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ГЕНЕТИКА

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ROLE OF THE *ABSA2$_{GH}$* GENE IN THE REGULATION OF MOENOMYCIN A PRODUCTION BY *STREPTOMYCES GHANAENSIS* ATCC 14672

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Gene *absA2$_{gh}$* encoding response regulator of two-component system AbsA2$_{gh}$1/2 was identified on the chromosome of *Streptomyces ghanaensis* – producer of moenomycins. Overexpression of *absA2$_{gh}$* in *S. ghanaensis* and heterologous expression in *S. coelicolor* led to decrease in MmA and actinorhodin production, respectively. In the same time, no significant changes in moenomycin production were revealed in *S. ghanaensis ΔabsA2$_{gh}$* mutant. Thus *absA2$_{gh}$* appears to influence moenomycin production under overexpression conditions, although it is not an essential regulator of this pathway.

Keywords: Streptomyces ghanaensis, moenomycin A, pleiotropic regulators, two-component system.

Members of the *Streptomyces* genus are well-known for their ability to produce a vast array of secondary metabolites, among them antibiotics, chemotherapeutic agents, immunosuppressants, and other agents of clinical use [5, 7, 17]. Genes encoding enzymes for the biosynthesis of antibiotics are clustered in distinct loci and are usually subject to complex regulation [3]. The study of antibioticbiosynthesis in the genetically well-characterized strain *Streptomyces coelicolor* gave insights into many aspects of the regulation of antibiotic biosynthesis. There are several hierarchically organized groups of regulatory genes affecting secondary metabolite production in streptomycetes: genesthat control both antibiotic production and sporulation, genes that control the production of several antibiotics, and pathway-specific regulators that control the productionof one antibiotic [17]. Most but not all secondary metabolite clusters contain pathway-specific regulatory genes whoseexpression usually depends on higher-level regulatory genes [3]. For example, *Streptomyces ghanaensis* clusters of genes for biosynthesis of phosphoglycolipid antibiotic moenomycinA (MmA) do not contain pathway-specific regulatory genes [12]. MmA drew the researcher's attention due to uniqueness of its structure and mechanism of action. MmAis the only known compound that inhibits peptidoglycan glycosyltransferases (PGTs), its inhibitory effect can be observed at nanomolar concentrations [12, 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 development of new antibiotics. MmA is more active than vancomycin against many gram-positive pathogens, including methicillin- and vancomycin-resistant pathogens. However, due to poor pharmacokinetics (e.g., a very long half-life) MmA is unsuitable for the clinical use [6]. Therefore, MmA is considered a blueprint for development of phosphoglycolipid analogs to fight multidrug-resistante pathogenic bacteria. Extremely low levels of MmA production and lack of knowledge about regulation of MmA biosynthesis has prompted us to study pleiotropic regulatory genes. The understanding of the genetic mechanisms that limit moenomycin biosynthesis is essential for construction of MmA-overproducing strains.

Having the sequence of *S. ghanaensis* genome we were able to identify the ortholog of *absA2* two-component system from *S. coelicolor* [1, 4] on *S. ghanaensis* chromosome. The *absA*-like operons are found primarily in actinomycetes and are often embedded in the biosynthetic gene clusters of secondary metabolites [14]. In *S. coelicolorabsA*locus encodes a two-component system consisting of the sensorkinase AbsA1 and the response regulator AbsA2and negatively regulates biosynthesis of four antibiotics [2]. AbsA1 phosphorylateAbsA2 on response to the yet-unknown signals from environment, phosphorylated AbsA2-P act as repressorof gene expression [16]. In particular, it was demonstrated that AbsA2 binds the promoter regions of *actII*-ORF4, *cdaR*, and *redZ*, which encode pathway-specific activators for actinorhodin, calcium-dependent antibiotic, and undecylprodigiosin, respectively. The binding of AbsA2 to eachgene is enhanced by phosphorylation [11].

In this study, we report the identification of *S. ghanaensisabsA2* ortholog, $absA2_{gh}$, and the investigation of the effects of $absA2_{gh}$knockout and overexpression on MmA production by *S. ghanaensis*.

