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Distinct Regulation of the *sprC* Gene Encoding *Streptomyces griseus* Protease C from Other Chymotrypsin Genes in *Streptomyces griseus* IFO13350

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Abstract The *sprC* gene encodes *Streptomyces griseus* protease C (SGPC), a bacterial chymotrypsin-like serine protease. Because the published data on *sprC* was not complete, we cloned and analyzed a new DNA fragment spanning downstream to upstream of the *sprC* gene from *S. griseus* IFO13350. The cloned 2.3-kb DNA fragment was placed on a high-copy number plasmid and introduced into *Streptomyces lividans* TK24. Chymotrypsin activity of the transformant was 8.5 times higher than that of the control after 3 days of cultivation and stably maintained until 9 days of cultivation, which clearly indicated that the cloned 2.3-kb fragment contained the entire *sprC* gene with its own promoter. When the same construct was introduced in the *S. griseus* IFO13350 (wild strain) and its two mutant strains in the A-factor regulatory cascade, Δ*adpA* and HO1, the chymotrypsin activity increased fivefold only in the Δ*adpA* strain. Transcriptional analysis based on RT-PCR revealed that the *sprC* gene is normally transcribed in both strains; however, earlier transcription was observed in the wild strain compared with the Δ*adpA* strain. A gel mobility shift assay showed that the AdpA protein did not bind to the promoter region of *sprC*. All these data clearly indicate that the expression of *sprC* is not dependent on the AdpA protein, but is distinctly regulated from other chymotrypsin genes composing an AdpA regulon. Earlier morphological differentiation was observed in *S. lividans* TK24, and *S. griseus* IFO13350 and HO1, transformed with the expression vector. The transformant of *S. griseus* Δ*adpA* formed markedly larger colonies. Antisense repression of *sprC* resulted in severe decrease of chymotrypsin activity, down to one-third of the control, and delayed morphological differentiation. All these data suggest that SGPC is related to normal morphogenesis in *S. griseus*.

Key words: *sprC*, *Streptomyces griseus*, SGPC, serine protease, bacterial chymotrypsin

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Streptomycetes are Gram-positive soil bacteria that produce many kinds of secondary metabolites and proteases, and exhibit unique characteristics of morphological differentiation developing into spores [3]. *Streptomyces griseus* is a producer of not only streptomycin and anthracyclines [11], but also Pronase, a commercially available product of proteases [33]. Owing to its industrial importance, *S. griseus* has become a representative model strain whose regulatory cascade has been intensively studied for secondary metabolism and morphogenesis [3, 4]. A-factor (2-isocapryloyl-3R-hydroxymethyl-gamma-butyrolactone) acts as a switch for secondary metabolite formation, such as streptomycin and yellow pigment production, and aerial mycelium formation at a very low concentration by inducing the expression of a specific activator protein: AdpA [13]. AdpA recognizes and binds to a specific promoter region and activates the transcription of target genes. Many genes controlled by AdpA have been elucidated and are grouped as members of the AdpA regulon [29, 31].

To date, several genes thought to be controlled by AdpA have been identified, such as *sprA*, *sprB*, *sprC*, and *sprD*, which encode *S. griseus* protease A (SGPA), *S. griseus* protease B (SGPB), *S. griseus* protease C (SGPC), and *S. griseus* protease D (SGPD), respectively [7, 12, 27, 28]. SGPA, SGPB, SGPC, and SGPD are closely related and are classified into a chymotrypsin-like serine protease [28, 30]. The *sprA*, *sprB*, and *sprD* genes are composed of the AdpA regulon in *S. griseus*, and their expression is dependent on A-factor at the transcription level via AdpA [29]. These three genes are efficiently overexpressed in *S. lividans*, with concomitant stimulation of actinorhodin production or morphological changes; however, their overexpressions are tightly regulated in *S. griseus* by unknown factor(s) regardless of the presence of A-factor [5, 6, 21]. Recently, the expressions of *sgmA* encoding a metalloprotease and *sprT/U* encoding trypsin-like serine proteases were reported as new members of the

