [19, 20]. More recently, the current authors also reported that the overexpression of the *sprD* gene from *S. griseus* IFO13350 induced the production of pigmented antibiotic, actinorhodin, in *S. lividans* TK24 without any morphological change, thereby implying that SGPD is closely involved in the physiological differentiation in the host strain [3]. In the current study, the *sprA* and *sprB* genes were successfully overexpressed in *S. lividans* TK24, and the effects of the overexpression of these proteases including the *sprD* and *sprT* genes on the physiological and morphological differentiation are discussed.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

The *S. lividans* TK24 with a low level of milk protein-hydrolyzing proteolytic activity was obtained from the John Innes Institute, United Kingdom. The *S. griseus* ATCC10137 was obtained from the American Type Culture Collection and *S. griseus* IFO13350 from the University of Tokyo, Japan. The *E. coli* strains JM109 and DH5α were used for subcloning. The *Streptomyces-E. coli* shuttle vector pWHM3 was obtained from C. R. Hutchinson (University of Wisconsin, U.S.A.). All bacterial strains and plasmids used in the current study are listed in Table 1.

Media and Culture Conditions

The *E. coli* DH5α strain was maintained on the M9 minimal agar and cultured in an LB medium at 37°C with agitation [22]. The *Streptomyces* strains were maintained on an R2YE agar and *S. lividans* TK24 grown in an R2YE liquid broth at 28°C was used for the preparation of the protoplasts and isolation of plasmid DNAs [12]. Various media listed in Table 2 were used to study the effects of the medium compositions on the morphological and physiological differentiations of the *S. lividans* TK24 transformant.

Enzymes and Chemicals

The restriction endonucleases and other DNA modifying enzymes were purchased from Takara Shuzo Inc. Japan,

Table 1. Bacterial strains and plasmids.

Designation	Relevant characteristics	Source or reference
Strains		
S. lividans TK24	*Str*-6	Hopwood [12]
S. griseus IFO13350	Wild type; Streptomycin producer, A-factor$^+$	IFO[a]
S. griseus ATCC10137	Wild type; Streptomycin producer	ATCC[b]
E. coli DH5αF'	*sup*E44 Δ*lac*U169(ϕ80*lacZ*ΔM15) *hsd*R17 *rec*A1 *end*A1 *gyr*96 *thi*-1 *rel*A1	Bethesda Research Laboratories
Plasmids		
pWHM3	High-copy, tsr^r, amp^r, *E. coli-Streptomyces* shuttle vector	Hutchinson
pWHM3-A	pWHM3 with *sprA* gene from *S. griseus* ATCC10137	In this study
pWHM3-B	pWHM3 with *sprB* gene from *S. griseus* ATCC10137	In this study
pWHM3-D	pWHM3 with *sprD* gene from *S. griseus* IFO13350	[3]
pWHM3-T	pWHM3 with *sprT* gene from *S. griseus* ATCC10137	[19]

[a]: Institute of Fermentation, Osaka, Japan.
[b]: American Type Culture Collection, U.S.A.

Table 2. *Streptomyces* growth media used in current study.

Medium	Ingradient	Amount (g/l)
R1R2 [12]	Sucrose	61.8
	Glucose	6.0
	K_2SO_4	0.15
	$MgCl_2 \cdot 6H_2O$	6.07
	Casamino acid	0.06
	Yeast extract	3.0
	Trace element	1.2
	L-Proline	0.9
	L-Asparagine	0.6
	Agar	13.2
	K_2HPO_4 (0.5%)	6 ml
	$CaCl_2 \cdot 2H_2O$ (3.68%)	48 ml
	TES (5.73%, pH7.2)	60 ml
R2YE [12]	Sucrose	103
	K_2SO_4	0.25
	$MgCl_2 \cdot 6H_2O$	10.12
	Glucose	10
	Casamino acid	0.1
	Yeast extract	5
	K_2HPO_4 (0.5%)	10 ml
	$CaCl_2 \cdot 2H_2O$ (3.68%)	80 ml
	L-Proline (20%)	15 ml
	TES (5.73%, pH7.2)	100 ml
	Trace element	2 ml
Benedict (pH 7.0) [3]	Yeast extract	2
	Malt extract	2
	Casamino acid	4
	Maltose	10

and the other fine chemicals were from Sigma Chemical Co. The primers for the PCR-based cloning were purchased from Atman BioScience Inc., Korea.

DNA Manipulations

The DNA preparation and manipulation were performed using the method of Maniatis *et al.* in *E. coli* [22] and Hopwood *et al.* in *Streptomyces* [12]. The DNA samples were digested with restriction endonucleases and ligated with T4 DNA ligase according to the supplier's recommendations. The plasmid DNAs and their digests were analyzed by horizontal agarose gel electrophoresis using a TAE buffer system [12].

