

Genetic factors that influence moenomycin production in streptomycetes

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Abstract Moenomycin, a natural phosphoglycolipid product that has a long history of use in animal nutrition, is currently considered an attractive starting point for the development of novel antibiotics. We recently reconstituted the biosynthesis of this natural product in a heterologous host, *Streptomyces lividans* TK24, but production levels were too low to be useful. We have examined several other streptomycetes strains as hosts and have also explored the overexpression of two pleiotropic regulatory genes, *afsS* and *relA*, on moenomycin production. A moenomycin-resistant derivative of *S. albus* J1074 was found to give the highest titers of moenomycin, and production was improved by overexpressing *relA*. Partial duplication of the *moe* cluster 1 in *S. ghanaensis* also increased average moenomycin production. The results reported here suggest that rational manipulation of global regulators combined with increased *moe* gene dosage could be a useful technique for improvement of moenomycin biosynthesis.

Keywords *Streptomyces* · Moenomycin · Regulation · Heterologous hosts · Global regulators

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Introduction

Significant effort has been put into understanding the biology of secondary metabolism of actinomycetes in order to accelerate and simplify the generation of industrial over-producers from typically low-producing wild-type strains [3, 5]. The regulation of antibiotic production is a complex multigene trait where genetic circuits of different rank, and environmental and intracellular cues are intricately combined to tune the expression of certain metabolic pathways [4, 13]. Although the general architecture of such regulation seems to be conserved across species, its effects on production of a given molecule cannot always be predicted and have to be addressed experimentally [7].

We are interested in moenomycin A (MmA; Fig. 1), a phosphoglycolipid antibiotic that inhibits peptidoglycan biosynthesis. For almost four decades MmA has been used as an animal growth promoter without a notable rise in MmA-resistant microflora. Now, when chemotherapy of human infections faces a serious shortage of efficient drugs, MmA has attracted the attention of both chemists and biologists as a promising model for the development of novel classes of antibiotics to treat multidrug-resistant pathogens [2, 17, 31, 33]. Exploiting its promise requires having access to significant quantities of moenomycin intermediates for further modification. We have previously identified the genes for MmA biosynthesis in the producing organism, *S. ghanaensis*, and have shown that a variety of Mms can be successfully produced in *S. lividans* TK24 [23]. However, low Mm production levels in both the natural producer, *S. ghanaensis*, and the heterologous host call for the development of better Mm-producing strains. Since no apparent regulatory genes were found in the MmA biosynthetic gene (*moe*) cluster [21], and the genetic factors that control its production are not understood, we have

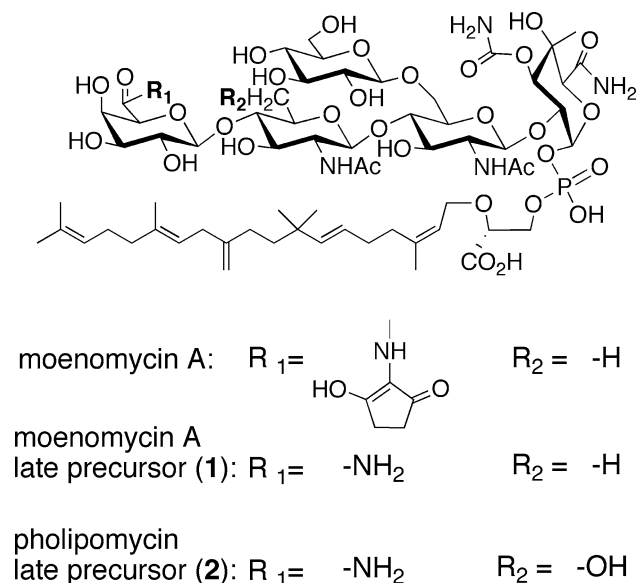


Fig. 1 Structures of moenomycins relevant to this study. MmA and compound **1** are accumulated by *S. ghanaensis* strains, while the production of compound **2** is directed by cosmids moeno38-5 and moeno38-1 in heterologous hosts [21]

examined several different strategies to improve biosynthesis in model moenomycin-producing strains. Our initial studies, reported below, support the idea that manipulating global antibiotic regulatory genes and gene dosage in suitable strain backgrounds can lead to useful improvements in production levels for other studies.