AdpA regulon in the same strain [15, 16, 32]. In addition, the *sgmA* disruptant showed delayed sporulation in *S. griseus*, implying the importance of proteases in morphological differentiation [16]. Although genetic and molecular studies on *sprA*, *sprB*, and *sprD* chymotrypsin genes have been conducted, a detailed molecular study of the *sprC* gene has not yet been performed. We therefore describe the cloning, overexpression, and molecular study of the *sprC* gene in this paper.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

The *S. griseus* IFO13350, *S. griseus* HO1, and *S. griseus* Δ*adpA* were all obtained from Prof. S. Horinouchi of the University of Tokyo, Japan [13]. *Escherichia coli* DH5αF' was used for the general cloning experiments and pT7-T vector was used for the cloning of PCR products. The *Streptomyces-E. coli* shuttle vector pWHM3 was used for overexpression in *Streptomyces* strains, and plasmid pUWL201 was used for the inactivation of the *sprC* gene by the antisense RNA transcript [17].

Media and Culture Conditions

Escherichia coli was maintained and cultivated at 37°C in Luria-Bertani (LB) medium [26]. *Streptomyces* strains were maintained on R2YE agar medium [17] and cultivated in R2YE liquid broth at 28°C for the preparation of protoplasts, isolation of plasmid DNAs and RNAs, and protease assay.

Enzymes and Chemicals

Restriction endonucleases and other DNA-modifying enzymes were purchased from Takara Shuzo Inc. (Tokyo, Japan), and other chemicals were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Primers for PCR-based cloning were purchased from DyneBio Inc. (Seoul, Korea).

DNA Manipulations

DNA preparation and manipulation were performed using the method of Sambrook *et al.* [26] for *E. coli* and Kieser *et al.* [17] for *Streptomyces*. DNA samples were digested with restriction endonucleases and ligated with T4 DNA ligase according to the supplier's recommendations. Plasmid DNAs and their digests were analyzed by horizontal agarose gel electrophoresis with the TAE buffer system.

Transformation Procedure

Competent cells of *E. coli* for transformation were routinely prepared according to the frozen storage protocol, and transformation was performed as described by Hanahan [10]. Protoplasts of *Streptomyces* were prepared as per Okanishi *et al.* [25]. *Streptomyces* protoplasts were transformed using the PEG-mediated transformation method [17].

Cloning of the *sprC* Gene

Streptomyces griseus IFO13350 was the original source of genomic DNA for the cloning experiments, and genomic DNA was isolated following a standard procedure [17]. Genomic cosmid libraries were prepared in Supercos-1 (Stratagene, La Jolla, CA, U.S.A.) according to the manufacturer's protocol using 30–40-kb genomic DNA partially digested with Sau3AI. The cells with genomic cosmid libraries were incubated on LB agar plates containing ampicillin. To amplify the 1.4-kb *sprC*-containing DNA fragment from the genomic DNA, two 25-bp primers were designed based on the distal ends of the reported nucleotide sequence from *S. griseus* IMRU3499 (GenBank Accession No. L29018). The PCR products amplified using the degenerate primers were labeled with a nonradioactive DNA-labeling and detection kit (Boehringer Mannheim, Mannheim, Germany) and used for hybridization. The genomic library was screened by PCR using the same primers and confirmed by Southern hybridization. Positive clones were subcloned and nucleotide sequences were determined using an ABI model 373 DNA sequencer (Applied Biosystems Inc., Foster City, CA, U.S.A.).

Construction of Expression Vector for the *sprC* Gene

The 1.4-kb fragment containing the *sprC* gene was cloned by PCR as described above. The 2.3-kb fragment for the complete *sprC* gene was also cloned by PCR with the following primers designed from our sequence data: forward primer, 5'-TGAAGCTCGCGCAACAACCGGGCCA-3'; reverse primer, 5'-CGTCGCACTCCGGGGTCGGGGCCAG-3'. The 1.4-kb and 2.3-kb PCR products were then cloned into T-vector, digested with EcoRI-HindIII restriction enzymes, and ligated into pWHM3 digested with the same restriction enzymes, resulting in the recombinant plasmids pWHM3-C1.4 and pWHM3-C2.3, respectively (Fig. 2).

