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ГЕНЕТИКА

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STUDY OF *STREPTOMYCES GHANAENSIS* ATCC14672 GENES *SSFG_03627* AND *SSFG_01411* ENCODING PUTATIVE MEMBRANE PROTEINS

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Streptomyces ghanaensis ATCC14672 produces moenomycin A, a potent phosphoglycolipid antibiotic that targets bacterial peptidoglycan glycosyltransferases (PGTs). The genetic and biochemical aspects of assembly of moenomycin molecule is well understood. Yet, it remains unknown how moenomycins are transported from the cell into an environment, and whether the active export of moenomycin might be important for resistance of producer to its own potentially toxic secondary metabolite. There is overall structural and functional similarity between moenomycin and Lipid II an essential precursor to peptidoglycan (common lipid-glycoside scaffold, both bind PGTs). We hypothesized that proteins responsible for Lipid II transport across the *S. ghanaensis* membrane, analogous to MurJ in *Escherichia coli*, could also be involved in moenomycin export. In this work we describe initial genetic characterization of two MurJ-like *S. ghanaensis* ATCC14672 genes, *SSFG_03627* and *SSFG_01411*. We revealed that gene *SSFG_03627* is essential and could be deleted from *S. ghanaensis* ATCC14672 genome only in presence (*in trans*) of the second *SSFG_03627* gene copy. Attempts to complement *murJ*-deficient *E. coli* NR1154 strain with either *SSFG_03627* or *SSFG_01411* gene were unsuccessful. Manipulations of *SSFG_03627* gene copy number had complex influences on moenomycin production in *S. ghanaensis* ATCC14672.

Keywords: *Streptomyces*, moenomycin, antibiotic resistance, Lipid II flippases

S. ghanaensis is the producer of moenomycin A (MmA) – founding member of a small family of phosphoglycolipid antibiotics that inhibit the growth of mostly Gram-positive bacteria, including many vancomycin- and methicillin-resistant strains. The structure of MmA features elements rarely found in the secondary metabolites of bacteria and it is the only known natural antibiotic that inhibits the transglycosylation step of bacterial cell wall synthesis through binding to the peptidoglycan transglycosylases (PGTs) [8]. This makes MmA an interesting target for drug development.

Genetic control of moenomycin biosynthesis is well studied, it is quite complex, with 17 genes being involved [7, 8]. These genes (*moe*) form two clusters located distantly to each other. There are no regulatory or resistance genes within either of the *moe* clusters, so there is a broad interest of studying other mechanisms involved in moenomycin biogenesis. One of possible research targets is to study how this molecule gets through the *S. ghanaensis* membrane, and what moenomycin resistance mechanisms are. It has been shown that moenomycin inhibits PGTs, the enzymes involved in the essential penultimate step of bacterial cell wall biosynthesis through binding in the active site cleft of PGTs. In this mechanism MmA and Lipid II, an essential precursor of peptidoglycan, compete for binding to PGT [3]. If so, we suggest that enzymes involved in Lipid II translocation across *S. ghananesis* membrane might also be involved in MmA export from cells, and contribute to *S. ghanaensis* resistance to MmA. Only recently genes and

enzymes for Lipid II translocation have been discovered in model organisms *E. coli* and *Bacillus subtilis* [9]. However, nothing is known about Lipid II translocation in actinomycetes and *S. ghanaensis* as well. Therefore, in our work we pursued several goals. First, we identified, *in silico*, homologs of *E. coli* Lipid II flippase in *S. ghanaensis*. Second, we constructed plasmids for overexpression of the identified genes in *Streptomyces*, and studied the effects of increased gene dosage on moenomycin production, colony morphology and overall antibiotic resistance. Third, we attempted to knock out the gene for one of the identified MurJ homolog. Fourth, we explored whether *S. ghanaensis* ATCC14672 *murJ* homologs are able to complement conditionally deficient *murJ*- strain of *E. coli* NR1154. As a result of these experiments, we show that the studied genes influence moenomycin production but not antibiotic resistance, although their exact biological roles in *S. ghanaensis* remain poorly understood.

