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Accessibility
Pleiotropic regulatory genes \textit{bldA}, \textit{adpA} and \textit{absB} are implicated in production of phosphoglycolipid antibiotic moenomycin

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1. Summary

Unlike the majority of actinomycete secondary metabolic pathways, the biosynthesis of peptidoglycan glycosyltransferase inhibitor moenomycin in \textit{Streptomyces ghanensis} does not involve any cluster-situated regulators (CSRs). This raises questions about the regulatory signals that initiate and sustain moenomycin production. We now show that three pleiotropic regulatory genes \textit{bldA}, \textit{adpA} and \textit{absB}—exert multi-layered control over moenomycin biosynthesis in native and heterologous producers. The \textit{bldA} gene for tRNALeu\textsuperscript{UAA} is required for the translation of rare UUA codons within two key moenomycin biosynthetic genes (\textit{moeO5} and \textit{moeE5}). It also indirectly influences moenomycin production by controlling the translation of \textit{adpA} and, probably, other as-yet-unknown repressor gene(s). \textit{AdpA} binds key \textit{moe} promoters and activates them. Furthermore, \textit{AdpA} interacts with the \textit{bldA} promoter, thus impacting translation of \textit{bldA}-dependent mRNAs—that of \textit{adpA} and several \textit{moe} genes. Both \textit{adpA} expression and moenomycin production are increased in an \textit{absB}-deficient background, most probably because \textit{AbsB} normally limits \textit{adpA} mRNA abundance through ribonucleolytic cleavage. Our work highlights an underappreciated strategy for secondary metabolism regulation, in which the interaction between structural genes and pleiotropic regulators is not mediated by CSRs. This strategy might be relevant for a growing number of CSR-free gene clusters unearthed during actinomycete genome mining.

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2. Introduction

Moenomycins (Mms) are a small family of secondary metabolites of actinomycete origin that display a number of remarkable traits in terms of their chemistry and biology [1]. Classified as phosphoglycolipids, they result from a unique assembly of glycoside- and isoprene-derived moieties bridged by 3-phosphoglyceric acid—an unprecedented building block in secondary metabolism (SM). Moenomycins strongly interfere with the growth of mainly Gram-positive bacteria, including VRE and MRSA pathogens, through direct inhibition of peptidoglycan glycosyltransferases (PGTs). High potency of these antibiotics and their unique mode of action explain much of the industrial and academic interest in them. We have recently identified genes for moenomycin production (moe gene cluster) by Streptomyces ghanaensis ATCC14672 and harnessed them for generation of a number of useful phosphoglycolipid analogues [2]. However, moenomycin production by either S. ghanaensis or heterologous hosts must be significantly increased before combinatorial biosynthesis can be a reliable source of novel moenomycins for biological tests or chemical modifications. We therefore set out to explore the regulation of moenomycin production by S. ghanaensis, with the ultimate goal of using the gained knowledge for strain improvement.

In the vast majority of studied cases, the transcriptional regulators of actinomycete SM gene clusters form a two-tiered network, with genes for cluster-situated regulators (CSRs) and global (or pleiotropic) regulators scattered over the genome and unlinked to SM pathways [3,4]. Global regulators affect the expression of more than one SM pathway by modulating the expression of CSR genes. SM pathways often have more than a single associated CSR, in which case one of the CRs is an ultimate regulator of antibiotic production (responsible for activation of structural antibiotic biosynthesis genes), while others may act either singularly, on the ultimate regulatory gene, or pleiotropically, on unrelated and unlinked genes. It should be emphasized that ‘topology-based’ classification of regulators (cluster-situated versus global) does not predict function. That is, a CSR gene may encode any of the following: (i) an ultimate regulator; (ii) a true pleiotropic regulator [5] or ultimate regulator with ‘cross-talk’ properties [6]; or (iii) a regulator of a distal gene cluster [7]. As one of the hallmarks of actinomycete SM gene clusters, CSRs have attracted the interest of researchers, particularly as a tool to develop antibiotic overproducers, and they are often considered an essential layer of transcriptional control over secondary metabolite production [8].