Materials and methods

The wildtype *S. ghanaensis*ATCC 14672 strain was from ATCC. *Escherichia coli* DH5α was used for routine subcloning. *E. coli* ET12567 pUB307^{+} was used to perform intergeneric conjugation from *E. coli* to *Streptomyces* species [9]. Transformation of *E. coli* by electroporation, and general molecular genetics techniques, were as in [15]. *Bacillus cereus* ATCC 19637 was used as test-culture in agar diffusion method. *Streptomyces* strains were maintained on oatmeal agar medium and soy-mannitol medium. All *E. coli* strains were cultured at 37°C in Luria broth or on Luria Broth agar supplemented with the appropriate antibiotics when needed. For conjugation, spores of *S. ghanaensis* and *S. coelicolor* strains were harvested from a lawn grown on soy-mannitol [8] and oatmeal medium (g/L: oatmeal flour – 34, agar – 20, pH 8.0 prior to autoclaving).For estimation of actinorhodin production *S. coelicolor* was grown in YMPG medium(yeast extract – 4 g/l; peptone – 1 g/l; maltose extract – 10 g/l; glucose – 10 g/l; $MgCl_2{\times}6H_2O$ – 2 g/l) and on MMGT medium (L-asparagines – 0,5g/l; K_2HPO_4 – 0,5g/l; $Mg_2SO_4{\times}7H_2O$ – 0,2g/l; $FeSO_4{\times}7H_2O$ – 0,01g/l; casamino acids – 6g/l; glucose – 5g/l; agar – 18g/l). When necessary, the medium was supplemented with antibiotics (ampicillin 100 μg/ml, apramycin 50 μg/ml, kanamycin 50 μg/ml). X-gal and IPTG were used as described elsewhere for blue-white colony selection in the case of the pUC57, pKC1139.

Isolation of genomic DNA from *Streptomycetes* and plasmid DNA from *E. coli* were carried out using standard protocols [8]. Restriction enzymes and molecular biology reagents were used according to recommendation of suppliers (NEB, MBI Fermentas). PCR was performed using Pfu DNA polymerase (MBI Fermentas) and primer pairs specific to each individual gene.

For disk diffusion test *Streptomyces* strains were grown in tryptic soy broth 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 28°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 of moenomycin production was done as described in [10]. *Actinorhodin*was extracted as described in [8].

Results and discussion

Gene $absA2_{gh}$ encodes protein of 221 amino-acid with predicted molecular mass 22,86 kDa. Computer analysis of the deduced protein product of $absA2_{gh}$with the BlastP program identified

a number of response regulator proteins having significant similarity to $AbsA2_{gh}$. The proteins showing the greatest identity to $AbsA2_{gh}$ were as follows: SBI_05979 from *S. bingchengensis* BCW-1 (80% of identity), SVEN_5106 (72%) from *S. venezuelae* ATCC 10712, SCLAV_4354 (67%) from *S. clavuligerus* ATCC 27064 and SSDG_06202 (72%) from *S. pristinaespiralis* ATCC 25486. As evident from the CDD search results and PSIPRED analysis (http://bioinf.cs.ucl.ac.uk/psipred/) $AbsA2_{gh}$ contains two conserved motifs:one of them N-terminal REC-domain (signal receiver domain) or CheY-like phosphoacceptor domain that is phosphorylated by histidine kinase – $AbsA1_{gh}$ (Fig. 1).

Response regulator proteins control theadaptive response in two-component regulatory systems and signal receiver domains contain several highly conserved residues composing "pocket" with phosphorylation occurringat the second aspartate residue. ClustalW2multiplealignment of $AbsA2_{gh}$ with response regulator proteins revealed three conservedresidues: D9, D54 and K104, where D54 is a putative site of phosphorylation. Based on the sequence of C-terminal DNA-binding domain, $AbsA2_{gh}$ can be classified into LuxR-like family of proteins. This domain contains a helix-turn-helix motif and may control DNArecognition and binding (Fig. 1).

