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INFLUENCE OF PLEIOTROPIC REGULATORY GENES *absB*, *relA*, *afsS* ON SIOMYCIN PRODUCTION BY *STREPTOMYCES SIOYAENSIS* Lv81

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Streptomyces sioyaensis Lv81 (=NRRL-B5408) produces thiopeptide antibiotic siomycin A (SiA). Use of wild type SiA producer is restricted because of low SiA titers. In this work we explored the utility of three heterologous pleiotropic regulatory genes for enhancement of SiA production. These genes under investigation are *absB* (codes for RNAse III), *relA* (for (p)ppGpp synthetase) and *afsS* (for 67-aminoacid peptide with chaperone-like activity). Gene *absB* was amplified from *S. ghanaensis* genome, genes *relA* and *afsS* – from the genome of *S. coelicolor*. Constructs containing abovementioned genes were transferred into *S. sioyaensis* cells. Significant increase in SiA titers was detected in case of *absB* and *afsS* genes. Thus, both genes appear to be involved in the positive regulation of SiA production. No increase in SiA titers were detected in case of *relA* overexpression. Our results suggest that pleiotropic regulatory genes are useful tools for siomycin production improvement.

Key words: secondary metabolism, thiopeptide antibiotics, siomycin, pathway-specific regulators, pleiotropic regulators.

Streptomyces sioyaensis Lv81 (=NRRL-B5408) is a producer of siomycin complex, a group of related thiopeptide antibiotics with siomycin A (SiA) being the major one [2, 13] (Fig. 1). SiA and its close congener, thiostrepton, exhibit potent antibacterial activities; they are active against various gram positive bacteria including *Mycobacterium tuberculosis* and many clinical resistant strains of *Staphylococcus*. They also display antiplasmodial, antitumor and immunosuppressive activities and are currently used in veterinary medicine [17, 18].

In spite of attractive bioactivities of SiA, its use is restricted because of low water solubility [2]. Chemical and/or biological engineering of SiA analogs is a promising way to get around this problem [8]. The availability of SiA overproducers would greatly facilitate the genetic engineering of SiA biosynthesis. Currently, there are no described SiA overproducers and SiA biosynthesis in the wild type is very low. Therefore, we set out to explore genetic approaches towards improvement of SiA production, which could be used alone or in combination with classical strain selection strategies.

Manipulations of regulatory gene network governing SiA production would be the most obvious way for increase SiA titers. Recently siomycin biosynthetic gene cluster was characterized and no pathway-specific regulatory genes were detected in it [10]. Consequently, the only gene-centred way to enhance antibiotic production is through manipulations of so-called global or pleiotropic regulators, involved in the control of more than one secondary metabolic pathway. These genes are usually not clustered with the genes for antibiotic biosynthesis and their identification was a result of decades-long efforts on isolation and characterization of specific classes of mutants. No data on *S. sioyaensis* pleiotropic gene regulatory network is available. Therefore, we decided to start with heterologous expression experiments of well known pleiotropic regulatory genes. Since the general architecture of the global regulation seems to be conserved across the species [12], it is reasonable to expect that *S. sioyaensis* should harbour some of the most

