УДК 575.224+577.21

STIMULATION OF NOGALAMYCINE PRODUCTION BY COEXPRESSION OF *SNORA* AND *RELA* GENES IN *STREPTOMYCES NOGALATER* LV 65

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Anthracycline antibiotics are an important group of anticancer compounds widely used in tumor chemotherapy. They are used to treat different types of diseases such as leukemias, lymphomas, breast, uterine, ovarian and lung cancers. Investigation of the regulatory mechanisms of antibiotic production in Streptomyces is of great interest, as these data provide a potential platform for generation of industrially important strains to increase the production of their secondary metabolites. Streptomyces nogalater Lv65 is a producer of an anthracycline antibiotic nogalamycine. Derivatives of this antibiotic are used in chemotherapy of tumors. Here we focused on expression of snorA and relA genes in S. nogalater Lv65. A set of plasmids for the expression of snorA and relA genes were generated. Conjugation procedures of the S. nogalater Lv65 and UV33, S. echinatus and S. peucetius wild-type strains with pKCAII and pVWBA1 were carried out from E. coli ET12567 (pUB307). An increasing in the nogalamycin production was observed when pKCAII and pVWBA1 plasmids were introduced into the Lv65 and UV33 strains. We believe that this indicates a crucial role for snorA and relA in nogalamycin biosynthesis under some nutritional conditions. This is in agreement with the general knowledge about aforementioned regulatory genes. Expression of snorA and relA genes in S. echinatus Lv 22 and S. peucetius subsp. caesius — producers of aranciamycine and doxorubicin respectively, stimulates antibiotic production in the above strains.

Keywords: ANTIBIOTIC BIOSYNTHESIS, *STREPTOMYCES NOGALATER*, NOGALA-MYCINE, SARP PROTEINS

СИНТЕЗ НОГАЛАМІЦИНУ ЗА УМОВ КОЕКСПРЕСІЇ ГЕНІВ *SNORA* ТА *RELA* В *STREPTOMYCES NOGALATER* LV 65

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Антрациклінові антибіотики є групою протипухлинних сполук, що широко використовуються в хіміотерапії ракових захворювань. Вони використовуються для лікування низки захворювань, таких як лейкози, лімфоми, рак матки, яєчників, а також раку легень. Дослідження регуляторних механізмів синтезу антибіотиків у стрептоміцетів є важливою проблемою сучасної мікробіології та біотехнології. Ці дані дають потенційну платформу для одержання промислово важливих штамів бактерій, що дозволяє збільшити виробництво їхніх вторинних метаболітів. Streptomyces nogalater Lv65 є продуцентом антрациклінового антибіотика ногаламіцину. Хімічні модифікації цього антибіотика сьогодні використовуються в хіміотерапії ракових захворювань. Робота присвячена експресії генів snorA і relA у клітинах S. nogalater. Сконструйовано низку плазмідних молекул ДНК для експресії генів snorA і relA, що є задіяними у регуляції вторинного метаболізму у актиноміцетів. Плазміди pKCAII та pVWBA1 перенесені в клітини штамів S. nogalater Lv65 та UV33, а також штамів S. echinatus та S. peucetius із використанням міжродової кон'югації з E. coli ET12567 (pUB307). За умов коекспресії

регуляторних генів у складі олігокопійної плазміди рКСАІІ та інтегративної pVWBA1 спостерігається зростання синтезу ногаламіцину в штамах Lv65 та UV33. Очевидно, що це вказує на важливу роль генів snorA і relA у біосинтезі ногаламіціну за культиваційних умов представлених досліджень та узгоджується із загальними уявленнями про їхню участь у регуляції вторинного метаболізму у актиноміцетів. Гетерологічна експресія snorA і relA у складі рКСАІІ та pVWBA1 в клітинах S. echinatus LV 22 та S. peucetius subsp. caesius підвищує продукцію аранциаміцину та доксорубіцину, відповідно.