Materials and Methods

Strains used and constructed in this work are listed in Table 1. Solid oatmeal (40g/l oat flour, 20g/l agar) and soy-mannitol media [5] were used to grow *Streptomyces* and plate matings. *B. cereus* and *E. coli* strains were grown in liquid or agar LB medium supplemented with appropriate antibiotic (if needed) at either 30 or 37 °C, respectively. For visual and microscopic lawn examination, strains were grown on oatmeal, soy-flour and TSB media. Vectors pKC1132 [5], pHYG, pGCymRP21, pTES, pUWLCre [11] were used for genetic engineering experiments. Genomic DNA from *Streptomyces* and plasmid DNA from *E. coli* were isolated using standard protocols [5]. Restriction enzymes and molecular biology reagents were used according to recommendation of suppliers (NEB, MBI Fermentas). Genome sequence of *S. ghanaensis* ATCC14672 was accessed through NCBI website.

Several plasmids were constructed in course of the work. 5-kb segment of *S. ghanaensis* ATCC14672 genome, centered on gene *SSFG_03627*, was amplified from *S. ghanaensis* ATCC14672 chromosome with primers mviNgh-largeXbaIup (AAATCTAGACATGACCAAGGCCTCCAC) and mviNgh-largeEcoRIrp (AAA GAATTCCTGCATCCGGTTCCGTTCC). Sites for restriction endonucleases (RE) are underlined in primer sequences. This segment was treated with RE XbaI and EcoRI and cloned into respective sites of pKC1132 to give pOOB100a. Gene *SSFG_03627* within pOO100a was replaced with hygromycin resistance cassette from pHYG using recombineering (primers mviNgh-red-hyg-up: GCCATGCCGCGGGCAACTCGGGCCACCCCGAGTCGGATCCCCGTA- GAGATTGGCGATCCC and mviNgh-red-hyg-rp: TCAGCGTCCCAGGCGTCCGCGGACCATGCCGA- CCAGAGACAGGCGCCGGGGGCGGTGTC). The final construct for *SSFG_03627* knockout was labeled as pOOB101c. Gene *SSFG_03627* was amplified with primers mviNgh-XbaIup (AAATCTAGACCGTTGAGGATGTCGTGG) and mviNgh-EcoRIrp (AAAGAATTCAGCGTCCCAGGCGTCC) and cloned into XbaI-EcoRI-digested vector pTES to give expression plasmid pOOB99a. For inducible expression of *SSFG_01411* 2.5-kb segment of *S. ghanaensis* ATCC14672, genome, contained the *SSFG_01411* gene, was amplified with primers ssfg_06665_up1 (AAAGATATCTAGAGTCCACCGCAAGATCCTCGC) and ssfg_06665_rp (AAAGGATCCGA ATTCACCGCACCCCCGGCAGGC). This segment was treated with XbaI+EcoRI and cloned into SpeI+EcoRI sites of pGCymRp21, under control of inducible P21-cmt promoter, to give pJS2. Also *SSFG_03627*, amplified with primers mviNgh-XbaIup (AAATCTAGACCGTTGAGGATGTCGTGG) and mviNgh-EcoRIrp (AAAGAATTCAGCGTCCCAGGCGTCC), was treated with XbaI+EcoRI and cloned into SpeI+EcoRI sites of pGCymRp21 to give pJS1.

Moenomycin was extracted from *S. ghanaensis* strains after five days of cultivation in liquid TSB medium and analyzed as described in [6]. Antibiotic resistance was analysed with 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.

