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Generation and study of the strains of streptomycetes – heterologous hosts for production of moenomycin

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Abstract

Moenomycins (Mm) are family of phosphoglycolipid natural products that is considered a blueprint to develop new class of antibiotics. The natural Mm producer, *Streptomyces ghanaensis* (ATCC14672), produces very low amounts of moenomycin, fueling the investigations on genetic approaches to improve its titers. Heterologous expression of moenomycin biosynthesis gene cluster (*moe*) would be one of the ways to reach this goal. Here we report the generation of a number of novel heterologous streptomycete hosts producing nosokomycin A₂ (one of the members of Mm family), and determine their potential for the antibiotic production. The *rpoB* point mutation in the model strain of *Streptomyces* genetics, *S. coelicolor* (strain M1152) significantly improved nosokomycin A₂ production compared to parental strains (M145 and M512), while double *rpoBrpsL* mutation in the same species (strain M1154) decreased it. Our results point to the previously unanticipated epistatic interactions between mutations that individually are known to be highly beneficial for antibiotic production. We also showed here for the first time that facultative chemolithotrophic streptomycete *S. thermospinosporus* and chloramphenicol producer *S. venezuelae* can be used as the hosts for *moe* genes.

Introduction

Shortage in development of novel antibiotics over the last two decades has created numerous challenges for healthcare worldwide, and renewed the interest in many underdeveloped classes of natural products. Moenomycins (Mm; Fig. 1), phosphoglycolipid natural products of streptomycete origin, are one such class. Mm directly inhibit peptidoglycan glycosyltransferases (PGTs), enzymes involved in penultimate step of bacterial cell wall biosynthesis [1]. There are no currently drugs on the market that would act through PGT inhibition. For more than four decades of use in animal nutrition, Mm have not led to significant increase in moenomycin resistance among clinically relevant bacteria [2]. Mm

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are extremely active against cocci, with an average minimal inhibitory concentration (MIC) 10–1000-fold lower (depending on strain) than that for vancomycin, another currently used antibiotic. These remarkable traits notwithstanding, development of Mm into a drug is complicated by its very low oral bioavailability and long half-life in the bloodstream – two properties, that are thought to be determined by the log isoprenoid chain in Mm structure (рис. 1) [3]. It therefore important to explore all possible ways to manipulate Mm structure in order to obtain improved analogs. Currently submerged fermentation of Mm producers is the only economically justified way to produce this class of compounds, although available strains accumulate small amounts of Mm.

Recently the gene cluster for Mm production (*moe*) has recently been cloned from *Streptomyces ghanaensis* (ATCC14672), and its biosynthesis has been deciphered. All *moe* genes, except for three controlling the formation of A-ring (fig. 1) and its attachment to glycoside portion of Mm, are located within *moe* cluster 1 [3]. Yet, it is not clear to date how the regulation of Mm is accomplished. This, to a large extent, slows down all genetic engineering of moenomycin variants.

Production of several different moenomycins have also been achieved in heterologous strains, such as *S. lividans* TK24, *S. coelicolor* M145 and *S. albus* J1074 [4]. Although the amounts of Mm produced by heterologous hosts do not exceed that of ATCC14672, the ease of genetic manipulations, faster growth, and simpler schemes of compound purification are the chief advantages of heterologous systems. We therefore decided to investigate a wider set of *Streptomyces* strains as a platform for Mm production, with the aim to obtain new insights into the genetic mechanisms of regulation as well as to identify improved heterologous hosts. In this work we focused on production of a nosokomycin A₂ (NoA₂; Fig. 1). This is one of the structurally simpler members of Mm family, and it is suitable for further chemical or enzymatic modification [5]. We have chosen six streptomycete strains (described in Results section in more details) for our heterologous expression experiments. Among other results, we revealed an unexpected genetic interaction between *rpoB* and *rpsL* mutations that diminished moenomycin titers. We also show here for the first time that chemolithotrophic streptomycete can be manipulated genetically and used for heterologous production of antibiotics.

Materials and methods

Strains and cosmid DNAs

Escherichia coli ET12567 (pUZ8002) was used for conjugative DNA transfer from *E. coli* to *Streptomyces*. *Bacillus cereus* ATCC 19637 was used as a test-culture in Mm activity bioassays. *S. ghanaensis* dH5 is a *moeH5*-deletion derivative of ATCC14672; it accumulates exclusively nosokomycin A (NoA; Fig. 1), a compound identical in potency to NoA₂. As hosts for heterologous expression we used following *S. coelicolor* strains: M145 (plasmidless (SCP1[−] SCP2[−]) derivative of wild type A3(2) [6]), M1152 *S. coelicolor* M145, (*act red cpk cda rpoB*[C1298T]), M1154 (*act red cpk cda rpoB*[C1298T] *rpsL*[A262G]), as well as *S. albus* J1074 (wild type) [7], *S. venezuelae* ATCC10712 (wild type) [8, 9], *S. thermospinosporus* NRRL-B24318 (= AT10^T = DSM 41779^T) (wild type) [10].

