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IDENTIFICATION AND CHARACTERIZATION OF *STREPTOMYCES GHANAENSIS* ATCC 14672 REGULATORY GENES *NSDAGH* AND *ATRAGH*

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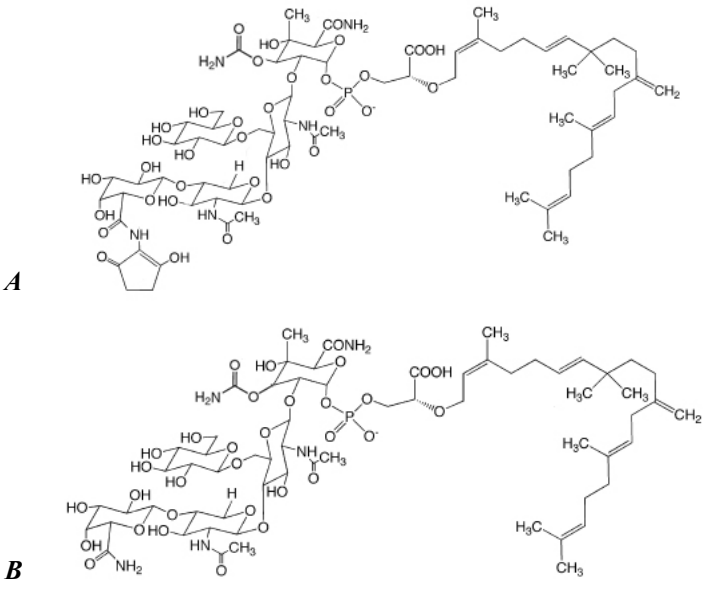
Genes *nsdAgh* and *atrAgh* encoding putative regulators of secondary metabolism were identified in the partially sequenced *Streptomyces ghanaensis* – producer of moenomycins. Both genes were cloned individually into *Streptomyces* expression vectors and overexpressed in *S. ghanaensis* and *S. coelicolor* strains. No significant changes in production of moenomycin A in *S. ghanaensis* and actinorhodin in *S. coelicolor* were revealed, implying that the aforementioned genes do not exert significant effects on moenomycin and actinorhodin production under overexpression conditions.

Key words: Streptomyces ghanaensis, moenomycin A, pleiotropic regulators, phylogenetic analysis.

Streptomyces ghanaensis produces the phosphoglycolipid antibiotic moenomycin A (MmA; Fig. 1) – the only natural product known to directly target the extracellular peptidoglycan glycosyltransferases (PGTs) involved in bacterial cell wall biosynthesis [13]. PGTs are highly conserved bacterial enzymes that catalyze the polymerization of the two-sugar subunits of bacterial peptidoglycan. These enzymes are considered excellent targets for the develop-



A

B

Fig. 1. Structure of moenomycin A (*A*) and its precursure 6B-hydroxy-6B-de[N-(2-hydroxy-5-oxo-1-cyclopenten-1-yl)amino]-moenomycin A (*B*).

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ment of new antibiotics. The unique traits of moenomycins are its unusual structure and activity against methicillin- and vancomycin-resistant pathogens. Although it was used in animal nutrition for decades the development of MmA-resistant microflora in vivo has not been documented. Due to poor pharmacokinetics (e.g., a very long half-life) MmA is unsuitable for the clinical use in. The long lipid chain of MmA is thought to cause its poor pharmacokinetics. The total synthesis of moenomycin has been completed and the *moe* cluster has been cloned, providing the possibilities for biosynthesis of MmA derivatives [12]. Recent studies show that genetic manipulations of MmA producers can be a source of novel moenomycins, although they are produced at extremely low levels. Therefore, it is important to design MmA-producing strains that will overproduce MmA analogs.

The majority of antibiotics are the products of complex biosynthetic pathways that are activated in a growth phase-dependent manner. The activation of antibiotic biosynthesis is genetically controlled at several levels. Pleiotropic regulatory genes exert their control over two or more antibiotics in the same organism. For example, products of genes *afsK*, *afsR*, and *afsS* in *S. coelicilor* are involved in the regulation of the production of actinorhodin, undecylprodigiosin, and calcium-dependent antibiotic [2, 19]. The MmA biosynthetic pathway is highly unusual because of the lack of pathway-specific regulatory genes, as is evident from the results of *moe* cluster sequencing and heterologous expression. Thus identification of pleiotropic regulatory genes influencing MmA production is of great interest. The availability of the genome sequences for the moenomycin producer, *S. ghanaensis*, should help explore the global regulators for MmA production. Our preliminary *in silico* analysis showed that the *S. ghanaensis* genome contains a number of orthologs of well-studied *S. coelicolor* regulators.