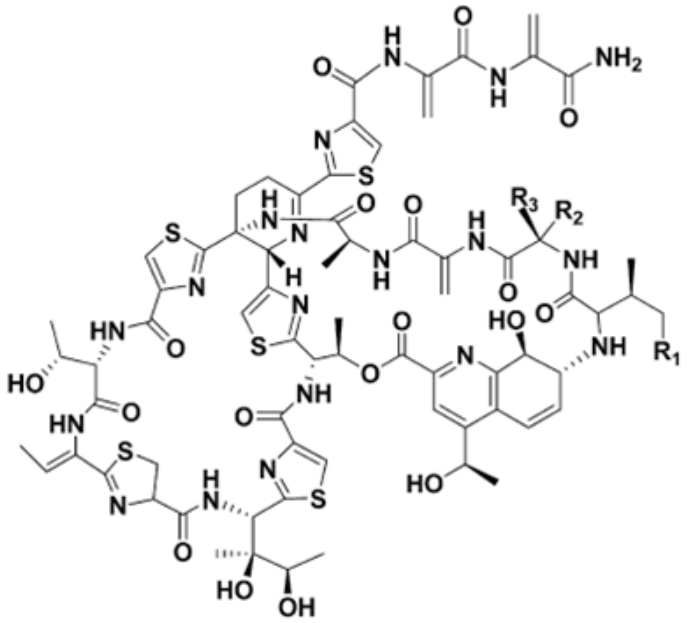

Fig. 1. Chemical structure of siomycin A ($R_1 = CH_3$; $R_2 = CH_3$; $R_3 = H$) and thiostrepton ($R_1 = H$; R_2, $R_3 = CH_2$) [10].

conserved elements of it. For this work we have chosen three genes, which were established as global regulators in model strains *S. coelicolor* A(3)2 and *S. griseus* [1, 3].These genes are: *absB* (encoding the ribonuclease III, a double-stranded RNA-specific endoribonuclease), *relA* ((p) ppGpp-synthetase) and *afsS* (small protein, putative chaperone; its product is connected with *actII*-ORF4 and *redD* expression regulation) [4, 9, 15]. For our work we used *afsS* and *relA* genes of *S. coelicolor* and *absB* gene of *S. ghanaensis*. Our work supports the idea that heterologous pleiotropic regulators are promising tools for rational improvement of SiA producer.

Materials and methods

Strains and plasmids used in this work are listed in Table 1. *Streptomyces sioyaensis* Lv81 (=NRRL-B5408) was used as SiA producer. *Escherichia col*i DH5α and ET12567 (pUB307) were used for routine subcloning and intergeneric conjugation, respectively. *Sarcina lutea* was used as a SiA sensitive strain in bioassays. *Streptomyces lividans* TK24 (pMO16) was used in bioassays as thiopeptide biosensor system [13]. Replicative vectors pKC1139 and pKC1218E contain pSG5 (moderate copy number) and SCP2 (low copy number) origins of replication, respectively [1, 7]. Vectors pSOK804 and pIJ6085contain *int–attP* fragments of actinophages VWB and φC31, respectively. All used vectors contain *aac(3)IV* gene, that confer resistance to apramycin [12].

Oatmeal medium was used to obtain spores of *S. sioyaensis* Lv81 and *S. lividans* (pMO16) as well as to plate intergeneric matings. Isolation of plasmid DNA from *E. coli* was carried out using standard protocols [1, 7, 14]. Restriction enzymes and molecular biology reagents were used according to recommendation of suppliers (NEB, MBI Fermentas). For the isolation of total DNA, *S. sioyaensis* strains were grown in SG medium that was described in Luzhetskyy et al. 2001 [11], for 3 days in presence of apramycin (25 μg/ml) whenever needed. *E. coli* was grown according to [7]. For *tipAp* induction experiments, *S. lividans* (pMO16) was plated on LB agar supplemented with kanamycin (50 μg/ml). *Streptomyces* and *S. lutea* strains were incubated at 28°C. *S. lutea* was grown on LB agar.

Intergeneric conjugation experiments were carried out as described in [13]. SiA production was measured with the help of antibiotic disc diffusion test. Strains were grown in SG medium for 6 days on a rotary shaker. 100 mg of biomass of each sample were extracted with 100 μl of dimethylsulphoxide (DMSO) for 12 hours on a shaker. 10 μl of each extract were poured on standard paper disks and dried at 37ºC. Previously prepared 5 ml of so-called soft agar (0.7%)

were mixed with 1 ml of freshly prepared suspension of *S. lutea* cells (10^9 CFU) and poured on LA plates. Dried paper discs were put on LA plates with suspension of *S. lutea* cells. Definitive plates were incubated for 14–16 hours and after that diameters of *S. lutea* growth inhibition zones were measured. Dry weight of each strain grown in SG medium was measured as follows: 1 ml of culture suspension was transferred to a 1.5 ml plastic tube and washed with 0.5 ml of distilled water twice. Cell precipitate was dried at 100ºC during 2 hours and weighed. Experiments with thiopeptide-inducible system were carried out as described in [13].