Cosmid moeno 38-6 (Fig.2) was used heterologous expression experiments. Its construction is fully described in [11]. It includes the *attP-int* fragment of actinophage ϕ C31 genome, enabling site-specific integration of the cosmid into *attB* ^{ϕ C31} [12]. The cosmid contains the entire major *moe*-cluster except for *moeR5moeS5* genes, and *moeH5* was substituted with kanamycin resistance gene *neo*. Because of the absence *moeH5*, *moeR5moeS5* genes, moeno38-6 directs the biosynthesis of NoA₂ [3, 11]. All strains and plasmids are maintained in the Collection of cultures of microorganisms-producers of antibiotics of Ivan Franko National University of Lviv.

Culture conditions

B. cereus and *E. coli* strains were cultured as described [13]. To produce spores the streptomycetes were grown on oatmeal agar [14] or MYM [15] from 2 to 8 days (depending on the strain) at 28 °C, (*S. ghanaensis* и *S. thermospinosporus* – при 37 °C). To examine NoA₂ biosynthesis, moeno38-6⁺ *Streptomyces* strains were grown in liquid R5A [4] or TSB (BD, USA) and on solid agar soybean medium (g/l: soy flour – 20, NaCl – 5, agar - 16). Conjugal matings streptomycetes were grown on oatmeal or soymeal media. Heterologous host strains carrying moeno38-6 were grown in the presence of kanamycin (50 μ g ml⁻¹) and hygromycin (100 μ g ml⁻¹).

Verification of the strains containing cosmid moeno 38-6 and analysis of nosokomycin A₂ production

Transconjugants were checked for the stability of kanamycin- and hygromycin-resistance after three passages under nonselective conditions (oatmeal agar without antibiotics). Then we used PCR to prove the presence of moeno 38-6 in the chromosome of the strains. For this purpose, we used primers *moeO5up* and *moeO5rev* [11] designed to amplify the key gene for Mm biosynthesis, *moeO5*. Using total DNA as a template, in all cases we observed the amplicon of expected size (1.3 kb), confirming the presence of moeno38-6 in transconjugants.

A set of bioassays was used to determine antibiotic activity of the actinomycete strains: productivity index (IP) – ratio of diameter of growth inhibition zone around the clone to the diameter of the latter [16]; agar plug method [17], antibiotic disc diffusion assay [4]. All LC-MS data were acquired on a Bruker Esquire 3000 ESI-MS machine as described in [4]. Following compounds (shown on Fig. 1) were monitored via LC-MS in the extracts: NoA, [M-H]⁻ = 1485.6 Da; NoA₂, [M-H]⁻ = 1501.6 Da.

Results

Choice of novel heterologous hosts for NoA₂ production

We previously showed that *S. albus* J1074 and *S. coelicolor* M145 can be used for heterologous production of nosokomycin B₂ (fig. 1) [4]. The first of the mentioned strains exhibited relatively high Mm production level, approaching that of native strain, ATCC14672, and we also decided to use this strain for NoA₂ production. *S. coelicolor* M145 was characterized by very low NoB₂ production. Two strains, M1152 and M1154 have been recently constructed on the basis of M145 with antibiotic overproducing

properties [9]. In both strains endogenous gene clusters for biosynthesis of actinorhodin, undecylprodigiosin, CPK and CDA have been deleted. Furthermore, in strain M1152 there point mutation S433L has been introduced into the gene *rpoB* for RNA polymerase subunit. The strain M1154 carries *rpoB* mutation along with mutation in ribosomal protein S12 (*rpsL*; [K88E]). These pleiotropic mutations are known to increase antibiotic production by various actinobacteria [18–21], and we decided to test their influence on Mm production. Next, we have chosen *S. venezuelae* (ATCC10712) because of its fast dispersed growth and simplicity of genetic manipulations (particularly amenability to electroporation and high frequency of transconjugants in matings with *E. coli*) [8, 9]. Finally, we expressed *moe* genes in thermophilic carboxydophilic *S. thermospinisporus* (NRRL-B24318) [10] as a first step in evaluation of a possibility use of chemolitotrophic bacteria for Mm production.