Transformation Procedure

Competent cells of the *E. coli* strains for transformation were routinely prepared according to the frozen storage protocol and the transformation was done using the method described by Hananhan [6].

The protoplasts of *S. lividans* TK24 were prepared as described by Okanishi *et al.* [27]. Cells were grown in 100 ml of R2YE containing 0.5% glycine in a 500 ml baffled flask and incubated for 18 to 24 h. The cells obtained were treated with 20 ml of P buffer containing 80 mg of lysozyme, then the resulting protoplasts were suspended in P buffer at a concentration of ~10^9/ml and frozen at −70°C. The *Streptomyces* protoplasts were transformed using 100 µl of the protoplasts (~10^8), 1 µg of the plasmid DNA in 20 µl of a TE buffer, and 500 µl of 25% PEG1000 in the P buffer [11]. The samples (100 µl) were plated in 2.5 ml of a 0.6% soft R2YE agar on R2YE regeneration plates. After incubation at 28°C for 18 to 24 h, the plates were overlaid with 2.5 ml of a 0.6% soft R2YE agar containing 25 µg/ml of thiostrepton. The transformants were visible after incubation for an additional 3 to 5 days at 28°C.

Overexpression of *sprA*, *sprB*, *sprD* and *sprT* Genes in *S. lividans* TK24

Three genes that encode SGPA, SGPB and SGT were cloned and kindly supplied by Prof. S.-M. Byun at KAIST, Korea [18], while the *sprD* gene for SGPD was directly cloned using the primer based PCR method as already reported [3, 14]. Because genes originating from Streptomycetes can not be properly expressed in an *E. coli* host system, the genes were introduced into the high-copy number plasmid pWHM3, an *E. coli-Streptomyces* shuttle vector. A 3.2 kb DNA fragment containing the *sprA* gene was obtained after digestion with *BamHI/XbaI* restriction enzymes and then ligated with pWHM3 digested with the same restriction enzymes to give pWHM3-A. pWHM-B was constructed by the ligation of the a 2.8 kb DNA fragment containing the *sprB* gene with pWHM3 using *KpnI/XbaI* restriction enzymes. The construction of the recombinant plasmids for the *sprT* and *sprD* genes was explained in the previous papers [3, 19]. All the recombinant plasmids were purified from the *E. coli* cells and then used for the protoplast transformation into *S. lividans* TK24. The transformants of *S. lividans* TK24 selected by thiostreptone were tested on a skim milk plate for the secretion of protease. A clear zone representing the degradation of the milk protein, surrounded each transformant that contained pWHM3-A, pWHM3-B, pWHM3-D and pWHM-T.

Sample Preparation for Protease Assay

The transformants of *S. lividans* TK24 harboring the each recombinant plasmid were grown in 100 ml of an R2YE medium in 500 ml baffled flasks at 28°C with vigorous shaking at 250 rpm. After 2 days of cultivation, 10 ml of the culture broth was used for inoculation into 100 ml of various liquid media, as listed in Table 2, in 500 ml baffled flasks under the same condition. Five ml of the culture broth was sampled everyday and centrifuged at 5,000 rpm for 10 min. The supernatant was then used to measure the protein concentration and protease activity, and the cell pellet was used to quantify the protein after cell disruption by sonication. The protein concentrations were measured according to the method of Bradford and bovine serum albumin was used as the standard [2].

Determination of Chymotrypsin Activity

The chymotrypsin activity was measured spectrophotometrically based on the release of p-nitroaniline using N-succinyl-ala-ala-pro-phe-p-nitroanilide as the artificial chromogenic substrate [17]. A reaction mixture containing 890 µl of a reaction buffer [100 mM Tris-HCl (pH 8.0), 10 mM $CaCl_2$] and 10 µl of 30 mM N-succinyl-ala-ala-pro-phe-p-nitroanilide in DMSO was used to react with 100 µl of the enzyme suspension. After 15 min, the chymotrypsin activity was measured at 405 nm. One unit of chymotrypsin was defined as the amount of enzyme corresponding to a 0.1 increase in absorbance under the above conditions

Determination of Trypsin Activity

The trypsin activity was measured spectrophotometrically based on the release of p-nitroaniline by the enzymatic hydrolysis of the artificial chromogenic substrate, N-α-benzoyl-DL-arginine-p-nitroanilide [17]. The assay reaction mixture, which was composed of 890 µl of a reaction buffer [50 mM Tris-HCl (pH 8.0), 20 mM $CaCl_2$] and 10 µl of 50 mM N-α-benzoyl-DL-arginine-p-nitroanilide was prewarmed for 5 min at 37°C, rapidly mixed with 100 µl of the enzyme suspension, and incubated for 15 min. The reaction was stopped by the addtion of 400 µl of 30% acetic acid in dioxane, and the absorbance at 405 nm was recorded. One unit of trypsin was defined as the amount of enzyme corresponding to a 0.1 increase in absorbance under the above conditions.