Construction of Antisense Repression Vector for *sprC*

To study the function of the *sprC* gene in *S. griseus*, a recombinant plasmid was designed to inactivate *sprC* activity by transcribing the antisense strand of *sprC*. The 1.7-kb internal fragment of *sprC* generated by BamHI-EcoRI restriction enzymes was inserted into pUWL201, a *Streptomyces-E. coli* shuttle vector, in an orientation so that the antisense strand of *sprC* would be transcribed by the *ermE* promoter. The resulting plasmid was named pUWL201-antiC.

Sample Preparation for Protease Assay

The transformants of *Streptomyces* harboring various recombinant plasmids were grown in 100 ml of R2YE media in 500-ml baffled flasks at 28°C with vigorous shaking at

250 rpm. After 2 days of cultivation, 10 ml of culture broth was inoculated into 100 ml of R2YE liquid media in 500-ml baffled flasks under the same conditions. Five ml of culture broth was sampled every day and centrifuged at 5,000 rpm for 10 min. The supernatants were used for protein concentration and protease activity measurements, and the cell pellets were used for the quantitation of proteins after cell disruption by sonication. Protein concentrations were measured by the Bradford method with bovine serum albumin used as the standard [1].

Determination of Chymotrypsin Activity

Chymotrypsin activity was measured spectrophotometrically by the release of *p*-nitroaniline using *N*-succinyl-ala-ala-pro-phe-*p*-nitroanilide as an artificial chromogenic substrate [20]. The reaction mixture containing 890 µl of 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 for reaction with 100 µl of enzyme solution in the same manner. After reacting for 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 in the above conditions.

RT-PCR Analyses

Total RNA was prepared from *S. griseus* IFO13350 and Δ*adpA* cultured in R2YE broth at 28°C for 48, 72, and 96 h (RNeasy plant mini kit; Qiagen, Hilden, Germany), and then first-strand cDNA was synthesized with the total RNA using the Superscript III first-strand synthesis system (Invitrogen, Carlsbad, CA, U.S.A.) following the manufacturer's protocol, and used for PCR. *sprC* mRNA was detected by RT-PCR with *sprC*-specific primers. A pair of *hrd* gene-specific primers [2] was used for a control PCR. The gene-specific primers used were as follows: *sprC*-5', 5'-CGCGCAACAACCGGGCCAGC-3'; *sprC*-3', 5'-GGCG-GACGCCACGCCGGTGA-3'; *hrd*-5', 5'-TCGATGAGC-GCCATCACAGACTCG-3'; *hrd*-3', 5'-TCGGCCCATTT-CACGTATGAG-3'.

Results and Discussion

Cloning of the *sprC* Gene from *S. griseus* IFO13350

The *sprC* gene encoding SGPC has already been identified on the basis of its homology to SGPB in *S. griseus* IMRU3499 [27]. The *sprC* gene encodes a 457-amino acid prepro-mature protein, and the 255 carboxyl-terminal amino acids are secreted as the mature enzyme. The amino-terminal domain is homologous to *S. griseus* proteases A, B, and E and the α-lytic protease of *Lysobacter enzymogenes*, but the carboxyl-terminal domain is not homologous with any known protease, although it shares extensive homology with chitin-binding domains of *Bacillus circulans* chitinases A1 and D [27].

For a functional study of SGPC, the 1.4-kb DNA fragment containing the *sprC* gene was cloned by PCR, based on the reported DNA sequence from *S. griseus* IMRU3499 (GenBank Accession No. L29018). Based on the G/C plot analysis [14], the reported sequence was assumed not to contain the proper promoter region as was previously reported [27]. When the 1.4-kb fragment was inserted into pWHM3, a *Streptomyces–E. coli* shuttle vector, and introduced into *S. lividans*, the transformant did not exhibit a major increase in chymotrypsin activity, indicating the absence of the promoter region in the 1.4-kb fragment (Fig. 2). We therefore cloned the entire *sprC* gene, including the promoter region.