Materials and methods

Bacterial strains and plasmids

Escherichia coli DH5 α (Life Technologies, Carlsbad, CA, USA) was used for routine subcloning. *E. coli* ET12567 (pUB307) was used to perform intergeneric conjugation from *E. coli* to *Streptomyces* strains. *Bacillus cereus*

ATCC19637 was used as a test-culture for antibiotic disc diffusion assays. Commonly used *S. coelicolor* M145, M512, *S. lividans* TK24, 1326 [14], and *S. albus* J1074 [6] strains were tested here as Mm producers. Plasmids pKD46 and pKD4 were a gift from Prof. J. Beckwith (Harvard Medical School, USA); they were used to carry out RedET-mediated gene replacement as described in following paragraph. *Streptomyces* expression vectors pKC1218E, pIJ6902, pSOK101 and cosmid moeno38-1 were described previously [13, 21]. *S. coelicolor* *relA* expression construct pIJ6085 was provided by Prof. M. Bibb (JIC, Norwich, UK). List of recombinant molecules constructed and used along with resulting strains are listed in Table 1.

Culture conditions and general methods

Standard genetic techniques for *E. coli* and *Streptomyces* and for DNA manipulations were used as described [14, 25]. *Streptomyces* strains were grown in liquid R5A [26] or TSB media [14] for Mm production. *E. coli* and *Bacillus* strains were grown in LB supplemented with appropriate antibiotics. Oatmeal medium [18] was used to obtain spores of streptomycetes and to plate intergeneric matings. Where needed, streptomycete strains were incubated in the presence of antibiotics: kanamycin (50 $\mu\text{g ml}^{-1}$), apramycin (50 $\mu\text{g ml}^{-1}$), or hygromycin (100 $\mu\text{g ml}^{-1}$). T4 DNA ligase and restriction enzymes were used according to the recommendations of suppliers (NEB, Beverly, MA; MBI Fermentas, Burlington, ON, Canada). Other DNA manipulations were carried out following standard procedures specified by the manufacturers (NEB; MBI Fermentas; Invitrogen, Carlsbad, CA, USA; Bio-Rad, Hercules, CA, USA).

Moenomycin extraction, purification, and quantitative analysis

One-hundred-milliliter flasks with 15 ml of seed medium (TSB) were inoculated with frozen spore suspensions

Table 1 Plasmids and *Streptomyces* strains constructed in this study

Plasmid/strain	Genotype/description
pONO5P	ϕ C31-based vector, <i>moeO5p</i> -driven expression of <i>neo</i>
pSOKmoeO5p	pSOK101 containing 500-bp promoter region of <i>moeO5</i>
pKCafS	pKC1218E containing <i>S. coelicolor</i> <i>afsS</i> gene under <i>ermEp</i> *
moeno38-5	Contains major portion of <i>moe</i> cluster 1, carries hygromycin and kanamycin resistance markers (Hyg ^r Km ^r)
<i>S. ghanaensis</i> <i>afsS</i> ⁺ / <i>relA</i> ⁺ / <i>moe38-5</i> ⁺	<i>S. ghanaensis</i> ATCC14672 carrying pKCafS or pIJ6085, or moeno38-5, respectively
TK24/1326 moeno38-5 ⁺	<i>S. lividans</i> TK24 or 1326 strain carrying moeno38-5
M145/M512 moeno38-5 ⁺	<i>S. coelicolor</i> M145 or M512 strain carrying moeno38-5
J1074 moeno38-5 ⁺	<i>S. albus</i> J1074 carrying moeno38-5
R1 moeno38-5 ⁺ / <i>relA</i> ⁺ / <i>afsS</i> ⁺	<i>S. albus</i> R1 expressing: moeno38-5 alone/moeno38-5 + <i>relA</i> (pIJ6085)/moeno38-5 + <i>afsS</i> (pKCafS)

¹ Details on construction of plasmids and strains can be found in “Materials and methods”

