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EXPERIMENTAL ARTICLES

Role of the *snorA* Gene in Nogalamycin Biosynthesis by Strain *Streptomyces nogalater* Lv65

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Abstract—*Streptomyces nogalater* Lv65 (= IMET 43360) is a producer of the anthracycline antitumor antibiotic nogalamycin. In this work, some aspects of the regulation of nogalamycin production by this strain were studied. Insertional inactivation of the *snorA* gene in the chromosome of the nogalamycin producer was carried out; as a result, strain *S. nogalater* A1 was obtained. This is the first successful gene knockout in *S. nogalater*. It was demonstrated that strain A1 is characterized by the absence of synthesis of nogalamycin and its precursors, as well as by the inability to form spores. As a result of the knockout complementation with an entire copy of the *snorA* gene, resumption of the nogalamycin synthesis by strain *S. nogalater* A1 was observed; in the case of the wild-type strain *S. nogalater* Lv65, insertion resulted in an increase in the antibiotic synthesis. Obtained results indicate that the *snorA* gene is involved in positive regulation of nogalamycin biosynthesis.

Keywords: antibiotics, nogalamycin, *Streptomyces nogalater,* SARP regulators, *snorA*.

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Mycelial bacteria of the genus *Streptomyces* are important subjects of modern microbiology and industrial biotechnology, primarily as producers of the majority of known antibiotics [1]. Antibiotic biosynthesis is controlled by many regulatory elements at different levels. This is associated with the onset of antibiotic production at a strictly defined growth stage of the culture, as well as with the toxicity of metabolites for the host cells [1]. Regulatory proteins, activators of gene expression, including expression of the genes responsible for the biosynthesis of antibiotics in streptomycetes, play the key role in the induction of the biosynthesis of a particular product of secondary metabolism. Most proteins of this group belong to the SARP (*Streptomyces* Antibiotic Regulatory Protein) family. These proteins include ActII-ORF4, RedD, MtmR, LndI, DnrI, and CcaR, which activate transcription of the structural genes responsible for antibiotic biosynthesis [2–7]. To date, a substantial amount of data has been collected indicating that proteins of the SARP family represent the last level of control in the cascade system responsible for the regulation of the secondary metabolism in actinomycetes [2]. These proteins receive signals from the regulators operating at higher levels and thereby induce expression of the structural genes of antibiotic biosynthesis. Our understanding of the processes involved in the regulation of the biosynthesis of different antibiotics would make it possible to obtain overproducers of these compounds.

Strain *Streptomyces nogalater* Lv65 (= IMET 43360) is a producer of nogalamycin, an antracycline antitumor antibiotic (Fig. 1). This antibiotic is of great importance, since its derivatives are widely used in cancer chemotherapy [8]. The genes controlling nogalamycin synthesis in *S. nogalater* were identified and cloned [9]. The authors used the results of the bioinformatic analysis of their sequences, as well as of some heterologous expression experiments, to assess the possible functions of these genes [9, 10].

Experiments with targeted inactivation of genes in the chromosome of the nogalamycin producer have not been performed yet, probably due to the complex-

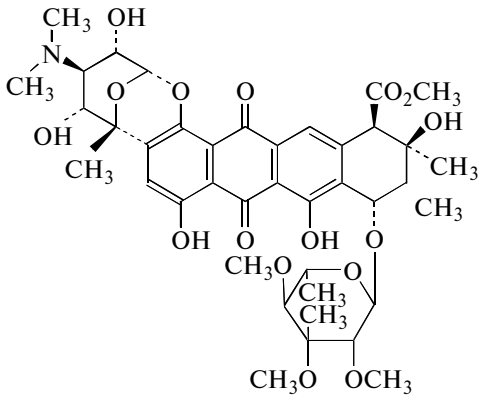

Fig. 1. Structural formula of nogalamycin.

