Genome engineering approaches to improve nosokomycin A production by *Streptomyces ghanaensis* B38.3

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Abstract

Here we describe our efforts to improve the levels of phosphoglycolipid antibiotic nosokomycin A production by *Streptomyces ghanaensis* ATCC14672 via genome engineering approaches. Introduction of two extra copies of leucyl tRNA (UUA) gene *bldA* and one copy of moenomycin biosynthesis gene cluster led, on average, to three-fold increase in nosokomycin A titers (up to 1.5 mg/L). Our results validate genome engineering approach as a viable strategy to improve moenomycin production.

Keywords: *Streptomyces ghanaensis*; moenomycins; genome engineering; integrative vectors.

Introduction

Streptomyces ghanaensis (ATCC14672) is producer of moenomycins (Mm), a small family of phosphoglycolipid antibiotics. The latter are the only known direct inhibitors of peptidoglycan glycosyltransferases that act in nanomolar concentration range against many Gram-positive pathogens, including multidrug resistant cocci [1]. Uniqueness of structure and mode of action of Mm attract attention to phosphoglycolipids as a blueprint for development of novel antibiotics [2]. Over the last 10 years, we genetically engineered streptomycetes to make a number of moenomycin analogs [1, 3]. Particularly, we constructed *S. ghanaensis* B38.3 which accumulated almost exclusively nosokomycin A (NoA; Fig. 1). NoA represents the simplest unmodified pentasaccharide phosphoglycolipid with a free carboxy group. The latter is an attractive chemical handle for further chemical derivatization. However, B38.3 produces trace amounts of NoA, making purification of the latter a challenging task [4]. We therefore set out to improve NoA production by B38.3 via genome engineering. Out of a wide variety of the latter [5], we focused on site-specific recombination approaches.

Our previous investigations revealed that AraC/XylS regulator AdpA and leucyl tRNA (UUA) gene *bldA* are among the major positive regulators of Mm biosynthesis [6]. Here we generated several B38.3 derivatives carrying additional copies of *adpA*, *bldA*, *moe* cluster or combinations thereof, and determined their NoA titers. Thus, the uniqueness of this study lies in systematic study of several different regulators (in combination and alone) on moenomycin yields.

Streptomyces ghanaensis B38.3 was used throughout the study. *Escherichia coli* WM6026 [7] was used for conjugal transfer of plasmids into *Streptomyces*. *Bacillus cereus* ATCC19637 was used as moenomycin-sensitive test culture in bioassays. Cosmids and plasmids are listed in Electronic Supplementary Materials (ESM), Table S1. Standard microbiological and molecular biology techniques were used throughout the work [8].

Marker eviction and LC-MS-based moenomycin quantification have been carried out as in [4, 6]. In LC-MS, we monitored NoA anion (1485.6 Da $(M-H)^-$) and mean value of its mass peak area in B38.3 strain was taken for 100%. The extracts were spiked with equal amounts of moenomycin A (to a final concentration of 1 μM) as an internal standard.

As a starting point for genome engineering strain B38.3 has been chosen. We hypothesized that introduction of additional copies of regulatory genes *bldA* and *adpA*, as well as increased copy number of *moe* genes may lead to increase in NoA production, as we observed it previously for the moenomycin A producer [6]. Our strategy involved the use of actinophage VWB- and ϕC31-based integrative vectors (pTOS and pTES, repsectively) which allow single copy stable maintenance of the cloned genes. They contain recombination sites *rox* and *loxP*, respectively, allowing vector sequences elimination subsequent to integration [4]. This enables re-use of apramycin resistance marker gene *aac(3)IV*. We showed previously that in *S. ghanaensis* integration of VWB-based vectors restores $attB^{VWB}$ in the right integration arm *attR* [9]. Therefore, elimination of the first pTOS vector sequence has set the ground for the integration of second copy of pTOS. Using the aforementioned vectors, regulatory genes *bldA*, *adpA* and *moe* gene cluster-containing cosmid moeno38-6 [3], we generated seven derivatives of B38.3 as summarized in Fig. S1 (ESM), and described below.

In the first stage we transferred additional copy of *adpA* (plasmid pOOB92a), *bldA* (pTOSbldA) or modified *moe* cluster directing the production of nosokomycins (moeno38-6) individually into B38.3. Both plasmid- and cosmid-carrying strains (see Fig. S1, ESM) were verified via re-transformation of *E. coli* with the total DNA of the transconjugants [9]. In all cases we confirmed the identity of the plasmids isolated from B38.3 and *E. coli* donor strains (see, for example, Fig. S2, ESM). In the second stage we introduced recombinase aDre-expressing plasmid pUWLDre into B38.3 carrying native copy of *bldA* gene along with *bldA* from *S. albus* J1074 (B38.3_2bldA) and selected for colonies that have lost *aac(3)IV*. The vector eviction was confirmed via PCR with primers complementary to vector sequences. The generated strain was referred to as

B38.3_2bldAm and it served as a platform in the last stage of B38.3 genome manipulation. Particularly, pTOSbldA was transferred into B38.3_2 bldAm and one Am^r colony, referred to as B38.3_3bldA, was selected for further characterization. We also transferred into B38.3_2bldAm *adpA*-expressing plasmid pOOB92a to yield B38.3_2bldAm+adpA. As expected, plasmid pOOB92a in B38.3_2bldAm+adpA led to increased *adpA* gene expression (Fig. S3, ESM). Analogously, an extra copy of *moe* gene cluster, on the cosmid moeno38-6, was transferred into B38.3_2bldAm. All generated strains exhibited equal growth rates and sporulation abundance.

