CHARACTERIZATION OF SANNAMYCIN A-NONPRODUCING MUTANTS OF *Streptomyces sannanensis*

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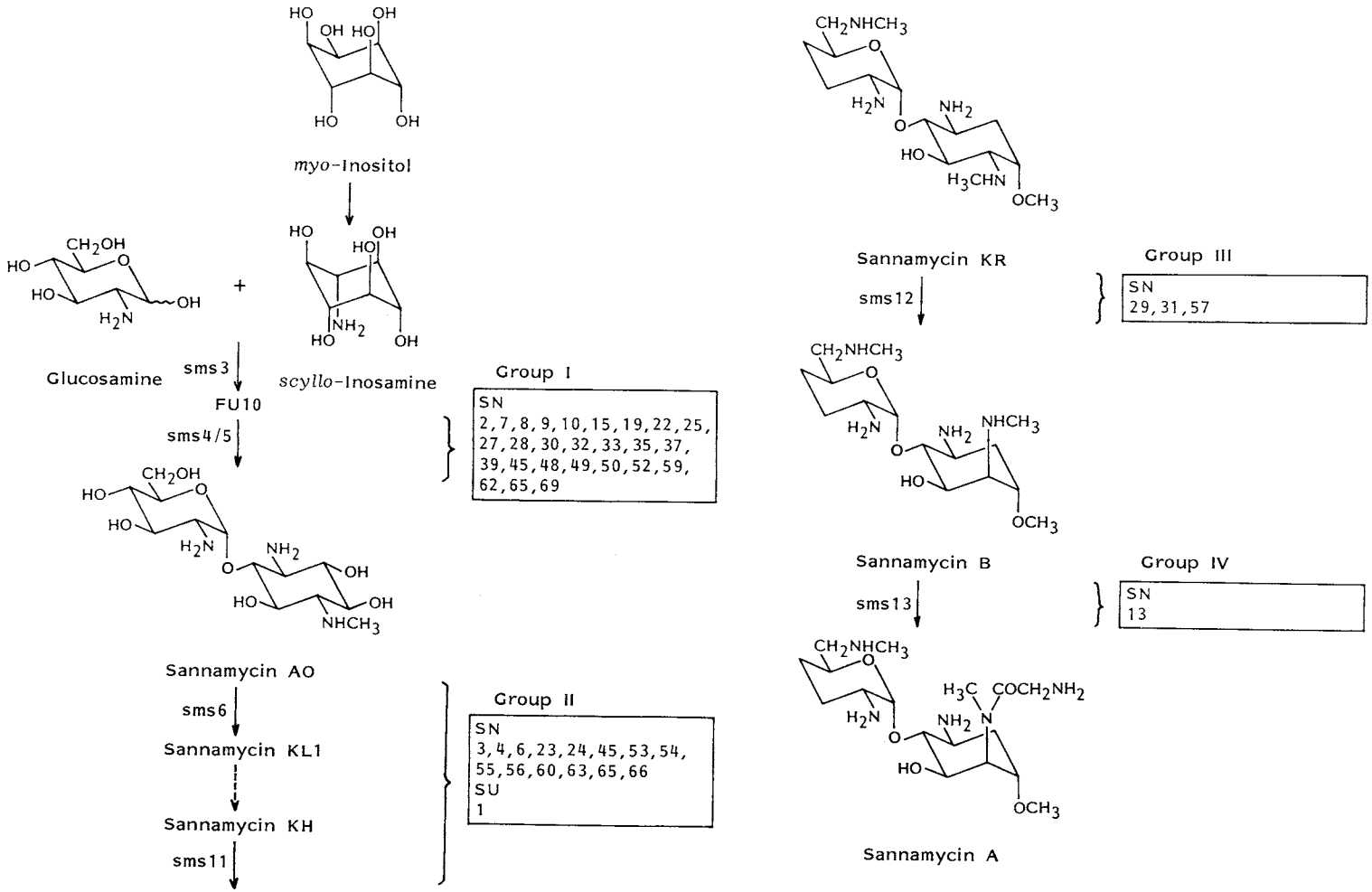
Gene cloning in *Streptomyces* has permitted the identification of a number of antibiotic-biosynthesis genes[1]. Complementation of nonproducing mutants is commonly used to identify specific antibiotic-biosynthesis genes. A serious difficulty is that many antibiotic-producing actinomycetes cannot be transformed efficiently by the conventional methods, *e.g.*, those developed for *Streptomyces lividans*[2]. *Streptomyces sannanensis* IFO 14239 is a case in point. We have examined conditions for protoplasting, regeneration and transformation, to establish a self-cloning system for *S. sannanensis*[3]. In the present study, we report the isolation of sannamycin A-nonproducing mutants of *S. sannanensis* and their classification on the basis of their ability to convert fortimicin A-biosynthetic precursors.