A genomic library was constructed from the genomic DNA of *S. griseus* IFO13350, and one positive clone was identified by PCR and Southern hybridization. The 565-bp upstream and 312-bp downstream regions of the reported *sprC* gene were completely sequenced and registered in GenBank (GenBank Accession No. AF515832). Frame-plot analysis of the entire sequence revealed the presence of a complete reading frame with a putative promoter region, and the deduced amino acid sequence of the coding region was the same as that of the reported *sprC* gene (Fig. 1).

Overexpression of the *sprC* Gene in *S. lividans* TK24

To confirm that the cloned DNA contains the *sprC* gene with its own promoter, an expression vector, pWHM3-C2.3, was constructed by inserting the 2.3-kb DNA fragment into pWHM3, and introduced into *S. lividans*

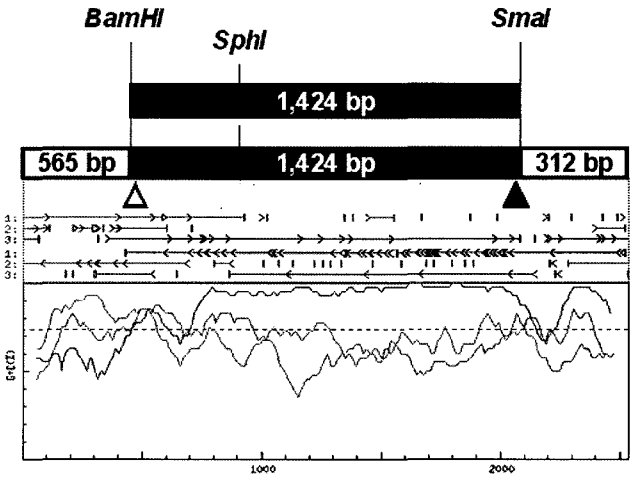

Fig. 1. Comparative restriction maps of the 1.4-kb DNA fragment with the newly cloned 2.3-kb fragment.
(Upper) The coding region of *sprC* and newly sequenced regions are depicted with black and open boxes, and the positions of initiation and termination codons are depicted with open and filled triangles, respectively. The number in the box indicates the length in nucleotides. **(Below)** Frame analysis of the 2.3-kb fragment by FramePlot version 2.2.1 shows the presence of a complete ORF.

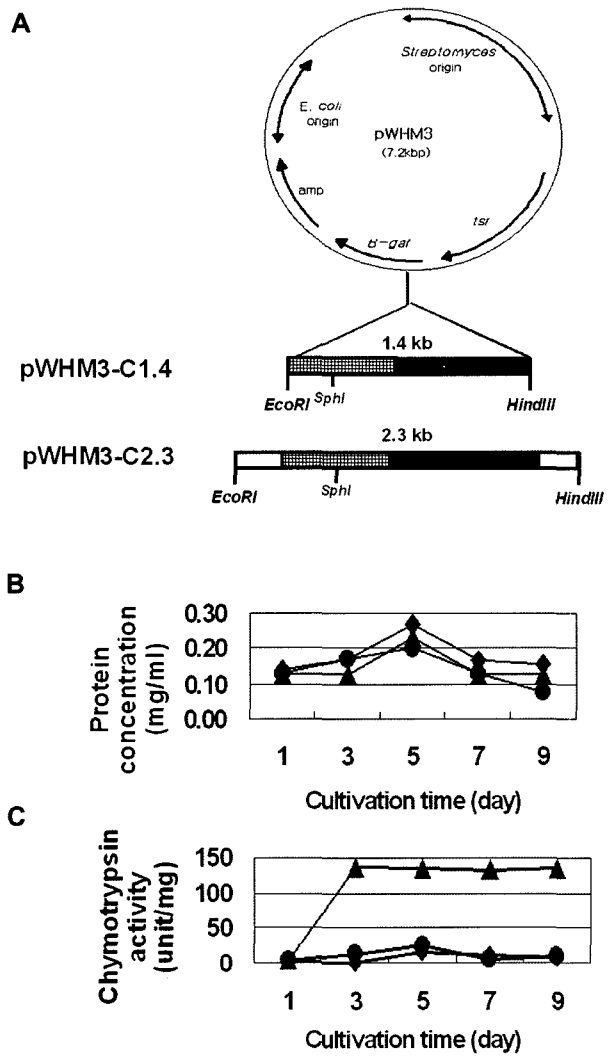

Fig. 2. Construction of expression vectors for *sprC* (**A**), and growth curves (**B**) and chymotrypsin activities (**C**) of the transformants.

