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COMPARISON OF ACTIVITY OF THREE HETEROLOGOUS PROMOTERS IN *STREPTOMYCES GHANAENSIS* ATCC14672

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Streptomyces ghanaensis ATCC14672 produces moenomycin A, the only known natural product that directly inhibits peptidoglycan glycosyltransferases. It is desirable to develop reliable genetic tools for this strains which would allow to generate ATCC14672 derivatives producing novel moenomycins. Using β-glucuronidase reporter system, here we studied the activity in ATCC14672 of the three heterologous promoters extensively used in *Streptomyces* genetics – *gylP1/P2, aac(3)IVp* and *ermEp.* The promoter of glycerol utilization operon *gylP1/P2* displayed the highest transcriptional activity, while *aac(3)IVp* the weakest one. Activity of the latter was 2 and 3 times lower than that of *gylP1/P2* on the first and fourth day of growth, respectively. The activity of *ermEp* was somewhat higher than that of *aac(3)IVp*, but did not exceed the strength of *gylP1/P2.*

Keywords: Streptomyces ghanaensis, moenomycin A, β-glucuronidase, reporter system.

Among the producers of clinically important compounds the representatives of the genus *Streptomyces* take a prominent place [2]. One of them is *Streptomyces ghanaensis* ATCC14672, a producer of the phosphoglycolipid antibiotic moenomycin A (MmA), which inhibits the cell wall formation of gram-positive microorganisms. It stands out due to an extremely high activity, the uniqueness of action and absence of reported cases of resistance among pathogens [12, 13]. However, the wild-type strain synthesizes MmA in small quantities [1, 12, 13]. It is necessary to have suitable tools for genetic manipulation of the strain for its improvement. Efficient expression vectors are one of the most useful tools required for such purposes. To obtain such vectors, it is necessary to identify a set of the promoters that display high expression level in ATCC14672. However, relative strength of heterologous promoters in this strain has not been studied so far. Therefore, we decided to study the activity of several popular heterologous promoters in moenomycin producer using a recently described β-glucuronidase reporter system [11]. Based on RNAseq results, this reporter is thought to provide reliable readout of transcriptional activity of the promoters [14]. For experiments we picked up three promoters: *gylP1/P2* from glycerol utilization operon of *S. coelicolor*, *ermEp* derived from erythromycin resistance gene *ermE* of *Saccharopolyspora erythraea* (encodes rRNA methyltransferase) and *aac(3)IVp* from aminoglycoside acetyltransferase gene *aac(3)IV* of *Klebsiella pneumoniae*. The aim of our work was to compare the efficiency of aforementioned promoters in different growth timepoints.

Material and methods

In this work we used the MmA producer *S. ghanaensis* ATCC14672 (wild type) and its recombinant strains *S. ghanaensis* (pMT3226GusA), *S. ghanaensis* (pSETamGusA), *S. ghanaensis* (pSETermGusA) and *S. ghanaensis* (pGUS). The strains were grown on solid oatmeal medium

