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Genes for biosynthesis of butenolide-like signalling molecules in *Streptomyces ghanaensis*, their role in moenomycin production

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Abstract

Moenomycins (Mm) – phosphoglycolipid compounds produced by *Streptomyces ghanaensis* ATCC14672 – are considered a promising model for development of novel class of antibiotics. In this regard it is important to generate Mm overproducing strains which would be a basis for economically justified production of this antibiotic. In this work a set of genes for synthesis and reception of low-molecular weight signaling molecules (LSM) in ATCC14672 were described and their significance for Mm production was studied. The ATCC14672 genome carries structural and regulatory genes for production of LSMs of avenolide and γ -butyrolactone families. Additional copies of LSM biosynthetic genes *ssfg_07848* and *ssfg_07725* did not alter the Mm production level. ATCC14672 LSMs are not capable of restoring the sporulation of butyrolactone-nonproducing mutant of *S. griseus*. Likewise, while the heterologous host *S. lividans* 1326 produced Mm, its mutant M707 (deficient in the butyrolactone synthase gene *scbA*) did not. Thus, while the natural level of LSMs production by ATCC14672 does not limit Mm synthesis, the former is essential for the synthesis of moenomycins.

Keywords

Streptomyces ghanaensis; moenomycin; A-factor; avenolide

Introduction

Prokaryotes are engaged in extensive intercellular interactions mediated by the synthesis, secretion and reception of low molecular weight signaling metabolites (molecules; LSMs) [1]. “Chemical communication” is known for a long time in bacteria, and it underlies the quorum sensing, a special kind of regulation of gene expression in bacteria that depends on population density and concentrations of the LSMs [1, 2]. These communication systems are present among streptomycetes – members of the class *Actinobacteria*, main industrial producers of antibacterial compounds, and they are based on butenolide scaffold [3–6]. In these bacteria, LSMs are involved in pleiotropic regulation of morphogenesis and secondary metabolism [7–10]. At the moment 14 LSMs of γ -butyrolactone type have been identified,

and they are produced by seven streptomycete species. General mechanism of action of these LSMs is based on binding of repressor protein; as a result, the latter releases promoter region and thus activates gene transcription [8, 9]. A-factor (2-(6'-methylheptanoyl)-3R-hydroxymethyl-4-butanolide) is the best studied member of γ -butyrolactone family. A-factor – positive regulator of streptomycin synthesis and morphogenesis in *Streptomyces griseus* [10].

Recently second group of butenolide-like LSMs has been found in streptomycetes, that structurally are furan derivatives. Methylenomycin furans of *S. coelicolor* and avenolides of *S. avermitilis* fall into this group; they are involved in regulation of production of antibiotics methylenomycin and avermectin, respectively [11, 12]. According to bioinformatics data [11], some of the genes for biosynthesis of avenolide-like LSMs are located in the genomes of several other streptomycetes, particularly in *Streptomyces ghanaensis* ATCC14672. This strain is interesting because of its ability to produce phosphoglycolipid antibiotics moenomycins (Mm), in particular moenomycin A (MmA, fig. 1), which is major component of Mm mixture accumulated by ATCC14672. MmA directly inhibits peptidoglycan glycosyltransferases, vital enzymes involved in bacterial cell wall formation [13]. No other classes of antibiotics are described so far that would exhibit the same or similar mode of action. Initial level of Mm production by ATCC14672 is minute. This prompts to investigate the mechanisms of regulation of Mm biosynthesis. In this regard, the transcriptional and translational regulatory mechanisms are studied relatively well [13, 14]. Yet, the role of LSMs in Mm biosynthesis remained unstudied. We note that heterologous expression of Mm biosynthetic genes (*moe*) in *S. coelicolor*, *S. lividans* and *S. albus* has been employed previously to study the genetics of regulation [13, 14]. This is explained by the difficulties of making gene knockouts in ATCC14672, and relatively high productivity of heterologous hosts, as well as by the fact that the genomes of the latter carry genes homologous to pleiotropic regulators of secondary metabolism of *S. ghanaensis*. Therefore, in this work we pursued following aims: to annotate all structural and regulatory genes involved in formation of communicative systems of *Streptomyces ghanaensis*; to study the role of LSMs in Mm production via overexpression of certain LSM-related genes; to reveal the influence of deletion of γ -butyrolactone synthase gene *scbA* of *S. lividans* M707 on heterologous production of Mm by this strain.