Table 1

Strains and plasmids used in this work

Bacterial strains and plasmids	Description	Source or reference
Escherichia coli DH5α	*supE*44Δ*lacU*169(φ80*lacZ*ΔM15)	MBI Fermentas
E. coli ET 12567 (pUB307)	*hsdR*17 *recA*1*endA*1*gyrA*96 *thi*-1 *relA*1 *dam*-13::Tn9(Cm^r) *dcm*-6 *hsdM*; carries conjugative plasmid pUB307; Cm^r, Km^r	C. P. Smith, UMIST, UK
Sarcina lutea Lv65	SiA-sensitive test-culture	Microbial Culture Collection of Antibiotic Producers of Ivan Franko National University of Lviv Ukraine (MCCAP)
Streptomyces sioyaensis Lv81	Wild type SiA producer	MCCAP
S. sioyaensis Rif49S	Rifampicin resistant mutant of *S. sioyaensis* Lv81	This work
S. sioyaensis 100	Streptomycin resistant mutant of Rif49S	This work
S. lividans TK-24 (pMO16)	Str-6 $SLP2^-$ $SLP3^-$; harboring plasmid pMO16;	[13]
pKCafsS	pKC1218E derivative with 2.9 kb fragment containing the entire *afsS* and a part of *afsR* genes under the control of *ermEp*	[12]
pKCabsB	pKC1218E derivative with 1.0 kb fragment containing *absB* gene	This work
pKC1139absB	pKC1139 derivative with 1.0 kb fragment containing *absB* gene	R. Makitrynskyy, IFNUL, Ukraine
pSOK804absB	pSOK804 derivative with HindIII/EcoRI – cloned *absB* gene	This work
pIJ6085relA	pIJ8600-based construct with 2.08 kb part of *relA* cloned under the control of *tipAp*	[15]

Results and discussion

For expression, genes *relA*, *afsS* and *absB* were cloned into one or several integrative or replicative plasmids mentioned above. Genes *relA* and *afsS* originate from *S. coelicolor*. In plasmids pIJ6085relA and pKCafsS these genes are located under the control of *tipA* and *ermE* promoters (see Table 1 and Fig. 2). Gene *absB* was amplified from *S. ghanaensis* genome as a part of 1.0 kb fragment and cloned into pKC1139. Both *S. coelicolor* and *S. ghanaensis* are close to *S. sioyaensis* phylogenetically, as judged from comparison of 16S rRNA [3]. For the constitutive expression, *absB* was excised from pKC1139absB as a part of 1.0 kb XbaI/EcoRI fragment and cloned into pKC1218E vector digested with the same enzymes. Then 1.2 kb HindIII/EcoRI fragment containing *absB* gene with *ermE* promoter was ligated with HindIII/EcoRI digested pSOK804 vector. Thus we have obtained two constructs for *absB* gene expression: one based on site-specific integrative (pSOK804*absB*) and another – on replicative (pKC1139*absB*) vector. For the detailed information concerning vectors composition see Fig. 2. These two constructs allow us to test the influence of *absB* copy number on SiA production.