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and in liquid TSB [3, 8]. Reporter plasmids pMT3226GusA, pSETamGusA and pSETermGusA were constructed through cloning of *ermEp*, *gylP1/P2* and *aac(3)IVp* fragments into pGUS to create transcriptional fusion of the promoter to *gusA* gene. The details of plasmids construction are reported in [5]. The plasmids were transferred, via conjugation, from *E. coli* ET12567 (pUZ8002) to ATCC14672 according to [10]. *E. coli* ET12567 (pUZ8002) was grown on LA medium with kanamycin (Km) at concentration of 50 µg/ml. The media for growth of recombinant actinomycetes and *E. coli* strains were amended with apramycin (Am) and Km at concentration of 50 µg/ml [8]. The activity of the studied promoters was as follows. 25 ml of the seed medium were inoculated with 3 ml from a two days-old preculture of *S. ghanaensis* strains containing reporter plasmids. Cells were grown for 20 or 94 hours at 37°C. 5 ml of the culture were harvested by centrifugation (4,000×*g* for 10 min), resuspended in lysis buffer (50 mM phosphate buffer [pH 7.0], 5 mM dithiothreitol [DTT], 0.1% Triton X-100, 4 mg ml^{-1} lysozyme). Lysis was performed at 37°C for 45 min (the completeness of the lysis by that time was confirmed microscopically). Lysates were centrifuged at 4,000 rpm for 10 min. Then, 0.5 ml of lysate was mixed with 0.5 ml of dilution buffer (50 mM phosphate buffer [pH 7.0], 5 mM DTT, 0.1% Triton X-100) supplemented with 5 µl 0.2 M *p*-nitrophenyl-D-glucuronide. The OD_{415nm} was measured every minute for a total of 20 minutes of incubation at room temperature. As control, a 1:1 mixture of lysate and dilution buffer was used. All measurements were repeated three times. The glucuronidase activity is calculated in m-units per mg of total protein in the lysate (mU mg^{-1}). Total protein was determined by Bradford Protein assay (BioRad, USA). One m-unit is referred to such amount of GusA that is able to produce in one minute 1 µM of *p*-nitrophenol from the substrate in a total volume of 1 mL.

Results and discussion

The transconjugant colonies of *S. ghanaensis* ATCC14672 carrying the aforementioned reporter plasmids were selected for the resistance to apramycin. We checked the presence of the glucuronidase (GusA) activity of *S. ghanaensis* (pMT3226GusA), *S. ghanaensis* (pSETamGusA), *S. ghanaensis* (pSETermGusA) and *S. ghanaensis* (pGUS) with flooding of 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) solution on the lawns of the strains. The strains with pMT3226GusA, pSETermGusA and pSETamGusA became dark blue because of chemical transformation of X-Gluc to 5,5'-dibromo-4,4'-dichloro-indigo due to GusA activity, while the strain carrying control plasmid pGus (promoterless *gusA*) remained white (Fig. 1). These data confirmed that strains indeed carried functional *gusA* gene.

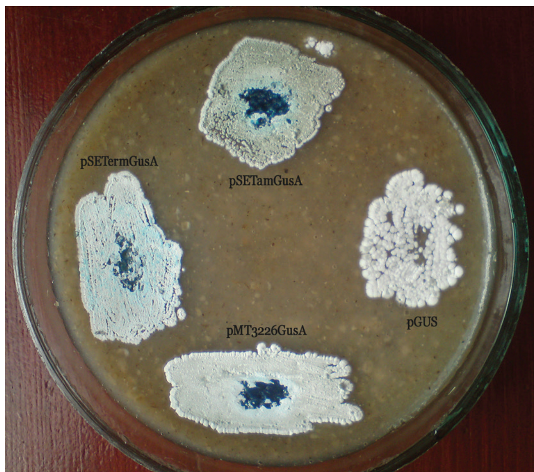

Fig. 1. The lawns of *S. ghanaensis* (pMT3226GusA), *S. ghanaensis* (pSETamGusA), *S. ghanaensis* (pSETermGusA) and *S. ghanaensis* (pGUS) onto which a drop of X-Gluc solution was applied.

To quantitatively describe the efficiency of the studied heterologous promoters, *S. ghanaensis* transconjugants were grown in TSB at 37ºC for 20 and 96 hours. At these timepoitns mycelium was washed, resuspended with reaction buffer, lysed and then chilled on ice**.** The glucuronidase activity was analyzed by adding the GusA substrate, X-Gluc (p-nitrophenyl-β-D-glucuronide) solution to the mycelium lysate and immediately measuring the optical density of the sample at 415 nm [9]. These results are represented on fig. 2 and fig. 3.

Glycerol promoter *gylP1/P2* showed the highest transcriptional activity. The *aac(3)IVp* was 2 and 3 times weaker than the *gylP1/P2* on 20 and 96 hour of growth, respectively. The *ermEp* showed higher activity than the *aac(3)IVp*, although it did not exceed that of *gylP1*/*P2*.

