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YTRA–LIKE REGULATORY GENE *SSFG_05654* AFFECTS MORPHOGENESIS AND ANTIBIOTIC RESISTANCE OF *STREPTOMYCES GHANAENSIS* ATCC14672

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Gene *SSFG_05654* encodes a transcriptional regulator of YtrA subfamily within the genome of moenomycin producer *Streptomyces ghanaensis* ATCC14672. The gene was replaced with apramycin resistance cassette. The resulting mutant *S. ghanaensis* 05654::Am was slightly delayed in aerial hyphae formation and spore pigment production when growing on oatmeal agar medium. On tryptic soy agar the mutant grew poorly and showed Bld-like phenotype. Antibiotic resistance assay revealed that *S. ghanaensis* 05654::Am is more sensitive to β-lactam antibiotics such as penicillin and ampicillin. SSFG_05654 disruption had no effect on the level of moenomycin production.

Keywords: Streptomyces ghanaensis, HTH transcriptional factors, morphogenesis, moenomycin.

Transcriptional factors containing helix-turn-helix DNA recognition element fall into many different families. GntR family is one of the most abundant and widely distributed in bacterial genomes. It comprises about 8500 proteins regulating versatile biological processes such as cell development, primary and secondary metabolism, plasmid transfer, motility and virulence [8, 21]. In general, they consist of conserved N-terminal DNA-binding domain (D-b) and C-terminal effector-binding/oligomerization (E-b/O) domain. E-b/O domain is very variable and, based on its secondary structure, all GntRs are divided into seven subfamilies – FadR, HutC, MocR, DevA, PlmA, AraR and YtrA [17, 23].

The YtRA subfamily remains little studied among bacteria [4, 17]. The founding member of the subfamily, YtrA from *B. subtilis*, was suggested to be involved in acetoine utilization by repressing its ABC transporters [25]. However, subsequent studies indicated that YtrA was not induced by acetoin and acetoin catabolism is determined by *acoABCL* operon [19]. Recent study revealed that YtrA regulator is required for induction of antibiotic stress response. YtrA recognizes and binds to inverted repeats upstream of *ytrA* and *ywoB* operons repressing their transcription until ligand, lipoglycopeptide antibiotic ramoplanin, binds YtrA and releases *ywoB* from repression [19]. Few YtrA-like proteins were annotated in genomes of *M. tuberculosis* (Rv1152), *M. smegmatis* (MSMEG_5174) and *M. marinum* (MMAR_4301) but their function is yet unknown [23, 24, 10].

To date, only one regulator of YtrA subfamily was investigated in streptomycetes. LndYR influences sporulation and landomycin E production by *Streptomyces globisporus*. It is situated near landomycin biosynthesis genes cluster and its targets are ABC transporter genes *lndW-lndW2*. The other as-yet-unidentified LndYR targets are probably involved in morphological differentiation of the strain [14].

In course of our *in silico* analysis of *Streptomyces coelicolor* YtrA-like sequences we identified one, SCO1728, which appeared to be universally conserved in *Streptomyces*, as well as

other actinobacteria, including *Mycobacterium*. This conservation indirectly points to essential function of the protein. However, *sco1728* knockout had no significant effects on *S. coelicolor* growth, antibiotic resistance profile, morphogenesis and secondary metabolism [22]. We therefore decided to investigate whether the knockout of *sco1728* ortholog in another streptomycete, would display any recognizable phenotype. We have chosen *Streptomyces ghanaensis* ATCC14672 [2, 3, 15, 16] for this purpose, because it encodes ortholog of Sco1728, SSFG_05654, and the latter has a genome context different from that observed for *sco1728* or its orthologs from the other well studied streptomycetes (see Results for more details). Therefore, our aim was to disrupt *ssfg_05654* and characterize the resulting *S. ghanaensis* mutant which would provide new insights into function of *sco1728* orthologous group.