Ключові слова: БІОСИНТЕЗ АНТИБІОТИКІВ, *STREPTOMYCES NOGALATER*, НОГАЛА-МІЦИН, SARP-БІЛКИ

СТИМУЛИРОВАНИЕ ПРОДУКЦИИ НОГАЛАМИЦИНА
ПУТЕМ КОЕКСПРЕСИИ ГЕНОВ *SNORA* И *RELA* У *STREPTOMYCES NOGALATER* LV 65

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Антрациклиновые антибиотики являются важной группой противоопухолевых соединений, широко используемых в химиотерапии опухолей. Они используются для лечения различных видов заболеваний, таких как лейкозы, лимфомы, рака матки, яичников, а также легких. Исследование регуляторных механизмов биосинтеза антибиотиков у Streptomyces представляет научный интерес, так как эти данные дают потенциальную платформу для получения промышленно важных штаммов, что позволяет увеличить производство их вторичных метаболитов. Streptomyces nogalater Lv65 является продуцентом антрациклинового антибиотика ногаламицина. Химические модификации этого антибиотика сегодня широко используются в химиотерапии опухолевых заболеваний. Данная работа сосредоточена на коэкспрессии генов snorA и relA в клетках S. nogalater Lv65. В работе получены плазмиды рКСАІІ и pVWBA1 для экспрессии регуляторных генов. Плазмиды рКСАІІ и pVWBA1 доставлены в клетки штаммов S. nogalater Lv65 и UV33, а также штаммов S. echinatus и S. peucetius с использованием междуродовой конъюгации из E. coli ET12567 (pUB307). Перенос этих векторов в клетки штаммов S nogalater Lv65 и UV33 активировал продукцию ими ногаламицина. Очевидно, что это указывает на важную роль snorA и relA в регуляции биосинтеза ногаламицина в условиях культивирования, представленных в этой работе. Это согласуется с общими знаниями о вышеупомянутых регуляторных генах. Гетерологическая коэкспрессия генов snorA и relA в клетках S. echinatus LV 22 и S. peucetius subsp. caesius стимулировала продукцию аранциамицина и доксорубицина соответственно.

Ключевые слова: БИОСИНТЕЗ АНТИБИОТИКОВ, *STREPTOMYCES NOGALATER*, НОГАЛАМИЦИН, SARP-БЕЛКИ

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. Most of the antibiotics are produced by complex biosynthetic pathways encoded by clustered genes [1, 2]. The gene clusters are usually regulated by pathway-specific transcriptional regulators that are located in these clusters. In addition, various global regulatory genes have been identified, which affect antibiotic production indirectly and have pleiotropic roles in stress response and morphological differentiation [1]. Most of these pleiotropic regulatory genes have been shown to influence the activity of the pathway-specific regulatory genes. Expression of both types of regulatory gene is influenced by a variety of physiological and environmental factors, including growth rate, signaling molecules,

imbalances in metabolism and various physiological stresses [1].

The final decision about onset of antibiotic production is made by transcriptional factors which genes are clustered with the respective antibiotic biosynthesis genes [3]. Members of this big group of proteins resemble the OmpR transcriptional regulator of *Escherichia coli* phosphate regulon particularly in the region of DNA-binding domain formed by "winged" helix-turn-helix structure [1–3]. Therefore, these transcriptional factors were grouped into SARP family [1]. SARP genes were found within almost all biosynthesis gene clusters governing aromatic polyketides production. These genes are usually clustered with antibiotic biosynthesis genes and activate or repress their expression. Manipulations of the SARP genes and pleiotropic regulatory genes have been shown to increase the production of antibiotics by streptomycetes [1, 3–5, 6].

Streptomyces nogalater Lv65 is a producer of an anthracycline antibiotic nogalamycin [4]. Derivatives of this antibiotic are used in chemotherapy of tumors. Among anthracycline derivatives, nogalamycines showed superior cytotoxicity and antitumor activity and also proved to be effective against breast cancer clinically [4]. Certain aspects of the regulation of nogalamycin production have been characterized [7–13].

The goal of the present work is coexpression of *snorA* and *relA* genes in *S. nogalater* Lv65. We expect that manipulations with these regulators will provide a potential platform for manipulating with industrially important strains to increase production of their secondary metabolites.

Materials and methods

Streptomyces nogalater Lv65, nogala-mycine producer, was used as a source of chromosomal DNA. Growth was carried out on trypticase soya broth (TSB; Oxoid) or R5A medium [14]. Spores of *Streptomyces* strains were harvested from a sporulated lawn grown on oatmeal agar plates [15]. The *Streptomyces* and *E. coli* strains and plasmids used in the present work are described in *Table 1*.

Apramycin-resistant, strains were tested for loss of apramycin resistance by culturing the colonies on HA plates with and without apramycin. Resistant colonies were subjected to Southern blotting.