In contrast to the model described above, moenomycin biosynthesis does not involve CSRs [9]. No CSR genes are found in the moe cluster; the presence of essential moenomycin-specific regulatory gene(s) elsewhere in the S. ghanaensis genome is unlikely given that we were able to recreate moenomycin production in several heterologous hosts [10]. Although CSR-free SM gene clusters in actinomycetes have been considered the exception rather than the rule [11,12], the number has increased steadily as numerous whole genomes have been sequenced and analysed [13–16]. These gene clusters represent a poorly understood archetype of regulation of actinomycete SM, where CSRs are not involved. In silico analysis of moe genes revealed the presence of TTA leucine codons in two key moe genes, moeOS and moeES. TTA is the rarest codon in actinobacteria [17] and, in streptomycetes, it is generally found in genes with auxiliary functions (SM, aerial mycelium and spore formation, cryptic). In Streptomyces coelicolor, mature tRNA^{Leu}_{TTA} (specified by bldA gene) is only formed during late stationary growth, defining the onset of hyphae and antibiotic production [18,19]. BldA regulation occurs via the presence of UUA codons within CSR genes [20]. Recent work on ippomycin biosynthesis has provided initial evidence that bldA also regulates the translation of structural SM genes [21]. We hypothesize that bldA regulates moenomycin production at the level of translation of mRNA of the key structural moe genes. However, it is unlikely that bldA is the only regulator of moenomycin production given the importance of transcriptional control over SM (vide supran). Indeed, our previous moe promoter titration studies pointed to the existence of transcriptional activator(s) of moe gene expression [10]. In this study, we show that AdpA_{Sg}, an S. ghanaensis orthologue of well-known S. coelicolor and Streptomyces griseus master regulator AdpA [22–24], is an important and direct activator of moe gene expression. The translation of UUA-containing adpA_{Sg} mRNA is dependent on bldA-encoded tRNA, although this dependence is not absolute. Finally, we provide circumstantial evidence that AdpA_{Sg} expression is regulated at the posttranscriptional level through the action of the absB_{Sg} gene, encoding an orthologue of S. coelicolor RNase III [25]. Together these data outline the involvement of three interacting global regulatory genes, absB–adpA–bldA, in control of a CSR-free secondary metabolic pathway. The first gene, absB, directly regulates adpA expression, bldA regulates the translation of both adpA and moenomycin structural genes and adpA directly influences moenomycin production. The regulatory influence of these genes on moenomycin production is effective in S. ghanaensis as well as several heterologous hosts. Our data and data from recent literature allow us to propose that AdpA and BldA may constitute a central regulatory component relevant to many SM pathways lacking cluster-situated, pathway-specific regulatory genes.

3. Results

3.1. In silico analysis of Streptomyces ghanaensis

gene suggests the involvement of AdpA in moenomycin production

Recent studies portrayed the transcription factor AdpA as one of the most versatile regulators of Streptomyces biology [24,26–29], including the expression of CSR-free secondary metabolic gene clusters [16]. In S. coelicolor and S. griseus, AdpA is known to influence other regulators, such as tRNA^{Leu}_{TTA} (BldA) and RNaseIII (AbsB). The latter regulates AdpA abundance via ribonucleolytic cleavage of its mRNA. As the moenomycin biosynthetic cluster does not contain any specific regulatory genes, it is an excellent test bed to investigate the possibility of combined SM regulation from AdpA, AbsB and BldA. Our laboratory previously identified an orthologue of absB in S. ghanaensis [10]. The absB-containing chromosomal regions of S. coelicolor and S. ghanaensis are syntenous. Presumably, absB_{Sg} belongs to the transcriptional unit which comprises three genes: SSFG_02131.1, SSFG_02130.1 and SSFG_02129.1 (absB_{Sg}) (figure 1).
In our in silico analysis [10] of *S. ghanaensis*, we identified an AdpA orthologue in *S. ghanaensis* and designated it as adpA<sub>gh</sub>. The coding sequence of adpA<sub>gh</sub> contains one TTA codon (figure 1), at the same position as other adpA<sub>gh</sub> orthologues [23,30–32]. Genes for several AdpA<sub>gh</sub> paralogues are present in the *S. ghanaensis* genome (see the electronic supplementary material, table S1). Additionally, a single copy of the tRNA<sub>Leu</sub><sup>UAA</sup> gene was identified in the *S. ghanaensis* genome relevant to this study. Triangles indicate position of AdpA-binding sites as predicted in silico (figure 1).