Antibiotic Assays

Assays for the detection of actinorhodin and undecylprodigiosin were performed as previously described [21]. A stationary culture of *S. lividans TK24* was transferred to 100 ml of R2YE in a 500 ml baffled flask, and incubated at 30°C on a reciprocal shaker. Portions (5 ml) of the culture broth sampled after different intervals were extracted with 5 ml of chloroform for 30 min at room temperature with shaking. Then, 5 ml of 1 N NaOH was added, and the tubes vortexed and spun in a microcentrifuge for 15S. The aqueous phase was found to contain actinorhodin, which was blue at an alkaline pH, and the A_{590} of the aqueous phase was determined. Meanwhile, the chloroform phase was found to contain undecylprodigiosin, which was yellow. For the absorbance measurements of undecylprodigiosin, the chloroform layer was acidified with HCl and the A_{530} of the now-red chloroform phase was then determined.

Mycelial growth and colony morphology: The *in vivo* effects of protease overexpression were determined. Spores or protoplasts prepared from *S. lividans* TK24 containing each gene were grown on solid R2YE, R1R2 and Benedict media. The morphological changes in the colonies and the pigment production were examined after 3 to 7 days of growth at 28°C and photographed.

Results and Discussion

Overexpression of Proteases in *S. lividans* TK24

Three chymotryspins, SprA (SGPA), SprB (SGPB) and SprD (SGPD), were compared with regards to their amino-acid sequences deduced from each nucleotide sequence (Fig. 1). The SGPA and SGPB showed an overall homology of 54% and regional homology of 50% and 43% in their signal peptides and pro-peptides, respectively, based on their amino acid identity [7]. In particular, the carboxy-terminal domains have 75% homologous, which suggests that both originated from the gene duplication of a common ancestral gene. The SprD (SGPD) proteins showed an overall homology of 38% (147 amino acids among 391 amino acids) with SGPA and SGPB based on their amino acid identity.

In previous reports, the current authors described the overexpression systems for the *sprD* and *sprT* genes originated from *S. griseus* in *S. lividans* TK24 [3, 19]. To overexpress the *sprA* and *sprB* genes in *Streptomyces*, the high-copy number plasmid pWHM3, an *E. coli-Streptomyces* shuttle vector, was double digested and ligated with the entire *sprA* and *sprB* genes that were digested with the appropriate restriction enzymes as indicated in Fig. 2. The resulting recombinant plasmid pWHM3-A and pWHM3-B prepared from *E. coli* were introduced into *S. lividans* TK24 using the protoplast transformation method. All the transformants were tested on a skim milk plate for the production of protease in the presence of thiostreptone. Many transformants were selected that exhibited large and obviously cleared proteolytic zones. The transformants were cultured in an R2YE broth containing 20 µg/ml of thiostrepton, then the chymotrypsin proteolytic activity was analyzed

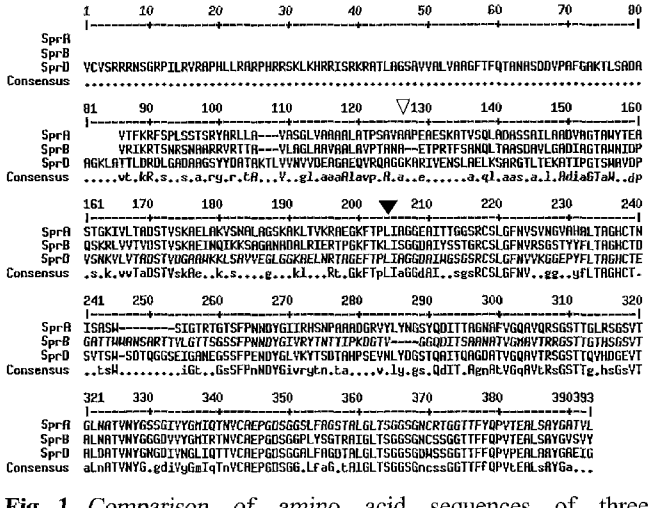

Fig. 1. Comparison of amino acid sequences of three chymotrysins, SprA (SGPA), SprB (SGPB), and SprD (SGPD).
The numbering to the right of the sequences is relative to the first amino acid coded by the gene. The junctions between the pre- and proregions and the pro- and mature regions are indicated by an open and closed triangle, respectively.