Materials and methods

In this work a wild type moenomycin producer *S. ghanaensis* ATCC 14672 was used. *Bacillus cereus* ATCC 19637 was used as a moenomycin-sensitive test culture in antibiotic disc diffusion assays of moenomycin production by ATCC14672. *Escherichia coli* DH5α [20] was used for routine subcloning. *E. coli* ET12567 harboring conjugative plasmid pUZ8002 [11] was used to perform intergeneric conjugation from *E. coli* to ATCC14672. *E. coli* BW25113 [6] harboring plasmid pIJ790 was used to carry out RedET-mediated gene replacement [6]. Solid oatmeal (40g/l oat flour, 20g/l agar) and soy-mannitol media [11] were used for strains maintenance, *Streptomyces* spore suspension harvesting, and plating *E. coli – Streptomyces* matings [1]. *B. cereus* and *E. coli* strains were grown in liquid or agar LB medium supplemented with appropriate antibiotic (if needed) at either 28 and 37°C, respectively. For visual and microscopic lawn examination, strains were grown on oatmeal, soy-flour and TSB media. Plasmids pKC0702 [11] and pIJ774 [6] were used for *ssfg_05654* expression and amplification of *aac(3)IV* cassette for recombineering experiments, respectively.

Genomic DNA from *Streptomyces* and plasmid DNA from *E. coli* were isolated using standard protocols [11, 20]. Restriction enzymes and molecular biology reagents were used according to recommendation of suppliers (NEB, MBI Fermentas). Genome sequence of *S. ghanaensis* ATCC14672 and sequence of SSFG_05654 protein is accessible through NCBI website.

Moenomycin production by *S. ghanaensis* strains was studied after five days of cultivation in liquid TSB medium as described by Makitrynskyy et al. [12]. Antibiotic resistance was analysed by using the disc diffusion method.

For scanning electron microscopy (SEM), small pieces of 7-day-old sporulating lawn were cut off the oatmeal agar plate samples, vacuum-dried and directly analyzed on a Jeol JSM-T220A scanning microscope.

Orthologs prediction was carried out via reciprocal BLASTP analyses. Analysis of genomic context of ssfg_05654 was based on gene annotation provided by NCBI Genome server. Phylogenetic analysis of SSFG_05654 homologs was carried out using maximum-likelihood algorithm at www.phylogeny.fr.

Results and discussion

Gene *ssfg_05654* encodes a small putative protein of 128 amino acids with predicted molecular weight 13.43 kDa. Reciprocal Blast searches identified a number of orthologs in sequenced streptomycete genomes. Phylogenetic tree (Fig. 1) is composed of the proteins showing the highest level of identity to SSFG_05654: SCO1728 *S. coelicolor* (87%), STVIR_6947 *S. viridochromogenes* (85%), SHJG_3180 *S. hygroscopicus subsp. jinggangensis* 5008 (85%), SSEG_05154 *S. sviceus* ATCC 29083 (84%), SZN_24780 *S. zinciresistens* K42 (86%), SAV_6565

S. avermitilis MA-4680 (84%), SCAB_72381 *S. scabiei* 87.22 (82%).

Chromosomal segment containing *ssfg_05654* is highly syntenous across streptomycete genomes. The genes adjacent to *ssfg_05654* are annotated as the ones encoding acetyltransferase (*ssfg_05653*), cellulose-binding protein (*ssfg_05650*), lipase (*ssfg_05649*), protease (*ssfg_05647*). The listed proteins are most likely used by the strain to provide nutrients for growth. Genes *ssfg_05657* and *SSFG_05658* encode succinoglycan biosynthesis protein and glycosyltransferase, respectively, probably responsible for exopolysaccharide production. Although similar gene arrangement is common for many *Streptomyces*, we revealed, using extensive BLAST searches, that *S. coelicolor* genome does not encode orthologs of SSFG_05657 and 05658. We also noted, that, unlike most YtrA subfamily regulatory genes, *ssfg_05654* is not part of an operon including ABC transporter gene.