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Strains and plasmids used in this study

Strain, plasmid	Characteristics	Source
E. coli DH5α	F⁻(φ*80d*Δ(*lacZ*)*M15 recA1 endA1 gyrA96 thi1 deoR* (*lacZYA-argF*)*U169*	MBI Fermentas
E. coli ET12567 (pUB307)	*dam-13*::Tn9(Cml^r) *dcm-6 hsdM*	Microbial Culture Collection of Antibiotic Producers, Ivan Franko National University of L'viv, Ukraine
S. nogalater Lv 65	Nogalamycin producer, wild type	Same
S. nogalater A1	*S. nogalater* Lv65 with insertional inactivation of the *snorA* gene	Constructed in this work
S. nogalater EA	*S. nogalater* Lv65, carrying the plasmid pKCA	Same
S. nogalater SRN73	UV-induced derivative of *S. nogalater* Lv65	Microbial Culture Collection of Antibiotic Producers, Ivan Franko National University of L'viv, Ukraine
pBlue*snorA*, 5.4 kbp	pBluescriptIIKS carrying a 2.5-kbp fragment of the *S. nogalater* chromosome containing the *snorA* gene	Constructed in this work
pKC1139, 6.5 kbp	*lacZ* α *ori*^{pUC19} *oriT*^{RP4} *ori*^{pSG5} *aac(3)IV*	[14]
pKCA, 9.0 kbp	pKC1139 carrying a 2.5-kbp *Eco*RI/*Hind*III fragment of pBlue*snorA*	Constructed in this work
pHP45 Ω, 4.5 kbp	pUC19 carrying the spectinomycin-resistance gene (Ω*aadA*)	J.-L. Pernodet, École Polytechnique, Orsay, France
pKCA::*aadA*, 11.0 kbp	pKCA with the BamHI fragment cloned from the plasmid pHP45 Ω and inserted at the BamHI site	Constructed in this work

ity of the methods used for transferring recombinant DNA into the producer cells [11]. Optimization of the method for insertion of recombinant DNA into the cells by *Escherichia coli* ET12567 (pUB307)–*Streptomyces* conjugation made it possible to study the genetic control of nogalamycin synthesis [11].

Earlier, a new gene designated as *snorA* was identified in the gene cluster responsible for nogalamycin biosynthesis [10]. Comparison of the possible product of this gene with the amino acid sequences available in the GenBank database showed homology to the regulators of the SARP family [10].

The goal of the present work was to determine the functions of the *snorA* gene by its targeted inactivation in the chromosome, as well as by the insertion of additional copies of this gene into the cells of strain *S. nogalater* Lv65.

MATERIALS AND METHODS

The strains and plasmids used in this study are listed in the table. Strain *Streptomyces nogalater* Lv65 and its derivatives were grown on the oatmeal and minimal media [12], as well as in the liquid TSB and SG media [13, 14] at 28°C; strains *Escherichia coli* ET12567 pUB307 [13], *E. coli* DH5α (MBI Fermentas), and *Sarcina lutea* were grown on LA and LB media at 37°C [13].

E. coli–S. nogalater conjugation was carried out as described in [11, 12]. The antibiotic apramycin (50 μg/ml) was used to select transconjugants; nalidixic acid (100 μg/ml) was used to eliminate the donor strain. In control experiments, the frequencies of spontaneous apramycin-resistant mutants of *S. nogalater* were determined. The stability of plasmid DNA inheritance in transconjugant clones was determined as a ratio of the number of colonies that retained resistance to the antibiotic after three transfers under nonselective conditions to the total number of colonies. *E. coli* transformation was carried out using the standard calcium chloride method [13].