A. Restriction maps of expression vectors pWHM3-C1.4 and pWHM3-C2.3. The black box indicates the *sprC* coding region. The organization of the structural gene, with pre-pro-peptide (202 amino acids, ▦) and mature protease (255 amino acids, ■) is shown in the map. Abbreviations: *amp*, ampicillin resistance; *tsr*, thiostrepton resistance; β-gal, β-galactosidase. **B**. Growth curve of the transformant in protein concentration (mg/ml) as a function of cultivation time. **C**. Chymotrypsin activity in units per mg of cellular protein of the transformant as a function of cultivation time. Transformants were grown at 28°C in R2YE medium and collected to measure protein concentration and chymotrypsin activity, as described in Materials and Methods. *Streptomyces lividans* TK24 harboring pWHM3 (◆-◆), pWHM3-C1.4 (●-●), and pWHM3-C2.3 (▲-▲), respectively.

TK24 (Fig. 2A). The transformant was cultured in R2YE medium containing 50 μg/ml thiostrepton, and a 5-ml sample was taken every day for measuring the cellular protein concentration and chymotrypsin activity. Although no significant difference in growth occurred (Fig. 2B), the chymotrypsin activity produced by the transformant reached its maximum level (136 units/mg protein) at 3 days of cultivation, and was 8.5 times higher than that of the control. This activity was stably maintained until 9 days of cultivation (Fig. 2C). The chymotrypsin activity of the transformant with pWHM3-C1.4 that contained the shorter *sprC* region, however, showed no difference compared with the control (Fig. 2C). These results clearly indicate that the 1.4-kb DNA fragment is not sufficient to encode the complete *sprC* gene to the point that it can be expressed, but the 2.3-kb fragment covers the entire *sprC* gene including its own promoter region.

Overexpression of the *sprC* Gene in *S. griseus*

Because the pWHM3-C2.3 was successfully used for overexpressing the *sprC* gene in *S. lividans* TK24, the same construct was introduced into *S. griseus* strains from which the *sprC* gene originated.

Streptomyces griseus is one of the best studied strains among the streptomycetes, especially regarding the regulation of secondary metabolism and morphogenesis by A-factor. A-factor induces aerial mycelium formation and secondary metabolite biosynthesis by binding to the ArpA receptor protein, which results in the release of the ArpA repressor from the ArpA-DNA complex [13]. From this, the *adpA* gene for an A-factor-dependent transcriptional activator is turned on, and AdpA activates multiple genes required for morphological development and secondary metabolism in a programmed manner. *Streptomyces griseus* HH1 is an A-factor-deficient mutant strain derived from strain IFO13350 by NTG-mutagenesis, which resulted in the loss of sporulation and the ability to produce streptomycin [11, 20]. *Streptomyces griseus* HO1, which produces streptomycin and forms aerial mycelium and spores, was derived from *S. griseus* HH1 by the single amino acid replacement of ArpA (A-factor receptor protein A). *Streptomyces griseus* Δ*adpA*, a disrupted mutant of *adpA* (A-factor dependent protein A) that is activated by the release of ArpA from the operator, can neither produce streptomycin nor form aerial mycelium in the same manner as HH1 [24].

The recombinant plasmid pWHM3-C2.3 was introduced into three strains of *S. griseus*: IFO13350 (wild-type), HO1, and Δ*adpA*. The level of chymotrypsin activity produced by *S. griseus* HO1 was 2.4-fold higher than that of *S. griseus* IFO13350 (1,627 and 690 units/mg protein, respectively); however, the *adpA*-negative strain (Δ*adpA*) showed chymotrypsin activity at a level one-fifth that of the wild strain (133 units/mg protein) at 9 days of cultivation. These results could be explained by the AdpA activator protein being constitutively produced, and thus, many protease-encoding genes of the A-factor regulon may be overexpressed in *S griseus* HO1 in contrast to the *adpA*-negative strain, resulting in increased protease activity in *S. griseus* HO1 (Fig. 3). In fact, AdpA can bind to the promoter region of *sprA, sprB*, and *sprD* chymotrypsin genes and regulates their expression at the transcription