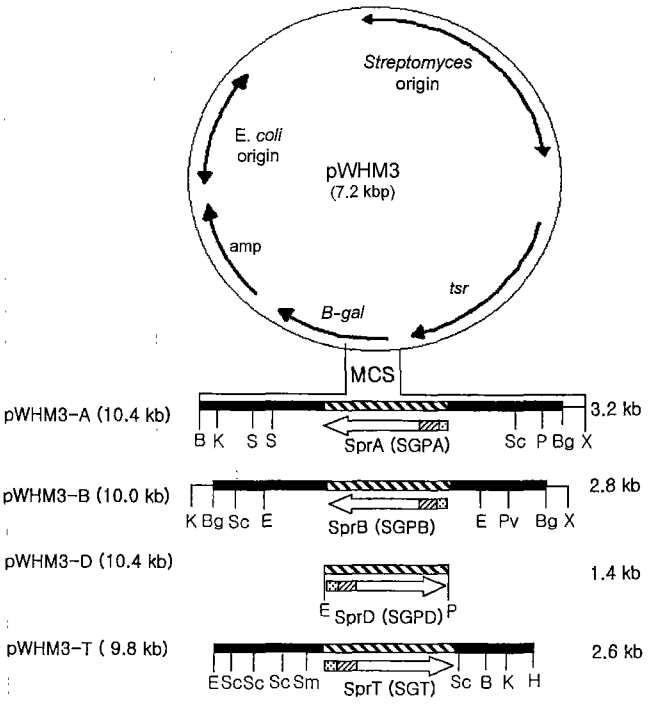

Fig. 2. Restriction maps of expression vector pWHM3-A, pWHM3-B, pWHM3-D, and pWHM3-T containing insert of entire *sprA*, *sprB*, *sprD* and *sprT* genes, respectively.
The restriction enzyme sites used for cloning are placed at both ends of the inserts. The DNA fragments derived from *S. griseus* are indicated by thick lines, while the parts from the multicloning sites introduced by the DNA manipulation procedure are depicted by thin lines. The coding region of the each gene is represented using a dashed line. The organization of the structural genes, with pre peptide (⊡), pro peptide (▨) and mature protease (□) is shown below the maps. Abbreviations: amp, ampicilline resistance; tsr, thiostreptone resistance; β-gal, β-galactosidase; B, *BamHI*; Bg, *BglII*; E, *EcoRI*; H, *HindIII*; K, *KpnI*; P, *PstI*; Pv, *PvuI*; S, *SalI* ; Sc, *SacI*; Sm, *SmaI*; X, *XbaI*.

using the artificial substrate, N-succinyl-Ala-Ala-Pro-Phe-ρ-nitroanilide. The release of the chromophoric substrate, a ρ-nitroanilide group, was monitored as an activity of a chymotrypsin protease.

The growth curve of each transformant was represented as the cellular protein concentration (mg/ml of culture), see Fig. 3A. The growth rate of *S. lividans* transformed with pWHM3, pWHM3-B, pWHM3-D and pWHM3-T were almost similar, yet the transformant with pWHM3-A reached its maximum cell mass after 6 days of culture. The chymotrypsin activity in the transformant with pWHM3-A increased sharply after 6 days of cultivation and reached a maximum level of 10 units/mg after 8 days of cultivation in the R2YE broth (Fig. 3B). In contrast, the chymotrypsin activity in the cells transformed with pWHM3-B increased sharply after 4 days of cultivation and the productivities of the chymotypsin remained at 14 units/mg up to 10 days of cultivation in the same medium. The production of SGPD and SGT started in the stationary phase of cell growth and continued to increase until the later stage of growth as in