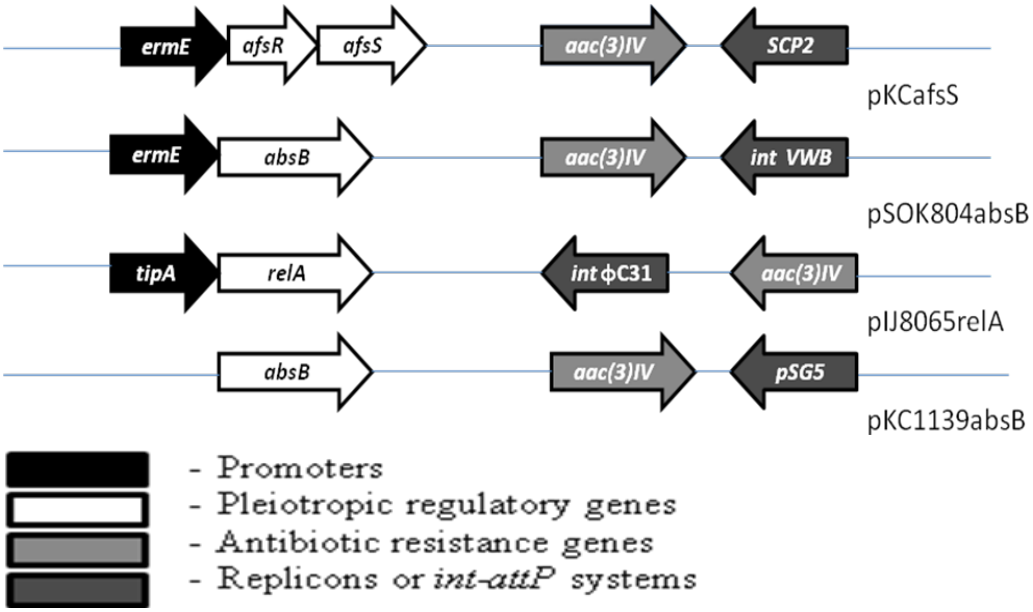

Fig. 2. Schemes of the plasmids used in this work. For convenience, plasmids are shown in linear form. Names of the genes and genetic elements on the figure – see the text and Table 1.

Aforementioned plasmids were successfully transferred into *S. sioyaensis* cells via intergeneric *Streptomyces-E. coli* conjugation. *E. coli* DH5α cells were transformed with total DNA extracted from the transconjugant strains. We successfully obtained transformants in each case, and, through physical mapping of plasmid DNA, were able to confirm the identity of the latter with initial plasmids. Hence, the fact of plasmid transfer was confirmed. We did not detect any changes in sporulation, biomass accumulation or morphology of the transconjugants when comparing to the initial strain. To reveal the deference in biosynthetic levels of transconjugants, we have extracted SiA from strains and carried out a set of experiments with *S. lutea* and *S. lividans* TK-24 (pMO16) as the test cultures. The latter strain has ability to grow on kanamycin-supplemented plates only in the presence of thiopeptide antibiotics. Thus *S. lividans* TK-24 (pMO16) can help to confirm the thiopeptide nature of antibacterially active substances extracted from transconjugants. All data concerning these tests is summarized in Table 2.

Table 2

Diameters of *S. lutea* growth inhibition zones and *S. lividans* TK-24 (pMO16) growth induction zones by the extracts from plasmid-bearing transconjugant strains

Strain	Diameter of *S. lutea* growth inhibition zones, *mm*	Diameter of *S. lividans* TK-24 (pMO16) growth induction zones, *mm*	Dry weight of the biomass of SiA producer, *mg/ml*
S. sioyaensis Lv81	8.5 ± 0.5	8.5 ± 1.0	4.7 ± 0.5
S. sioyaensis pIJ6085relA	9.0 ± 1.0	8.5 ± 0.5	4.8 ± 0.4
S. sioyaensis pSOK804absB	11.0 ± 0.5	10.0 ± 1.0	4.0 ± 0.7
S. sioyaensis pKC1139absB	13.0 ± 0.5	12.0 ± 1.0	3.6 ± 1.1
S. sioyaensis pKCafsS	18.0 ± 1.0	18.0 ± 0.5	4.8 ± 0.9
S. sioyaensis Rif49S	12.0 ± 0.8	11.0 ± 0.5	3.9 ± 01.0
S. sioyaensis Rif49S pKCafsS	18.0 ± 1.0	18.0 ± 1.0	3.8 ± 0.5
S. sioyaensis 100	16.0 ± 0.5	15.0 ± 1.0	3.6 ± 1.1
S. sioyaensis 100 *pKCafsS*	20.0 ± 1.0	20.0 ± 1.0	4.1 ± 0.5