We mined the promoter regions of adpA<sub>gh</sub>, bldA<sub>gh</sub>, absB<sub>gh</sub> and moe clusters for the presence of AdpA operator sequences [33]. As expected, such sequences were revealed within adpA<sub>gh</sub> and bldA<sub>gh</sub> (figure 1). AdpA operator-like sites were identified within many intergenic regions of the moe cluster 1 (data not shown). Particularly, promoter regions of the key genes moeE5, moeK5 and moeO5, responsible for production of the earliest monosaccharide MmA intermediate [2], contain three, two and one such sites, respectively (figure 1). The presence of an AdpA orthologue in the *S. ghanaensis* genome and its respective operator sequences within the moe cluster indicated that it may have a role in the regulation of moenomycin production.

### 3.2. Moenomycin production is completely abolished in *Streptomyces ghanaensis* adpA and bldA mutants, and increased in the absB mutant

Deletion of adpA<sub>gh</sub> in the *S. ghanaensis* chromosome completely abolished moenomycin production, as determined by LC-MS (figure 2) and bioassays. No mass peaks corresponding to the earliest known moenomycin precursors [2] were found in the extracts of adpA<sub>gh</sub> mutant (ΔadpA<sub>gh</sub>), showing that moenomycin production was blocked at the initial first steps. Knockout of adpA<sub>gh</sub> had a significant influence on the morphological development *S. ghanaensis*. On solid media, a phenotype of *S. ghanaensis* ΔadpA<sub>gh</sub> resembled that of the ‘bald’ (bld) mutants described for streptomyces (figure 3 and [34]). AdpA proteins in other species are key developmental regulators, and their deletion has been reported to lead to substantial morphological defects [26,32,35].

The moenomycin production and morphology in the ΔadpA<sub>gh</sub> were restored to the wild-type state upon introduction of an intact copy of adpA<sub>gh</sub> (plasmid pSETAdpA-exp). Insertion of an extra copy of adpA<sub>gh</sub> under the control of a strong constitutive promoter erm<sup>Ep</sup> (plasmid pTSEadpA-exp),
caused a 2.5-fold increase in moenomycin production compared with the control strain (figure 2).

Like the ΔadpAgh, S. ghanaensis ΔbldAgh did not produce MmA or any of its intermediates (figure 2). Deletion of bldAgh impaired morphological development of S. ghanaensis (figure 3); in particular, aerial mycelium formation was considerably delayed compared with the wild-type strain (figure 3).

The introduction of a native copy of bldAgh (plasmid pSETbldA) restored the moenomycin production and normal timing of morphogenesis, implying that the ΔbldAgh phenotype is solely due to the absence of tRNALeu_UAA. The introduction of a second copy of bldA (pSET152bldA) into the wild-type strain led to a slight (1.6-fold on average) but reproducible increase in moenomycin production (figure 2).

The transcription and translation of several moe genes was analysed in further detail to determine whether the bldA mutation affected moenomycin production directly (by blocking the translation of UUA-containing moeO5 and moeE5 mRNAs) or indirectly (by arresting adpAgh expression). Semi-quantitative RT-PCR analysis of moeO5, moeE5 and moeGT4 showed that their transcription was not decreased in ΔbldAgh; in fact, it appeared to be increased (figure 4). Western blots revealed that MoeE5 protein is present in the cell-free lysate of the wild-type strain, but not in that of ΔbldAgh (figure 4), indicating a direct regulatory influence on the expression of TTA-containing moe genes by tRNALeu_UAA.

The RNase III-deficient mutant (ΔabsBgh) produced on average 2.7 times more moenomycin compared with the parental strain (figure 2). On solid media, ΔabsBgh differed subtly from the wild-type (figure 3). Chromatograms of the methanol extracts from the three aforementioned mutants and the wild-type demonstrated little qualitative difference beyond the moenomycin-related peaks (see the electronic supplementary material, figure S1). Nevertheless, new mass peaks seemed to occur in both ΔbldAgh and ΔadpAgh extracts; detailed characterization of these peaks was not pursued.