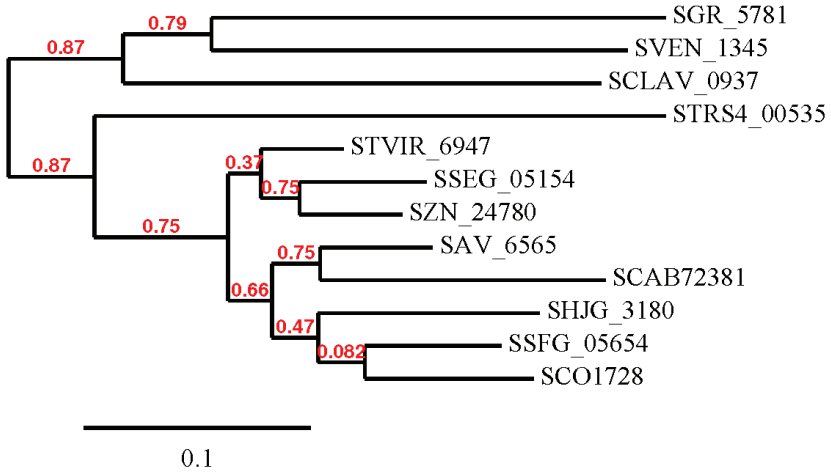

Fig. 1. Unrooted maximum-likelihood phylogram showing the relationships between SSFG_05654 and its nearest relatives from *Streptomyces* genus. Confidence aLRT values are shown on the nodes. The tree was built with the help of phylogeny.fr v.2 server [5]. Protein abbreviation – see the text. The scale bar indicates number of amino acids substitution per position.

The *ssfg_05654* in *S. ghanaensis* chromosome has been replaced with apramycin resistance cassette derived from pIJ774 [6]. For this purpose, the *ssfg_05654* sequence with 1500 bp flanking regions was amplified from chromosome with primer pair SSFG_05654for (AAATCTAGAAACTCGTCCTCGTCCAGCCG) and SSFG_05654rev (AAAGGATCCT-GACCAAGGGGTACGCGGTG). The amplicon was digested with endonucleases BamHI and XbaI and cloned into respective sites in pKC0702 yielding pKCSSFG_05654. The plasmid was verified by restriction mapping. To generate construct with replaced gene, RedET technology has been implicated [6]. Apramycin resistance cassette was amplified from pIJ774 using following primer pair SSFG_05654Am_for (GTGCGGGACAATCGGGCCGTGACCTTGAAGATC-CATATGATATCTCTAGATACCG) and SSFG_05654Am_rev (CTACTCCCCGTAGACCG-CCCGCAGGGCGTCCCGCACGGC AACAAAAGCTGGAGCTC). The amplicons were introduced through electroporation into BW25113 (pIJ790) strain harboring pKCSSFG_05654. The resulting gene disruption construct pKCSSFG_05654::aac(3)IV was conjugally transferred from *E. coli* ET12567 (pUZ8002, pKCSSFG_05654::aac(3)IV) into *S. ghanaensis*.

Transconjugants were selected for resistance to hygromycin (50 μg/ml). To promote single and double cross-over between the plasmid and respective region of the ATCC14672 genome,

the initial trasnconjugants were incubated at 40°C for 3 days in TSB and then plated onto oatmeal agar containing apramycin. Apramycin-resistant colonies were screened for hygromycin sensitivity, an indicative of loss of vector sequences. The expected colonies were identified and *ssfg_05654* replacement with *aac(3)IV-oriT* cassette was verified via PCR (data not shown).

After five days of growth on oatmeal medium strain *S. ghanaensis* SSFG_05654::Am looks like the wild type. It was able to form aerial hyphae with subsequent spore formation but showed a slightly delayed production of a green spore pigment. However, scanning electron microscopy did not reveal differences between mature spore of both strains (shape, size and amount was same, Fig. 2A) when growing on oatmeal agar. At the same time, growth of the wild type and ssfg_05654 mutant strains on TSB-agar plates is markedly different. *S. ghanaensis* SSFG_05654::Am exhibited the so called "*bald*" (Bld) phenotype [7–9, 11] – it did not form aerial mycelium and spores (Fig. 2B). Scanning electron microscopy showed that the mutant produced sparse hyphae and aerial mycelium (Fig. 2F). There are a few GntR regulators known to be involved in streptomycete morphogenesis regulation, although clear mechanism of action is yet to be investigated. For instance, the *devA* mutant of *S. coelicolor* produces rare aerial hyphae that further develop aberrant spore chains [9]. The *agl3R* mutant shows Whi-phenotype (impaired spore pigmentation). This gene acts to repress its own transcription as well as immediately adjacent ABC transporters for carbohydrate uptake [7]. YtrA-like regulator LndYR affects spore formation and antibiotic production in *S. globisporus* 1912 [14]. Thus, GntR regulators appear to be connected with developmental processes in streptomycetes.