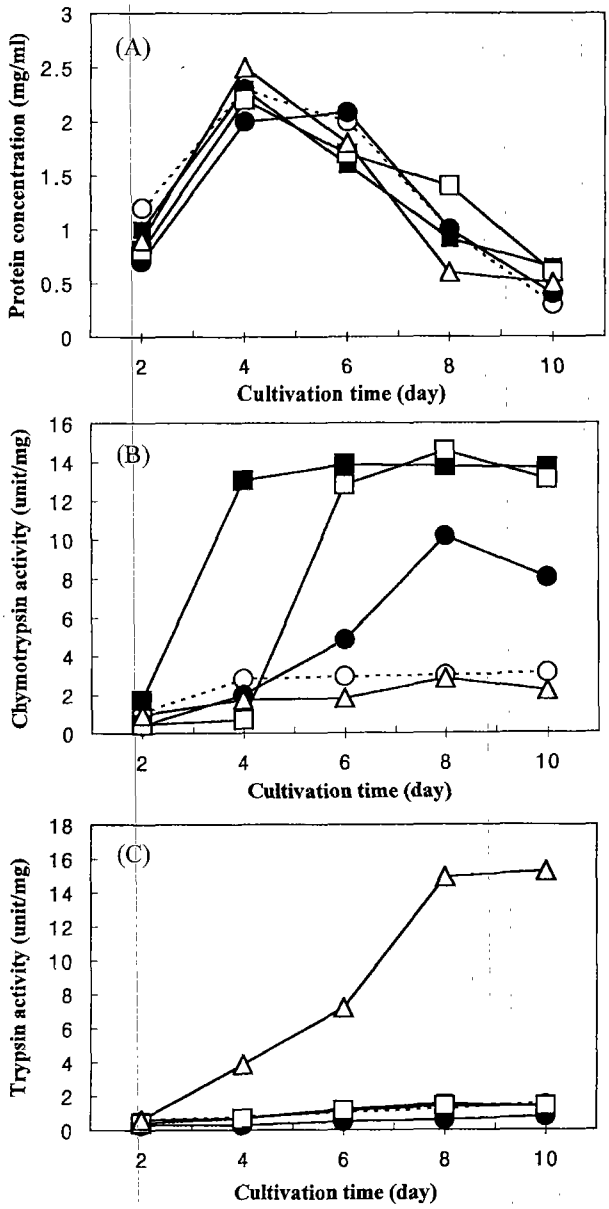

Fig. 3. Comparison of the growth curve (A), chymotrypsin activity (B), and trypsin activity (C) produced by each transformant of *S. lividans* TK24 in R2YE medium.
(A) The concentration of cellular protein in the transformants was measured using the Bradford's method after the cells were disrupted by sonication and expressed in mg/ml. (B) The chymotrypsin activity of the cultural filtrate prepared from the transformant was expressed in unit/mg of cellular protein. (C) The trypsin activity of the cultural filtrate prepared from the transformant was also expressed in unit/mg of cellular protein, as described in 'Materials and Methods'. ○-○, Control with pWHM3; ●-●, transformant with pWHM3-A; ■-■, transformant with pWHM3-B; □-□, transformant with pWHM3-D; △-△, transformant with pWHM3-T.

the cases of SGPA (Fig. 3A, 3B, 3C). Judging from the growth curves and protease production, SGPB would appear to be a primary protease, whereas SGPA, SGPD and SGT are secondary proteases.

Effects of *sprA* Gene on Antibiotic Production in *S. lividans* TK24

Because of the industrial significance, many scientists have investigated strain improvement for the mass production of antibiotics. From the intensive studies on antibiotic biosynthesis, it has been discovered that the regulation of secondary metabolite formation is affected by many factors, such as nutrient limitations, environmental changes, global regulatory factors and pathway-specific regulatory factors [4, 8, 10, 13, 23]. *S. coelicolor* A3(2) is a good developmental system for studying the regulation of antibiotics synthesis, since two of its four antibiotics are pigmented [9]. The biosynthetic genes that produce the colored antibiotics actinorhodin and undecylprodigiosin are clustered as separate operons in the chromosomal DNA. *S. lividans* is a strain closely related to *S. coelicolor*, and also has biosynthetic gene clusters for the production of the above pigmented antibiotics. However, these gene clusters are present as sleeping genes in *S. lividans* and under usual conditions the strain is not able to produce these antibiotics [13]. During this experiment, the transformant including the *sprA* gene was found to produce a massive amount of the blue-pigmented antibiotic, actinorhodin, on R1R2 and R2YE solid media (Fig. 5B-b). Therefore, it appear that the overexpression of the *sprA* gene in *S. lividans* stimulated the expression of the gene cluster for the pigmented antibiotic production. When the transformants were cultured in an R2YE liquid medium, the productions of actinorhodin and undecylprodigiosin increased by 1.7 and 1.5 times, respectively (Fig. 4A, 4B). Although the extent of the antibiotics production was relatively lower than that expected in the liquid medium, this was seemingly due to the higher background of the quantitation method used. However, it was evident that only the transformant with pWHM3-A produced a massive amount of the antibiotics in a solid medium (Fig. 5B).