Bioinformatic analysis indicated that absBgh and two upstream genes (SSFG_02131.1 and SSFG_02130.1) are separated by 2 and 19 bp, indicative of transcriptional operon organization (figure 1). For complementation of S. ghanaensis ΔabsBgh, a series of integrative plasmids with different portions of this putative operon were constructed (for details, see §5). Only the plasmid containing absBgh in cis with the two upstream genes (pSOKabsBgh-III; figure 1) decreased moenomycin production to the wild-type level, suggesting that the absBgh is the last gene in a tricistronic message. Additional complementation experiments were designed to confirm that absBgh alone is sufficient to restore the wild-type phenotype. absBgh under the control of ermEp (pSOKabsBgh-exp) was integrated into the S. ghanaensis ΔabsBgh chromosome, and the resulting strain produced 2.5 times less moenomycin than the wild-type strain. Introduction of the same plasmid (pSOKabsBgh-exp) into the wild-type strain resulted in significantly decreased antibiotic biosynthesis (figure 2).
3.3. GusA reporter systems reveal the interactions of regulators with moe genes and each other

The recently described β-glucuronidase (GusA) reporter system [36] was applied to investigate the functional connection between the aforementioned pleiotropic regulators and moe genes. First, we measured transcription from the promoter of key structural gene moeE5 (moeE5p) in all of the S. ghanensis mutants. The wild-type strain had relatively high levels of transcription from moeE5p (see, for comparison, the activity of other SM gene promoters [36]), but we failed to detect transcription in the ΔbldAgh strain (figure 5). The moeE5 transcription was increased more than twofold and threefold from wild-type levels in moeEp (moeE5p) and ΔbldAgh strains, respectively (figure 5), in agreement with RT-PCR data (figure 4). While the pattern of moeEp activity in ΔbldAgh and ΔabsBgh is as anticipated [25], increased levels of moeE5p transcript in the ΔbldAgh are somewhat unexpected. A plausible explanation is that moeE5p might be a target of an as-yet-unknown repressor(s) positively regulated by BldA, in which case the deletion of bldA would remove the repressive signal. To further delineate the involvement of bldA in the translational regulation of moenomycin production, we analysed GusA activity of translational fusions of gusA to moeE5 (plasmid pmoeE5trans) and adpAgh in a ΔbldAgh background. We found no GusA activity in ΔbldAgh carrying moeE5–gusA fusion (figure 6), underscoring the essentiality of bldA for translation of the two UUA codons in moeE5 mRNA. Surprisingly, GusA activity was detected in the ΔbldAgh strain carrying adpAgh–gusA fusion, although it was much weaker (15-fold) than that in wild-type strain (figure 6). This observation can be attributed to mistranslation of adpAgh UUA codon in the absence of tRNA_{Lys, UAA} [37,38]. As the expression of AdpA in other cases has been shown to be strictly dependent on BldA [23,32,39], our data set a precedent for this important group of pleiotropic activators.

Next, we analysed adpAgh transcription. In comparison to the wild-type strain, adpAgh levels (figure 5) increased 2.3-fold in S. ghanensis ΔabsBgh and were almost undetectable in the ΔadpAgh strain (figure 5). We also measured the level of translation when adpAgh and the entire adpA gene were fused to gusA (padpAtransl; see §5) and found it in ΔabsBgh to be double that of wild-type (figure 6). Thus, AdpAgh acts as a positive activator of its own expression and its activity is increased in the absence of ribonucleolytic activity of AbsBgh. This conclusion is supported by observations in other streptomycetes [25,40]. Similar to our moeEp data, adpAgh activity was also significantly increased in the ΔbldAgh strain (figure 5), suggesting the existence of an unidentified bldA-dependent repressor(s) of AdpAgh-regulated promoters. There was no difference between absBgh transcriptional activity in ΔadpAgh and wild-type strains, indicating that AdpAgh does not influence the transcription of absBgh.

At the same time, we revealed almost complete cessation of bldAgh transcription in the ΔadpAgh strain (figure 5).