A plasmid for complementation has been constructed to verify that the observed phenotype is not caused by unanticipated polar effects or genome rearrangements. The entire *ssfg_05654* sequence with its own promoter was amplified from chromosomal DNA using following primers: SSFG_for (AAATCTAGATGTGTCCGGGATGCACGTAC) and SSFG_rev (AAAGGATCCGGGCTGTACCTGTAGCCGTA). The PCR product was digested with endonucleases BamHI and XbaI and ligated with respective sites of pIJ82 to yield pIJSSFG_05654. A plasmid pIJSSFG_05654 was conjugally transferred into *S. ghanaensis* giving transconjugants *S. ghanaensis* pIJSSFG_05654. The complemented stain had growth rate and morphological features identical to that of the wild type (Fig. 2G, H).

In *B. subtilis* YtrA protein was shown to regulate cell envelope stress responses to cell wall antibiotics [19]. Thus, we decided to check the susceptibility of *S. ghanaensis* SSFG_05654::Am to different antibiotics, including β-lactams and glycopeptides, since the latter target peptidoglycan synthesis. An antibiotic disk diffusion assay was carried out. We tested inhibitors of transcription (rifampicin), translation (streptomycin, kanamycin, gentamycin, erythromycin, lincomycin), peptidoglycan precursor biosynthesis (fosfomycin), membrane disruptors (polymixin, bacitracin), binders of cell wall precursors (teicoplanin, ristocetin) and beta-lactams that target peptidoglycan transpeptidases (benzylpenicillin, cefalotin, cephalexin, carbenicillin, ampicillin). The results were recorded after 1 and 3 days of growth of the strains on TSB agar plates in presence of antibiotic discs. We revealed that the antibiotic resistance profile of the wild type and mutant strains was identical for all groups of antibiotics being tested except for β-lactams. Namely, after 24 h of incubation *S. ghanaensis* SSFG_05654::Am displayed significant susceptibility to benzylpenicillin, ampicillin and cephalexin (diameters of growth inhibitions zones were 25±1, 23±1, and 18±1 mm, respectively) in comparison to the wild type (no growth inhibition zone at all). On third day of growth the inhibition zone around the aforementioned antibiotic discs was decreased (on average, from 25–18 mm to 15–10 mm), although the growth within it was not abundant. We confirmed increased sensitivity of