Among the many regulatory genes, *nsdA* negatively affects antibiotic production and sporulation [11]. Gene *nsdA* is present and conserved in many actinomycetes, such as *S. hygroscopicus* 10B22, *S. spiecies* FR-008, *S. lividans* ZX64, *S. aureofaciens* 211, *S. albus* JA3453, *S. hygroscopicus* 5008, *S. avermitilis* NRRL8165 and *S. coelicolor* M145 [18]. It is possible, that *nsdA* may have some general effects on antibiotic biosynthesis and, therefore, may affect MmA production. AtrA is a transcriptional activator for actinorhodin biosynthesis [17]. Expression of *S. coelicolor* AtrA (AtrAc) in *S. griseus* causes a DNA binding-dependent reduction in streptomycin production and in the mRNA levels of *strR* and genes of streptomycin biosynthesis. It was investigated that, AtrAc is able to bind *in vitro* to the promoter of *strR*, a transcriptional activator unrelated to *actII-ORF4*, that is the final regulator of streptomycin production in *S. griseus*. Also, AtrAc stimulates the production of actinorhodin in *S. lividans*, but has no effect on the production of oxytetracycline or rimocidin by strains of *S. rimosus* [8]. Gene *aveI* – ortholog of *atrAc*, negatively regulates the avermectin biosynthesis by *S. avermitilis* while enhancing actinorhodin production by *S. coelicolor* [4]. At the same time, *atrAc* exert negative effect on avermectin biosynthesis by *S. avermitilis*. AtrA occurs in all streptomycetes, its DNA binding domain is extremely well conserved (>90% amino acid identity), which is compelling phylogenetic evidence against a specific function in *S. coelicolor*. There is evidence, that AtrA controls a more diverse regulon including metabolic genes, and recent DNA binding experiments revealed binding sites in the *cda* gene cluster, suggesting a pleiotropic (activating?) function for AtrAc in the control of antibiotic production. Furthermore, induced expression of *atrAc* can bypass the effects of hyper-repressive alleles of pleiotropic regulators such as *absA1* [19].

In this study, we report the identification of *S. ghanaensis* pleiotropic regulatory genes homologous to *nsdA* and *atrA* and the investigation of the effects of overexpression of *nsdAgh* and *atrAgh* genes on MmA production.

Strains used in this work: *S. ghanaensis* ATCC 14672 – MmA producer; *S. coelicolor* M145; *Escherichia col*i DH5α was used for routine subcloning; *E. coli* ET12567 (*dam-13*::Tn*9* (Cmr), *dcm-6*, *hsdM*) harbouring the conjugative plasmid pUB307 was used to perform intergeneric conjugation from *E. coli* to *Streptomyces* species [1]. *B. subtilis* was used as test-culture in agar diffusion method. For plasmid DNA isolation, *E. coli* strains were grown as described in [10] and [15]. *Streptomyces* strains were maintained on oatmeal agar medium and soy-mannitol medium [10]. For estimation of actinorhodin production *S. coelicolor* was grown on MMGT medium (g/l): L-asparagine – 0,5, K_2HPO_4 – 0,5, $MgSO_4 \cdot 7H_2O$ – 0,2, $FeSO_4$ – 0,01, casamine acids – 6, glucose – 5l, agar – 10–18. *E. coli* ET12567 (pUB307) carrying plasmids for conjugal transfer was grown on LB medium containing kanamycin (25 μg/ml) and apramycin (50 μg/ml) for 16 h. For conjugation, spores of *S. ghanaensis* and *S. coelicolor* strains were harvested from a lawn grown on soy-mannitol and oatmeal medium [10]. Isolation of plasmid DNA from *E. coli* was carried out using standard protocols [10]. Restriction enzymes and molecular biology reagents were used according to recommendation of suppliers (NEB, MBI Fermentas). PCR was performed using Pfu DNA polymerase and primer pairs specific to each individual gene. For disk diffusion test *Streptomyces* strains were grown in tryptic soy broth (TSB) for 5 days on a rotary shaker. 200 mg of wet weight of each sample were extracted with an equal volume of methanol. Extract were dried and dissolved in methanol. The top agar containing the bacterial cultures of *B. subtilis* were poured and distributed evenly onto the LB agar plates. The plates were transferred into incubator at 37°C. The presences of inhibition zones on LB plates were observed and the diameters of each inhibition zone were measured and recorded. For moenomycin production HPLC-MS analysis *S. ghanaensis* strains were grown at 28°C for 5 days in TSB on a rotary shaker. 0,9 g of wet weight of each sample were extracted with an 5 ml of methanol. Moenomycins were purified from the extracts by using a 100 mg/1,5 ml C18 SPE cartridge (Alltech) and were analyzed by LC-MS on an Agilent 1100 series LC/MSD machine as previously was described in [5]. The mobile phases were stabilized with 0.1% NH_4OH. For phylogenetic analysis, the sequences of NsdAgh and AtrAgh were aligned using CLUSTAL W software [16]. Evolutionary distance matrices were constructed using the algorithm of Jones-Taylor-Thornton [9] and evolutionary trees for the datasets were inferred from the neighbourjoining method [6]. The stability of relationships was assessed by performing bootstrap analysis of neighbour-joining data based on 100 resamplings.