Escherichia coli was grown in Luria-Bertani (LB) medium at 37° C, and plasmids were introduced into *E. coli* by transformation done by standard procedures [15]. pBluescriptKS was purchased from Amersham Biosciences. pVWB, a non-replicative vector carrying an apramycin resistance deteminant, was described by Bierman et al. [15]. For *E. coli* strains harboring plasmids, ampicillin was added at a final concentration of 100 mg/ml. Antibiotic activity of *S. nogalater* strains was studied by diffusion in agar using *Sarcina lutea* as a test-culture. *S. nogalater* strains were grown in liquid SG medium and the antibiotics were extracted from the culture liquid by chloroform (1 : 1). The extracts were dried at 37 °C, the dry residue was dissolved in methanol, and applied to petri dishes with 0.7% agar containing *Sarcina lutea* (109 CFU). The plates were incubated at 28° C for 12 and 72 h. Strain productivity was evaluated by the ratio between the diameter of *Sarcina lutea* growth inhibition zone to the dry mass of the mycelium from which the antibiotic was extracted.

Conjugation procedures of the *S. nogalater, S. echinatus* and *S. peucetius* wild-type strains with pKCAII and pVWBA1 was carried out from *E. coli* ET12567 (pUB307) as described [16]. Donor *E. coli* ET12567 (pUB307) strain was grown on LB agar supplemented with apramycin (50 μg/mL) and kanamycin (50 μg/mL) for 12 h at 37 °C. *S. nogalater* spore suspension (harvested from 7 days-old lawn), according to a standard protocol, was heat-treated for 10 min at 50 °C. Donor and recipient cells were mixed in a 1:1 ratio and spread on oatmeal medium. The plates were incubated (12 h, 28 °C) and then covered with 1 ml of water containing 1.5 mg of apramycin and 1.5 mg of nalidixic acid. Transconjugants were counted after 5 days. The frequency of transconjugant occurrence was calculated as a ratio of the number of transconjugants to the titer of recipient strain spores.

Standard methods for DNA isolation and manipulation were performed as described by [15]. Genomic DNA was isolated from Streptomyces strains by lysozyme treatment and phenol/chloroform extraction as described elsewhere [14].

Thin layer chromatography (TLC) of the antibiotic extracts was performed on Silufol UV254 silica gel plates in the solvent system chloroform–methanol–ethanol–distilled water (120 : 25 : 6 : 4.5). Detection of antibiotics on the plates was performed under visible and ultraviolet light (λ 254 nm).

Southern blot analysis was performed on Hybond-N membranes (Amersham Biosciences) with digoxigenin-labelled probes by using DIG high prime DNA labelling and detection kit II (Roche Molecular Biochemicals) [15]. Hybridization filters were washed off under standard conditions.

Table 1

Strains and plasmids

Bacterial strain or plasmid	Description	Source or reference
E. coli DH5α	*supE44 ΔlacU169(φ80lacZΔM15) hsdR17 recA1endA1gyrA96 thi-1 relA1*	MBI Fermentas
E. coli ET12567 (pUB307)	*dam-13*::Tn9(Cml^r) *dcm-6 hsdM*	Microbial culture collection of antibiotic producers, Lviv, Ukraine
S. echinatus Lv 22 (=DSM 40730)	Producer of aranciamycin	The same
S. nogalater Lv 65	Producer of nogalamycin	The same
S. peucetius subsp. *Caesius*	Producer of doxorubicin	The same
pVWB	*E. coli/Streptomyces* shuttle vector with φBT1 *attP/int* integrative system, *aac(3)IV*	Microbial culture collection of antibiotic producers, Lviv, Ukraine
pVWBA1	pVWB derivative harboring *snorA* and *relA* genes cloned in the EcoRI, *aac(3)IV*	This work
pKC1218E	*E. coli/Streptomyces* shuttle expression vector with PermE and SCP2 replicon, *aac(3)IV*	[15]
pKCAII	pKC1218E derivative with a fragment harboring *snorA* and *relA* genes gene, *aac(3)IV*	This work

Results and discussion

Previously, we have studied the effect of additional copies of the *snorA* gene on nogalamycin synthesis in *S. nogalater* Lv65. Nogalamycin production was increased after insertion of the additional copies of the *snorA* gene (at least under our experimental conditions) [5, 6]. *relA* (*S. coelicolar* A3(2)) also stimulates antibiotic production in *S. nogalater.* In the present study *snorA* and *relA* genes were simultaneously coexpressed in the pKC1218-based plasmid pKC1218E under constitute ErmEp promoter and in the inegrative plasmid pVWB.