S. sioyaensis strain carrying pIJ6085relA plasmid virtually did not differ from wild type in SiA production, as evident from antibiotic disc diffusion and *tipAp* induction tests (Table 2). In case of *absB* gene, a considerable increase in SiA production can be recorded. Our *tipA*p induction tests are in agreement that antibiotic activity of *absB*-expressing strains is due to increase in thiopeptide production. More SiA is produced by strain carrying pKC1139absB. It has been shown previously [13] that pSOK804 vector has one site of integration in the *S. sioyaensis* genome, while pSG5-based pKC1139 is present at 20 to 50 copies per cell [7]. Therefore, increased *absB* dosage favors SiA production, implying positive role of this gene in SiA biosynthesis.

There are several possible explanations as to why *absB* influenced SiA production and *relA* didn't. Both *absB* and *relA* gene are considered pleiotropic regulators involved in different cellular processes. RelA protein, known as (p)ppGpp-synthetase, usually is synthesized during the amino acids starvation of the cell or as a response to multiple stress conditions. In this work, *relA* gene expression is expected to be constant from the onset of siomycin biosynthesis because of induction of *relA* transcription from *tipAp* with SiA. It is possible that such conditions are not appropriate for SiA biosynthesis or *relA* gene expression naturally has little or no connection to SiA biosynthesis. It has been shown for some streptomycetes that expression of additional copies of *relA* gene can lead to antibiotic overproduction [15], while other streptomycetes overproduce antibiotics in the absence of *relA* [5]. In our case *relA* appears to be dispensable for SiA biosynthesis.

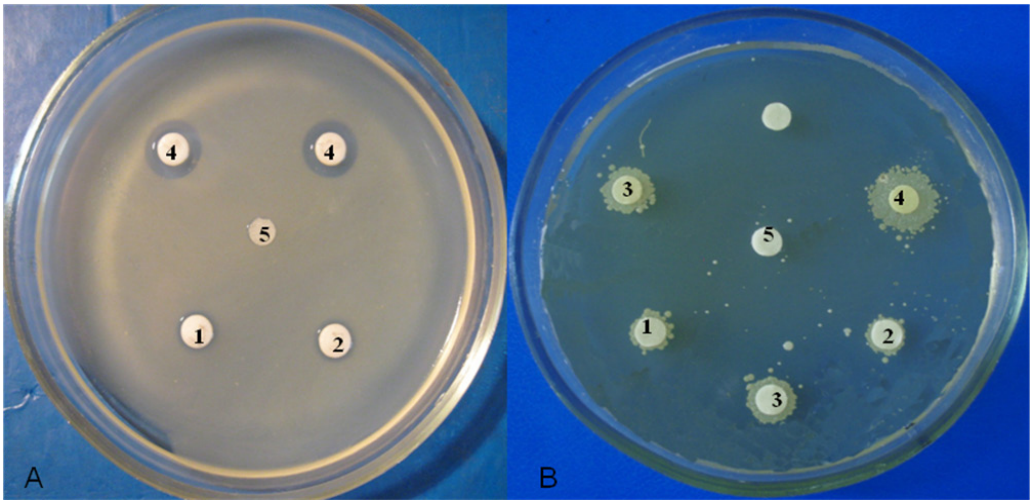

Fig. 3. A – disk diffusion bioassay with *S. lutea* used as a test-culture; B – disk diffusion bioassay with *S. lividans* TK-24 (pMO16) used as a test-culture. 1 – *S. sioyaensis* Lv81; 2 – *S. sioyaensis* pIJ6085*relA*; 3 – *S. sioyaensis* pSOK804*absB*; 4 – *S. sioyaensis* pKC1139*absB*; 5 – DMSO.

