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NOTE

Enhanced Production of the Fluorinated Nucleoside Antibiotic Nucleocidin by a *rif*^{R}-Resistant Mutant of *Streptomyces calvus* IFO13200

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Nucleocidin, a fairly broad antibacterial and trypanocidal agent produced by *Streptomyces calvus*, has the unique structure of 4′-α-fluoro-5′-*O*-sulfamoyl adenosine. This nucleoside antibiotic has been a target for organic synthesis in past decades; however, microbial large-scale production has not been established due to low yield and poor reproducibility. To activate the dormant secondary metabolism of *S. calvus*, we examined the effect of an *rpoB* mutation that was induced by ultraviolet light irradiation. The resulting rifampicin-resistant strains showed remarkably improved antibiotic activity, which was extracted by n-butanol and identified by Electron-Spray-Ionization Mass Spectrometry. DNA sequencing identified double mutations, C1309A and C1318A, in the *rpoB* gene (according to the numbering of *Streptomyces coelicolor rpoB*). The resulting amino acid substitutions, H437N and R440S, corresponded to two of the previously reported amino acid substitutions that allowed the activation of dormant actinorhodin production in *S. lividans* 66.

Nucleocidin is a nucleoside antibiotic, in which fluorine is covalently bound to the 4′-carbon of adenosine through a C-F bond (Fig. 1). The antibiotic was first isolated as an anti-trypanosome antibiotic from the fermentation broth of *Streptomyces calvus* (Hewitt *et al.*, 1956; Thomas *et al.*, 1956). Its fluorinated nucleoside structure was eventually elucidated by ^{19}F-nmr and mass spectrometry (Morton *et al.*, 1969). Nucleocidin exhibits broad anti-bacterial activity against gram-positive and gram-negative bacteria, including some pathogenic bacteria, such as *Streptococcus pyogenes var. hemolyticus* (Stephen & Gray, 1960; Tobie, 1957). Nucleocidin and the analogous nucleoside antibiotics, ascamycin and dealanyl-ascamycin, constitute classes of nucleoside antibiotics that commonly have a structure comprising a sulfonamide group at the ribose-C5′ position (Isono *et al.*, 1984a; Isono *et al.*, 1984b). The sulfonamide-bearing nucleoside antibiotics were shown to inhibit the ribosome-dependent translation process *in vitro* (Florini *et al.*, 1966), suggesting that their mode of action depends on the potent inhibition of protein synthesis. However, recent studies suggested that the potent activity by this class of nucleosides is more likely due to inhibition of pantothenate synthetase (Tuck *et al.*, 2006) or the inhibition of histidine triad nucleotide-binding protein, which catalyzes the hydrolysis of the phosphoramide linkage between AMP and an amino group (Krakowiak *et al.*, 2004). The sulfamoyl-group bearing nucleoside antibiotics were shown to mimic the structure of an adenosine phosphoramidate intermediate, which is involved in the positive regulation of RNA polymerase activity in eukaryotic cells (Korsisaari & Makela, 2000).

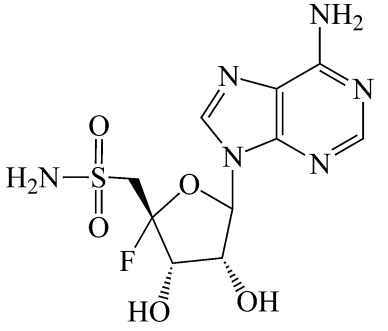

Fig. 1. Structure of nucleocidin

The mode of action of this unique class of nucleoside antibiotics has been studied in detail, and many studies focused on synthesizing analogs and derivatives of nucleocidin and ascamycin (Jenkins *et al.*, 1971; Jenkins *et al.*, 1976; Maruyama *et al.*, 1992; Shuman *et al.*, 1969; Ubukata *et al.*, 1985; Ubukata & Isono, 1986; Ubukata *et al.*, 1988; Verheyden *et al.*, 1975). In contrast, the biosynthetic mechanism of this unique nucleoside compound has never been elucidated. The present study is focused on enzymes involved in nucleocidin production, which involves an intriguing issue of biological fluorination. Fluorinated metabolites are rare in nature. An enzyme that catalyzes the C-F bond formation was first discovered by O'Hagan and coworkers, who reported that the *flA* gene product, fluorinase, catalyzes the formation of 5′-fluoro-5′-deoxy-adenosine from fluoride and S-adenosyl-methionine (SAM) (O'Hagan *et al.*, 2002; Schaffrath *et al.*, 2002). The enzymatic fluorination proceeds with an inversion of the

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stereochemistry at the C5′ of SAM, supporting an S_N2 type mechanism, which requires at least partial de-solvation of the fluoride anion (Zhu *et al.*, 2007). Elucidation of the mechanism of biological fluorination must start with the activation of dormant genes and the purification of the enzymes that catalyze nucleocidin production.