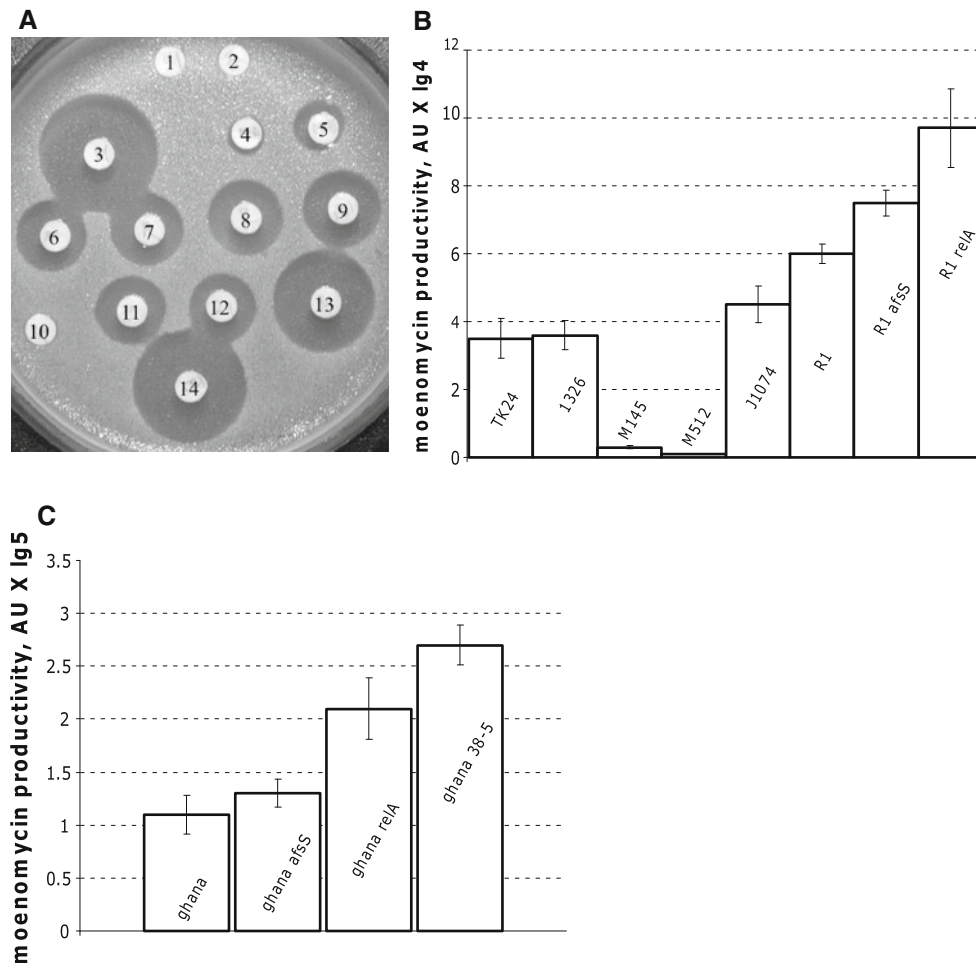


Fig. 2 Levels of moenomycin production by various streptomycete strains. **a** *B. cereus* growth inhibition around paper discs saturated with methanol extracts from 100 mg (wet weight) of biomasses of different Mm producers and control strains: *S. albus* J1074 (1), *S. coelicolor* M145 (2), 2 μ g of MmA (3), M512 moeno38-5⁺ (4), M145 moeno38-5⁺ (5), J1074 moeno38-5⁺ (6), R1 moeno38-5⁺ (7), R1 moeno38-5⁺ *afsS*⁺ (8), R1 moeno38-5⁺ *relA*⁺ (9), TK24 (10), TK24 moeno38-5⁺ (11), 1326 moeno38-5⁺ (12), *S. ghanaensis* (13), *S. ghanaensis relA*⁺ (14). **b** Moenomycin productivity of heterologous strains expressing cosmid moeno38-5. Column labels: TK24, 1326—*rpsL* and wild-type strains

of *S. lividans*, respectively; M145, M512—wild-type and Act⁻Red⁻ strains of *S. coelicolor*; J1074, R1—parent and MmA-resistant strains of *S. albus*, respectively; R1*afsS* and R1*relA*—R1 strains overexpressing *afsS* and *relA* genes, respectively. **c** Moenomycin productivity of different *S. ghanaensis* strains. Column labels: ghana—wild-type (ATCC14672); ghana *afsS*, ghana *relA*, and ghana 38-5—*S. ghanaensis* strains overexpressing *afsS*, *relA*, and cosmid moeno38-5, respectively. The Mm production values of *relA*-expressing strains and *S. ghanaensis* carrying duplicated *moe* cluster 1 are statistically significant, since they did not overlap with 2 σ range of the control strains