Generation and verification of heterologous hosts for NoA₂ biosynthesis

The cosmid moeno38-6 (Fig.2) was transferred conjugally from *E. coli* ET12567 (pUZ8002) into six aforementioned streptomycetes: *S. albus* J1074, *S. coelicolor* M145, M1152, M1154, *S. venezuelae* ATCC10712 and *S. thermospinisporus* NRRL-B24318. The hygromycin-resistant (Hy^r) transconjugants have been obtained readily for all strains, although their frequency varied greatly, from 10⁻³ for J1074 and ATCC10712 to 10⁻⁷ for M1154 and NRRL-B24318. Using primers for *moeO5* gene, we obtained the amplicons of expected size (1.3 kb) from total DNA of the transconjugants, but not from the parental strains. The Hy^r phenotype of the transconjugants was 100 % inherited after 5 passages (200 colonies were tested) in the absence of hygromycin, indicating the stable integration of the cosmid into recipient's genomes.

We checked the Mm production by the heterologous hosts using LC-MS. All the transconjugants produced the expected compound, NoA₂, as exemplified on Fig. 3 by extract from moeno38-6⁺ M1152 and J1074 strains. Interestingly, although moeno38-6⁺ J1074 strain produced NoA₂, it was not a major Mm in the extract. Instead, tetrasaccharide intermediate to NoA₂ (lacking terminal galacturonic residue, see Fig. 1) dominated the extract, accounting roughly for 90 % of Mm content (Fig. 3). The parental strains did not produce Mm in the absence of moeno38-6 (data not shown).

Analysis of Mm production

Although LC-MS allows precise qualitative detection of Mm in the extracts, it is very time-consuming and nontrivial method for quantitative analysis. Moreover, when one plans to test many strains under numerous conditions, it becomes economically prohibitive. To quantify Mm production, we routinely used various bioassays tailored to the needs of the experiment [4, 22]. However no side-by-side comparison of these methods has ever been carried out. We therefore set out to address this question, which will help to develop a bioassay-based method of analysis of many clones, giving reliable prediction of the productivity under submerged fermentation conditions. There were tested two approaches based on growth of Mm producer on solid media, and one based on analysis of extracts from the biomass from liquid media. Results of these measurements are summarized on Fig. 4. LC-MS of the extracts from the biomass grown in liquid media was then employed for verification; these data turned out to coincide with IP bioassay data (fig. 4a), and we cite them below. *S. albus*

J1074 produced the highest Mm titers when growing on either solid or in liquid media. (1.5-fold increase in IP as compared to *S. ghanaensis* dH5). This is in contrast to *S. venezuelae* strain, which produced minute quantities of Mm in liquid R5A (0.1–1% of that observed for J1074), although it seemed to accumulate them more abundantly on soybean agar. Perhaps, it is explained by increased NoA₂ production on that medium, as well as co-production of chloramphenicol. (fig. 4a, b). We observed a rather high level of moenomycin production by *S. thermospinisporus*., which was within the range of productivity of M1152 moeno38-6⁺ strain. (fig. 4a).

Intriguing results could be inferred from the comparison of NoA₂ production levels of *S. coelicolor* M145, M1152 and M1154 strains. In comparison to M145 or M512 [4], the *rpoB* mutation (strain M1152) alone increased NoA₂ production 1.6-fold. Nevertheless, a combination of *rpoB* and *rpsL* mutations (strain M1154) decreased NoA₂ production 2- and 3-fold in comparison to M145 and M512, respectively. The *S. coelicolor* strains did not differ in growth rate or biomass accumulation.

Discussion

Here we report the generation and study of several novel heterologous strains for Mm. The choice of novel heterologous strains was based on their beneficial genetic or microbiological traits, as detailed in the first section of Results. As a model we used the cosmid moeno38-6 that directs the production of NoA₂. There is significant industrial interest in this molecule as a scaffold for further chemical or bioenzymatic derivatization, although its availability is very limited. Production of NoA₂ was previously shown by us in *S. lividans* TK24 [6], but its purification was complicated by the presence of endogenous metabolites of *S. lividans*, such as actinorhodin. This work therefore establishes for the first time a route to reliable fermentation-based access to NoA₂ as a final and major compound. On combining the results of different methods of analysis of NoA₂ production by the heterologous hosts, we gained useful insight into the biosynthesis of Mm and the methodology of their quantification.

We first discuss the methodological aspects of our work, which provide a necessary foundations for the analysis of the obtained data. Several different bioassays were tested as a way to obtain semiquantitative data on productivity of the generated heterologous hosts. Their further verification via LC-MC provided evidence that measurement of IP of colonies grown on solid media most precisely reflects the productivity of the strains under submerged fermentation conditions. Disc diffusion method was not so reliable, probably because it was difficult to guess the amount of compound applied to the disc. If it exceeded certain value, the diffusion of Mm could be compromised by micelle formation, a known problem for all lipid-containing metabolites [3]. The agar block method had several shortcomings too, such as low diffusion from the blocks, or unequal diffusion from the blocks of even slightly different heights, or because of changes in size and density of the blocks due to agarolytic activity of atinomycetes (the latter was observed for *S. coelicolor* M1152 and M1154 strains). This led us to consider IP measurement a method of choice for future experiments.