Recently, there have been an increasing number of reports suggesting that certain mutations in the *rpoB* gene, encoding the RNA polymerase β-subunit protein, or in the *rpsL* gene, encoding the ribosomal S12 protein, effectively enhanced secondary metabolite production in gram-positive bacteria. These phenomena have been applied to various secondary metabolites, such as polyketide antibiotics in *S. coelicolor* and *S. lividans* (Hu & Ochi, 2001; Shima *et al.*, 1996; Tamehiro *et al.*, 2003), tripyrrole antibiotic and lipopeptide in *S. lividans* (Hu *et al.*, 2002), and amino sugar antibiotics in *Bacillus subtilis* (Inaoka *et al.*, 2004). While the antibiotic resistance-guided mutation has been applied to various classes of secondary metabolites, there has been no application of *rpoB* mutation to increase any nucleoside antibiotic production; only one previous study reported an effect of the mutation of the *rpsL* gene on the nucleoside antibiotic fosmycin (Hosoya *et al.*, 1998). In the present study, we examined the effect of *rpoB*-mutation on nucleocidin production by *S. calvus*.

To produce nucleocidin, the wild-type strain, *S. calvus* IFO13200, was grown for five days at 30°C in 5 ml of 10 different media CM, F, G, H, K, N5, N, N′, P, PY, which are preferentially used for antibiotic production (Otake, 1986). Although all the media contained $CaCO_3$ in the original formula (as described by Otake), $CaCO_3$ was omitted in our study to avoid the formation of insoluble calcium fluoride. Growth inhibition activity against *Agrobacterium tumefaciens* was produced only in the N5 medium, which is composed of 2% (w/v) glucose, 2% maltose, 2% soybean flour, 14 mM Na_2HPO_4, 10 mM KF, and 0.2 mM $CoCl_2 \cdot 6H_2O$. The width of the inhibitory zone was as small as 1 mm. The other nine media did not allow production of the anti-bacterial activity, suggesting that nucleocidin was produced at levels below the detection limit.

Although over 30 strains were obtained by spontaneous mutation, all of them were poor producers of the anti-bacterial activity. Another set of rifampicin-resistant mutant strains were obtained by ultraviolet light irradiation. The wild-type strain grown overnight in 5 ml of Tryptic Soy broth medium (Becton Dickinson & Co) was spread on minimal plate medium (Hopwood, 1967), irradiated with ultraviolet light for 30 to 90 sec, and the cells allowed to grow in the dark. Surviving colonies were selected on the same medium containing rifampicin at a concentration of 50 or 400 µg/ml. Eighteen rifampicin-resistant mutants grew on the minimal medium containing 400 µg/ml rifampicin, and 12 of them produced an enhanced inhibitory zone of 5 to 14 mm. Mutant strain *S. calvus* R400, which reproducibly made the largest inhibitory zones, was chosen as the high producer strain and was used in the following studies. The R400 strain produced aerial mycelia as rigorously as the wild-type strain, and no obvious impairment in its morphological differentiation ability was observed.

Rifampicin inhibits initiation of transcription by binding to the β subunit of bacterial RNA polymerase (Campbell *et al.*, 2001; Wehrli *et al.*, 1968). In *E. coli*, rifampicin-resistance frequently results from a mutation in a certain region of the open reading frame of the *rpoB* gene (Jin & Gross, 1988; Singer *et al.*, 1993). Enhanced actinorhodin production by an *rif*^{R} mutant of *S. coelicolor* has been ascribed to specific mutations in the corresponding narrow region of the *rpoB* gene, which is designated as the rif-1 cluster (Hu & Ochi, 2001; Lai *et al.*, 2002). We cloned the rif-1 cluster region of the *rpoB* gene from the wild-type and R400 strains using a pair of primers designed from the conserved sequence of *rpoB* genes from *S. coelicolor* A3(2) (GenBank SCO939121) and *S. avermitilis* (GenBank BA000030). The DNA fragments corresponding to the rif-1 clusters were sequenced in both the forward and reverse directions. Comparison of the two genes identified double mutations C1309A and C1318A (according to the numbering for the *S. coelicolor rpoB* gene). The deduced amino acid substitutions, His437Asn and Arg440Ser, corresponded to two of the mutations that conferred rifampicin-resistance and activated dormant actinorhodin production in *S. lividans* 66 (Hu & Ochi, 2001).