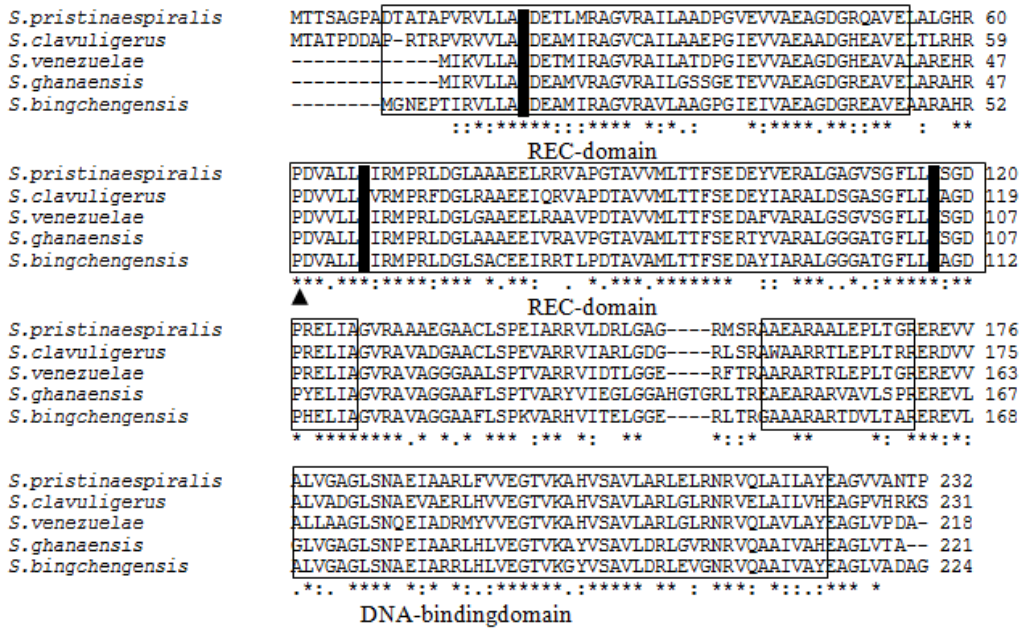

Fig. 1. Amino acid sequence alignments of *Streptomyces*AbsA2-like regulators.Conserved (asterisk) and homologous (colon) amino acids are marked underneath.Three highly conserved residues are highlighted in black, the putative site of phosphorylation is indicated with a triangle, black frames indicate N- and C-terminal conserved motifs.

For the $absA2_{gh}$ overexpression in *S. ghanaensis* a plasmid pKCabsA2gh was constructed. The upstream and downstream primers used for amplification of $absA2_{gh}$ gene from *S. ghanaensis* chromosomal DNA were: 5'-AAAGCTTCGGACAAGTCGGAGAGGAT-3' and 5'- AGGATCCCGGGCTCTAGCTAGAACGT-3', respectively. Primers were designed to generate 3 kb PCR products, each with unique HindIII and BamHI sites (underlined) at 3' and 5' ends, respectively, to facilitate directional subcloning into pKC1139. The resulting construct called pKCabsA2gh was introduced into *S. ghanaensis* strain obtaining *S. ghanaensis* $pKCabsA2gh^{+}$. Obtained excon-

jugants did not show significant differences in phenotypes in comparison with wild type strain. Also vector pKC1139 was transferred into *S ghanaensis* as a negative control. The extracts from the *S. ghanaensis* pKCabsA2gh$^+$ and *S. ghanaensis*pKC1139$^+$were analysed by LS-MC and it was shown an average 2,6 times decrease in MmA production compared to wild type strain (Fig. 2).