Materials and methods

Draft genome sequence of *S. ghanaensis* ATCC14672 and BLASTP program, both accessible via NCBI (www.ncbi.nlm.nih.gov), were used to search genes involved in biosynthesis and reception of LSMs and to perform pairwise alignments.

In this work we used strain *S. coelicolor* M145 (producer of actinorhodin; a derivative of wild type A3(2) strain lacking plasmids SCP1 and SCP2), wild type MmA producer *S. ghanaensis* ATCC14672 as well as its mutants: *adpA_{gh}*, impaired in aerial mycelium formation [14]; *moeO5* impaired in the first step of MmA biosynthesis [14]; *ssfg_05654* that has lost the ability to produce aerial mycelium on solid medium TSA [21]. We also use wild type *S. lividans* 1326 and its mutant M707 carrying the deletion of *scbA* (provided by Prof. E. Takano, Manchester university, UK). Mutant of *S. griseus* IFO13350 with impaired

biosynthesis of A-factor was provided by Prof. B. Matseliukh (D.K. Zabolotnyy Institute of Microbiology and Virology of NASU). Strains *Escherichia coli* DH5 α Ta ET12567 (pUZ8002), which were used for generation and transfer of recombinant plasmids, are described in [15]. *S. ghanaensis* ATCC14672, *S. lividans* 1326, M707 and their derivatives were grown on solid oatmeal and TSB media [13, 15], and in liquid TSB (tryptone – 17 g, glucose – 2.5 g, NaCl – 5g, K₂HPO₄ – 2.5, soy hydrolyzate – 10, H₂O) – to 1L). Strains of *E. coli* were grown according to standard protocols [15]. The media for growth of recombinant actinomycete and *E. coli* strains were amended with apramycin (Am) and kanamycin (Km) at concentration of 50 μ g/ml. Structural and regulatory genes for biosynthesis of LSMs of *S. ghanaensis* were cloned into vector pKC1139, which is based on temperature-sensitive pSG5 replicon [15]. Recombinant plasmids and cosmid moeno38-5 controlling nosokomycin B₂ synthesis (see fig. 1) were transferred into actinomycete strains via intergeneric conjugation from *E. coli* ET12567 (pUZ8002).

Total and plasmid DNA isolation, restriction endonuclease digestion, DNA ligation, DNA electrophoresis and *E. coli* transformation were carried out according to standard methods [15]. PCR was carried out as in [14] using PTC100 MiniCycler (“MJ Research”, USA). Intergeneric conjugation *E. coli* – *S. ghanaensis* was carried as in [13]. DNA sequencing was performed by Sanger method at the Biopolymers facility of Harvard Medical School (Boston, USA)

Mm purification from the biomass and its quantitative analysis via disc diffusion assay and liquid chromatography coupled to mass spectrometry was carried out according to [13].

Results and discussion

Bioinformatic analysis of the genes encoding LSM systems in *S. ghanaensis*

An *in silico* analysis showed the presence in *S. ghanaensis* the of the genes, encoding putative structural, regulatory and receptor proteins involved in metabolism of LSMs of butenolide types (γ -butyrolactones and avenolides). The identified genes of *S. ghanaensis* have homologues in the genomes of strains *S. coelicolor*, *S. griseus* and *S. avermitilis* (Table 1).

Protein product of gene *sfg_07725* – AfsA_{gh} - is the component of the first hormonal system. Like its homologues (Table 1), it contains FabZ domain that functions as thioesterdehydratase, and participates in fatty acid metabolism, whose intermediates are necessary for synthesis of A-factor [12, 16]. Gene *ssfg_07725* is located on one terminus of linear *S. ghanaensis* chromosome near the divergently situated gene *ssfg_07724*. The protein product of the latter is possible A-factor receptor. Gene *ssfg_01628* encodes TetR family protein homologous to ArpA-like receptor of *S. coelicolor*.

Gene *ssfg_07848* for presumable avenolide receptor is located in one cluster with genes *aco_{gh}* and *cyp* encoding acyl-CoA oxidase and cytochrome hydroxylase, respectively. They are located the terminal region of *S. ghanaensis* chromosome (opposite of A-factor biosynthetic genes), and homologous to genes for biosynthesis of avenolide of *S. avermitilis*. Translation product of *ssfg_07848* – a TetR-like protein, with characteristic secondary

structure of DNA-binding helix-turn-helix domain. Analysis of available at the moment streptomycete genomes showed that *S. ghanaensis*, unlike the vast majority of the other members of *Streptomyces*, possesses genes for two communicative systems – that of γ -butyrolactone and avenolide types.