(2×10^5 cfu). The flasks were incubated at 28°C for 48 h on a rotary shaker at 180 rpm. One ml of the resulting seed broth was used to inoculate the 300-ml flasks with 30 ml of fermentation medium (R5A for heterologous hosts and TSB for *S. ghanaensis* strains). The fermentation was carried out on a rotary shaker at 180 rpm for 5 days at 30°C. The fermented broth was centrifuged at 3,500 rpm for 10 min. The mycelium was washed twice with distilled water. Mm was extracted by stirring the biomass (1 g, wet weight) with 3 ml of methanol for 12 h. The extract was concentrated *in vacuo* and diluted to the final volume of 300 μ l. Then it was either directly used for antibiotic disc diffusion assay or purified for LC–MS analysis according to the described procedure [21–23]. For the antibiotic diffu-

sion assay, paper discs (\emptyset 5 mm, Whatman, Maidstone, UK) were impregnated with a portion of the extract and dried at 37°C for 1 h. Discs were placed on the plates with 0.7% soft agar containing *B. cereus* (10^8 cfu). The plates were incubated at 4°C for 1 h and then at 37°C for 17 h. Relative changes in moenomycin production by various strains were determined with the help of LC–MS as described [23]. For heterologous strains, the quantity of compound 2 (Fig. 1) was determined, whereas for *S. ghanaensis* strains Mm productivity is the sum of production of MmA and its intermediate 1 (Fig. 1). Mean values were found from at least four biological repeats and standard deviations (σ) are shown in Figs. 2 and 3. Several control experiments were carried out to ensure that multistep purification and analysis process

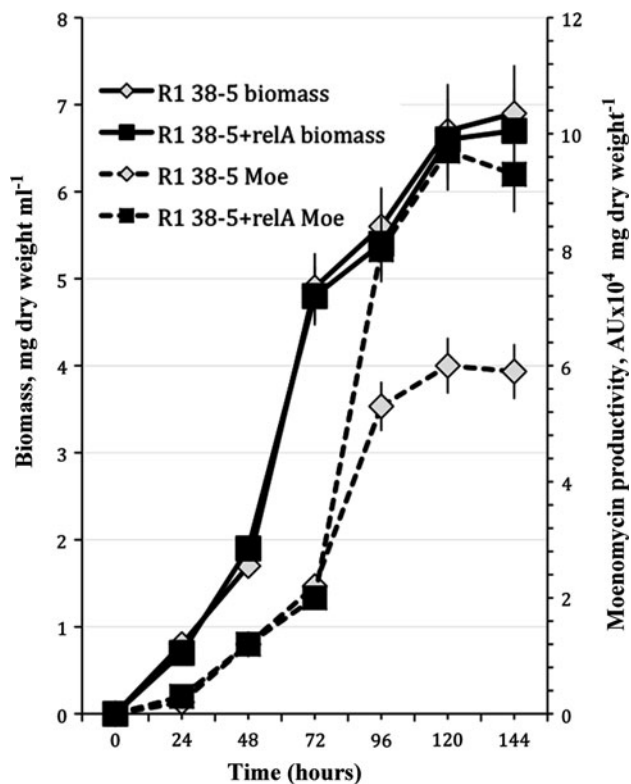


Fig. 3 Time course for biomass and Mm accumulation by *S. albus* R1 38-5 strains with and without *relA*. Solid lines biomass production, dashed lines specific Mm productivity, open diamonds R1 38-5 strain, solid rectangles R1 38-5 + *relA* strain. The productivity values are statistically significant as mentioned in the legend of Fig. 3

did not distort the measurements. Samples that contain increasing concentrations of MmA (in the 0.003–10 μ M range) were run through LC–MS and shown to give proportional increases in MmA-specific mass-peak area. Pure MmA was used as an internal standard for Mm extracts from heterologous hosts. The only samples that showed essentially identical MmA: 2 ratio upon LC–MS analysis were then taken into account. Under our LC–MS conditions [23], 1 AU of moenomycin productivity corresponds to 1580.6-Da peak area resulted from injection of 100 pM MmA; 10^5 AU corresponds to 10 μ M MmA.

Verification of the strains containing cosmid moeno38-5

Kanamycin- and hygromycin-resistant transconjugants carrying cosmid moeno38-5 were checked for the stability of inheritance of antibiotic resistance phenotype. Two hundred clones of each transconjugant were checked after three passages under nonselective conditions and all were found to retain the expected phenotype. PCR was employed to prove the presence of moeno38-5 in the chromosomes of streptomycete hosts. For this purpose, we used primers *moeO5up* and *moeO5rev* (Table 1S, Electronic Supplemental Materials,

ESM) specific for *moeO5* gene and those used to amplify *attR* ^{ϕ C31} [28], the “right” arm of moeno38-5 integration. The amplicons of expected size (1.3 and 0.6 kb for *moeO5* and *attR* ^{ϕ C31}, respectively) were amplified from all transconjugants in case of both primer pairs.