Approximately, 1.7 kb fragment carrying the entire *relA* gene and its flanking regions were cloned from [5]. The product was cloned into EcoRV site of pKCEAII to give pKCAII (*Fig. 1a*). The obtained construct contains *snorA* and *relA* genes controlled by ErmEp of *Saccharapolyspora erytreae*. Five Am^r transconjugants (an indicative of a pKCEAII) were selected out of 17 tested. All seven candidates exhibited the same phenotype. One of them (referred to as pKCEAII$^+$) was used throughout this work.

Integrative vector for *snorA* and *relA* genes overexpression was generated as follows. A 2.4 kb fragment carrying the entire *snorA* gene and its promoter regions was cloned from pKCEA to give pVWBA. This plasmid was used as a DNA source for construction of pVWBA1 (*Fig. 1b*). The *relA* gene was
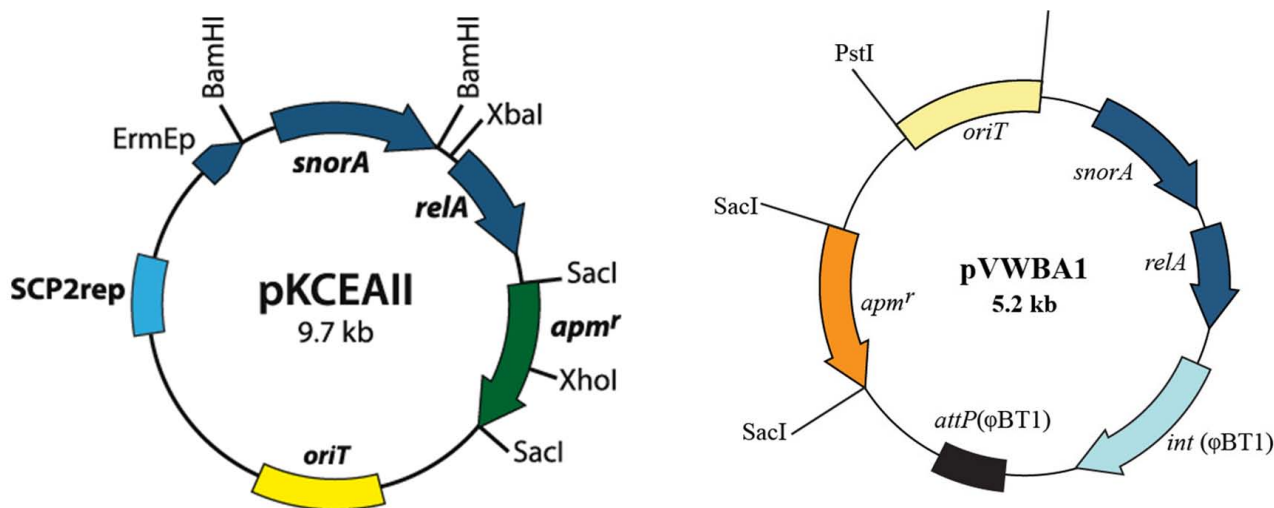
Fig. 1. Structure of pKCAII and pVWBA1 plasmids

introduced into the unique BamHI site within pVWBA. Obtained vector was transferred into streptomycetes using intergeneric conjugation from *E. coli* ET 12567. Two Am^r colonies (an indicative of a pVWBA1) were selected. One of them (referred to as pVWBA1$^+$) was used throughout this work.

Both pKCEAII$^+$ and pVWBA1 plasmids were transferred to *S. nogalater* Lv65 (wild-type straine) and *S. nogalater* UV33 (UV-induced overproducer of nogalamycin). Expression of