Gene *nsdAgh* encodes 498 amino-acid protein with a predicted molecular weight 54,8 kDa. A BlastP sequence homology search identified regulatory and putative regulatory proteins with significant similarity profiles. The proteins showing the greatest homology to NsdA were such regulators as, SCO5582 from *S. coelicolor* (90% of identity), NsdA from *S. bingchengensis* (90% of identity), NsdA from *S. fradiae* (89% of identity), putative regulatory protein SAV_2652 from *S. avermitilis* (88% of identity). Also, such bacteria as *Actinosynnema mirum* DSM 43827, *Salinispora archicola* CNS-205, and *Frankia* sp. EAM1pec contain NsdA homologues. NsdA contains a tetratricopeptide repeat (TPR)-like domain, which mediate protein-protein interactions [9]. Analysis of the amino acid sequence was performed by using the InterPro Scan (http://www.ebi.ac.uk/Tools/InterProScan/) search engine.

The *atrAgh* gene of *S. ghanaensis* encodes a protein of 227 amino-acid with a calculated molecular weight of 24,093 kDa. Initial analysis of AtrA was performed using SwissProt protein motif identification software (http://myhits.isb-sib.ch/cgi-bin/motif_scan). AtrAgh matches an entry in the Protein families (Pfam) database that corresponds to the N-terminal helix-turn-helix DNA-binding domain of *E. coli* TetR and related proteins. However, AtrAgh

does not contain ligand-binding domain and dimerization domain in contrast to TetR from *E. coli*. Therefore, the common feature of AtrA and TetR-family proteins is a DNA recognition domain [14].

BlastP searching indicated that the closest homologues of *atrA* from *S. ghanaensis* are such proteins: TetR family transcriptional regulator from *S. viridochromogenes* (81% of identity), SCO4118, TetR family transcriptional regulator from *S. coelicolor* (64% of identity), TetR family transcriptional regulator from *S. lividans* (64% of identity), SAV_4110, TetR family transcriptional regulator from *S. avermitilis* (77% of identity).

Phylogenetic analysis of these protein sequences (Fig. 2, 3), using the neighbour-joining method within the PHYLIP 3-6.9 package [6], showed that AtrAgh phylogenetic tree forms two clades which have common ancestor with transcriptional regulator from *S. hygroscopicus*, supported by 87% of bootstrap replicates. One includes TetR-family transcriptional regulators from *S. clavuligerus* ATCC 27064, *S. pristinaespiralis* ATCC 25486, *S. roseosporus* NRRL, *S. scabies, S. albus* J1074 and the other includes AtrAgh sharing highest similarity with proteins from *S. coelicolor, S. bingchengensis, S. fradie*. NsdA phylogenetic tree forms diverse phyletic lines. Members of one of these groups (*S. coelicolor, S. bingchengensis, S. fradie*) were found to share highest similarity with NsdA-g protein, supported by 93%