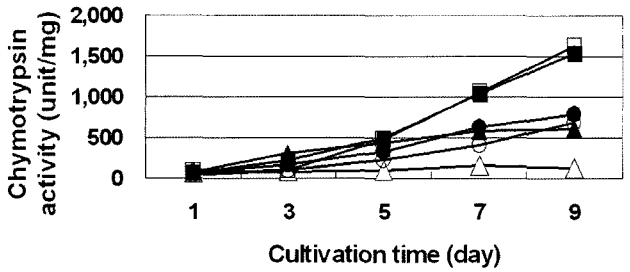

Fig. 3. Chymotrypsin activity in unit per mg of cellular protein of the transformant as a function of cultivation time.
Streptomyces griseus strains were grown at 28°C in R2YE medium, and then chymotrypsin activities of the culture broths were measured at a given cultivation time. ○-○, *S. griseus* IFO13350 (pWHM3); ●-●, *S. griseus* IFO13350 (pWHM3-C2.3); △-△, *S. griseus ΔadpA* (pWHM3); ▲-▲, *S. griseus ΔadpA* (pWHM3-C2.3); □-□, *S. griseus* HO1 (pWHM3); ■-■, *S. griseus* HO1 (pWHM3-C2.3).

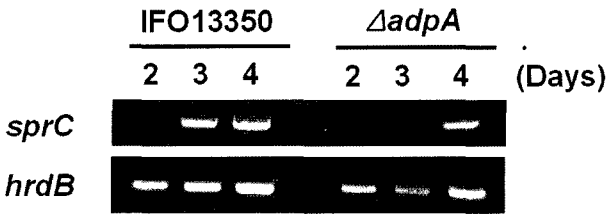

Fig. 4. Transcriptional analysis of *sprC*.
The time course of *sprC* transcription was followed by RT-PCR with RNA prepared from cells grown at 28°C for the indicated days in R2YE broth. As a control, transcription of *hrdB* was also determined with the same RNA samples. *hrdB*, which is transcribed throughout growth, was used to check the purity and amount of RNA used [2].

level [29]. Unexpectedly, the gene dosage effect was not observed in both wild phenotypic strains (*S. griseus* IFO13350 and HO1) transformed with pWHM3-C2.3. It is difficult to explain this repression, but it may be due to some factors that are present in wild phenotypic strains that tightly regulate the overexpression of *sprC*. Another possibility is posttranslational modification of SGPC by some protease inhibitors or proteases in wild phenotypic strains. In contrast, the introduction of pWHM3-C2.3 in the *ΔadpA* strain restored the level of chomotrypsin production to that of the wild strain, which strongly suggests that the expression of *sprC* is not dependent on the AdpA protein and that the *sprC* gene does not belong to the A-factor regulon.

Transcriptional Analysis of the *sprC* Gene in *S. griseus*

AdpA activates a number of genes required for morphological and physiological differentiation, resulting in an AdpA regulon [24]. The *strR* (a pathway-specific transcriptional activator for streptomycin biosynthetic genes), *adsA* (ECF sigma factor of RNA polymerase essential for aerial mycelium formation), *sgmA* (a metalloendopeptidase involved in degradation of substrate hyphae), *ssgA* (a small acidic protein for spore septum formation), and *amfR* (essential for aerial hyphae formation) genes have been identified as members of the AdpA regulon [24]. Recently, two trypsin genes (*sprT* and *sprU*), and three chymotrypsin genes (*sprA*, *sprB*, and *sprD*), were added to the AdpA regulon [15, 16, 29].