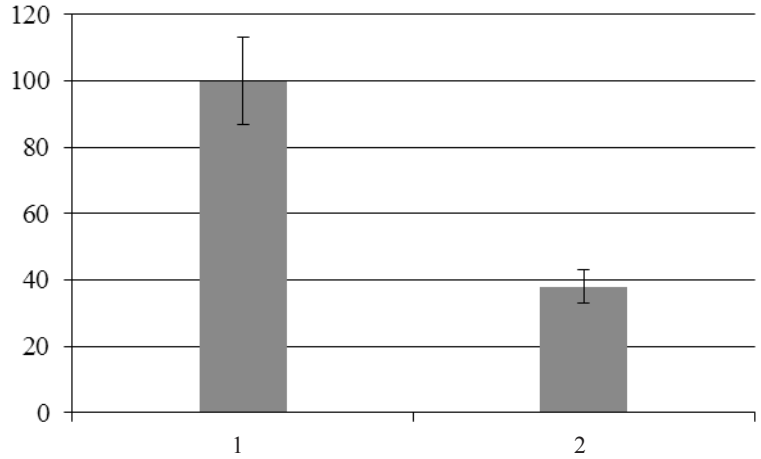

Fig. 2. Moenomycin production of *S. ghanaensis* strains: 1 – *S. ghanaensis* pKC1139$^+$; 2 – *S. ghanaensis* pKCabsA2gh$^+$ (in %; 100% corresponds to the production level of ATCC14672). Mean values were found from at least 3 independent experiments and normalized against equal amount of the biomass (dry weight). Error bars represent two standard deviations (± 2σ).

These results indicate negative role of $absA2_{gh}$in regulation of MmA biosynthesis.pKCabsA2ghplasmid wasalso introduced in *S. coelicolor* M145in order to see if the heterologous overexpression of regulatory protein from *S. ghanaensis* will have effect on the production of actinorhodin. Compared to the wildtype M145 strain, no change ofmorphological phenotypes was observed in *S. coelicolor* pKCabsA2gh$^+$ when grown on solid MMGT plate. In the same time, the recombinant strain produced much lessactinorhodin (Fig. 3).

Fig. 3. Photographs of *S. coelicolor* (1) and *S. coelicolor* pKCabsA2gh$^+$ (2) takenfrom the bottomof the MMGT plates to visualize the antibiotic production. Intense blue color of the leftmost plate is due to actinorhodin production.

Spectrophotometric analysis of actinorhodin production under submerged conditions showed that heterologous expression of $absA2_{gh}$ in *S. coelicolor* resulted in 1,7-fold decrease of pigmented antibiotic accumulation (Fig. 4).

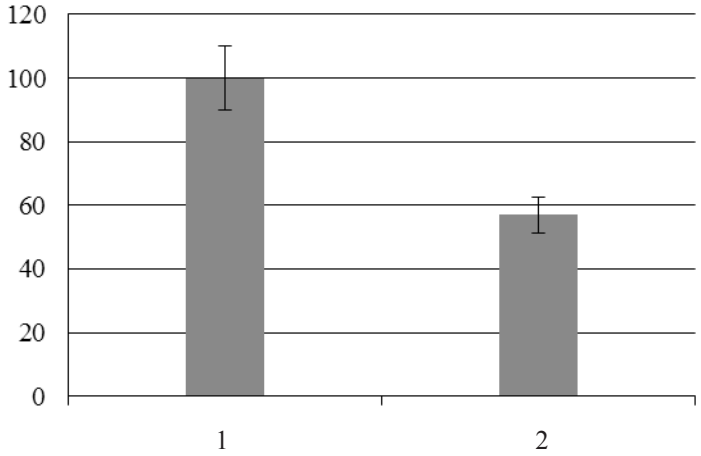

Fig. 4. Actinorhodin productionby*S. coelicolor* strains in liquid medium YMPG: 1 – *S. coelicolor* M145; 2 – *S. coelicolor* pKCabsA2gh^{+}.

Like AbsA2, response regulator from *S. ghanaensis* exhibits negative effect on actinorhodin biosynthesis. It was demonstrated that AbsA2 binds promoter of *actII-OFR4* – pathway-specific regulatory gene from *act*-cluster. Therefore, it is likely that $AbsA2_{gh}$ is capable of binding same binding sites in *S. coelicolor*as AbsA2. Significant sequence similarity in the region ofthe putative helix-turn-helix DNA binding motif supports this suggestion.