The spectrum of antibiotic resistance of *S. nogalater* was studied using the method of agar diffusion with the *Sarcina lutea* test culture. *S. nogalater* strains were grown in liquid SG medium; the antibiotic was then extracted from the culture liquid by the addition of chloroform (1 : 1). The extracts were evaporated at 37°C. The obtained dry powder was dissolved in methanol, placed in petri dishes with agarized media (0.7% agar) inoculated with *Sarcina lutea* (10^9 CFU), and incubated at 28°C for 12 and 72 h. The productivity intensity (PI) of the strains was deter-

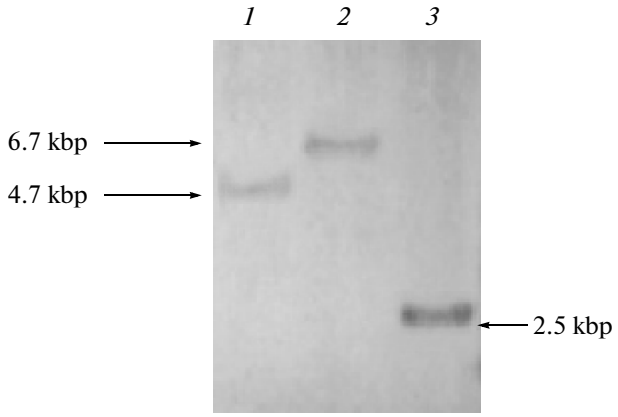

Fig. 2. The results of DNA–DNA-hybridization of the *Kpn*I restricts of the total DNA of strains *S. nogalater* Lv65 (*1*), and *S. nogalater* A1 (*2*); with a DIG-labeled *Eco*RI/*Hind*III probe of plasmid pBlue*snorA* (*3*).

mined as the ratio between the diameter of *S. lutea* growth inhibition zones and the weight of dry mycelium from which the antibiotic was obtained.

The obtained antibiotic extracts were loaded onto Silufol UV254 silica gel plates and analyzed by thin-layer chromatography (TLC) using the solvent system of chloroform : methanol : ethanol : distilled water (120 : 25 : 6 : 4.5). Detection of antibiotics was carried out under visible and UV ($\lambda = 254$ nm) light.

All molecular biological analyses were carried out according to the standard procedures [13–15]. Non-radioactive DIG-labeling of DNA probes, DNA–DNA hybridization, immunodetection, and colorimetric detection of hybridization signals were carried out using the DIG DNA Labeling and Detection Kit (Boehringer Manheim, Germany) according to the manufacturer's recommendations. Hybridization membranes were washed under strict conditions. Two primers, *snoA1* (5'-CGGAAGGCGTCGTG-GTCGGT-3') and *snoA2* (5'-GGGTGGGCAT-TGACCGGTCG-3') were used for amplification of the *S. nogalater* genome fragment carrying the *snorA* gene. The amplicon (2.5 kbp) was cloned into the EcoRV site of pBluescriptIIKS (the obtained plasmid is designated as *pBlue*snorA). It was then transferred into pKC1139 as an *Eco*RI/*Hind*III fragment. As a result, the plasmid pKCA was generated. To obtain a mutant allele of the *snorA* gene in the *Bam*HI site of the *snorA* gene of the plasmid pKCA, *aadA* (spectinomycin resistance gene) was cloned from the plasmid pHP45Ω. The obtained plasmid, pKCA::*aadA*, was used for experiments with insertional inactivation of *snorA* in the *S. nogalater* chromosome.

RESULTS AND DISCUSSION

To determine the role of the *snorA* product in nogalamycin biosynthesis, its targeted inactivation in the chromosome of *Streptomyces nogalater* Lv65 strain was carried out by its replacement by the mutant allele. For this purpose, the plasmid pKCA::*aadA* was transferred from *E. coli* ET12567 pUB307 into *S. nogalater* cells by conjugation. The possibility of successful conjugative transfer of the plasmid DNA in the *E. coli* ET12567 (pUB307) × *S. nogalater* crosses was previously described in [11]. The frequency of the formation of transconjugants carrying pKCA::*aadA* was 2.0×10^{-7}. To obtain an *S. nogalater* strain with the inactivated *snorA* gene, Apm^R transconjugants containing this plasmid were selected and cultivated on TSB medium at 39°C for 5 days. This temperature is nonpermissive for the replication of the plasmid pKCA::*aadA*, which contains a temperature-sensitive replicon of the plasmid pSG5 [14]. Under these conditions, pKCA::*aadA* is unable to replicate and would be either lost by transconjugants or integrated into their chromosomes via homologous recombination.