We seeded the strains into 300-mL flasks containing 35 mL of TSB medium and grown for five days at 37°C. Antibiotic was extracted from equal amounts mycelia, and quantified with LC-MS. B38.3 derivatives carrying extra copy of *moe* cluster, alone or in combination with *bldA* genes, exhibited significantly higher NoA titers as compared to B38.3 (Fig. 3).

To conclude, we succeeded in generation of *S. ghanaensis* B38.3 derivatives carrying more than one copy of the aforementioned regulatory or *moe* genes. We observed no increase in NoA production when two *adpA* copies were present in B38.3 genome. This was rather unexpected in light of previous data, where *adpA* under the same promoter that we used here led to 2.5-fold increase in moenomycin biosynthesis [6]. At the moment we cannot explain this observation; experiments addressing this issue are underway in our laboratory. Our results also revealed no positive effects of increased *bldA* gene copy number on NoA production. Therefore, although complete absence of $tRNA^{Leu}_{UAA}$ abolishes moenomycin production, increased dosage of *bldA* alone cannot improve the latter. In our experiment we merely increased the production of nascent tRNA, which is not yet translationally competent until extensive post-transcriptional modifications take place [10]. Probably, it would be necessary to fine-tune tRNA modifications in order to observe the effects of tRNA gene dosage on moenomycin production. The combination of extra copy of major *moe* cluster 1 and *bldA* had larger effects on NoA than *moe* cluster had. Therefore, it seems that increase in *moe* gene dosage is the most straightforward way to enhance Mm production.

Under conditions of increased *moe* cluster copy number *bldA* becomes a limiting factor for NoA production, as analysis of B38.3_2bdlAm+moe also suggests.

Conflict of interest. All authors declare that they have no conflict of interest.

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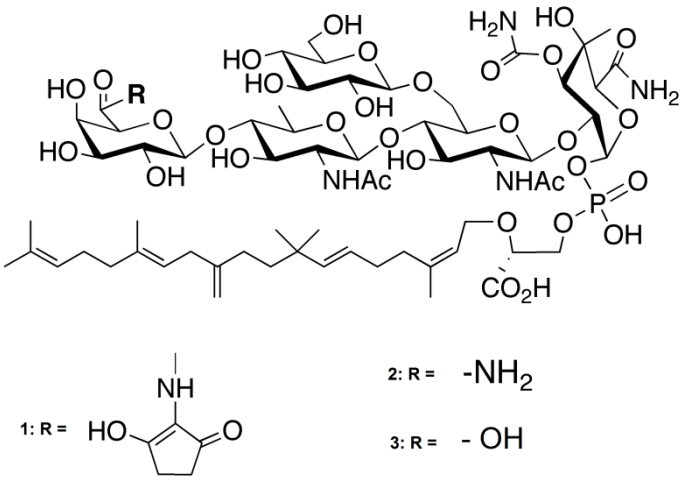
Legends to figures

Fig. 1. Chemical strucutures of moenomycins mentioned in the text: moenomycin A (MmA, 1), nosokomycin B (NoB, 2) and nosokomycin A (NoA, 3)

Fig. 2. Nosokomycin A titers accumulated by *S. ghanaensis* strains. The productivity was determined via LC-MS (see the main text). All strains accumulated equal amounts of the biomass in the end of fermentation (24±3 mg per 10 mL). Data are shown as mean values ± 2σ.



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169 Fig. 1

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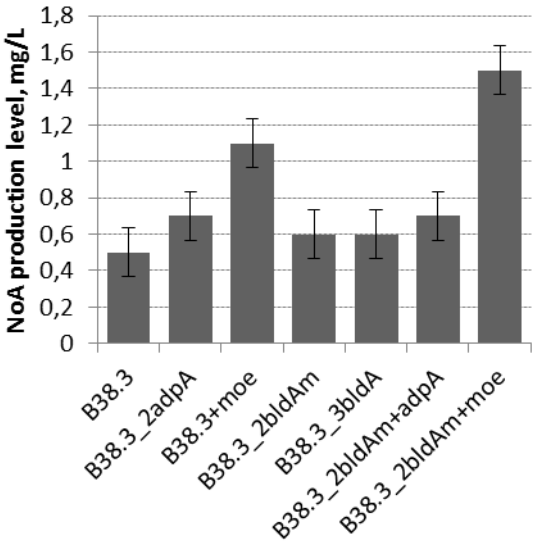

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