3.4. AdpAgh interacts with promoters of bldAgh, adpAgh and key moe genes

The GusA reporter data suggested that AdpAgh is a transcriptional activator that regulates its own expression as well as that of bldAgh and moe genes. To test this, we set out to demonstrate AdpAgh binding to moeO5, moeK5, moeE5, bldAgh, and adpAgh promoter regions using electrophoretic mobility shift assay (EMSA). A C-terminally His-tagged derivative of AdpAgh was overexpressed in Escherichia coli and purified to homogeneity (see the electronic supplementary material, figure S2). Increasing amounts of AdpAgh-His were incubated with radiolabelled DNA probes corresponding to the promoter regions of interest, and the complexes were separated by native gel electrophoresis. Purified AdpAgh-His was bound to the promoter regions of moeO5, moeK5, moeE5, bldA and adpAgh in quantities as low as 1.1–11.0 pmol. Increasing concentrations of AdpAgh resulted in more than one protein–DNA complex (padpAtransl; see §5) and found it in ΔabsBgh to be double that of wild-type (figure 6). This observation can be attributed to mistranslation of adpA UUA codon in the absence of tRNA_{Lys, UAA} [37,38]. As the expression of AdpA in other cases has been shown to be strictly dependent on BldA [23,32,39], our data set a precedent for this important group of pleiotropic activators.

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all AdpA operators will be occupied by the recombinant protein. Finally, we confirmed that AdpA gh binds to its own promoter (figure 7). Low concentrations of AdpA gh (4.4 pmol) caused the appearance of intermediate nucleoprotein complexes, whereas saturation of the reaction mixture with AdpAgh resulted in the formation of single band.

3.5. Absb, AdpA and BldA are important for moenomycin production by heterologous hosts

Previously, we demonstrated the successful expression of moe clusters in different streptomycetes [9,10]. To investigate whether the regulatory network we discovered in S. ghanaensis also operates in these heterologous hosts, we analysed the moenomycin production of the strains of S. coelicolor and Streptomyces lividans impaired in adpA, absB and bldA genes.

To determine the level of moenomycins biosynthesis on a DeltaabsB-gh background, a cosmid moeno38-5 [10] carrying the main part of moe cluster 1 and directing the production of nosokomycin B2 (NoB2) was introduced into S. coelicolor DeltaabsB strain J3410 [41]. S. coelicolor J3410 moeno38-5-gh was grown in parallel with a control strain S. coelicolor M145 moeno38-5-gh and NoB2 was quantified. On average, J3410 moeno38-5-gh accumulated 20% less biomass than M145 moeno38-5-gh and produced three times less NoB2 compared with the control strain (figure 8). These data correlate with the results of reporter experiments, where we observed a 1.5-fold decrease in moeE5 transcription in a DeltaabsB-gh strain compared with a control M145 strain (data not shown). Our results suggest that the AbsB RNase III-mediated

![Figure 5. Transcriptional activity of selected promoters in S. ghanaensis DeltaabsB, BldA and AdpA strains. WT, DeltaabsB, BldA and AdpA correspond to wild-type, absBgh, bldAgh and adpAgh null mutant strains, respectively, of S. ghanaensis expressing gusA from different promoters. The moeE5p, adpAp, absBp and bldAp correspond to promoters of moeE5, adpAgh, absBgh and bldAgh, respectively.](image)

![Figure 6. Translation of AdpAgh and MoeE5 is strongly affected on bldAgh-minus background. WT, DeltaabsB and DeltaBldA correspond to wild-type, absBgh and bldAgh null mutant strains, respectively, of S. ghanaensis expressing gusA fused to tested genes along with their promoters. AdpA and moeE5 correspond to genes adpAgh and moeE5, respectively. As a negative control, promoterless versions of the above genes were fused to gusA and introduced into respective strains; these constructs had marginal or no GusA activity.](image)
Figure 7. AdpA \textsubscript{gh} interacts with promoter of its own gene (a), bldA \textsubscript{gh} (b) and several moe genes (c). EMSA showing binding of purified AdpA \textsubscript{gh} to various promoter regions comprising in silico-predicted AdpA-binding sites. moeOS, moeK5p, moeESp, adpA\textsubscript{gh}p and bldA\textsubscript{gh}p correspond to promoter regions of moeOS, moeK5, moeES, adpA\textsubscript{gh} and bldA\textsubscript{gh}, genes, respectively. Bands corresponding to protein–DNA complexes (bound) and free DNA (free) are indicated. The final amount of AdpA\textsubscript{gh} (pmol) is indicated above each line.

Figure 8. Levels of nosokomycin B1 production by various streptomycetes expressing cosmids moeno38-5. Column labels: M145, J3410 and M851—wild-type, M851 and J1725 strains. NoB2 production was restored to M851 and J1725 upon introduction of adpA\textsubscript{gh} and bldA\textsubscript{gh} null mutant of S. lividans, respectively.