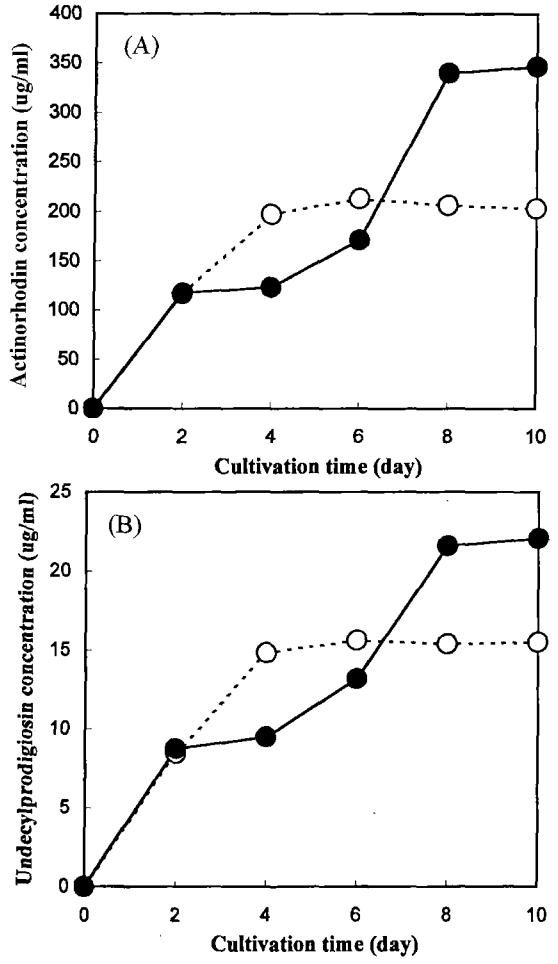

Fig. 4. Effects of SGPA overproduction on pigmented antibiotic production of *S. lividans* TK24.

The portions sampled as described in 'Materials and Methods' were scanned using a spectrophotometer to quantify the actinorhodin (A) and undecylprodigiosin (B). ○-○, Control with pWHM3; ●-●, transformant with pWHM3-A.

In a previous report, the activation of the actinorhodin biosynthetic gene cluster by the introduction of the *sprD* gene in *S. lividans* TK24 described [3]. Even though SGPA, SGPB and SGPD belong to the same chymotrypsin family with a higher similarity, their *in vivo* effects appeared different. Interestingly, SGPA and SGPB exhibited an overall homology of 54% based on their amino acid identity, while their carboxy-terminal domains were 75% homologous (Fig. 1). The close homology between the *sprA* and *sprB* genes suggests that both originated from the duplication of a common ancestral gene. However, the current results indicate that the *sprA* and *sprB* genes have different *in vivo* activity in *S. lividans*. At present, an explanation on how the proteases SGPA and SGPD awake the sleeping genes for secondary-metabolites formation can not be given. However, the current results clearly show that proteases are closely involved in physiological differentiation, such as the secondary metabolism in *Streptomyces*.

Effects of *sprA* and *sprT* Genes on Colony Morphology in *S. lividans* TK24

One of the main fields of *Streptomyces* study is the morphogenesis leading to aerial mycelium formation and sporulation. Many sigma-factors, peptides, and phosphorylating regulatory proteins are known to be involved in this morphological differentiation process [29, 33, 35]. Proteases from the chymotrypsin superfamily are a large group of enzymes and known to have many important intracellular functions including mammalian blood clotting cascades, the digestive activity of the pancreas, regulation of the cell cycle, and maturation and secretion of other proteins [30]. *S. griseus* can produce many extracellular proteases and at least four genes that are responsible for chymotrypsin-like proteases have been reported. When considering that secondary metabolism or cell morphogenesis occur in the