The transcription from *gylP1/P2* in *S. coelicolor* is known to be increased in the presence of glycerol, because the latter interacts with GylR repressor and makes dissociate from the promoter [4]. Glucose, in contrast, is a co-repressing molecule. We note that in ATCC14672 *gylP1/P2* does not seem to be repressed by GylR (its gene is located on pMT3226gusA), as the GusA activity is present in the extracts from the respective strain already after 20 hours of growth. In contrast to the published data [4], we observed that 1% (w/v) glycerol rather repressed than induced *gylP1/P2* (see fig. 2 and 3). On the 96th hour *gylP1/P2* activity was more vigorous, than on the 20th hour. It likely was caused by the exhaustion of glucose in the broth, which eased up *gylP1/P2*repression.

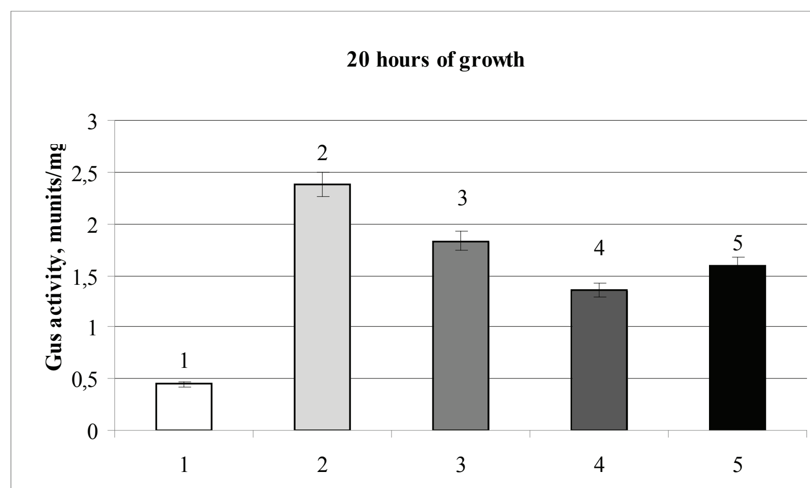

Fig. 2. Glucuronidase activity in cell lysates of *S. ghanaensis* mutants after 20 hours of growth: 1 – *S. ghanaensis* (pGUS) (control); 2 – *S. ghanaensis* (pMT3226GusA); 3 – *S. ghanaensis* (pMT3226GusA), with presence of 1% glycerol in the medium; 4 – *S. ghanaensis* (pSETamGusA); 5 – *S. ghanaensis* (pSETermGusA). Error bar: ±2σ.

After 96 h of growth, the activity of *aac(3)IVp* and *ermEp* was lower in comparison to that observed on 20th h. Perhaps it is caused by the shift of the culture to the stationary growth phase and the lysis of the cells.

In this work we provide experimental evidence that GusA reporter system is suitable to develop the tools for genetic manipulation in *S. ghanaensis.* In ATCC14672 cells the *gylP1/P2* promoter showed highest transcriptional activity level among three heterologous promoters being tested. Its absolute value was around 1–2 mU mg^{-1} of GusA activity, and it did not decrease over the period of 4 days of growth in TSB. Thus, *gylP1/P2* can be used in the future construction of vectors for gene overexpression in *S. ghanaensis*.

