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Functional Anaylsis of *sprD* Gene Encoding *Streptomyces griseus* Protease D (SGPD) in *Streptomyces griseus*

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Abstract The chromosomal *sprD* gene encoding *Streptomyces griseus* protease D (SGPD), a chymotrypsin-like protease, was disrupted in *Streptomyces griseus* by insertion of the neomysin-resistance gene. The production of chymotrypsin activity of *sprD* disruptant was not completely abolished, but delayed by 24 h, compared with that of wild-type strain. The aerial mycelial formation of *sprD* disruptant was retarded, and specifically the formation of spores was not observed in the central region of colonies. However, normal morphological development into spores was observed in the marginal region of colonies. In addition, the production of yellow pigment that might be dependent on A-factor was also decreased in the *sprD* disruptant, compared with that of the wild-type strain. Introduction of the *sprD* gene, which was placed on a high copy-numbered plasmid into *S. griseus* Δ*sprD*, partially restored the ability of morphological development, and a significant level of sporulation was observed. When the overexpression vector for *sprD*, pWHM3-D, was introduced in *S. griseus*, there was no significant change in the chymotrypsin activity or colonial morphology, in contrast to *Streptomyces lividans*, indicating the presence of a tight regulation system for the overexpression of the *sprD* gene in *S. griseus*.

Key words: *sprD*, SGPD, *S. griseus*, morphogenesis, secondary metabolism

Pronase is one of the commercially available products of proteases produced by *Streptomyces griseus*, which is a Gram-positive soil bacterium that produces many kinds of secondary metabolites and proteases [19]. To date, several genes such as *sprA*, *sprB*, *sprC*, and *sprD*, which encode for *S. griseus* protease A (SGPA), *S. griseus* protease B (SGPB), *S. griseus* protease C (SGPC), and *S. griseus* protease D (SGPD), have been cloned [6, 13, 16]. SGPA, SGPB, SGPC, and SGPD are closely related and classified into chymotrypsin-like serine proteases [19].

S. griseus is a representative strain that has been intensively studied for the regulatory cascade including A-factor (2-isocapryloyl-3R-hydroxymethyl-gamma-butyrolactone), which leads to morphogenesis and secondary metabolite formation [8]. A-factor acts as a switch for secondary metabolite formation, such as streptomycin and yellow pigment production, and aerial mycelium formation at a very low concentration in cooperation with its specific receptor protein, ArpA. Recently, the expressions of *sgmA* encoding a metalloprotease and *sprT* encoding a trypsin-like protease were reported to be regulated by the A-factor [10, 11]. In addition to A-factor, several regulatory cascades, including stringent factors and protein kinases, were reported to be involved in the regulation of antibiotics production and morphological differentiation of *Streptomyces* [8, 18].

In a previous study, we isolated the *sprA*, *sprB*, *sprD*, and *sprT* genes from *S. griseus,* and showed that the heterologous expression of the *sprD* gene induced the production of the pigmented antibiotic actinorhodin in *S. lividans* [2, 3]. These observations strongly suggested that the *sprD* gene might be very important in the physiological or morphological differentiation of *Streptomyces*, which prompted us to study the intracellular function of *sprD* by gene disruption and overexpression in its original strain. First, the disruption of the chromosomal *sprD* gene was carried out by a single crossover homologous recombination.

The 900 bp of internal region of the *sprD* gene was amplified by PCR [15], and two primers (forward primer; 5'-GAATTCGCGCCCCATCCTCCGTGTA-3', reverse

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primer; 5'-GGATCCAGTTGACCGTGGCGTCCAG-3') were designed with a little modification of the original sequence to have EcoRI and BamHI restriction enzyme sites, respectively. The PCR fragment was digested with EcoRI and BamHI, and ligated into pUC18 to give a recombinant plasmid, pUC18-D. The selection marker, a neomycin-resistance gene, was cut from pFD-NeoS plasmid [5] and inserted into BamHI/HindIII sites of the pUC18-D, which resulted in pUC18-Dneo, a streptomycetes suicide vector (Fig. 1A). Competent cells of *E. coli* strains for transformation were routinely prepared according to the frozen storage protocol, and transformation was done by the method described by Hananhan [4].