snorA and *relA* genes in pKCEAII$^+$ increase the nogalamycin production in the following strains (*Fig. 2*). We believe that this indicates a crucial role for *snorA* and *relA* in nogalamycin biosynthesis under some nutritional conditions. This is in agreement with the general knowledge about aforementioned regulatory genes. Manipulations of the SARP genes and *relA* have been shown to increase the production of antibiotics by streptomycetes. For example, introduction of *lanI* (SARP
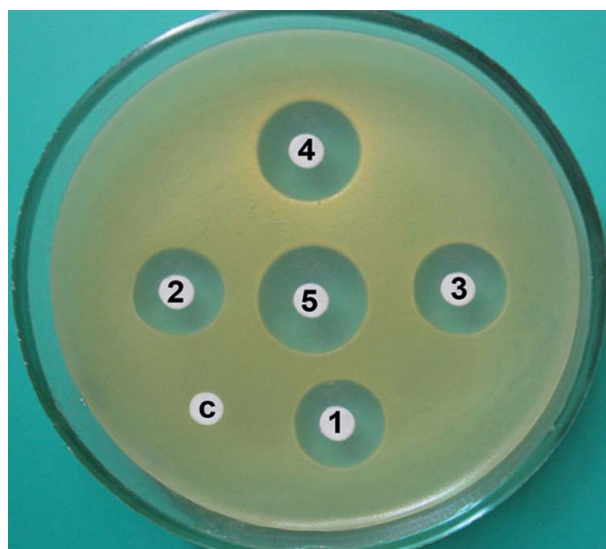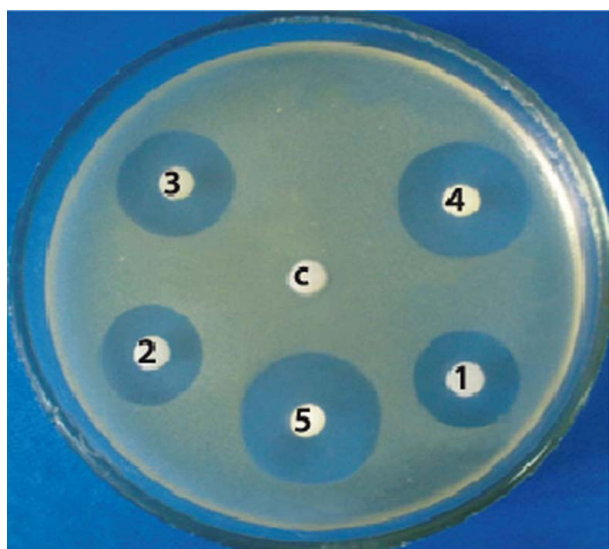

Fig. 2. a. Growth inhibition zones of the *S. lutea* test culture caused by the antibiotic extracts obtained from strains Lv65 (*1*), pKC1218E$^+$ (*2*), UV33 (*3*), Lv65 pKCEAII$^+$ (*4*), UV33 pKCEAII$^+$ (*5*), c — negative control (methanol, used as a solvent). The antibiotics were extracted from equal amounts of wet biomass. *b.* Growth inhibition zones of the *S. lutea* test culture caused by the antibiotic extracts obtained from strains Lv65 (*1*), pVWB$^+$ (*2*), Lv65 pVWBA1$^+$ (*3*), UV33 (*4*), UV33 pVWBA1$^+$ (*5*), c — negative control (methanol, used as a solvent)

regulator of landomycin A biosynthesis in *S. cyanogenus* S136) and *lndI* (*lanI* homologue controlling landomycin E biosynthesis in *S. globisporus*1912) activated production of aforementioned antibiotics [3].

Accumulation of ppGpp in streptomyces also causes the induction of complex changes in the pattern of gene expression with global cellular consequences. For example, in *S. coelicolor*, genetically the most characterized streptomycete that serves as a model organism for the genus, accumulation of ppGpp occurs transiently in response to amino acid limitation and requires the presence of both RelA and RelC [17–18].

Nogalamycine biosynthesis was also increased in *S. nogalater* Lv65 and *S. nogalater* UV33 strains harboring pVWBA1. pVWBA1 contains *int* gene of VWB phage which inegrates site-specifically into two sites of *S. nogalater* chromosome. Site-specific integration makes pVWBA1 a powerful tool for generation of stable overproducers of nogalamycin.

pVWBA1 and pKCAII were used for the coexpression of *snorA* and *relA* genes in heterological hosts. *S. echinatus* Lv 22 and *S. peucetius* subsp. *caesius* — producers of aranciamycine and doxorubicin respectively, were used in the present study. Both plasmids were transferred into *S. echinatus* and *S. peucetius* cells using intergeneric conjugation from *E. coli* ET 12567 (pUB307). Stimulation of aranciamycine and doxorubicin production was observed in pVWBA1$^+$ and pKCAII$^+$ strains.

Conclusions

Here we further investigated the possibilities of nogalamycine overproduction by combination of SARP and pleiotropic regulatory genes cloned in multy-copied and integrative vectors. Expression of *snorA* and *relA* genes cloned in pKCEAII$^+$ and pVWBA1 plasmids increase the nogalamycin production in *S. nogalater* strains. Both genes also stimulate aranciamycine and doxorubicin production in *S. echinatus* and *S. peucetius*. Our experiments provide a potential platform

for manipulating anthracycline producing streptomycetes to increase production of their secondary metabolites.

Prospects for further research. Understanding of the physiological and environmental conditions that trigger the expression of the *snorA* and *relA* genes, or that influence the activities of their products, and the corresponding signal transduction pathways that are responsible for the activation of secondary metabolism remains a main goal for the future research.

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