The data for overexpression in the *S. griseus ΔadpA* strain suggested to us that the *sprC* gene may not be a member of A-factor regulon, and transcriptional analysis of the *sprC* gene was performed to confirm this hypothesis. Total RNAs were extracted from *S. griseus* IFO13350 and *ΔadpA*, and the *sprC* transcripts were analyzed by RT-PCR (Fig. 4). The transcript was detected at 3 days of cultivation in both strains, but stronger transcription was observed in the wild strain at 3 days of cultivation compared with *ΔadpA*. However, the two strains gave similar strong signals for the *sprC* transcript at 4 days of cultivation in R2YE broth, which supports our idea that the *sprC* gene is not a member of the AdpA regulon. At present, it is difficult to give a plausible explanation for the abundant transcription of *sprC* at 3 days of cultivation. However, strains that can sporulate and synthesize secondary metabolites must normally be different in terms of their gene expression and physiological conditions, compared with AdpA-negative strains that cannot sporulate or synthesize the secondary metabolites. The difference in the cellular physiological state may induce earlier and abundant transcription of the *sprC* gene in the wild strain of *S. griseus*.

The promoter region of *sprC* did not give any positive signal for AdpA binding in the gel mobility shift assay (data not shown). In addition, we could not find any consensus sequence for the AdpA binding site (5'-TGGC[G/C]-G[A/T][A/T][T/C]-3') in the promoter region of the *sprC* gene [31]. We therefore concluded that the expression of *sprC* is regulated in a different way from that of the AdpA regulon in *S. griseus*.

Effects of Overexpression of the *sprC* Gene on Morphology

The colony of *S. lividans* transformed with pWHM3-C2.3 showed earlier morphological differentiation by day 1, and the degree of sporulation was denser than that of the control (Fig. 5, Upper). In addition, the production of actinorhodin, whose production is unusual in *S. lividans*, was also induced by the overexpression of *sprC* (data not shown). These results imply that the overexpression of *sprC* stimulates morphological and physiological differentiation in *S. lividans*.

Similarly, earlier and denser sporulation was also observed in *S. griseus* IFO13350 and HO1 strains transformed with pWHM3-C2.3 (Fig. 5, middle). The colonial size of the transformants was also increased by more than twofold in *S. griseus* strains (Fig. 5, middle and lower). These observations indicate that the overexpression of

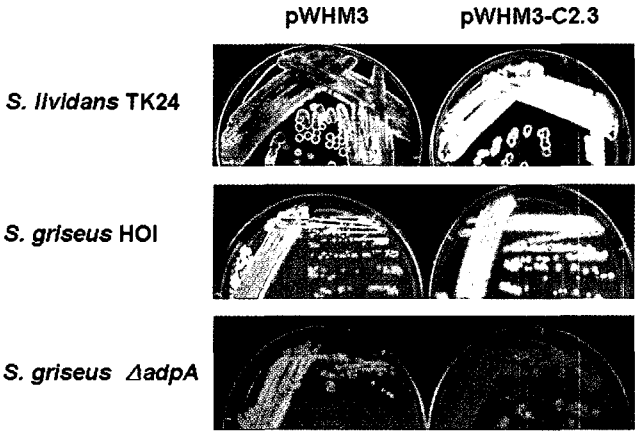

Fig. 5. Gene dosage effect of *sprC* on morphological differentiation of *Streptomyces lividans* and *S. griseus*.
Three strains, *S. lividans* TK24 and *S. griseus* HO1 and Δ*adpA* mutants, were grown at 28°C on R2YE agar medium for 6 days, and morphological changes were compared with the cells transformed with pWHM3-C2.3. Earlier and denser morphological differentiation in *S. lividans* and the formation of bigger colonies in *S. griseus* were observed in the transformants with pWHM3-C2.3.

sprC also affects the morphological differentiation in *S. griseus*.

Antisense Repression of *sprC*

To confirm whether the *sprC* gene plays critical roles in morphological differentiation of *Streptomyces*, the effect of *sprC* gene repression was examined by using the antisense RNA technique in *S. griseus*. The recombinant plasmid pUWL201-antiC that was designed for the strong transcription of the antisense strand of *sprC* by the *ermE* promoter was transformed into *S. griseus* IFO13350, and its ability to inhibit the production of chymotrypsin was measured (Fig. 6A). The chymotrypsin activity of the transformant with pUWL201-antiC decreased to less than one-third that of the control (vector only), indicating that the antisense strand is transcribed and effectively inhibits the expression of *sprC* (Fig. 6B). The transformant showed a delayed morphogenesis by day 2, which suggests that SGPC is necessary for normal morphogenesis (Fig. 6C).