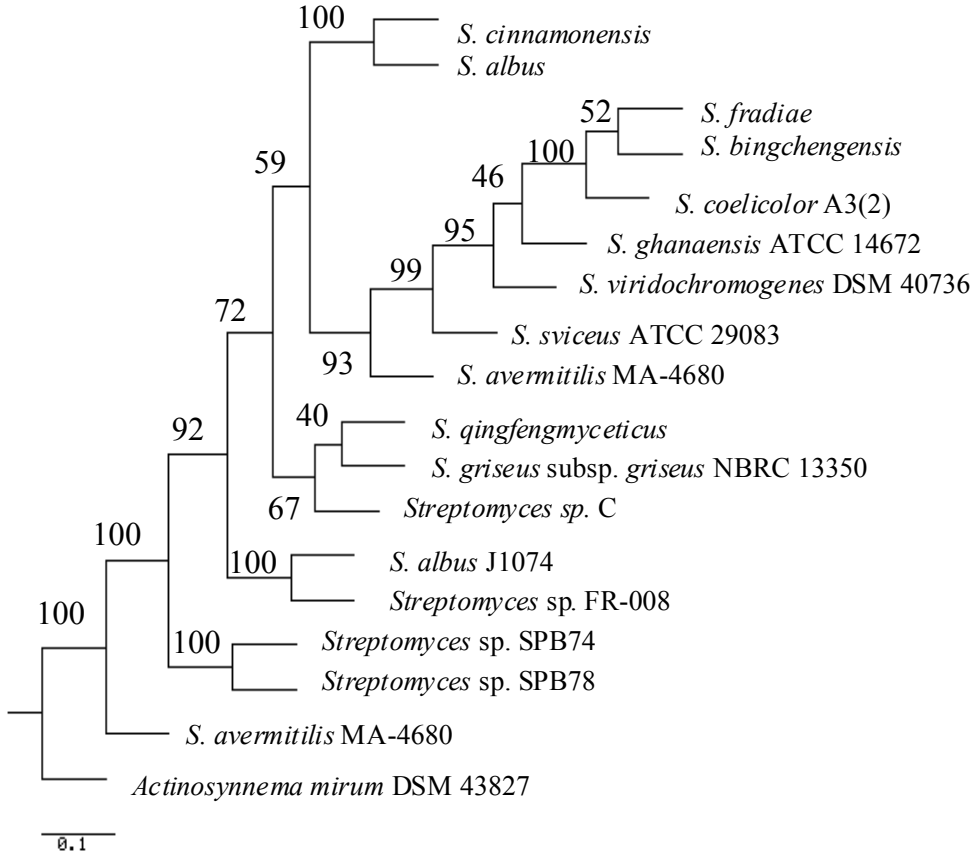

Fig. 2. Relationship of NsdAgh with its nearest phylogenetic relatives in the phylogenetic tree resulting from neighbourjoining method. Numbers adjacent to branches indicate bootstrap values (expressed as a percentage of 100 replicates). Bar, 0,1 substitutions per site.

of bootstrap replicates. Phylogenetic analysis showed that the tree includes paralogous proteins from *S. albus* genome (NsdA and regulatory protein) and *S. avermitilis* genome (SAV1236 and SAV2652).

A 2,8 kb DNA fragment containing *nsdAgh* was amplified by PCR from *S ghanaensis* and cloned into pKC1139 vector [3]. The presence of the *nsdAgh* fragment was confirmed by DNA sequencing. The resulting construct designed pKCnsdAgh was transferred into *S. ghanaensis* from *E. coli* by conjugation [10].

The upstream and downstream primers used for amplification of *atrAgh* gene from *S. ghanaensis* chromosomal DNA were: 5'- AAA TCT AGA TGT GCT CAA CGG ATC CGC-3' and 5'- AAA GAA TTC CTA TTC GGG CCG TGA GCG C-3', respectively. Primers were designed to generate 0,9 kb PCR products, each with unique XbaI and EcoRI sites (underlined) at 5' and 3' ends, respectively, to facilitate directional subcloning in pKC1139ermE [13]. The resulting construct called pKCatrAgh was introduced after passage through *E. coli* ET12567 into *S. ghanaensis* strain obtaining *S. ghanaensis* (+pKCatrAgh). Also vector pKC1139 was transferred into *S ghanaensis* as a negative control.