Plasmid construction

For *afsS* overexpression

pIJ4112 [11] was treated with *PstI* and the resulting 2.9-kb fragment containing the entire *afsS* and a part of *afsR* genes was cloned into *PstI*-digested vector pKC1218E [21]. The final construct (pKcafsS) contained gene *afsS* under the control of *ermEp**

For *moeO5p-neo* fusion

A 1.5-kb DNA fragment carrying the fd terminator, MCS, and promoterless neomycin resistance gene *neo* was amplified from pMO9 using primers *terforNheI* and *neorevMfeI* [24], and cloned into pGEM T-easy vector (Promega, Madison, WI, USA) to give pGN1. The 515-bp DNA fragment adjacent to the *moeO5* gene that includes the first 35 bp of the ORF was amplified with primers O5PFHindIII and O5PRXbaI (Table 1S, ESM) from cosmid moeno38-1 [21] and cloned into pSTBlue1 Acceptor vector (Novagen, Whitehouse Station, NJ, USA). The promoter region was retrieved with *HindIII-XbaI* and cloned into respective sites of pGN1. Obtained plasmid pGNO5P was digested with *NheI* and *MfeI* and the *neo* cassette fused to *moeO5p* was cloned into *EcoRI/XbaI* sites of pOOB5 [21] to give pONO5P. All constructs mentioned in this study were verified by sequencing at the Biopolymer Facility (Harvard Medical School).

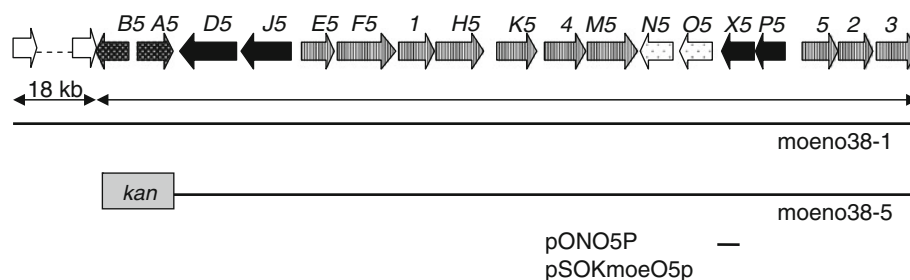
For *moeO5p* titration experiments

A 0.5-kb DNA fragment upstream of the *moeO5* start codon was amplified with aforementioned primers O5PFHindIII and O5PRXbaI. The resulting amplicon was digested with *HindIII* and *XbaI* and cloned into respective sites of high-copy number pIJ101-based vector [14] pSOK101 to give pSOKmoeO5p.

Cosmid moeno38-5

Cosmid moeno38-5 was constructed to ensure that the *moe* cluster-flanking 18-kb segment of *S. ghanaensis* chromosome (Fig. 4) does not cause any possible effects on Mm production by heterologous strains. The kanamycin resistance gene from plasmid pKD4 [8] was amplified with primers 38start-KD4 and *moeA5-P3* (Table 1S, ESM). The

Fig. 4 Genetic constructs used to express the segments of *moe* cluster 1. 18-kb region of cosmid moeno38-1 along with genes *moeB5moeA5* have been replaced with kanamycin resistance cassette *kan* (marked as grey rectangle)



resulting amplicon was used to replace the *moeA4moeB5* gene pair as well as the entire 18-kb “left arm” of moeno38-1 (Fig. 4) using the described λ Red approach [8]. We did not evict the *kanR* gene region from moeno38-5 because it did not exert any negative effects on MmA production. The replacement of the *moeA5* and *moeB5* genes with *kanR* in moeno38-5 was confirmed via diagnostic PCR (primers P2-KD4 and alsrev1 (Table 1S, ESM). Levels of Mm production by moeno38-5⁺ and moeno38-1⁺ *S. lividans* TK24 transconjugants were identical (data not shown), implying that no regulatory or structural *moe* genes were present in the deleted 18-kb region.