Table 1

Bacterial strains used in this work

Strain	Relevant characteristics	Source / Reference
E. coli DH10B	Routine cloning host; F^- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *end*A1 *ara*D139 Δ(*ara, leu*)7697 *gal*U *gal*K λ$^-$ *rps*L *nup*G.	Invitrogen
E. coli ET12567 pUZ8002	Strain for conjugative transfer of coresident plasmids; *dam13*::Tn9 (Cm^r) *dcm6 hsdM hsdR zjj*202::Tn10 (Tet^r) *recF143 galK2 galT22 ara14 lacY1 xyl5 leuB6 thi1 tonA31 rpsL136* (Str^r) *hisG4 tsx78 mtl1 glnV44* . pUZ8002 - Km^r	[5]
E. coli BW25113 pIJ790	F- , DE(*araD-araB*)567, *lacZ4787*(del)::*rrnB*-3, *LAM-* , *rph-1*, DE(*rhaD-rhaB*)568, *hsdR514*. Contains the λ RED recombination plasmid pIJ790.	[4]
E. coli NR1154	MurJ-depletion strain; MC4100 *ara*+ Δ*lysA*::*kan murJ* Ω(−14::*bla araC* P_{BAD}).	[9]
E.coli NR1154-99a	NR1154 derivative. Carries *SSFG_03627* gene (pOOB99a).	This work
E.coli NR1154 JS2	NR1154 derivative. Carries the *SSFG_01411* gene under control of cumate-inducible promoter (pJS2 plasmid).	This work
Bacillus cereus ATCC19637	Moenomycin-sensitive test culture.	ATCC
S. ghanaensis ATCC14672	Wild type moenomycin producer.	ATCC
S. ghanaensis YS1	ATCC14672 derivative. Carries the second copy of *SSFG_03627* gene integrated into $attB^{\varphi C31}$. Am^r	This work
S. *ghanaensis* YS2	YS1 derivative. pOOB99a vector sequences were excised from YS1 genome with pUWLCre plasmid. Am^S	This work
S.*ghanaensis* YS3	YS2 derivative. The native copy of *SSFG_03627* of YS2 genome was knocked out with plasmid pOOB101c. Hy^rAm^s	This work
S. *ghanaensis* JS1	ATCC14672 derivative. Carries the extra copy of *SSFG_03627* gene under cumate-inducible promoter (pJS1).	This work
S. *ghanaensis* JS2	ATCC14672 derivative. Carries extra copy of *SSFG_01411* gene under cumate-inducible promoter (pJS2 plasmid).	This work
S. *albus* SAM2	Derived from *S. albus* J1074 by deletion of *φ*C31 *pseB4.*	[1]
S. *albus* JS1	SAM2 derivative. Carries the extra copy of *SSFG_03627* gene under cumate-inducible promoter (pJS1 plasmid).	This work
S. *albus* JS2	SAM2 derivative. Carries the extra copy of *SSFG_01411* gene under cumate-inducible promoter (pJS2 plasmid).	This work

Genome sequences were accessed through NCBI. Orthologs prediction was carried out via reciprocal BLASTP analyses. Phylogenetic analysis was carried out using maximum-likelihood algorithm at www.phylogeny.fr; default parameters of phylogenetic reconstruction have been applied. Transmembrane domain prediction program TMHMM (http://www.cbs.dtu.dk/services/TMHMM/) was used to analyze putative Lipid II flippases.

Results and Discussion

***In silico* search for MurJ homologs encoded within *S. ghanaensis* ATCC14672 genome.** Using aminoacid sequence of *E. coli* MurJ, a putative flippase of Lipid II [9], we employed BLASTP to look for orthologs within *S. ghanaensis* ATCC14672 genome. Our initial search (in 2010) was based on analysis of partially sequenced *S. ghanaensis* ATCC14672 genome, and it returned protein SSFG_03627 (767 amino acids; 44 % similarity over 457-aa segment) as the reciprocal best BLASTP hit (RBH). According to program TMHMM, SSFG_03627 protein featured 14 transmembrane domains. This was consistent with a hypothesis about membrane

location of presumed Lipid II flippase. Recently we updated our ortholog search using more extensively sequenced *S. ghanaensis* ATCC14672 genome. With BLASTP program we searched RBH for SSFG_03627 in genomes of *Mycobacterium tuberculosis, B. subtilis and E. coli* K12 genomes. None of the hits was reciprocal this time. However, we identified another MurJ-like protein – SSFG_01411, that was RBH to all putative Lipid II flippases from the studied genomes, except for MurJ (Fig. 1). TMHMM program confirmed that SSFG_01411 had 14 possible transmembrane domains in its structure (data not shown).