Obtained exconjugants did not show significant differences in phenotypes in comparison with wild type strain. Subsequently, the agar diffusion method was used to analyze and evaluate the antibacterial activity against *B. subtilis* for each extract. From the observation and measurement of diameter of inhibition zones formed, it has been demonstrated that the extracts from the *S. ghanaensis* (+pKCatrAgh) and *S. ghanaensis* (+pKCnsdAgh) strains had no significant changes in antibiotic activity in comparison with extracts from *S. ghanaensis*

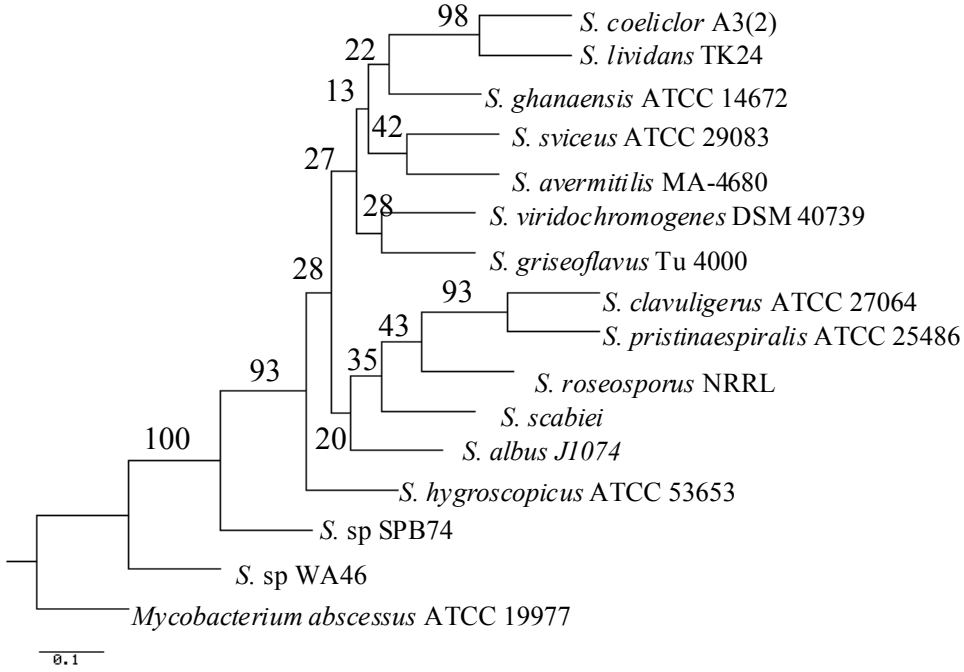

Fig. 3. Relationship of AtrAgh with its nearest phylogenetic relatives in the phylogenetic tree resulting from neighbourjoining method. Numbers adjacent to branches indicate bootstrap values (expressed as a percentage of 100 replicates). Bar, 0,1 substitutions per site.

(+pKC1139). Also LC-MS analysis was performed detecting abundance of two ions: moenomycin A $[M-H]^-$ of m/z 1580,6 Da and its precursor of m/z 1485,63 Da (Fig. 4).

pKCatrAgh and pKCnsdAgh plasmids were introduced in *S. coelicolor* M145, in order to see if the heterologous overexpression of regulatory proteins from *S. ghanaensis* will have effect on production of actinorhodin. However, differences in actinorhodin production were not revealed, while overexpression of NsdAc and AtrAc results in changes in antibiotic biosynthesis by *S. coelicolor* M145. As was proposed by Hong [8], it is likely that the ancestral AtrA and its targets have coevolved. Even though a homologue is able in a heterologous host to bind sites used by the native activator, this may not be sufficient for the former to stimulate transcription as it may be unable to make necessary contacts with RNA polymerase. The co-expression of such a homologue may antagonize activation by the native activator as a result of competition for DNA binding [8].

There are several possible explanations of these results. Apparently genes *nsdAgh* and *atrAgh* may have no regulatory effect on biosynthesis of MmA or they have no regulatory effect on MmA production under our laboratory conditions. As it was previously shown, *S. griseus* mutant Δ*atrA-g* produced a smaller amount of streptomycin than the wild-type strain on Bennett agar containing either 1% maltose or glycerol [7]. AtrAgh could have conditionally dependent effect on MmA production. Also, we cannot exclude the possibility that TetR-family transcriptional regulator AtrAgh is involved in regulation of cryptic cluster in *S. ghanaensis*.