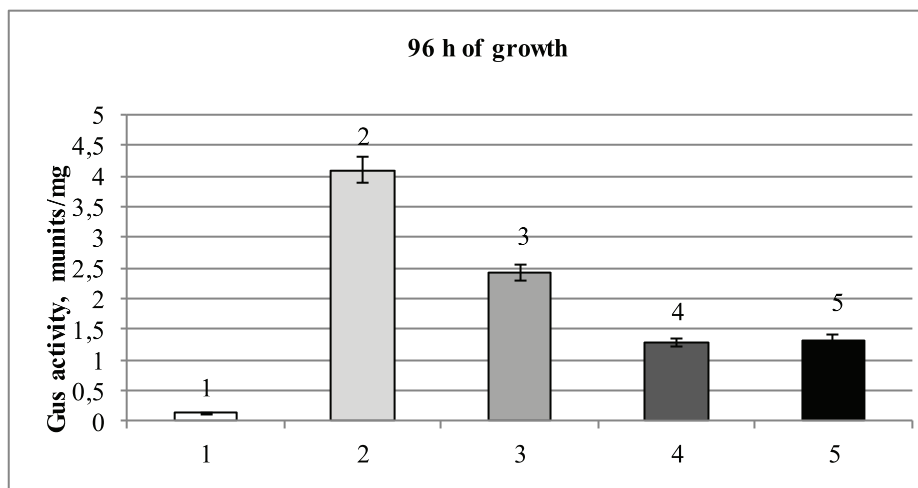

Fig. 3. Glucuronidase activity in cell lysates of *S. ghanaensis* mutants on the 96th h of growth: 1 – *S. ghanaensis* (pGUS) (control); 2 – *S. ghanaensis* (pMT3226GusA); 3 – *S. ghanaensis* (pMT3226GusA), with presence of 1% glycerol in the medium; 4 – *S. ghanaensis* (pSETamGusA), 5 – *S. ghanaensis* (pSETermGusA).

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ПОРІВНЯННЯ АКТИВНОСТІ ТРЬОХ ГЕТЕРОЛОГІЧНИХ ПРОМОТОРІВ У *STREPTOMYCES GHANAENSIS* ATCC 14672

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Streptomyces ghanaensis ATCC14672 продукує моеноміцин А, єдину відому природну сполуку, що прямо інгібує пептидогліканові глікозилтрансферази. Становить інтерес розроблення надійних генетичних знарядь, які дали би змогу конструювати похідні ATCC14672 зі зміненим біосинтезом моеноміцинів. Використовуючи β-глюкуронідазну репортерну систему, ми дослідили в ATCC14672 активність трьох гетерологічних промоторів, що широко використовуються у генетиці стрептоміцетів – *gylP1/P2, aac(3)IVp* та *ermEp*. Промотор оперона утилізації гліцеролу *gylP1/P2* виявляв найвищу активність, тоді як *aac(3)IVp* – найнижчу. Активність останнього була у 2 і 3 рази нижча ніж така *gylP1/P2* на першу і четверту доби росту, відповідно. Активність *ermEp* була трохи вища ніж *aac(3)IVp* , але не перевищувала сили *gylP1/P2.*

Ключові слова: Streptomyces ghanaensis, моеноміцин А, β-глюкуронідаза, репортерна система.

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СРАВНИТЕЛЬНЫЙ АНАЛИЗ АКТИВНОСТИ ТРЕХ ГЕТЕРОЛОГИЧЕСКИХ ПРОМОТОРОВ В *STREPTOMYCES GHANAENSIS* ATCC 14672

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Streptomyces ghanaensis ATCC14672 продуцирует моеномицин А, единственное известное природное соединение, прямо ингибирующее пептидогликановые гликозилтрансферазы. Представляет интерес разработка генетических инструментов, которые позволили бы конструировать штаммы ATCC14672 с измененным биосинтезом моеномицинов. Используя β-глюкуронидазную репортерную систему, мы исследовали в ATCC14672 активность трех гетерологических промоторов, которые широко используются в генетике стрептомицетов – *gylP1/P2, aac(3)IVp* и *ermEp.* Промотор оперона утилизации глицерола *gylP1/P2* показал самую высокую активность, тогда как *aac(3)IVp* – самую низкую. Активность последнего была в 2 и 3 раза ниже, чем таковая *gylP1/P2* на первые и четвертые сутки роста, соответственно. Активность *ermEp* была выше, чем *aac(3)IVp*, но не превышала силы промотора *gylP1/P2.*

Ключевые слова: Streptomyces ghanaensis, моеномицин А, β-глюкуронидаза, репортерная система.