For $absA2_{gh}$ inactivation plasmid pKCabsA2gh-neo was constructed. 1,5 kb DNA fragment containing $absA2_{gh}$ upstream region was amplified with primers Xbaup2 5'-AAATCTAGACCTC-GTGTCGTTCGTGTTC-3' and EcoRrev2 5'-AAAGAATTCGCTGTTCAGCCTCTCCTAC-3' (XbaI and EcoRI sites, respectively, are underlined). The PCR product was gel-purified, digested with XbaI and EcoRI, and ligated into linearized pUC57 vector using T4 DNA ligase to yield pUCabsA2gh-up. 1,4 kb DNA fragment containing $absA2_{gh}$ downstream region was amplified with primers Xbarev1: 5'-AAATCTAGACGGTGAAGGCGTACGTCAG-3' and Hindup1: 5'-AAAGCTTGATCTTGCAGTCACTGTCGG-3' (XbaI and HindIII sites, respectively, are underlined). The PCR product was gel purified, digested with XbaI and HindIII, and ligated into linearizedpUCabsA2gh-up plasmid to yield pUCabsA2gh-updown. Kanamycin cassette (*neo* gene) from pKD4 plasmid was cloned into XbaI-digested pUCabsA2gh-updown to give pUCabsA2gh-neo. The resulting plasmid pUCabsA2gh-neo was digested with HindIII and EcoRI, the fragment containing the $absA2_{gh}$*::neo* mutant allele was cloned into the same sites of pKC1139 to yield pKCabsA2gh-neo.

The resulting plasmid pKCabsA2gh-neo was introduced by transformation into the non-methylating strain *E. coli* ET12567 (pUB307) and then transferred into *S. ghanaensis* by intergeneric conjugation. *S. ghanaensis* pKCabsA2gh-neo^{+}was incubated for 6 days at 40°C, plated onto LB agar supplemented with kanamycin and grown for 4–5 days at 37°C.

Toselectfor apramycin-sensitiveandkanamycin-resistantmutantsof *S. ghanaensis* $absA2_{gh}$*::neo* thestrainwas platedonoatmeal agarmediumsupplemented withkanamycin and grownat 37°C for5 days to obtain sporulated lawn. The spores were diluted andplatedonoatmeal-medium. Separate colonies were checked for antibiotic phenotype. In this way, three clones out of the 270 checked turned out to be $Am^{S}Kan^{R}$. Replacementof the $absA2_{gh}$withkanamycin resistancegeneinAm^{S}Kan^{R}cloneswasverifiedby PCR. Bymorphology *S. ghanaensis* $absA2_{gh}$*::neo*did

not differfromwild type (Fig. 5). The test forantibiotic activityshowedthat the level ofmoenomycin production by the *S. ghanaensis absA2*$_{gh}$*::neo*was not significantlydifferent from theone of thewild-typestrain (Fig. 6).

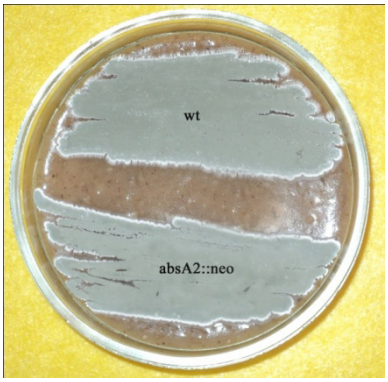

Fig. 5. Photograph of *S. ghanaensis* ATCC14672 (wt) and *S. ghanaensis absA2::neo (absA2::neo)*on oatmeal agar medium.

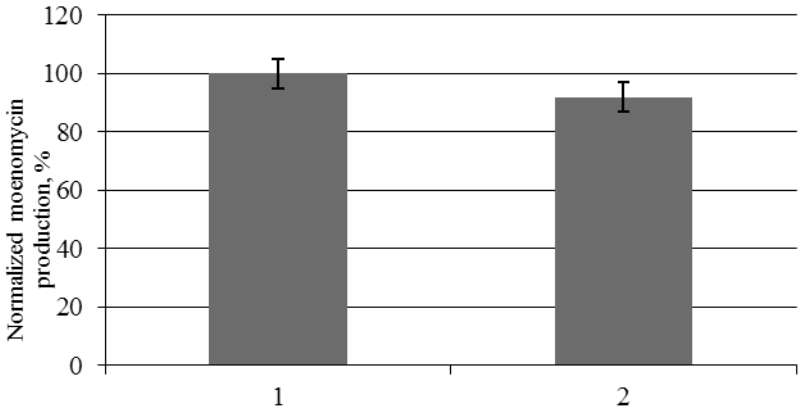

Fig. 6. Moenomycin production (as measured with disk diffusion test) by *S. ghanaensis* strains: 1 – *S. ghanaensis* ATCC14672; 2 – *S. ghanaensisabsA2*$_{gh}$*::neo*. Mean values were found from at least 3 independent experiments and normalized against equal amount of the biomass (dry weight). Error bars represent two standard deviations (± 2σ).