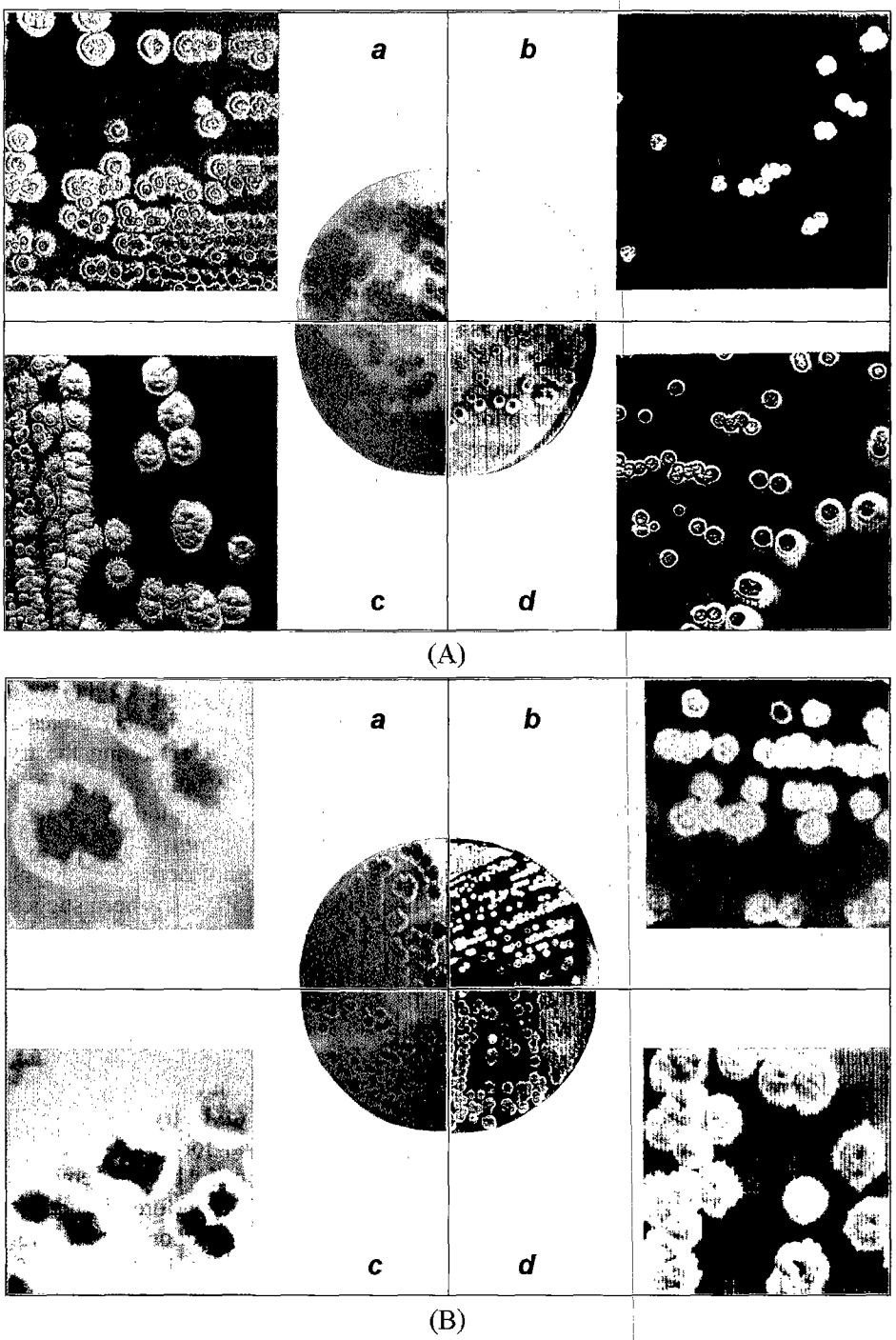

Fig. 5. Effects of protease overproduction on morphogenesis of *S. lividans TK24.*
Photographs of plates in Benedict medium (A) and R1R2 medium (B). For a comparison of the microbial growth and colony size, a part of the photograph taken of the entire plate is placed in the center, while the photographs of the colonies are placed in the margins. (A) Photographs of the surface of the plate taken after 5 days of cultivation in a Benedict medium. a, *S. lividans* TK24 harboring pWHM3 as the control; b, *S. lividans* TK24 transformed with pWHM3-A, exhibiting smaller and nonsporulating colonies as differentiated from the control and blue pigment production in the region of abundant growth (refer to Fig. 4); c, *S. lividans* TK24 transformed with pWHM3-B, where the colony morphology is almost identical with the control; d, *S. lividans* TK24 transformed with pWHM3-T, where white colored spores and the formation of clear pocks in the center can be clearly differentiated from the control. (B) Photographs of the surface of the plate taken after 5 days of cultivation in an R1R2 medium. a, *S. lividans* TK24 harboring pWHM3 as the control; b, *S. lividans* TK24 transformed with pWHM3-A, exhibiting smaller colonies with an oval shape and massive production of the blue pigment around the colonies. c, *S. lividans* TK24 transformed with pWHM3-B, where the colony morphology is almost identical with that of the control; d, *S. lividans* TK24 transformed with pWHM3-T, showing gray colored sporulation without pock formation as clearly differentiated from the control. The transformant with the pWHM3-D showed the same morphology as the control, therefore, the photographs were not illustrated in this figure.

later stage after cell growth has stopped in conjunction with proteases production, it would seem that the expression of the genes leading to differentiation and protease production may be closely related.

Recently, the current authors studied the proteases produced by *S. griseus* IFO13350 and its A-factor deficient mutant strain (blocked for sporulation and streptomycin production), *S. griseus* HH1 [17]. *S. griseus* showed a higher degree of cell growth and protease activity in proportion to its ability to produce a higher amount of A-factor. In particular, the specific activity of the trypsin of *S. griseus* IFO13350 was greatly enhanced, while that of the metalloprotease of *S. griseus* HH1 was significantly improved in the later stage of growth. Interestingly, when the serine protease inhibitor, pefabloc SC, and metalloprotease inhibitor, EDTA, were applied to strain IFO13350 to examine the *in vivo* effects of the protease inhibitors on the morphological differentiation, the formation of aerial mycelium and spores was delayed [17]. These observations seem to imply that certain proteases may be very important for morphological differentiation in *Streptomyces*.