For further selection of the cells in which double crossing over occurred, aliquots of the culture were plated onto the apramycin-containing medium. After verification of the phenotype of the obtained transconjugants, they were divided into two classes: spectinomycin- and apramycin-resistant (Apm^R Spc^R) transconjugants, obtained due to single crossing over, and spectinomycin-resistant and apramycin-sensitive (Apm^S Spc^R) transconjugants, obtained due to double crossing over (Fig. 2a). Among all the studied clones, one Apm^S Spc^R clone (designated as *S. nogalater* A1) was selected for further investigations. The phenotype of this strain was inherited under nonselective conditions throughout at least five transfers. The results obtained suggest that strain *S. nogalater* A1 could have originated from double crossing-over and contains the *snorA* gene disrupted by the insertion of the spectinomycin resistance gene *aadA*.

The fact of the *snorA* gene knockout in the *S. nogalater* Lv65 chromosome was confirmed by the results of DNA–DNA hybridization (Southern blot). We used a 2.5-kbp *Eco*RI/*Hind*III fragment of plasmid pKCA containing the intact *snorA* gene. The labeled probe hybridized with the *Kpn*I fragment (4.7 kbp) of the total DNA of *S. nogalater* Lv65 and with the *Kpn*I fragment (6.7 kbp) of the total DNA of *S. nogalater* A1. The 6.7-kbp fragment was obtained due to the integration of the *aadA* gene (2.0 kbp) into the *Bam*HI site of the *sno* cluster. The obtained results confirm that the *snorA* gene in the *S. nogalater* Lv65 chromosome was disrupted (Fig. 2b).

The average PI for *S. nogalater* Lv65 was 2.3, whereas the *S. nogalater* A1 metabolites from the culture liquid did not inhibit the growth of the test culture (Fig. 3a). The TLC extracts obtained from the culture liquid of strains *S. nogalater* A1 and *S. nogalater* Lv65 revealed that *S. nogalater* A1 was incapable of producing nogalamycin, which was the main metabolite in the extracts obtained from the cells of the wild-type *S. nogalater* Lv65 (Fig. 3b).