The pUC18-Dneo was denatured in alkaline condition by adding 2 μl of 1 N NaOH for 30 min, neutralized with 1 N HCl, and then used for the protoplast transformation of *S. griseus* IFO 13350. Protoplasts of *Streptomyces* were prepared as described by Okanishi *et al.* [17], by using the PEG-mediated protoplast transformation method [7]. The transformant was selected by overlaying neomycin solution

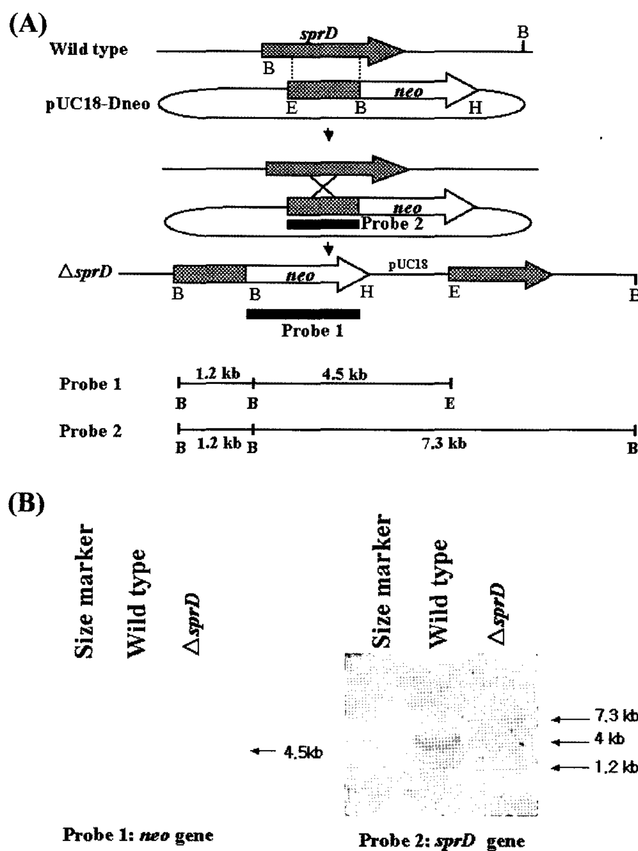

Fig. 1. Disruption of the chromosomal *sprD* gene.
A. Schematic representation of disruption of *sprD* on the chromosome of *S. griseus* IFO13350. The size of expected fragments of the disruptant with probe 1 and probe 2 is indicated. B. Southern hybridization to check the correct disruption of *sprD* with the indicated probe 1 (*neo* gene) and probe 2 (*sprD* gene) against the BamHI/EcoRI-digested and the BamHI-digested chromosome, respectively. Abbreviations: E, EcoRI; B, BamHI; H, HIndIII; *neo*, neomycin resistance.

(final concentration, 10 μg/μl) after incubation for 14 h at 28°C. It was expected that the disruptant of the *sprD* gene could be generated by a single crossover homologous recombination event between the internal fragment of the *sprD* gene in the chromosomal DNA and in the suicide vector.

After regeneration of the protoplasts, three candidates with neomycine resistance were obtained. Southern hybridization technique was adopted to confirm the disruptant [9]. When the neomycin resistance gene was applied as a probe (probe 1) to the chromosomal DNA digested with BamHI and EcoRI, a strong positive signal corresponding to 4.5 kb was detected only in the gene disruptant (Fig. 1B). When the 900 bp of internal region of the *sprD* was used as a probe (probe 2) for the BamHI-digested chromosomal DNAs, the hybridized signal with 1.2 kb could be detected with an extra weak signal corresponding to 7.3 kb (Fig. 1B). Judging from the single band with 4 kb that was detected in the wild-type strain, it was concluded that one additional BamHI site was present in an unidentified downstream region of the *sprD* gene, which yielded 4 kb and 7.3 kb signals in the wild-type strain and gene disruptant, respectively. From these results, the candidate was confirmed as the *sprD* disruptant and named as *S. griseus* Δ*sprD*.