Semiquantitative RT-PCR

Total RNA samples were isolated after 4 days of *S. albus* growth in R5A according to instructions of the manufacturer of the RNeasy kit (Qiagen, Valencia, CA). To avoid DNA contamination, samples were treated with RQ1 DNase (Promega). RNA concentration and purity were determined by measuring the ratio of OD₂₆₀/OD₂₈₀, and an equal amount of RNA from each studied strain was used for RT reactions. cDNA was obtained using AMV Reverse Transcriptase (Promega) and random hexanucleotide primers. PCR was performed using Go-Taq DNA polymerase (Promega) and primer pairs specific to *moeO5* and *moeE5* genes (350-bp segments, Table 1S, ESM). As a positive control, the *rrnA* primer pair specific to 16S rRNA of *Streptomyces coelicolor* (380-bp segment) was used. Negative controls were carried out with *rrnA* primers to confirm the absence of contaminating DNA in the RNA preparations. PCR products were analyzed by electrophoresis in 1.2% agarose gels, and band intensity was established using ImageJ1.36b software (National Institutes of Health, Bethesda, MD, USA).

Screening for global regulators in genomes of *S. ghanaensis* ATCC 14672 and *S. albus* J1074

To assess the presence of pleiotropic regulators of antibiotic production in the genomes of *S. ghanaensis* ATCC 14672 and *S. albus* J1074, draft genomic assemblies of these strains were downloaded from the Web site

of Broad Institute (http://www.broad.mit.edu/annotation/genome/streptomyces_group/). The Open Read Frame (ORF) prediction was performed by a self-training application Glimmer3 [9] with default settings. Protein sequences of 20 known global regulators of *S. coelicolor* A3(2) secondary metabolism were collected from GenBank with subsequent BLAST [1] comparison to the genomes of interest. The list of regulators and other details are given in the ESM. Apart from turning off “low-complexity filtering” in the BLAST suite, we have used default values. In a few cases, manual adjustment of predicted ORFs was needed (i.e., if the sequence alignment extended beyond ORF boundaries with high probability). The identity value in the table is derived from best high-scoring pair (HSP) from the BLAST output, not the whole proteins, however in most cases these coincide (or almost coincide). Due to the unfinished status of sequencing projects of both genomes, no data on exact location in the chromosome or fixed locus tags for the predicted regulators can be presented.

Results

Generation and analysis of novel heterologous hosts for Mm production

Although novel MmA analogs were successfully isolated from recombinant *S. lividans* strains [21, 23, 33], their low productivity (up to 500 $\mu\text{g l}^{-1}$) is a formidable challenge for purification of large amounts of Mms needed for various studies. Better heterologous moenomycin producers would significantly facilitate the chemoenzymatic synthesis of novel phosphoglycolipids. Since the generation of (and especially analysis of) Mm-producing strains is laborious, a simple method to find the optimal host(s) would be desirable. We reasoned that the efficiency of expression of a key gene for MmA production, *moeO5* [23] in different strains could be used to sort them according to their ability to produce Mm. The activity of the *moeO5* promoter has been established in promoter titration experiments. Particularly, the *S. ghanaensis* pSOKmoeO5p⁺ strain, carrying multiple *moeO5p* copies *in trans*, accumulated significantly smaller amounts of moenomycins in the mycelium

$[(0.3 \pm 0.1) \times 10^5 \text{ AU mg}^{-1} \text{ dry biomass}]$ as compared to the control strain $[(1.4 \pm 0.2) \times 10^5 \text{ AU mg}^{-1} \text{ dry biomass}]$. Hence, as-yet-unknown transcriptional activator(s) appear to interact with *moeO5p* and their activity is effectively “diluted” by plasmid-borne *moeO5p*. This result encouraged us to construct pONO5P that carries a transcriptional fusion of *moeO5p* to the promoterless kanamycin resistance gene *neo*. As a test case, we compared the kanamycin resistance levels that pONO5P confers to three strains widely used as heterologous hosts, namely *S. coelicolor* M512 (*ActII-ORF4*, *AredD*), *S. lividans* TK24, and *S. albus* J1074. The 10- μl aliquots containing approx. 2×10^4 cfu of three independent pONO5P⁺ transconjugants of each aforementioned strain were applied to TSB agar plates supplemented with different amounts of kanamycin (1, 2.5, 5, 7.5, 10, 15, 25, and 50 $\mu\text{g ml}^{-1}$). Only *S. albus* grew at the highest kanamycin concentration tested (50 $\mu\text{g ml}^{-1}$). Good growth of *S. lividans* TK24 was observed on TSB with 25 $\mu\text{g ml}^{-1}$ of kanamycin, whereas *S. coelicolor* M512 grew on 2.5 $\mu\text{g ml}^{-1}$. No growth of the M512 and TK24 control strains carrying empty vector, was observed at the lowest kanamycin concentration tested. In the case of the control J1074 strain, a slight growth was observed at 2.5 $\mu\text{g ml}^{-1}$ of kanamycin. Next, cosmid moeno38-5 [carrying no additional segments of the *S. ghanaensis* genome except for the *moeD5-moeGT3* portion of the *moe* cluster 1 (Fig. 4)], was introduced into different strains of *S. albus*, *S. coelicolor* and *S. lividans* J1074 to compare their Mm production levels. *S. albus* gave the highest Mm titer, while M512 had the lowest one. There was approximately a 30-fold difference in production levels between J1074 and M512, showing considerable variation between heterologous hosts (Fig. 2). Expression of the *moeO5p-neo* construct and Mm production in the strains being tested seem to correlate positively, therefore such reporter systems can potentially be used as a tool to screen for optimal strains and conditions for Mm production.