Of the entire set of strains being tested, *S. albus* J1074 at first appeared to be the most promising host for NoA₂ production. However, as LC-MS analysis showed, total antibiotic activity of J1074 moeno38-6⁺ resulted from accumulation of two different Mm - NoA₂ and its tetrasaccharide intermediate, and the latter is dominant in the mixture (Fig. 4). Either production of galacturonic acid (a building block of the terminal sugar residue in Mm) is a bottleneck step in J1074 metabolism, or expression of *moe* gene for glycosyltransferase MoeGT2 (attaches last sugar in Mm biosynthetic pathway [11]) is for some reason decreased in J1074.

Selection for certain streptomycin- and/or rifampicin-resistant mutants of antibiotic producers is well known and widespread method to improve their productivity [18, 23, 24]. The use of heterologous hosts genetically engineered to bear such mutations is one way around tedious process of selection and isolation of such mutants, and we took this approach in our work. Our results showed the utility of *rpoB* mutation for improvement of NoA₂ production by *S. coelicolor* M1152 moeno38-6⁺. The M1152 moeno38-6⁺ strain is currently being considered by us the most promising platform for NoA₂ production. Unexpectedly, combination of *rpoB* and *rpsL* (A262G) mutations decreased NoA₂ production (by 50%), below the level of parental strain M145. We showed previously that the same *rpsL* mutation in *S. lividans* had no effect on Mm production [4]. This work demonstrates for the first time that *rpsL* mutations could be not only beneficial or neutral with respect to secondary metabolism, but deleterious as well, particularly in combination with *rpoB* mutation. While each mutation alone increases antibiotic production (or is at least neutral), their combination produces clear epistatic effect. This might be caused by the overall negative influence of double *rpoBrpsL* mutation on growth of M1154 strain, as already has been noted by the researchers, who developed it [18]. Probably, unlike the production of polyketides or nonribosomal peptides, the biosynthesis of glycoside-rich molecules, such as moenomycins, is very strictly co-regulated with certain essential primary metabolic pathways, and any perturbation in the latter lead to decreases in antibiotic production. We note that in course of analysis of big collections of streptomycin and rifampicin resistant mutants only a few had significantly produced more antibiotic, whereas the majority accumulated the latter at or below the wild type level [23–25]. Our results provide a cautionary tale about the challenges in generating optimal expression host through accumulation of pleiotropic mutations [26, 27].

S. venezuelae has recently entered the arena of *Streptomyces* genetics as a novel model for fundamental studies and heterologous expression experiments [10, 11]. Indeed, this strain showed fast and fine growth under Mm production conditions, but it also co-produced chloramphenicol and other colored compound(s), confounding the results of bioassays. Therefore, real NoA₂ titer of *S. venezuelae* is low, suggesting unfriendly genetic background for Mm production, and/or nonoptimal nutritional conditions. This latter explain trace production of NoA₂ in R5A. In contrast, *S. thermospinosporus* did not excrete any colored metabolite or compound active against Mm test culture. It produces NoA₂ as well as M1152 does, indicating its good prospects as a platform for Mm production. There are no reports in the literature on the transfer of foreign DNA into *S. thermospinosporus*, or any other genetic manipulations with this chemolithotrophic strain. So our, data are first of its kind.

Here NoA₂ was produced heterotrophically, and we currently are testing the possibility of its biosynthesis under conditions of carboxydrotrophy and higher temperatures (>37 °C). Besides practical goal of making valuable molecules from cheap materials such as CO and CO₂, this work would help us understand the most fundamental links between primary and secondary metabolism.

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Fig. 1.

Structural formulae of moenomycins discussed in this work. Tetrasaccharide produced by *S. albus* J1074 moeno38-6⁺ lacks galacturonic residue (B)

Fig. 2.
Genetic organization of cosmid moeno 38-6 (see text for details)

Fig. 3. Extracted ion chromatograms corresponding to NoA₂ and its tetrasaccharide precursor in methanol extracts from moeno38-6+ M1152 and J1074 strains

Fig. 4.

Levels of Mm biosynthesis by parent and heterologous host strains as measured by IP measurements (a), method of agar blocks (b) and antibiotic disc diffusion assay (c). Mm production level of the parent strain is considered a 100%, which is approximately 1 mg/l of liquid medium TSB. Column labels: *S. gh.* – *S. ghanaensis* wild-type; *S. alb m* – *S. albus* moeno38-6; *S. ven m* – *S. venezuelae* moeno38-6; *S. th m* – *S. thermospinisporus* moeno38-6; *M 145 m* – *M 145* moeno38-6; *M 1152 m* – *M 1152* moeno38-6 – *rpoB* mutant; *M 1154 m* – *M 1154* moeno38-6 *rpoBrpsL* mutant. * - the bioactivity value represents total antibiotic activity of Mm and chloramphenicol.