The way of function of NsdA in *S. coelicolor* is not clear yet. The disruption *SCO4114* which had previously been shown to prevent premature sporulation and septation of *S. griseus*, had no obvious phenotypic effects on *S. coelicolor* [8]. Possibly, there are differences in the way that different members of this paralogous family are involved in regulation of antibiotic production. However, the NsdA orthologue SAV2652 from *S. avermitilis* is able to complement the phenotype of *S. coelicolor* Δ*nsdAc* to the wild-type level just like *nsdAc* from *S.*

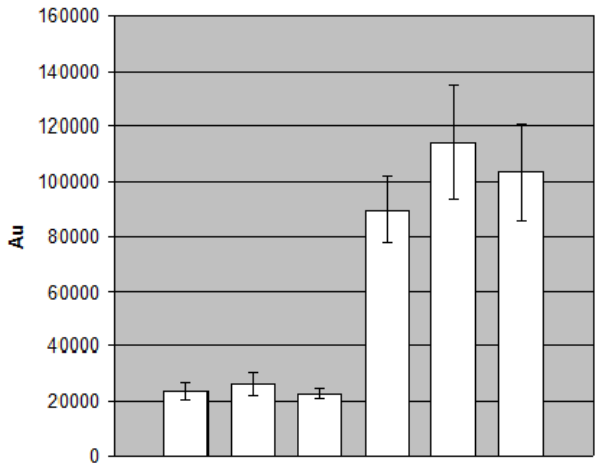

Fig. 4. HPLC-MS analysis (ESI, negative mode) of moenomycin production by *S. ghanaensis* harbouring plasmid pKC1139 and recombinant *S. ghanaensis* strains. 1, 2, 3 – abundance of moenomycin A $[M-H]^-$ ions (m/z 1580,6 Da) from extracts from *S. ghanaensis* (+pKC1139), *S. ghanaensis* (+pKCatrAgh), *S. ghanaensis* (+pKCnsdAgh), respectively; 4, 5, 6 – abundance of 6B-hydroxy-6B-de[N-(2-hydroxy-5-oxo-1-cyclopenten-1-yl)amino]-moenomycin A (m/z 1485,63 Da) from extracts from *S. ghanaensis* (+pKC1139), *S. ghanaensis* (+pKCatrAgh), *S. ghanaensis* (+pKCnsdAgh), respectively. Au – arbitrary units.

coelicolor can. The results of gene overexpression are not always definitive, and gene inactivation experiments will provide more information about functions of these genes in *S. ghanaensis*.

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ВИЯВЛЕННЯ І ВИВЧЕННЯ РЕГУЛЯТОРНИХ ГЕНІВ *NSDAGH* І *ATRAGH* У ШТАМІ *STREPTOMYCES GHANAENSIS* ATCC 14672

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У частково секвеновану геномі продуцента моеноміцинів *Streptomyces ghanaensis* ідентифіковано й охарактеризовано гени *nsdAgh* і *atrAgh*, які кодують імовірні регулятори вторинного метаболізму. Ці гени клоновано у стрептоміцетні вектори експресії та надекспресовано у штамах *S. ghanaensis* і *S. coelicolor*. Значних змін у продукції моеноміцинів в *S. ghanaensis* і актинородину в *S. coelicolor* не виявлено, що свідчить про відсутність впливу цих генів на синтез вказаних антибіотиків за умов їхньої надекспресії.

Ключові слова: Streptomyces ghanaensis, моеноміцин А, плейотропні регулятори, філогенетичний аналіз.

ОБНАРУЖЕНИЕ И ИЗУЧЕНИЕ РЕГУЛЯТОРНЫХ ГЕНОВ *NSDAGH* И *ATRAGH* В ШТАММЕ *STREPTOMYCES GHANAENSIS* ATCC 14672

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В частично секвенированном геноме продуцента моеномицинов *Streptomyces ghanaensis* идентифицированы и охарактеризованы гены *nsdAgh* и *atrAgh*, кодирующие вероятные регуляторы вторичного метаболизма. Эти гены клонированы в стрептомицетные векторы экспрессии и экспрессированы в штаммах *S. ghanaensis* и *S. coelicolor*. Значительных изменений в продукции моеномицинов у *S. ghanaensis* и актинородина у *S. coelicolor* не обнаружили. Это свидетельтствует о том, что эти гены не оказывают влияния на синтез указанных антибиотиков в условиях их повышенной экспрессии.

Ключевые слова: Streptomyces ghanaensis, моеномицин А, плейотропные регуляторы, филогенетический анализ.

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