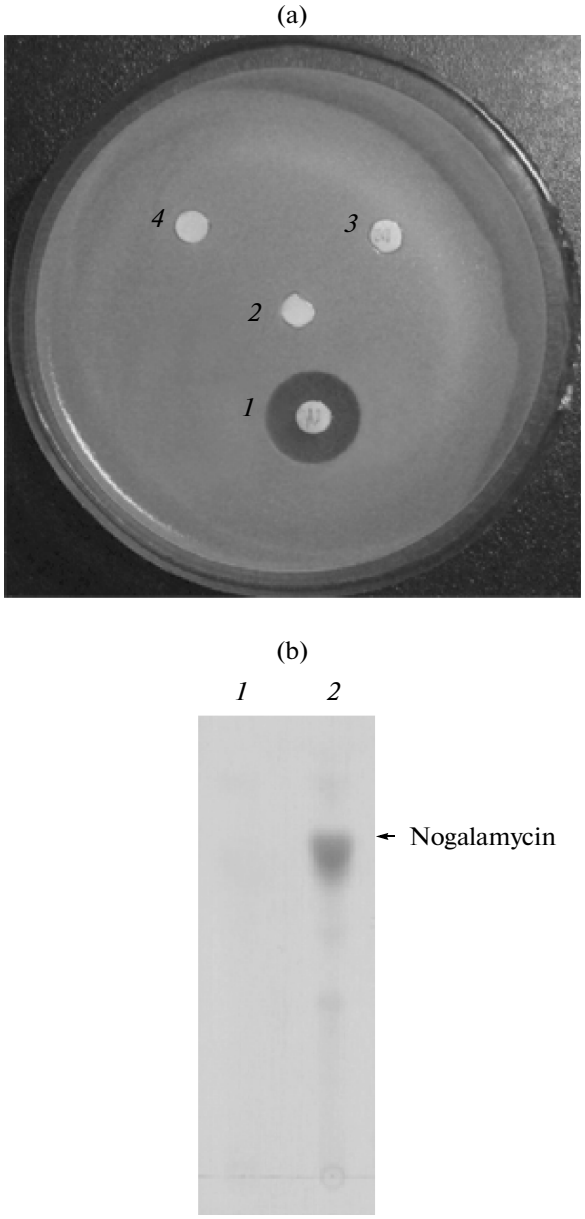

Fig. 3. Growth inhibition zones of the *S. lutea* test culture caused by the antibiotic extracts obtained from strains *S. nogalater* Lv65 (*1*) and *S. nogalater* A1 (*2* and *3*); *4*, negative control (methanol, used as a nogalamycin solvent) (a). The results of the TCL analysis of the extracts of *S. nogalater* A1 (*1*) and *S. nogalater* Lv65 (*2*) secondary metabolites (b). The antibiotics were extracted from the same amounts of wet biomass.

The *snorA* gene knockout affected the morphological characteristics of *S. nogalater* as well. Unlike the wild-type strain (Fig. 4, 1), strain A1 (Fig. 4, 2) did not produce spores on complete and minimal media. These results indicate the pleiotropic effect of *snorA::aadA* mutation, which confirms the suggestion about the regulatory function of the *snorA* gene.

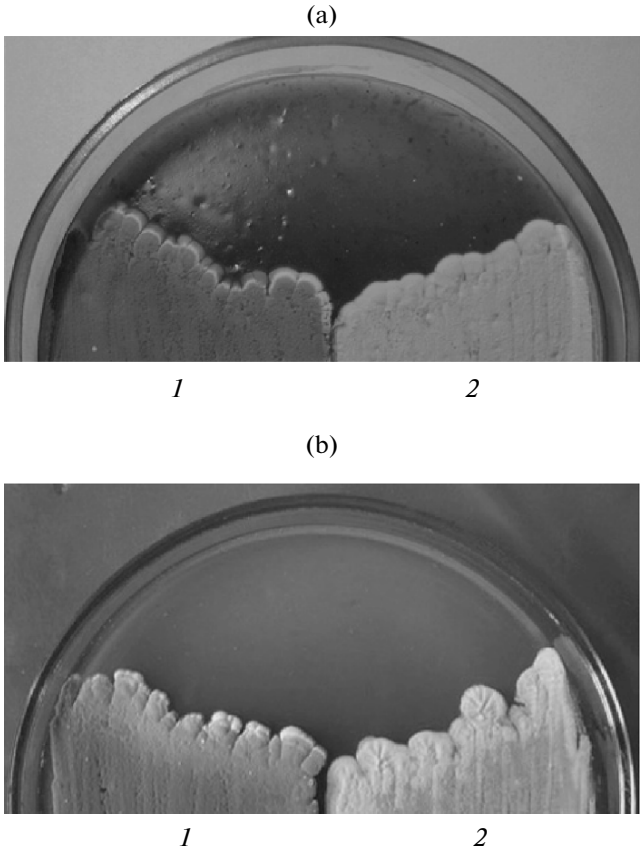

Fig. 4. Strains *S. nogalater* Lv65 (*1*) and *S. nogalater* A1 (*2*) growing on the oatmeal (a) and minimal (b) media. Incubation time, 6 days.