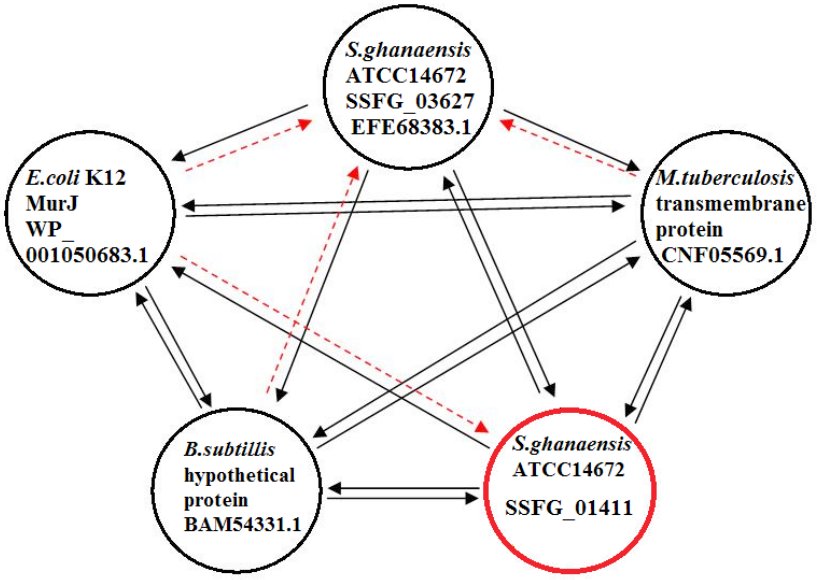

Fig. 1. Orthologous relationships (based on RBH strategy) within the group of putative Lipid II flippases from model strains and *S. ghanaensis* ATCC14672. Solid two-way arrows indicate RBH, dashed arrows indicate non-best hit (e.g., MurJ is RBH for SSFG_01411, but the latter is not RBH for MurJ). SSFG_01411 and SSFG_03627 are paralogues. In all cases *E* value in pairwise alignments was below 3^{-10}, implying that our findings were statistically significant

Studies of *SSFG_03627* and *SSFG_01411* in *S. ghanaensis* ATCC14762. Plasmid pOOB101c for *SSFG_03627* knockout (see Methods) was transferred into *S. ghanaensis* ATCC14672 and hygromycin-resistant (Hy^r), apramycin-sensitive (Am^s) clones (an indicative of *SSFG_03627* replacement with Hy^r cassette) were looked for. In spite of much effort, we failed to isolate viable Hy^rAm^s clones; all of them grew marginally and died after one passage. These facts pointed to essentiality of *SSFG_03627*. However, disruption of an essential gene should be possible if two copies of the latter are present in the genome. So we set out to delete native copy of *SSFG_03627* in *S. ghanaensis* ATCC14762 genome in presence of the second *SSFG_03627*, located *in trans*. The construction of *S. ghanaensis* ATCC14762 carrying two copies of *SSFG_03627* was therefore carried out as follows. First, second copy of *SSFG_03627* was integrated into $attB^{\varphi C31}$ of *S. ghanaensis* ATCC14672 genome using plasmid pOOB99a. The generated strain was referred to as *S. ghanaensis* YS1. Second, pOOB99a vector sequences were excised from *S. ghanaensis* YS1 using plasmid pUWLCre to give *S. ghanaensis* YS2 (Am^s). Third, native copy of *SSFG_03627* was knocked out of *S. ghanaensis* YS2 genome with plasmid pOOB101c. Hy^rAm^s clones were readily isolated, and one of them, marked as *S. ghanaensis* YS3, was verified by diagnostic PCR to carry the expected *SSFG_03627* knockout (data not shown).

Our results point that gene *SSFG_03627* is essential, because it could be facilely deleted from *S. ghanaensis* ATCC14672 genome in presence of extra copy of *SSFG_03627*.