In conclusion, we revealed in this study that*absA2*$_{gh}$overexpression in *S. ghanaensis* and *S. coelicolor* negatively affects moenomycin and actinorhodin biosynthesis, respectively. In contrast, *absA2*$_{gh}$ deletion had no effect on moenomycin production. The possible explanation of these results is that *absA2*$_{gh}$ is not essential for regulation of MmA production, at least under our laboratory conditions, or is involved in regulation of cryptic clusters in *S. ghanaensis*. However, its overexpression exhibit negative effect. It could be indirect influence, since elevated levels of AbsA2$_{gh}$ protein could result in nonspecific DNA-binding, thus repressing either activator genes for moenomycin biosynthesis or expression of *moe*genes. It was demonstrated for *S. coelicolor* that genes for AbsA1/2are embedded *cda* cluster and directly repress*cda*promoters [14]. In *S. ghanaensisabsA2*$_{gh}$ gene is not located in cluster of genes for secondary metabolite production.

Our results imply that regulatory network formoenomycin production differs from that for the majorityof secondary metabolites. More studies are needed for the elucidation ofthe regulatory aspects of biosynthesis of phosphoglycolipid antibiotics, which will eventually leadto new ways to construct recombinant strains that overproducemoenomycinsand to a better understanding ofthe complex regulatory networks involved in the productionof unusual natural products.

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РОЛЬ ГЕНА *ABSA2$_{GH}$* У РЕГУЛЯЦІЇ БІОСИНТЕЗУ МОЕНОМІЦИНУ А У *STREPTOMYCES GHANAENSIS* АТСС 14672

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Ген *absA2$_{gh}$*, що кодує регулятор відповіді двокомпонентної системи AbsA2$_{gh}$1/2, виявлено у хромосомі *Streptomyces ghanaensis* – продуцента моеноміцинів. Надекспресія *absA2$_{gh}$* у *S. ghanaensis,* а також гетерологічна експресія у *S. coelicolor* призвела до зниження синтезу моеноміцину А й актинородину, відповідно. Однак у мутантному штамі *S. ghanaensis ΔabsA2$_{gh}$* не виявлено істотних змін у синтезі моеноміцину А. Отже, хоча *absA2$_{gh}$* впливає на синтез моеноміцинів за умов надекспресії, він не є необхідним регулятором цього метаболічного шляху.

Ключові слова: Streptomyces ghanaensis, моеноміцин А, плейотропні регулятори, двокомпонентна система.

РОЛЬ ГЕНА *ABSA2$_{GH}$* В РЕГУЛЯЦИИ БИОСИНТЕЗА МОЕНОМИЦИНА А В *STREPTOMYCES GHANAENSIS* АТСС 14672

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Ген *absA2$_{gh}$*,кодирующий регулятор ответа двухкомпонентной системы AbsA2$_{gh}$1/2, был обнаружен в хромосоме *Streptomyces ghanaensis* – продуцента моеномицинов. Повышенная экспрессия *absA2$_{gh}$*в *S. ghanaensis*, а также гетерологическая экспрессия в *S. coelicolor* привела к снижению синтеза моеномицина А и актинородина, соответственно. Однако в мутантном штамме *S. ghanaensis ΔabsA2$_{gh}$* не обнаружены существенные изменения в синтезе моеномицина А. Можно заключить, что *absA2$_{gh}$* влияет на синтез моеномицина в условиях повышенной экспрессии, однако он не является обязательным регулятором этого метаболического пути.

Ключевые слова: Streptomyces ghanaensis, моеномицин А, плейотропные регуляторы, двухкомпонентная система.