S. griseus IFO 13350 and *S. griseus* Δ*sprD* were cultured in YMPD medium (yeast extract, 0.2%; meat extract, 0.2%; bactopeptone, 0.4%; NaCl, 0.5%; $MgSO_4 \cdot 7H_2O$, 0.2%; glucose, 1%; pH 7.2), and the chymotrypsin activity of the two strains was compared at every 12-h intervals after inoculation. Chymotrypsin activity was measured spectrophotometrically by the release of ρ-nitroaniline using N-succinyl-ala-ala-pro-phe-ρ-nitroanilide as an artificial chromogenic substrate [12]. 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-ρ-nitroanilide in DMSO was used with 100 μl of enzyme solution in the same manner. After the reaction for 15 min, the chymotrypsin activity was measured at 405 nm. One unit of chymotrypsin was defined as the amount of enzyme corresponding to 0.1 increase in absorbance in the above conditions. The total protein concentration was measured by the method of Bradford [1] to show the growth of wild-type strain and Δ*sprD*, indicating that their growths were not different (Fig. 2A). Meanwhile, the production of chymotrypsin activity of *sprD* disruptant was delayed by 24 h in the YMPD medium, as shown in Fig. 2A. However, its activity after 72 h of cultivation was the same in both strains. These results suggest that disruption of *sprD* does not give any difference in total chymotrypsin activity after the stationary growth stage, because other chymotrypsin-encoding genes, such as *sprA*, *sprB*, and *sprC*, might be overexpressed to compensate for the lost SGPD activity in *S. griseus* Δ*sprD*. Similar results were also observed in the case of *sprA* and *sprB* disruptants