Also, plasmid pJS1 was conjugally transferred into *S. ghanaensis* ATCC14672 strain. We thus generated *S. ghanaensis* JS1 strain, which carried the extra copy of *SSFG_03627* gene under cumate-inducible promoter [6]. In the same way, we generated *S. ghanaensis* JS2 strain with extra *SSFG_01411* copy under cumate-inducible promoter. Both plasmids were also transferred into *S. albus* SAM2.

The next purpose of our work was to investigate the phenotype of above mentioned strains. We checked their resistance to common antibiotics and revealed no differences in comparison to initial strains. No significant morphological changes were also detected. Because of both structural and chemical similarities of Lipid II and MmA, we suggested that the latter could be transported out of the cell by MurJ homologues in *S. ghanaensis*. We analysed the level of MmA accumulation separately in mycelium and supernatant of *S. ghanaensis* fermentation medium with the help of biochromatography. These results are summarized in Fig. 2. In *S. ghanaensis* YS3 strain (deletion of native *SSFG_03627* gene), MmA production was almost completely blocked. In the wild type background, presence of *SSFG_03627*, either under *ermEp* (pOOB99a, lanes 2M, 2S) or P21-*cmt* (lanes 4M, 4S) led to qualitative and quantitative changes in moenomycin production profile. Particularly, *SSFG_03627* expression from plasmids caused *S. ghanaensis* to accumulate in mycelium as-yet-unidentified antibacterials, while accumulation of MmA and second most abundant phosphoglycolipid, nosokomycin B [7] was not observed. In the same time, MmA was also absent in the supernatant from *S. ghanaensis* YS1 and *S. ghanaensis* JS1 strains as compared to *S. ghanaensis* ATCC14672. Presence of extra copy of *SSFG_01411* (*S. ghanaensis* JS2) had no effects on moenomycin production (lanes 5M, 5S, Fig. 2). Finally, we determined the resistance to moenomycin A in *S. albus* JS1 and JS2 strains; no differences were found as compared to the wild type strain.

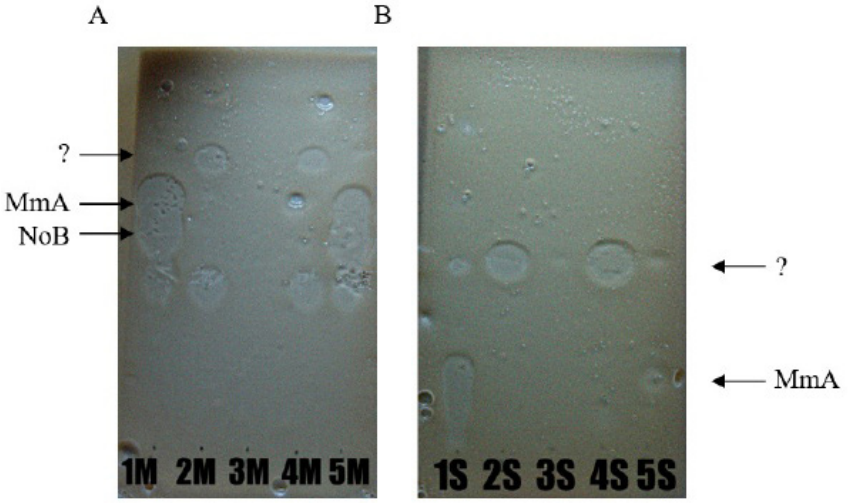

Fig. 2. Biochromatography of moenomycins extracted from mycelium (A) or supernatant (B) of following *S. ghanaensis* strains: ATCC14672 (lanes 1M(ycelium), 1S(upernatant)), YS1 (2M, 2S), YS3 (3M, 3S), JS1 (4M, 4S), JS2 (5M, 5S). Arrows mark bioactive spots corresponding to MmA and nosokomycin B (NoB). TLC plates shown on the photograph are representative of typical result of three independent repeats. Spot below NoB belongs to minor forms of moenomycin complex. Interrogation sign (?) marks unknown antibacterial compound, chemically unrelated to moenomycins