As typically observed for many other nucleoside antibiotics, the antibiotic activity produced by *S. calvus* R400 was efficiently extracted by n-butanol. It has been reported previously that nucleocidin could be dissolved in n-butanol at concentrations up to 0.25 µg/ml (Thomas *et al.*, 1956). The smallest volume of n-butanol required for complete extraction of antibiotic activity was one fifth of the volume of the culture broth. Fig. 2 shows the result of n-butanol

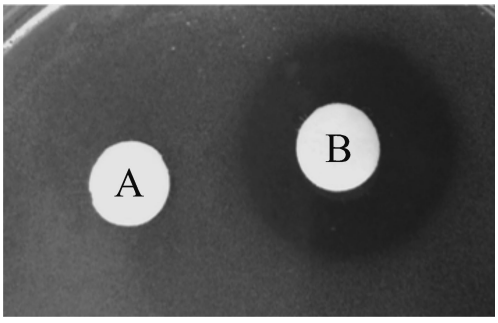

Fig. 2. Extraction of antimicrobial activity with n-butanol. The culture broth (5 ml) was extracted with 1 ml of n-butanol, and both the aqueous phase (A) and butanol extract (B) were concentrated *in vacuo* and lyophilized to dryness. Lyophilized samples were dissolved in 50 µl sterile water, loaded onto a paper disk, and dried under a stream of air. Bioassays were carried out with *Agrobacterium tumefaciens* EHA105 as the test organism.

extraction, by which anti-*A. tumefaciens* activity was completely transferred from the aqueous phase to n-butanol.

S. calvus R400 produced the antibiotic activity when the cells were grown in 5 ml of N5 medium, but large-scale production in a 50 ml-culture produced no antibiotic activity, even in extended cultivation periods beyond 5 to 10 days. We hypothesized that the biosynthetic enzymes were repressed during the large-scale culture or that there was a poor supply of metabolic intermediates, even in the presence of biosynthetic enzymes. To address this question, we examined whether cells grown in 50 ml of medium N5 were capable of producing nucleocidin when suspended in buffer without a carbon or nitrogen source. The resting cell systems were prepared from the mycelia of *S. calvus* R400 strain grown in 50 ml of the medium N5 for five days. The mycelia were washed twice with 50 mM potassium phosphate buffer, pH 7, and suspended in 50 ml of 50 mM potassium phosphate solution that was adjusted to pH 6. The cell suspensions were then incubated at 30°C under aeration at 200 rpm using a reciprocal shaker Inova 4230 (New Brunswick Scientific, United States). The cells were completely removed by centrifugation and the supernatant solution was extracted with 10 ml of n-butanol. The butanol was removed *in vacuo* and the water-soluble components were further lyophilized to dryness. The components were dissolved in 50 µl of sterile water for the bioassay.

Cells of *S. calvus* R400 grown in 50 ml of N5 medium were capable of producing antibiotic activity against *A. tumefaciens*, when the cells were transferred to the resting cell system. The compounds with antibiotic activity were extracted in n-butanol, and examined by a disc-diffusion assay. We examined the effect of initial pH (between pH 5 and 8) of the resting cell suspension on nucleocidin production. We found the largest inhibitory zone of 4 mm was produced when the incubation was started at pH 6 with 50 mM potassium phosphate. At pH 7, the inhibitory zone was 2.5 mm, and no inhibitory activity was produced by the resting cell systems at pH 5 or pH 8. The concentration of cells was another significant factor that affected nucleocidin production; the best production was achieved when approximately 3 g of wet cells, obtained from 100 ml of culture broth, was suspended in 50 ml of the above-mentioned buffer. In contrast to the narrow optimal KF concentrations for *S. cattleya* NRRL8057 (Tamura *et al.*, 2003), which produces mono-fluoroacetate and 4-fluorothreonine from inorganic fluoride, *S. calvus* R400 showed a rather broad range of optimum fluoride concentrations, from 1 to 4 mM. In fact, the R400 strain could produce anti-bacterial activity even without fluoride supplementation (Table 1). High antibiotic activity was obtained when potassium fluoride was added to the N5 medium in a rather wide range of 1 to 4 mM. Our result of antibiotics production without added KF (0 mM KF in Table 1) and the first report of nucleocidin discovery in which inorganic fluoride was not supplemented (Thomas *et al.*, 1956), suggest that *S. calvus* takes up fluoride ions from the culture medium to produce the fluorinated metabolite.