Spore suspension of *S. sannanensis* IFO 14239 (3×10^6 spores in 0.1 ml 0.7% NaCl solution) were irradiated with an UV lamp (15 W) at a distance of 20 cm for 30~60 seconds, or alternatively treated with 0.2~2.0 mg/ml of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) at room temperature for 30 minutes. The spores were spread on SY2 medium at 30°C for 3 days. SY2 medium consisted of yeast extract (Difco) 0.15% (w/v), peptone (Difco) 0.25%, malt extract (Difco) 0.15%, soluble starch 1.0%, glucose 0.5%, agar 2.2%, pH 7.2. Survival rates were 0.01~0.8% in UV-irradiation and 0.01~0.1% in MNNG-treatment, respectively. Survivors were transferred to SA medium. SA medium consisted of peptone (Difco) 0.02%, yeast extract (Difco) 0.02%, corn steep liquor 0.05%, NaCl 0.03%, $MgSO_4 \cdot 7H_2O$ 0.005%, corn starch 0.4%, agar 2.0%, pH 7.0. After incubation at 30°C for 3 days, the colonies were overlaid with B medium containing about 10^5 cells/ml of *Bacillus subtilis* ATCC 6633 at 42°C. B medium consisted of yeast extract (Difco) 0.2%, beef extract (Difco) 0.3%, Tryptone (Difco) 0.6%, Tris-HCl 1.2%, malate 0.12%, glucose 0.1%, agar 1.0%, pH 8.0. After incubation at 37°C for 16 hours, colonies not forming inhibitory zones were transferred to SY2 medium containing 50 μg/ml of fortimicin A (Kyowa Hakko Kogyo) and incubated at 30°C for 3 days to eliminate *B. subtilis*. Only one mutant, SU1, was obtained out of 4,545 survivors after UV irradiation. On the other hand, 80 nonproducing mutants, SN1 to SN80, were identified in 13,357 survivors of MNNG-treatment. Of these mutants, 14 were slow-growing, and 19 were "leaky" or reverted to productivity. In all, we obtained 48 stable nonproducing mutants.

The mutants were cultivated in SAM medium at 30°C for 3 days, and their metabolites in culture broth were analyzed by TLC[4]. SAM medium consisted of corn steep liquor 1.0%, yeast extract (Difco) 0.2%, NaCl 0.3%, $MgSO_4 \cdot 7H_2O$ 0.05%, Stabilose K (Matsutani Kagaku) 4.0%, soy bean meal (0.2%), pH 7.0. We found that a nonproducing mutant, SN13, accumulated sannamycin B (data not shown). Therefore, SN13 was considered to be blocked at the glycylation step of sannamycin B which corresponded to *fms13* of fortimicin A-biosynthesis in *Micromonospora olivasterospora*. SN13 was designated as "Group IV" mutant. We tried to classify the other mutants by co-synthesis tests on solid medium, but no combinations showed apparent restoration of antibiotic production. It could be that the mutants failed to accumulate sufficient precursor to be detected as co-synthesis activity.

We next examined the ability of mutants to convert biosynthetic precursors of fortimicin A in SA agar medium, since it had been shown that most precursors could be converted into subsequent intermediates in *S. sannanensis*[5]. Each mutant was grown on SA medium containing biosynthetic precursors of fortimicin A, *i.e.*, *myo*-inositol (100 μg/ml), *scyllo*-inosose (100 μg/ml), *scyllo*-inosamine (100 μg/ml), D-glucosamine (100 μg/ml), fortimicin AO (100 μg/ml) or fortimicin KR (15 or 50 μg/ml). After incubation at 30°C for 6 days, the colonies were overlaid with E medium containing about 10^5 cells/ml of *Escherichia coli* ATCC 26 at 42°C. E medium consisted of yeast extract (Difco) 0.25%, beef extract (Difco) 0.3%, Tryptone (Difco) 0.6%, Tris-HCl 1.2%, glucose 0.1%, agar 1%, pH 7.5, 8.0 or 8.5. After incubation at 37°C for 16 hours, appearance of inhibitory zones were observ-

Fig. 1. Classification of nonproducing mutants.



Proposed pathway of sannamycin A biosynthesis and structures of sannamycin A-biosynthetic precursors are presented. Dotted arrows indicate several biosynthetic steps. Mutants classified in this study are indicated according to group corresponding to the putative blocked biosynthetic step.

ed in some cases. No mutants produced antibacterial activities in the presence of *myo*-inositol, *scyllo*-inosamine or D-glucosamine. On the other hand, 26 mutants were restored to antibiotic formation when supplemented with fortimicin AO. These latter were assumed to be blocked at the steps before sms4/5, corresponding to fms4/5 converting step from FU10 to AO in *M. olivasterospora*. These mutants were designated as "Group I" mutants. On supplementing with fortimicin KR, 18 out of the remaining 21 mutants were restored to antibiotic production. They were presumably blocked at the steps between sms6 (corresponding to fms6; AO to KL1) and sms11 (corresponding to fms11; epimerization from fortimicin KH to KR). These mutants are designated as "Group II" mutants. As converting activity fortimicin B to A was detected in washed mycelia of the other three mutants, they correspond to mutants blocked at the step of sms12 (corresponding to fms12; KR to B). These mutants were designated as "Group III" mutants. Thus the 48 nonproducing mutants could be classified into four groups as shown in Fig. 1.

Group I mutants could be restored to production by supplementing with fortimicin AO; in spite of the fact that *S. sannanensis* could not convert fortimicin KH to KR (epimerization of 2-hydroxy group of fortamine moiety; the step of fms11) at the downstream step in fortimicin A biosynthesis[5]. This observation suggests that dehydroxylation of the 2-hydroxy group occurs at the step between sms6 and sms11 in *S. sannanensis*.

We found some mutants which lost their ability to sporulate among the leaky mutants. They might be defect in a factor regulating both secondary metabolism and differentiation like A-factor of *Streptomyces griseus*[6]. A low molecular weight factor, degnated as S-factor required for the fortimicin A production was found in *M. olivasterospora* (KASE; personal communication). We are concurrently analyzing the relationship of these pleiotropic mutants and S-factor.

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