S. coelicolor is naturally resistant to MmA, as are most streptomycetes strains we have examined, but *S. albus* 1074 is sensitive, with a survival rate of 0.001% at 1 $\mu\text{g ml}^{-1}$ of MmA (data not shown). Since MmA sensitivity could affect production levels in *S. albus*, we introduced cosmid 38-5 into the spontaneous MmA-resistant *S. albus* strain R1 (97% survival at 1 $\mu\text{g ml}^{-1}$ of MmA; data not shown) and compared growth and Mm accumulation in the R1 and J1074 transconjugants. The resistant strain showed a negligible increase in Mm production. Since MmA is primarily accumulated inside of the cells [23] and its target is located on cell surface, it is conceivable that certain (low) levels of MmA production can be achieved even by streptomycetes very sensitive to exogenously added MmA.

Since cross-regulation of antibiotic production has been previously reported for *S. coelicolor*, we examined whether

the heterologous production of Mms could be affected by the production of actinorhodin (Act) and undecylprodigiosin (Red). The Mm production levels in moeno38-5⁺ *S. coelicolor* M145 (Act⁺ Red⁺) were still low, but were reproducibly higher (three times) than in the Act- and Red-deficient variant moeno38-5⁺ M512 (Fig. 2a). Hence, some of the *act/red* genes or Act/Red themselves have a positive, albeit modest, influence on MmA biosynthesis.

We also investigated the influence of a mutation in the *rpsL* gene in *S. lividans* on Mm production levels. *S. lividans* TK24, used as the original heterologous host for Mm production, contains a point mutation (K43R) in the *rpsL* gene encoding ribosomal protein S12. This mutation is reported to enhance antibiotic production in various actinomycetes [27]. To determine the effect of this mutation on Mm production, we compared production of Mms in *S. lividans* TK24 (*rpsL* mutant) and the parent strain *S. lividans* 1326. Both antibiotic disc diffusion and LC-MS analyses (Fig. 2) showed that 1326 and TK24 accumulated comparable amounts of moenomycins. Hence, the *rpsL* K43R mutation does not significantly affect the level of Mm production.

In silico analysis and initial in vivo experiments show that certain global regulators may influence Mm production

The absence of pathway-specific regulatory genes associated with the *moe* cluster and its differential expression in various streptomycetes point to the possibility that some global antibiotic biosynthesis regulators govern MmA production. Such regulators are well studied in *S. coelicolor* [19] and we searched for their counterparts in partially sequenced genomes of *S. ghanaensis* and *S. albus* J1074. Our in silico analysis showed that both strains possess homologues of transcriptional factors, components of the A-factor signaling cascade and other proteins orchestrating secondary metabolism of *S. coelicolor* (Table 2S, ESM). Two *S. coelicolor* genes, *afsS* and *relA*, were chosen as a test case to obtain experimental evidence for their possible role in regulation of MmA production. These genes are known to cause the overproduction of secondary metabolites in *S. coelicolor*, *S. lividans*, and *S. avermitilis* [4, 11, 15, 30]. The exact function of *afsS* gene is unknown [16], while *relA* encodes (p)ppGpp synthetase [29]. Strain *S. albus* R1 moeno38-5⁺ *relA*⁺ showed a twofold increase in Mm production level compared to *S. albus* R1 moeno38-5⁺ carrying empty vector (Fig. 2). Overexpression of the *afsS* gene in *S. albus* R1 moeno38-5⁺ increased Mm production negligibly (1.25-fold; Fig. 2a, b). We introduced the regulatory genes described above into *S. ghanaensis* ATCC14672 and studied their effects on MmA production. Gene *afsS* had no impact on Mm biosynthesis, while the *relA*⁺ strain produced roughly twofold more Mm than control strain