Table 1. Effect of KF concentration on production of antibiotic activity.

KF (mM)	Inhibitory zone (mm)
0	7.5
1	10.0
2	9.5
3	9.0
4	9.0
5	6.5

N5 media were supplemented with potassium fluoride, and antibiotic activity was determined by growth inhibition of *A. tumefaciens* EHA105.

The finding that potent antibiotic activity was produced by the resting cell system suggests that the biosynthetic enzymes are maintained in the producer strain even after the large-scale culture. Therefore, we examined appropriate culture media for large-scale production; R400 strain was grown for 5 days in 50 ml of GYM (Ochi, 1987), GYM33 (Wang *et al.*, 2008), Potato dextrose medium, N5 medium, and N5P media. n-Butanol extracts from cells cultured in N5 and GYM33 media produced inhibitory zones of <1 mm and 2 mm, respectively. Large inhibitory zones (up to 14 mm) were obtained when the R400 mutant was grown in N5P medium. N5P medium contains 1.0% (w/v) D-glucose, 1.0% maltose, 1.0% soybean flour, 14 mM Na_2HPO_4, 10 mM KF, and 0.2 mM $CoCl_2 \cdot 6H_2O$ dissolved in potato broth (prepared by boiling 100 g potato slices in 1 L water for 20 min). The compound with antibiotic activity produced in the culture was extracted by n-butanol, and subjected to an electron spray ionization-mass spectrometry using a Quattro Ultima (Miromass Ltd, UK). A molecular mass of $m/z = 365.25$ [M+1] was obtained, corresponding to the theoretical formula mass of nucleocidin 364.3 (Fig. 3).

In this study, we have shown that the introduction of a certain *rpoB* mutation into a wild-type strain of *S. calvus* resulted in *rif*-resistant strains that overproduced the nucleoside antibiotic nucleocidin, the production of which was dormant in the type strain IFO13200. It has been pointed out that the ability of *Actinomycetes* to produce antibiotics, and other bioactive secondary metabolites, has been underestimated due to the presence of cryptic gene clusters (Hu *et al.*, 2002; Lai *et al.*, 2002). Such dormant genes could be activated by certain mutations (*rif*) in the *rpoB* gene that confer resistance to rifampicin (Inaoka *et al.*, 2004; Tala *et al.*, 2009). Nucleoside antibiotics constitute an important group of secondary metabolites with high selective toxicity to eukaryotic pathogens (Isono, 1988; Isono, 1991). The previous pioneering studies on the

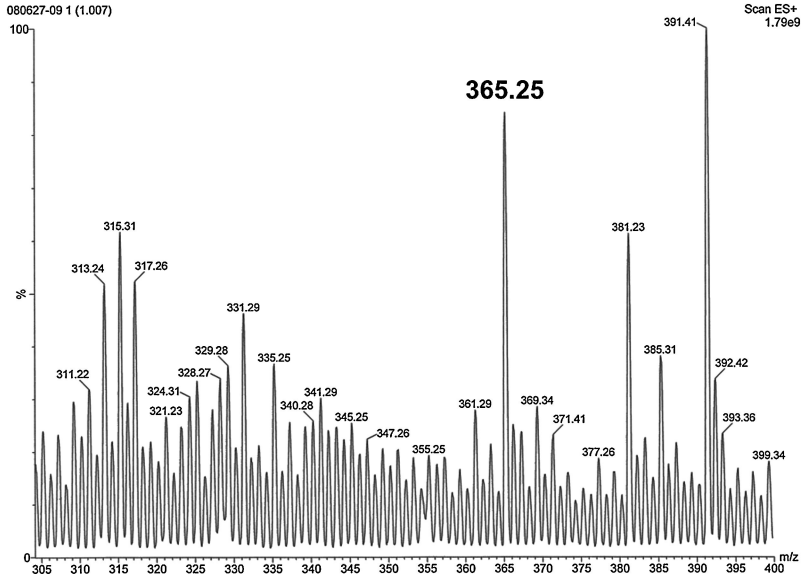

Fig. 3. ESI mass spectrometry of nucleocidin extracted with n-butanol.

effects of *rpoB*- and *rpsL12*-mutations on antibiotic production were limited to spontaneous mutations that avoided additional mutation besides the desired *loci*. Identifying the double C → A mutations in the *rif-1* cluster illustrated the effectiveness of UV light-induced mutagenesis over spontaneous mutation, which normally allows only a single mutation. It remains to be clarified whether one of the double base changes was sufficient or whether both mutations are required for the activation of dormant nucleocidin production.

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