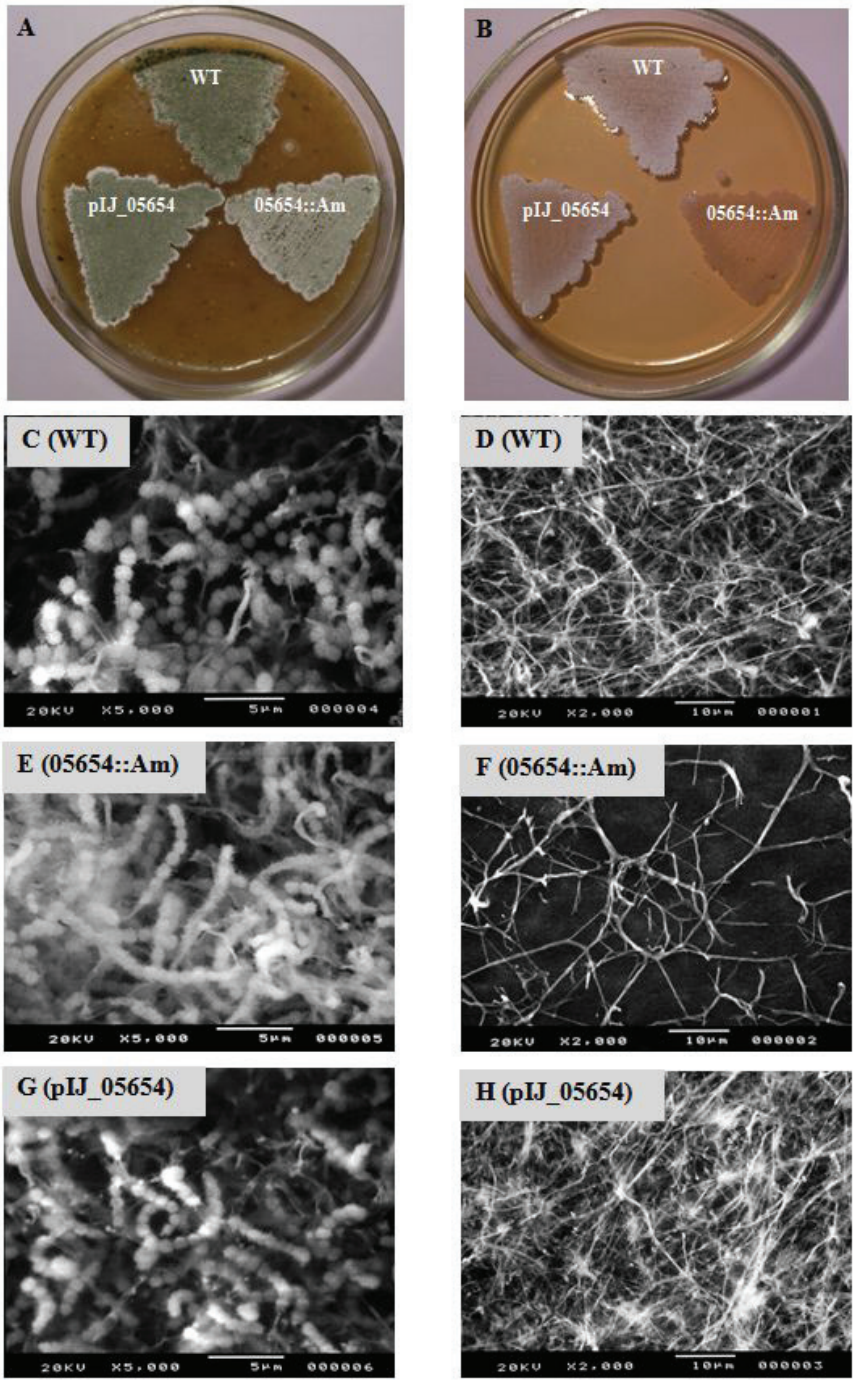

Fig. 2. Lawns of S. ghanaensis strains on oatmeal (A) and TSB (B) plates, and SEM images of the latter (C-H). Labels: WT – ATCC14672; SSFG_05654::Am – knockout strain; pIJ_05654 – complementation of the mutant. C and D, E and F, G and H represent the same lawn under ×5K and ×2K magnification, respectively.

SSFG_05654::Am to benzylpenicillin through analysis of strain's survival on TSB plates with increasing concentrations of this antibiotic (Fig. 3). Indeed, while the wild type displayed roughly 2 and 0.4% survival rate at 30 and 100 mcg/ml of benzylpencillin, respectively, the mutant showed 0.07% survival at 30 mcg/ml, and no survival at 100 mcg/ml. Since we plated around 3×10^6 cfu of the mutant on 100 mcg/ml antibiotic plates, this implies that survival rate at this concentrations will not exceed 0.00003%. Hence, at 100 mcg/ml of benzylpenicillin, the resistance of the mutant strain to the antibiotic is at least four orders of magnitude lower than that of initial strain. At the moment we can only speculate that SSFG_05654 regulates synthesis of exopolysaccharide layer of cell wall. Thus, β-lactam antibiotics can easier target peptidoglycan synthesis if mutant strain *S. ghanaensis* SSFG_05654::Am does not produce any additional extracellular polysaccharides.

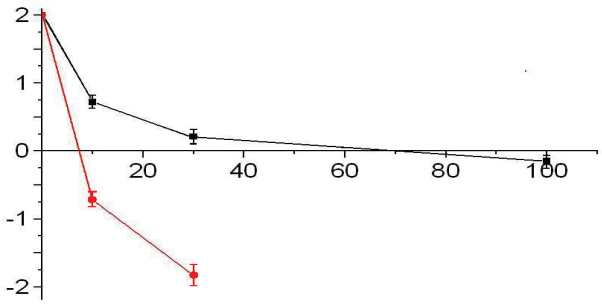

Fig. 3. Growth of *S. ghanaensis* wild type (A) and *S. ghanaensis* SSFG_05654::Am (B) in presence of increasing amounts of benzylpenicillin. X-axis – antibiotic concentrations (mcg/ml); Y-axis – lg % of survival. Error bars represent standard error.

We checked whether *ssfg_05654* knockout had effect on biosynthesis of moenomycin, the only secondary metabolite known to be produced by *S. ghanaensis* [13]. The wild type and mutant strains were grown in TSB, and equal amounts of the mycelia were used to extract the antibiotic. The bioassay revealed no significant difference in the levels of moenomycins present in the extracts. Hence, SSFG_065654 is not connected to secondary metabolism of *S. ghananesis*, at least under our experimental conditions.