Introduction of additional copies of *ssfg_07848* and *sfg_07725* into *S. ghanaensis* ATCC14672 cells does not influence Mm production level

To introduce extra copies of *ssfg_07848* and *sfg_07725* into *S. ghanaensis*, several pKC1139-based plasmid have been generated. Fragments of *S. ghanaensis* chromosome containing *ssfg_07848* and *sfg_07725* (654 bp and 957 bp in size, respectively) were amplified with primers decorated at the ends with EcoRI and HindIII recognition sites. To amplify *sfg_07725* we used primers: afsA-ghf (AAAGAATTCATAGCCTGCCAGCCGGTTGG) and afsA-ghr (AAAAAGCTTTCAGATCCAGGCGGCATCCGC). To amplify *ssfg_07848* we used primers avaR-ghf, avaRr-ghr (AAAGAATTCATCCAGATCAGAGGGCGCGC, AAAAAGCTTTCAGCTGCTCATCGGCTCCG, respectively). Amplified and gel-purified DNA fragments were digested with EcoRI and HindIII, and then purified and ligated to linearized vector pKC1139. *E. coli* DH5 α was transformed with the ligation mixture and recombinant plasmid were selected on indicator plates containing chromogenic substrate X-Gal. The cloning of aforementioned genes into the vector was verified via mapping and sequencing. The generated plasmids were labeled as pKCafsA_{gh} and pKCavaR_{gh}. They were transferred conjugally into *S. ghanaensis* ATCC14672 from *E. coli* ET12567 (pUZ8002). Colonies of the transconjugants *S. ghanaensis* (pKCafsA_{gh}) and *S. ghanaensis* (pKCavaR_{gh}) did not differ from control strain (*S. ghanaensis* carrying empty vector pKC1139) in growth rate, sporulation and antibiotic resistance. The transconjugants had the same level of Mm production as compared to the control strain (fig. 2).

LSMs produced by *S. ghanaensis* did not restore normal morphological development of *S. griseus* afsA mutant

Structural and regulatory genes for γ -butyrolactone biosynthesis in *S. ghanaensis* are similar to *afs* genes of *S. griseus*, which points to the structural relatedness (probably, identity) of LSMs, whose production they control. To check this suggestion, we performed the test on restoration of sporulation of *afsA* mutant of *S. griseus* with the strains of *S. ghanaensis*. In this test *S. ghanaensis* strains are secretors of LSMs, which, in case of their functional similarity to A-factor of *S. griseus*, would restore the aerial mycelium formation and sporulation to *S. griseus* IFO13350 mutant. As secretors we used following strains: *S. ghanaensis*: ATCC14672, *S. ghanaensis* (pKCafsA_{gh}), *S. ghanaensis moeO5* (deletion of key Mm biosynthesis gene, no Mm production), *S. ghanaensis adpA* (knockout of the master regulatory gene for secondary metabolism and morphogenesis), and model actinomycete *S. coelicolor* M145. When growing in the vicinity of the *afsA* mutant, none of the investigated streptomycetes was able to restore normal colony development to the *afsA* mutant when growing in the vicinity of the latter (fig. 3A). γ -butyrolactones produced by different actinomycetes are structurally similar. Yet, only one case was described when LSMs of one species restored the sporulation of another LSM-deficient species [4, 10]. In the same time, wild type *S. ghanaensis* strain partially restored the sporulation of *S.*

ghanaensis ssfg_05654 when growing on TSB agar (fig. 3B). This points to the ability of *S. ghanaensis* to produce and excrete LSMs. That is why our result, most probably, shows that LSMs produced by *S. ghanaensis* are quite different structurally from A-factor, and they cannot be recognized by the receptor proteins of *S. griseus*.