Complementation of *E. coli* NR1154 with putative *S. ghanaensis* flippase genes. We attempted to complement *murJ* depletion strain *E. coli* NR1154 with either *SSFG_03627* or *SSFG_01411* genes. Briefly, *murJ* expression in *E. coli* NR1154 is under the control of arabinose-inducible promoter. Hence, growth (measured spectrophotometrically at OD_{600}) of NR1154 depends on on the presence of arabinose in the medium. The plasmids pOOB99a (carries *SSFG_03627*) and pJS2 (*SSFG_01411*) were introduced through electroporation into *E. coli* NR1154 to obtain *E. coli* NR1154-99a and *E. coli* NR1154 JS2 strains respectively. Growth of the parental strain and both *E. coli* NR1154-99a and *E. coli* NR1154 JS2 strains was analyzed in presence of either arabinose or rhamnose; the latter, a nonmetabolizable sugar, caused cell lysis of *murJ* depletion strains. We observed no complementation in case of either plasmids. Unexpectedly, we observed growth of *E. coli* NR1154 strain in the absence of arabinose, which might point to some unanticipated changes in *E. coli* NR1154 genome.

To summarize, we have identified, *in silico*, two genes, candidates for Lipid II flippase in *S. ghanaensis.* We provide circumstantial evidence that one of them, *SSFG_03627*, is essential, at least under laboratory conditions. This gene also suppresses moenomycin production when natural expression mode is altered (e.g. when native copy is deleted or additional copy of *SSFG_03627* is introduced). All these data point to important role of Ssfg_03627 protein in *S. ghanaensis* biology, which might be related to transport of lipid-like substances across membrane. This work also underscores the importance of balanced transport processes for moenomycin production.

At the moment little can be told about function of the second gene, *SSFG_01411*. Its overexpression had no impact on the properties that we examined (resistance, morphology, antibiotic titers). More detailed investigation of this gene will be needed to uncover the role of this gene.

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ВИВЧЕННЯ ГЕНІВ *STREPTOMYCES GHANAENSIS* ATCC14672 *SSFGH_03627* ТА *SSFGH_01411*, ЩО КОДУЮТЬ ІМОВІРНІ МЕМБРАННІ БІЛКИ

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Streptomyces ghanaensis ATCC14672 – продуцент моеноміцину А, фосфоліколіпідного антибіотика, що діє на бактерійні пептидогліканові глікозилтрансферази (PGTs). Генетичні та біохімічні аспекти синтезу молекули моеноміцину залишаються не цілком зрозумілими. Досі невідомо, як моеноміцини транспортуються назовні з клітини, і чи може активний транспорт моеноміцину бути важливим для набуття резистентності продуцента до власного потенційно токсичного вторинного метаболіта. Спостерігається загальна структурна і функціональна подібність між моеноміцином і ліпідом II – життєво важливим попередником пептидоглікану (схожа ліпідно-глікозидна основа, обидва зв'язують PGTs). Ми припускаємо, що продукти гена(ів), задіяні у транспорті ліпіду II через мембрану *S. ghanaensis* ATCC14672, аналогічно до MurJ *Escherichia coli*, можуть брати участь в експорті моеноміцину. У цій роботі ми виконали початкову генетичну характеристику двох ідентифікованих нами *murJ*-подібних генів *S. ghanaensis* ATCC14672, а саме *SSFG_03627* і *SSFG_01411*. Ген *SSFG_03627* – життєво важливий, і його може бути делетовано з генома *S. ghanaensis* ATCC14672 тільки за наявності додатково внесеної копії гена *SSFG_03627*. Спроби комплементувати *murJ*-дефектний штам *E. coli* NR1154 вищезгаданими генами *S. ghanaensis* були невдалими. Маніпуляції з числом копій гена *SSFG_03627* мали складний вплив на продукцію моеноміцину в *S. ghanaensis* ATCC14672.

Ключові слова: стрептоміцети, моеноміцин, стійкість до антибіотиків, фліппази ліпіду II