Accordingly, the *in vivo* effects of protease overexpression on morphological changes were studied (Fig. 5A, 5B). When the transformant of *S. lividans* TK24 with pWHM3-A was grown in a Benedict medium, a much smaller colony (about one fourth of the normal colony) without sporulation and with a squeezed oval shape was formed (Fig. 5A-b), however, the transformant with pWHM3-B was almost identical with the control (Fig. 5A-c). The transformant with pWHM-T showed a smaller white colony with a clear nonsporulated central zone like a volcano (Fig. 5A-d). The same experiments were also carried out in R1R2 and R2YE media (Fig. 5B). The transformant with pWHM3-B produced a large circular colony with gray spores in the marginal area and a central pock that was identical to that of the control in an R1R2 medium (Fig. 5B-c). Similar to the result in a Benedict medium, the transformant with pWHM3-A formed a smaller and squeezed oval shaped colony with brown spores (Fig. 5B-b), while transformant with pWHM3-T produced slightly smaller sized colony with a dense layer of gray spores (Fig. 5B-d). In an R2YE medium, the transfomants with pWHM3-A and pWHM3-T showed different colony shapes that were more wrinkled and have deeper pocks than those of the control and the transformant with pWHM3-D (data not shown). The transformant with pWHM3-D did not exhibit any significant change in the media tested (data not shown). Therefore, the above described observations support the idea that certain proteases such as SGPA and SGPT play very important roles in the morphological differentiation of *Streptomyces*.

The importance of serine proteases in the morphological differentiation of *Streptomyces* can be found in many previous reports. A *sawD* gene that encoded the homologous to the serine protease of *Caulobacter cresceatus* with a conserved serine-catalytic active site (GPSAG) was already cloned from a total DNA library of *Streptomyces ansochromogenes* [5]. This study of a *sawD*-disrupted mutant demonstrated that sawD may be related to sporulation and especially to spore septation in *S. ansochromogenes*. In addition, the preliminary results indicated that the *sawD* gene may also be involved in pigment biosynthesis and nikkomycin biosynthesis in *S. ansochromogenes*. Two exocellular nucleases with molecular masses of 18 and 34 kDa, which are nutritionally regulated and reach their maximum activity during aerial mycelium formation and sporulation, were previously detected in *Streptomyces antibioticus* [26]. The *S. antibioticus* 18-kDa nuclease is produced by proteolytic processing from a less active protein precursor. The protease responsible has been identified as a serine protease, plus the inhibition of both of the nucleases or the protease impairs aerial mycelium development in *S. antibioticus*.

Trypsin and metalloprotease also have been reported as important proteases in the differentiation of *Streptomyces*. In the previous report, the current authors described the *in vivo* effects of various proteases inhibitors and the importance of trypsin and metalloprotease in the differentiation of *S. griseus* [17]. Controlling the trypsin activity using protease inhibitor would seem to be an efficient way to regulate the differentiation process in *Streptomyces* [15]. *Streptomyces exfoliatus* SMF13 sequentially produces leupeptin, leupeptin-inactivating enzyme (LIE) and trypsin-like protease (TLP). It has been suggested that leupeptin is inactivated by hydrolysis of peptide bond between leucine and leucine and between leucine and argininal of leupeptin by the leucine-specific protease, LIE. The aerial-mycelium growth and aerial spore formation of *S. exfoliatus* SMF13 is inhibited by the addition of bestatin, an inhibitor of LIE. This inhibition of the morphological differentiation is due to the inhibition of the trypsin-like protease (TLP) activity, which is essential for aerial-mycelium formation and is inhibited specifically by the remaining leupeptin that is not inactivated. Therefore, it has been concluded that the physiological function of LIE is to inactivate leupeptin when TLP activity is required for aerial-mycelium formation. [15, 16]. An extracellular proteolytic complex with metalloprotease and trypsin-like activities defining the differentiation of a substrate mycelium into the aerial hyphae and then spores has also been reported in *Streptomyces albogriseolus* 444 [24].

In addition to serine protease and metalloprotease, other types of proteases are also involved in the differentiation process in *Streptomyces*. The genes of *S. coelicolor* A3(2) encoding the catalytic subunits (ClpP) and regulatory subunits (ClpX and ClpC) of the ATP-dependent protease family Clp are one example [34]. The disruption of the *clpP1* gene in *S. lividans* and *S. coelicolor* blocks differentiation

in the substrate mycelium step. The overexpression of *clpP1* and *clpP2* accelerates aerial mycelium formation in *S. lividans*, *S. albus* and *S. coelicolor*. The overproduction of ClpX accelerates actinorhodin production in *S. coelicolor* and activates its production in *S. lividans*.

At present, it is very difficult to understand how these proteases actually participate in the differentiation process of *Streptomyces*. However, it is clear that certain proteases play very important roles in the physiological and morphological differentiation in *Streptomyces*. To elucidate the exact mechanism, the target protein of the proteases will be studied in the near future.

Acknowledgments

This work was supported by 2000 Research Fund of Myongji University and the Ministry of Education through BK21 program.

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