The biggest increase in SiA production was recorded for the *S. sioyaensis* pKCafsS strain (Table 2). So far as expression of *afsS* gene has shown the most notable results, we have decided to transfer pKCafsS construct to high-producing *S. sioyaensis* strains obtained in our laboratory. Two strains were chosen for this purpose: *S. sioyaensis* Rif49S and *S. sioyaensis* 100 (Table 1). These strains are spontaneous mutants selected for increased resistance to rifampicin and streptomycin. It is known for other streptomycetes that acquisition of antibiotic resistance mutations (especially certain streptomycin and rifampicin resistance mutations) enables cells to overproduce antibiotics, [9, 16]. *S. sioyaensis* Rif49S strain was obtained as a spontaneous mutant resistant

to 0.1 μg/ml of rifampicin. Heterologous expression of *afsS* gene in this strain increased SiA production to the level observed for wild type carrying pKCafsS (Fig. 4). We offer several explanations to this fact. Possibly, influence of the *afsS* gene expression was in some way altered in Rif49S mutant due to as-yet-unknown changes in its genome. It is also possible that there is upper level of (potentially autotoxic) SiA production by the studied strains, which cannot be overcome through mere expression of regulatory genes. We have carried out one more experiment to address this question. Gene *afsS* was transferred to *S. sioyaensis* 100 strain that was obtained as a derivative of *S. sioyaensis* Rif49S showing high streptomycin resistance. In case of *S. sioyaensis* 100 pKCafsS strain, the highest SiA biosynthetic levels were observed in comparison to all strains analyzed in this study (Fig. 4). Thus, the SiA titers observed in Rif49S expressing *afsS* is not due to reaching the upper level of SiA biosynthesis.

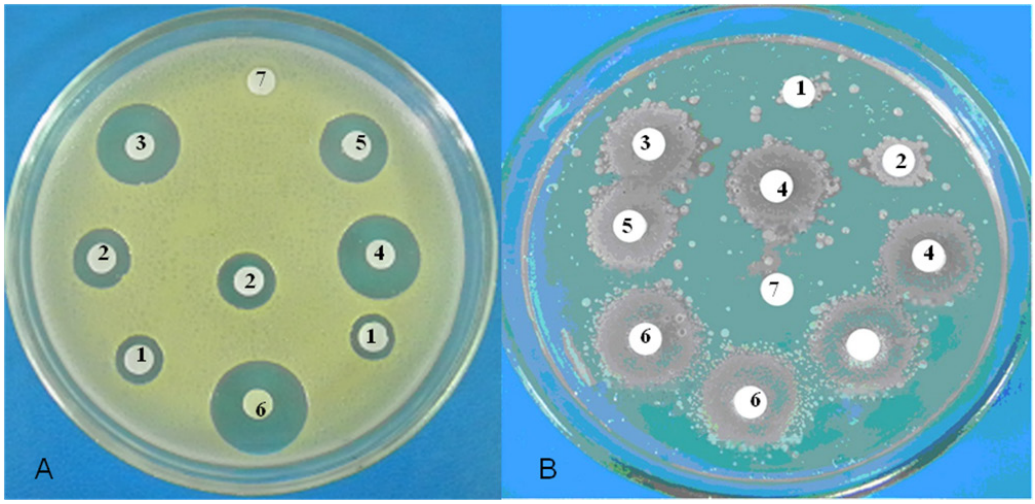

Fig. 4. A – Disc diffusion bioassay with *S. lutea* used as a test-culture; B – *tipAp* induction assay bioassay with *S. lividans* TK-24 (pMO16) used as a test-culture: 1 – *S. sioyaensis* Lv81; 2 – *S. sioyaensis* Rif49S; 3 – *S. sioyaensis* pKC*afsS*; 4 – *S. sioyaensis* Rif49S pKC*afsS*; 5 – *S. sioyaensis* 100; 6 – *S. sioyaensis* 100 pKC*afsS*; 7 – DMSO.