Influence of deletion of *scbA* gene on moenomycin production by *S. lividans*

In both *S. coelicolor* and *S. lividans* genes *scbA* and *scbR* encode γ -butyrolactone synthase and receptor protein with DNA-binding properties, respectively [17]. These genes play key role in LSM synthesis and regulatory pathways the LSMs are part of. Mutations in these genes have different effects on production of secondary metabolites. Particularly, *scbA* deletion caused early start of production of actinorhodin and undecylprodigiosin in *S. coelicolor* [2], while in *S. griseus* deletion of *afsA*, (*scbA* orthologue) led to complete cessation of streptomycin production [6]. Presumably, opposite effects of these mutations are caused by peculiarities of mechanisms of hormonal regulation in the aforementioned species. We showed previously that *S. lividans*, carrying cosmid moeno38-5 is able to produce nosokomycin B₂ (member of Mm family, see fig. 1) on a relatively high level [13]. Taking into account the difficulty of genetic manipulations of *S. ghanaensis*, absence of information on chemical structure of LSMs and their production level in native Mm producer, we decided to investigate the role of LSMs in Mm biosynthesis in heterologous *S. lividans* strains: 1326 (wild type) and M707 (deletion of *scbA*). The cosmid moeno38-5 was transferred into the both strains. Methanol extracts of the biomass of 1326 moeno38-5⁺ and M707 moeno38-5⁺ were analyzed with bioassays and high-performance liquid chromatography coupled to mass spectrometry. Extract of 1326 moeno38-5⁺ contained the nosokomycin B₂, while M707 moeno38-5⁺ extract showed neither activity in bioassays nor mass-peaks expected for nosokomycin B₂ (see fig. 2). Therefore, *S. lividans* 1326 *scbA* gene is a positive regulator of Mm biosynthesis.

In conclusion, we suggest that moenomycin biosynthesis, not regulated at the pathway-specific level [13, 18], is still a subject for global regulation from LSM-dependent pathways. *S. ghanaensis* genome analysis witnesses the presence of gene sets for synthesis and reception of two butenolide-like classes of LSMs, that of γ -butyrolactone and avenolide families. Experiments are being carried out in our laboratories to determine the fact of accumulation of avenolide-like compounds by *S. ghanaensis* cells and to reveal their role in Mm biosynthesis. In the same time, we already can state that LSMs of γ -butyrolactone family are necessary for Mm production. This statement is supported by inability of M707 moeno38-5⁺ strain to produce nosokomycin B₂. However, natural level of synthesis of LSMs in *S. ghanaensis* is likely to be high enough in order not to limit the Mm production. This is evident from the fact that overexpression of several LSM production-associated genes did not lead to increase in Mm titers. Our results set the groundwork for more detailed investigations of function and interaction of pathways to LSMs in *S. ghanaensis*.

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Fig. 1.

Structural formulae of moenomycins mentioned in the text. Moenomycin A – main product in the moenomycin biosynthetic pathway of *S. ghanaensis* ATCC14672. Nosokomycin B₂ is accumulated by *S. lividans* 1326 upon introduction of cosmid moeno38-5

Fig. 2.

Level of Mm production by *S. ghanaensis* and *S. lividans* strains. 100 % production is the level of wild type (ATCC14672), approximately 1mg/L in TSB. For *S. lividans* strain 1326 the nosokomycin B₂ productivity was around 0.2 mg/L in TSB. The data are mean values of three independent experiments referred back to equal amounts of dry biomass.

A

B

Fig. 3.

Results of tests on chemical complementation of impaired morphogenesis in streptomycetes.

A. Complementation of *afsA* mutant of *S. griseus* by different strains. 1 – *S. griseus afsA*, 2 – *S. coelicolor* M145, 3 – *S. ghanaensis* (pKCafsA_{gh}), 4 – *S. ghanaensis ghanaensis adpA*, 5 – *S. ghanaensis moeO5*, 6 – ATCC14672. B. Complementation of impaired morphogenesis of *ssfg_05654* mutant *S. ghanaensis* (1) with wild type *S. ghanaensis* ATCC14672 (2).

Table 1
Genetic components of LSM systems of *S. ghanaensis* and their homologues in *S. coelicolor*, *S. griseus* and *S. avermitilis*

<i>Streptomyces ghanaensis</i> ATCC14672			Homologues			
No	Protein	Function	Gene	Protein	Strains	Similarity %
1	Ssfg_07724	Butyrolactone receptor	<i>scbR</i>	A-factor receptor	<i>S. coelicolor</i>	46
2	Ssfg_07725 (AfsA _{gh})	Regulator/Necessary for LSM synthesis	<i>afsA</i>	A-factor sythesis	<i>S. griseus</i>	44
3	Ssfg_07843	TetR-like regulator	<i>scbR</i>	TetR-like A-factor receptor	<i>S. coelicolor</i>	45
4	Ssfg_07848 (AvaR _{gh})	Autoregulatory receptor protein	<i>avaR</i>	Avenolide receptor	<i>S. avermitilis</i> MA-4680	75
5	Ssfg_07849	Acyl-CoA oxidase	<i>aco</i>	Acyl-CoA oxidase	<i>S. avermitilis</i> MA-4680	48
6	Ssfg_01628	TetR-like A-factor receptor	<i>cprA</i>	ArpA-like receptor	<i>S. coelicolor</i>	76