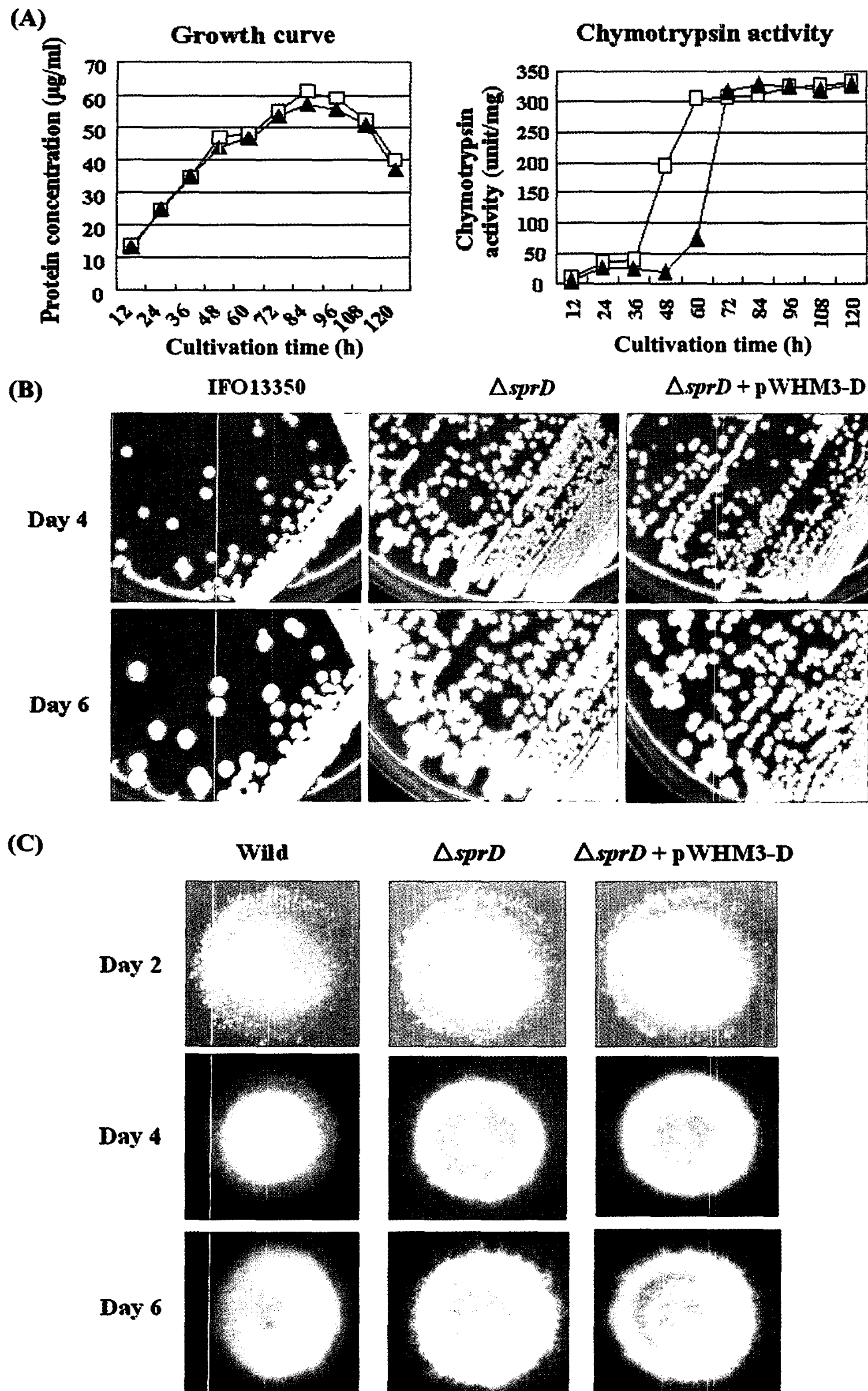

Fig. 2. Chymotrypsin activity and morphological change in the *sprD* disruptant.
A. Chymotrypsin activity of the Δ*sprD* mutant as a function of cultivation time. (*right*) Growth curve in protein concentration (μg/ml). (*left*) Chymotrypsin activity in unit per mg of cellular protein. Strains were grown at 28°C in YMPD medium. □–□, *S. griseus* IFO13350 as a control; ▲–▲, Δ*sprD* mutant. **B.** Photographs of the surface of the plate. **C.** Photographs of the surface of the colony. Three strains, including *S. griseus* IFO13350 (wild-type), Δ*sprD* mutant, and Δ*sprD* mutant transformed with pWHM3-D, were grown at 28°C on YMPD agar medium.

(personal communication with Prof. S. Horinouchi, The University of Tokyo).

Phenotypic differences between wild-type and *sprD* disruptant on YMPD medium were observed. The aerial mycelium formation of *sprD* disruptant was retarded significantly, and the frequency of sporulation became especially rare, compared with that of the wild-type strain (Fig. 2B). In particular, the morphology of colony of *sprD* disruptant was dramatically changed. Whereas the colony surface of the wild-type strain showed a smooth surface with equal distribution of spores on the entire surface (Fig. 3C), that of *sprD* disruptant seemed to be bold in the central region of the colony; furthermore, the distribution of the spores were restricted in the marginal surface of the colony (Fig. 2C). The restoration of the *sprD* disruptant was attempted by the introduction of pWHM3-D plasmid that has the entire *sprD* gene on a high copy number plasmid (Fig. 3A). Although complete restoration of the morphological change was not observed, a significant degree of sporulation was restored in the central region of the colony (Fig. 2C). These results clearly indicate that the *sprD* gene plays some essential roles in the morphological differentiation, such as colony shape and morphogenesis, especially in the internal region of the colony.

In addition, the production of yellow pigment was also decreased in the *sprD* disruptant, compared with the wild-type strain, in YMPD liquid medium and R2YE solid medium (data not shown). However, there was no significant difference in the production level of streptomycin in the two starins (data not shown).