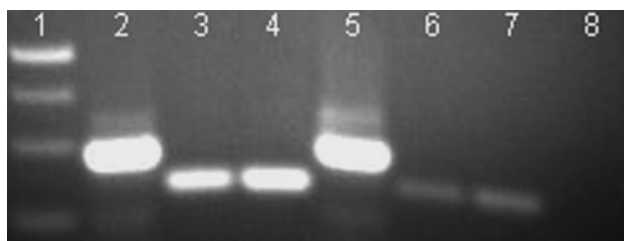


Fig. 5 RT-PCR analysis of *moeO5* and *moeE5* transcription in *S. albus* R1 38-5⁺ and R1 38-5⁺ *relA*⁺ strains during Mm production phase (96 h). Lanes: 2, 5—*rrmA* transcript from parent and *relA*⁺ strain, respectively; 3, 4—*moeE5* and *moeO5*, respectively, from R1 38-5⁺*relA*⁺; 6, 7—*moeE5* and *moeO5*, respectively, from R1 38-5⁺; 8—negative control (*rrmA* amplification from RNA sample in absence of RT); 1—1-kb ladder

S. ghanaensis (pIJ6902) (Fig. 2). Of all combinations tested, *relA* expression in *S. albus* had the biggest positive impact on Mm production, and we investigated this case in more detail. Although growth curves of *relA*⁺ and control strains were virtually identical, there is marked increase in specific Mm productivity of *relA*⁺ variant starting from the end of the 4th day, and declining slightly on the 6th day. This is paralleled by increased transcription of two key *moe* genes, *moeO5*, and *moeE5*, on the 4th day of fermentation of the 38-5⁺*relA*⁺ strain, as judged by semiquantitative RT-PCR (Fig. 5).

Increased *moe* gene dosage influences MmA production by *S. ghanaensis*

Massive amplification of antibiotic biosynthesis genes is often one of the outcomes of empirical strain improvement programs [10, 32]. To test whether an increased dosage of *moe* genes could be used as a tool to enhance MmA biosynthesis, we integrated cosmid moeno38-5 into the *S. ghanaensis* *attB*^{φC31} site. The resulting strain thus carries at least two copies of most of *moe* cluster 1 genes. We revealed that introduction of moeno38-5 into *S. ghanaensis* led, on average, to a twofold increase in Mm production (Fig. 2c).

Discussion

MmA is considered a promising lead to combat vancomycin- and methicillin-resistant pathogens because of its high potency, uniqueness of structure, and mode of action [12, 17, 20]. Better knowledge of the factors that limit Mm production by streptomycetes would certainly help resolve the issue of intrinsically low Mm yields. Here we describe a simple reporter system that may be used to find an efficient heterologous host for the production of moenomycins. In a test case, screening of several well-studied streptomycetes turned up heterologous host, *S. albus*, as being better than

S. lividans or *S. coelicolor* in terms of Mm production level and ease of antibiotic purification (because of the fast and dispersed growth and absence of pigmented metabolites). Of note, we revealed a dramatic difference in Mm-producing capacities of several species being tested, highlighting the importance of host-specific genetic factors for antibiotic production. More extensive screening with the help of the described reporter system may reveal even better strains.

We also addressed for the first time the possibility of the use of heterologous regulatory elements for MmA improvement. As the *relA* expression shows, at least some of the well-known pleiotropic regulatory genes might exert significant effects on Mm production, probably through direct or indirect modulation of expression of structural *moe* genes. This result encourages further investigations on the possible use of *S. coelicolor* global regulators to enhance Mm biosynthesis. Finally, duplication of *moe* cluster segments may increase antibiotic yields. We envision that proper combination of the discovered genetic factors with optimized fermentation media and purification procedures can significantly improve the production of novel moenomycins by recombinant strains. It is not known at the moment whether positive effects observed in our work (e.g., *relA* expression and *moe* gene dosage) will be additive. Although the results presented here are for model/wild-type strains, their application to known industrial strains or other potential heterologous hosts is anticipated to exert similar effects on Mm production.

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