In conclusion, this study has revealed that YtrA subfamily SSFG_065654 of *S. ghanaensis* ATCC14672 is a pleiotropic regulator implicated in morphological differentiation and antibiotic resistance under certain growth condition. The protein is needed for mycelium formation while growing on TSB medium but not on oatmeal medium. YtrA-like regulators are metabolite/condition-responsive proteins that modify genes expression tin response to certain environmental challenges. It seems that SSFG_065654 responds to nutrient status of the cells, because the mutant phenotype was observed on TSB agar, containing aminoacid-rich soy hydrolyzate, but not on oligotrophic, polysaccharide-based oatmeal medium. The mutant strain is more sensitive to β-lactam antibiotics that could occur due to absent or impaired synthesis of exopolysaccharide layer, likely controlled by the genes *ssfg_05657, 05658* in the vicinity of the *ssfg_05654* gene. Failure to produce this layer (or its diminished amount) may provide easier access to transpeptidases, targets of β-lactams on the surface of the cell. Alternatively, SSFG_05654 can be involved in regulation of antibiotic stress response, like it has been recently described for *B. subtilis* YtrA protein. Finally, our work provides a cautionary tale about the challenges in ascribing a function to the group of orthologous genes on the basis of study a single gene and *in silico* analysis. The proteins encoded by *ssfg_05654* and *sco1728* are clearly very similar and phylogenetically close to each other (see Fig. 1), and yet their effects on respective host bacteria are very different.

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YTRA–ПОДІБНИЙ РЕГУЛЯТОРНИЙ ГЕН *SSFG_05654* ВПЛИВАЄ НА МОРФОГЕНЕЗ І СТІЙКІСТЬ ДО АНТИБІОТИКІВ У *STREPTOMYCES GHANAENSIS* ATCC14672

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Ген *ssfg_05654* продуцента моеноміцину *Streptomyces ghanaensis* ATCC14672 кодує транскрипційний регулятор підродини YtrA. Отримано мутантний штам *S. ghanaensis* 05654::Am, у котрому ген *ssfg_05654* заміщено на касету стійкості до апраміцину. Порівняно з вихідним штамом під час росту цього мутанта на вівсяному середовищі спостерігається затримка формування повітряного міцелію та синтезу спорового пігменту. Водночас на триптон-соєвому середовищі мутант виявляє Bld-подібний фенотип, утворюючи лише поодинокі гіфи повітряного міцелію. Штам *S. ghanaensis* 05654::Am більш чутливий до β-лактамних антибіотиків пеніциліну й ампіциліну, ніж дикий тип. Нокаут гена *ssfg_05654* не впливає на рівень синтезу моеноміцину.

Ключові слова: *Streptomyces ghanaensis*, транскрипційні фактори HTH, морфологічна диференціація, моеноміцин.

YTRA-ПОДОБНЫЙ РЕГУЛЯТОРНЫЙ ГЕН *SSFG_05654* ВЛИЯЕТ НА МОРФОГЕНЕЗ И УСТОЙЧИВОСТЬ К АНТИБИОТИКАМ *STREPTOMYCES GHANAENSIS* ATCC14672

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Ген *ssfg_05654* продуцента моеномицина *Streptomyces ghanaensis* ATCC14672 кодирует транскрипционный регулятор подсемейства YtrA. Получен мутант *S. ghanaensis* 05654::Am, у которого *ssfg_05654* замещен генной кассетой устойчивости к апрамицину. По сравнению с исходным штаммом, при росте этого мутанта на овсяной среде наблюдается задержка формирования воздушного мицелия и синтеза спорового пигмента. В то же время на триптон-соевой среде мутант проявляет Bld-подобный фенотип, образуя лишь одиночные гифы воздушного мицелия. Штамм *S. ghanaensis* 05654::Am более чувствителен к β-лактамным антибиотикам пенициллину и ампициллину, чем дикий тип. Нокаут гена *ssfg_05654* не влияет на уровень синтеза моеномицина.

Ключевые слова: *Streptomyces ghanaensis*, транскрипционные факторы HTH, морфологическая дифференциация, моеномицин.