It has been proposed that many hydrolytic enzymes, such as proteases, nucleases, and lipases, are required for the degradation and reuse of cytoplasmic contents of substrate mycelium at the beginning of aerial mycelium formation [22]. Several reports have supported the close relationship between extracellular serine proteases and morphological differentiation [8, 9, 18, 19, 23]. However, the serine protease responsible for morphological differentiation has not yet been described. Even the multiple mutants, such as *S. griseus* Δ*sprABD* and Δ*sprTU*, were found to form aerial hyphae and spores normally [15, 29]. It was reported, however, that the mutant Δ*sgmA* showed a delayed differentiation by day 1 in *S. griseus*

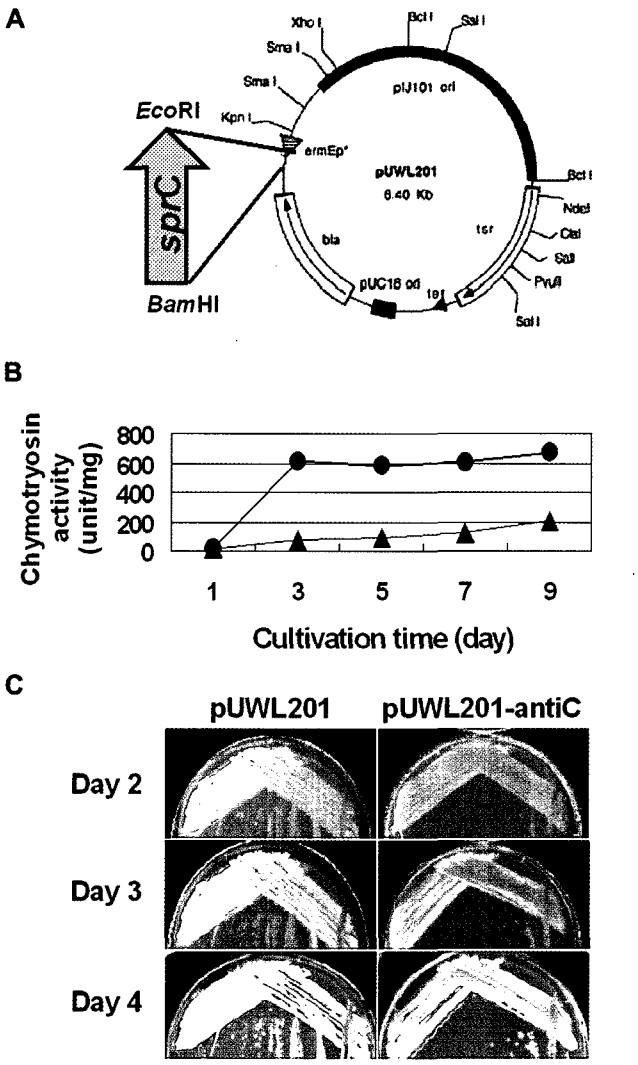

Fig. 6. Construction of antisense repression vector for *sprC* (**A**), and effects of antisense repression on SprC activity (**B**) and the morphological differentiation (**C**) of *Streptomyces griseus* IFO13350.
A. Construct of an antisense repression vector, pUWL201-antiC. The antisense repression vector was constructed by inserting a 1.7-kb internal fragment of *sprC* so that the antisense RNA of *sprC* could be transcribed from the strong *ermE* promoter in *Streptomyces*. **B**. Effects of antisense repression on SprC chymotrypsin activity. The transformation of pUWL201-antiC resulted in a severe decrease of chymotrypsin activity in *S. griseus*, which suggested that antisense repression is an effective way to inactivate *sprC* activity. **C**. Effects of antisense repression the on morphological differentiation of *S. griseus* IFO13350. Antisense repression of *sprC* resulted in delayed morphological differentiation.

[16], which is very similar to our results for *sprC* repression. The presence of many chymotrypsins and trypsins in *S. griseus* may compensate for the defects in protease activity in the mutants, which may lead to difficulties in finding the phenotypic differences in these mutants. Combinatorial studies on proteases might provide some clues as to the role of the proteases in morphological differentiation.

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