Previously, we reported that the introduction of the *sprD* gene, which was placed on pWHM3-D, induced overproduction of chymotrypsin activity 7 times more than that of control, and concomitantly triggered biosynthesis of the pigmented antibiotic, actinorhodin, in *S. lividans* TK24 [3]. These results suggested that overexpression of *sprD* could affect some physiological or morphological differentiation of *Streptomyces*, which made us introduce the *sprD* gene in *S. griseus* strains.

In *S. griseus*, A-factor switches on aerial mycelium formation and secondary metabolite biosynthesis. The A-factor can bind to the ArpA and make the receptor protein lose its ability as a repressor by releasing it from the ArpA-DNA complex [8]. By this event, 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. The recombinant plasmid pWHM3-D (Fig. 3A) was introduced into four strains of *S. griseus*, such as IFO 13350 (wild-type), HH1 (A-factor-deficient mutant), HO1 (A-factor receptor protein inactivated mutant), and ΔadpA (A-factor-dependent protein-A-disrupted mutant). There was little difference in the cell growth curves (data not shown) and chymotrypsin activities between the control

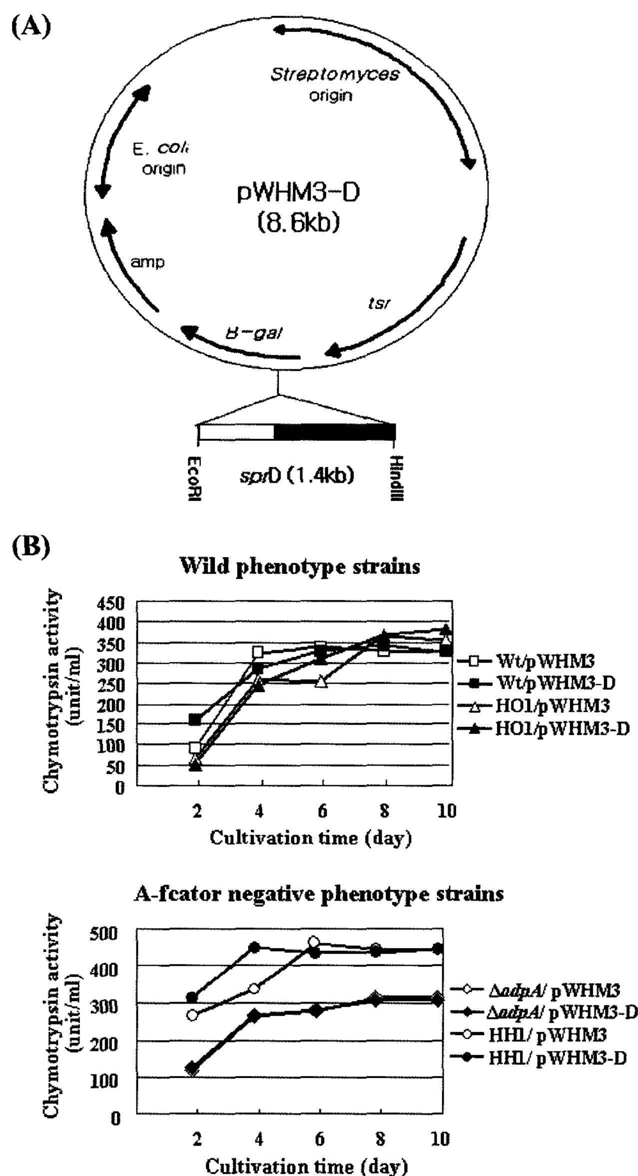

Fig. 3. Restriction map of expression vector pWHM3-D and production of the SGPD in *S. griseus*.
A. Restriction map of expression vector pWHM3-D. The thick line indicates the *sprD* coding region. The organization of the structural gene, with pre-pro peptide (204 amino acids, ■) and mature protease (187 amino acids, □) is shown below the map. Abbreviations: *amp*, ampicilline resistance; *tsr*, thiostreptone resistance; β-gal, β-galactosidase. **B.** Comparison of SGPD activity produced by *S. griseus* transformed with pWHM3-D. *S. griseus* strains, including IFO13350 (wild-type), HH1 (an A-factor-deficient mutant), HO1 (an inactivated mutant in A-factor receptor protein A), and ΔadpA (a disrupted mutant of A-factor-dependent protein A), were transformed and cultured in YMPD broth, as described in Materials and Methods.