Heterologous expression of *afsS* gene has shown the best results concerning the improvement of SiA biosynthetic levels. This fact can be explained by the probable existence of gene in the *S. sioyaensis* genome homologous to *S. coelicolor afsS* and its close involvement in regulation of SiA production. Exact mechanisms of these processes remain unclear, but it is evidently that pleiotropic regulatory genes can be used as tools for improvement of SiA production.

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ВПЛИВ ПЛЕЙОТРОПНИХ РЕГУЛЯТОРНИХ ГЕНІВ *absB, relA* ТА *afsS* НА БІОСИНТЕЗ СІОМІЦИНУ ШТАМОМ *STREPTOMYCES SIOYAENSIS* Lv81

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Streptomyces sioyaensis Lv81 (=NRRL-B5408) – продуцент тіопептидного антибіотика сіоміцину А (SiA). SiA синтезується штамом дикого типу в малих кількостях, що обмежує використання цього штаму. Мета нашого дослідження – вивчити шляхи підвищення біосинтезу SiA за допомогою гетерологічної експресії у *S. sioyaensis* плейотропних регуляторних генів *absB* (кодує РНК-азу III), *relA* ((p)ppGpp-синтетаза) та afsS (67-амінокислотний пептид, що володіє шаперон-подібними властивостями). Ген *absB* ампліфіковано із геному *S. ghanaensis*, гени *relA* та *afsS* – із геному *S. coelicolor*. Рекомбінантні ДНК, що містили вищезгадані гени, перенесено у клітини *S. sioyaensis*. У випадку генів *absB* та *afsS* виявлено значне зростання продукції SiA транскон'югантними штамами, що свідчить про позитивну регуляцію біосинтезу SiA цими генами. Експресія гена *relA* не впливала на біосинтез SiA. Ці результати вказують на перспективність використання плейотропних регуляторних генів для підвищення біосинтезу SiA у штамі дикого типу *S. sioyaensis*.

Ключові слова: вторинний метаболізм, тіопептидні антибіотики, сіоміцин, шлях-специфічні регулятори, плейотропні регулятори.

ВЛИЯНИЕ ПЛЕЙОТРОПНЫХ РЕГУЛЯТОРНЫХ ГЕНОВ *absB, relA* ТА *afsS* НА БИОСИНТЕЗ СИОМИЦИНА ШТАММОМ *STREPTOMYCES SIOYAENSIS* Lv81

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Streptomyces sioyaensis Lv81 (=NRRL-B5408) – продуцент тиопептидного антибиотика сиомицина А (SiA). Штамм дикого типа синтезирует небольшое количество SiA, поэтому использование этого штамма ограничено. Целью нашего исследования было изучение путей повышения уровня биосинтеза SiA при помощи гетерологической экспрессии в *S. sioyaensis* плейотропных регуляторных генов *absB* (кодирует РНК-азу III), *relA* ((p)ppGpp-синтетаза) и *afsS* (67-аминокислотный пептид, владеющий шаперон-подобными активностями). Ген *absB* был клонирован из генома *S. ghanaensis*, гены *relA* и *afsS* – из генома *S. coelicolor*. Для этого рекомбинантные ДНК, содержащие упомянутые гены, были перенесены в клетки *S. sioyaensis*. В случае генов *absB* и *afsS* обнаружено значительное увеличение уровней биосинтеза SiA трансконъюгантными штаммами, что свидетельствует о позитивной регуляции биосинтеза SiA этими генами. Экспрессия гена *relA* не влияла на биосинтез SiA. Эти результаты указывают на перспективность использования плейотропных регуляторных генов для повышения биосинтеза SiA в штамме дикого типа *S. sioyaensis*.

Ключевые слова: вторичный метаболизм, тиопептидные антибиотики, сиомицин, путь-специфические регуляторы, плейотропные регуляторы.