The absence of nogalamycin synthesis and changes in the spore formation by strain *S. nogalater* A1 may be due to both the *snorA* gene inactivation and the polar effect of the *aadA* insertion into the *sno* genes in the 3' direction. This effect is possible, since the *aadA* gene used to carry out the *snorA* gene knockout is flanked by the transcription/translation terminators of bacteriophage T4 [14]. If the *sno* genes located in the 3' direction from the *snorA* gene are within one transcription unit, the *snorA* knockout will block transcription of these genes and may have adverse effects on antibiotic synthesis. Therefore, we have carried out complementation of a mutation in *S. nogalater* A1 with the intact *snorA* gene from the plasmid pKCA. This plasmid was transferred from *E. coli* ET12567 pUB307 into *S. nogalater* A1 cells by conjugation. Analysis of the TCL extracts obtained from strain *S. nogalater* A1 pKCA$^+$ indicated the presence of nogalamycin and its precursors. Hence, due to complementation of the *snorA::aadA* mutation, nogalamycin synthesis and spore formation were restored, which indicates that the phenotype of *S. nogalater* A1 is the result of the *snorA* gene inactivation, rather than of the polar effect of this mutation.

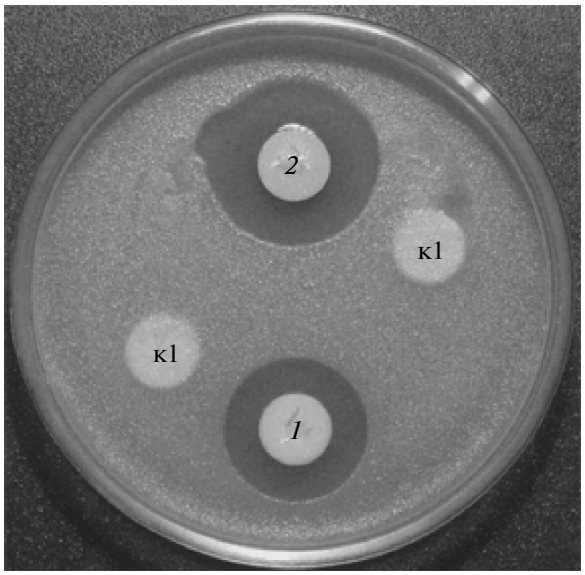

Fig. 5. Growth inhibition zones of the *S. lutea* test culture caused by the antibiotic extracts obtained from strains *S. nogalater* Lv65(*1*) and *S. nogalater* EA (*2*); K1 and K2 are negative controls (methanol, used as a nogalamycin solvent). The antibiotics were extracted from the same amounts of wet biomass.

Along with gene knockouts, the insertion of additional copies of the regulatory genes into the wild-type cells is an efficient method of studying the functions of these genes [2, 3, 5, 7]. We studied the effect on nogalamycin synthesis of additional copies of the *snorA* gene transferred into *S. nogalater* Lv65. For this purpose, the pKCA vector was used (table). It was transferred from *E. coli* ET12567 into *S. nogalater* Lv65 cells by conjugation. As a result, apramycin-resistant (Apm^R) clones of *S. nogalater* were obtained at a frequency of 4.0×10^{-7}. Since the obtained transconjugants exhibited the same phenotype, we selected one of them for further investigation and designated it as *S. nogalater* EA.

Analysis of the extracts obtained from these strains indicated that nogalamycin synthesis increased by 20% on average as compared to the wild-type strain (Fig. 5). This indicates that nogalamycin production increased after insertion of the additional copies of the *snorA* gene.

Hence, the absence of nogalamycin synthesis due to the *snorA* gene knockout, the results of complementation analysis, and the insertion of additional copies of the *snorA* gene (within the plasmid pKCA) into *S. nogalater* Lv65 cells, as well as the results of the bioinformatic analysis, suggest that this gene is involved in the positive regulation of nogalamycin biosynthesis. The obtained results are typical of the SARP proteins of many known actinomycetes [3–7]. For instance, insertional inactivation of the *lndI* and *lanI* genes controlling the biosynthesis of landomycins E and A, respectively, results in complete inhibition of the production of these antibiotics by bacteria. Biosynthesis of antibiotics is restored by the insertion of additional copies of these genes into the knockout actinomycete strains.

We suggest that investigation of the regulation of nogalamycin biosynthesis may make it possible to gain some insight into the genetic mechanisms responsible for nogalamycin production by *S. nogalater*. Moreover, the obtained information is an important step in the gene-engineering construction of nogalamycin overproducers.

ACKNOWLEDGMENTS

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