(vector only) and transformants with pWHM3-D of all four kinds of strains, indicating that the gene dosage effects are clearly repressed in all four strains (Fig. 3B). The level of chymotrypsin activities of *S. griseus* IFO 13350 and HO1 were almost the same, which might be reasonable because *S. griseus* HO1 is phenotyphically identical with

IFO 13350 (Fig. 3B, upper). The chymotrypsin activity of *S griseus* HH1 was higher than those of *S. griseus* IFO 13350 and HO1, but it is impossible to interpret this data at present, because the strain HH1 was generated by the NTG mutagenesis that could lead to multiple random mutations in the chromosomal DNA (Fig. 3B, lower). In contrast, the *adpA*-negative strain (Δ*adpA*) gave significantly lower chymotrypsin activity than that of wild-type strain, which strongly suggests that the expression of *sprD* or some other chymotrypsin genes might be dependent on A-factor (Fig. 3B, lower). In a similar way, the *sprA* and *sprB* genes from *S. griseus* were successfully overexpressed in *S. lividans* TK24, but their overexpression in *S. griseus* failed also [14]. These results strongly suggest that the expressions of *sprA, sprB*, and *sprD* genes are tightly regulated in *S. griseus* by the factor(s) that is not present in *S. lividans*. Therefore, the possible regulatory cascade by A-factor and other factors for the expression of chymotrypsin genes should be elucidated in the future.

In addition, no phenotypic change was observed in the *sprD* transformant, since those of *sprA* and *sprB* transformants were the same [14]. These observations are in contrast to the observations that heterologous expression of *sprA, sprB,* and *sprD* genes in *S. lividans* affected morphological or physiological differentiation of the host [2, 3, 20]. The basal protease activity produced by *S. lividans* was equivalent to of 2% lower than that by *S. griseus* (data not shown). Therefore, *S. lividans* seems to be sensitive to the level of overexpressed protease so that some physiological and morphological changes could be induced. In *S. griseus*, however, the oevrexpression of chymotrypsin-encoding genes is tightly regulated, and the phenotypic changes cannot be induced.

The first evidence for the involvement of protease in the morphogenesis of *Streptomyces* was obtained from the *in vivo* treatment of protease inhibitors on *S. griseus* [12]. In particular, a serine protease inhibitor, pefabloc SC, and metalloprotease inhibitor, EDTA, severely inhibited the formation of aerial mycelium and spores, implying the importance of serine proteases and metalloproteases in the morphological development of *Streptomyces*. A *Streptomyces griseus* trypsin gene (*sprT*) and a zinc-containing metalloendopeptidase gene (*sgmA*) have been identified as new members of A-factor regulon that are regulated by binding of AdpA to AdpA-binding sites in the promoter regions [10, 11]. In the present study, the *sprT* and *sgmA* genes were transcribed in an AdpA-dependent manner, and their transcription was markedly enhanced at the time of aerial mycelium formation. Although the *sprT* and *sgmA* genes were expected to be responsible for morphological development, there was no phenotypic change in the disruptants, except delayed aerial hyphae formation by half a day in the *sgmA* disruptant. Therefore, we suggest that the *sprD* gene is one of the serine proteases that are involved in the morphological differentiation of *S. griseus*. The exact function and mechanism of the *sprD* product in the developmental process and the regulatory cascade for *sprD* expression by A-factor or other factors should be intensively studied in the near future.

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