4. Discussion

The vast majority of natural product biosynthetic gene clusters contain one or more CSR genes. Expression of the latter is shown in many cases to be dependent on global pleiotropic regulators, for example AdpA [26,42]. Once produced, CSR proteins directly activate the transcription of structural biosynthetic genes [3,4,43]. However, a growing body of data suggest that cluster-situated layers of regulation are not an obligatory component of actinomycete secondary metabolic pathways. The elucidation of the genetic organization of the erythromycin biosynthetic cluster in the early 1990s provided the first evidence of an SM pathway lacking CSRs [11,12,44]. The list of ‘CSR-free’ gene clusters continues to grow; they direct the production of secondary metabolites, as chemically diverse as polyketides (erythromycin), both ribosomal and non-ribosomal peptides (thiostrepton, albonourins, pacidamycins) [45,46], nucleoside analogues, phosphoglycolipids [1,14,15,47,48] and acarbose-like natural products [49,50].

It is important to understand whether the expression of different ‘CSR-free’ gene clusters has a common mechanism(s) or principle of regulation. In this study, we show that expression of one such gene cluster, that for moenomycin production, is directly governed by two pleiotropic regulators, one of which is likely to be also under the influence of a third regulator. The described regulatory network is summarized in figure 9. Here, two pleiotropic regulators AdpA and BldA are involved in direct and multi-layered control over moenomycin production, whereas another protein, AbsB, limits AdpA abundance via ribonucleolytic activity. We would like to underscore the reciprocity of functional interactions enabling strict control over moenomycin production. The pleiotropic transcriptional regulator AdpA directly binds to the promoter regions of antibiotic biosynthetic genes as well as its own promoter. BldA contributes to the availability of developmentally regulated tRNA\textsubscript{uuu} \textsuperscript{UAU}, the absence of which limits the translation of both adpA and moe structural genes. Finally, absB-encoded RNaseIII influences antibiotic production by modulating AdpA abundance in addition to other, poorly understood way(s) evident from our heterologous expression experiments. This kind of regulatory network was initially elucidated in model streptomycetes, S. coelicolor and S. griseus [39], where it also governs antibiotic production. However, unlike these model cases, the influence of the studied regulators on moenomycin production does not appear to be mediated by CSRs.

According to available genomic data, absB, adpA and bldA orthologues are omnipresent in Streptomyces genomes, providing the necessary foundation for their evolution as a regulatory system that bypasses CSRs. Of the three regulators, BldA directly regulating CSR-free pathways has been extensively studied in other systems [21,47], while the involvement of AdpA was most substantially confirmed in the case of grisemycin biosynthesis [16,51]. The presence of AdpA operator sequences in the promoters of structural genes is another important indication of its role in the regulation of CSR-free pathways. A cursory in silico analysis indicates that the gene clusters for the biosynthesis of thiostrepton,
pacidamycin, albonoursin, acarbose and puromycin all contain putative AdpA operator sequences within certain intergenic regions. Some of these clusters include structural genes containing TTA codons as well (tunicamycin, albonoursin, erythromycin and puromycin clusters). Echoing the idea put forward by Higo et al. [24], we think that the low DNA-binding specificity of AdpA may be the key to the evolution of its control over CSR-free antibiotic biosynthetic gene clusters. In fact, AdpA was shown to form the largest bacterial regulon known to date with over 500 genes under its direct control. AdpA, like no other pleiotropic transcriptional factor of Streptomyces, would therefore be capable of putting laterally acquired antibiotic biosynthesis gene clusters under growth phase-dependent control. We note that another moenomycin biosynthetic gene cluster, located within the giant plasmid pSCL4 of S. clavuligerus ATCC27064 [52], may provide complementary evidence for the importance of AdpA control over the secondary metabolome. In fact, AdpA was shown to form the largest bacterial regulon known to date with over 500 genes under its direct control. AdpA, like no other pleiotropic transcriptional factor of Streptomyces, would therefore be capable of putting laterally acquired antibiotic biosynthesis gene clusters under growth phase-dependent control. We note that another moenomycin biosynthetic gene cluster, located within the giant plasmid pSCL4 of S. clavuligerus ATCC27064 [52], may provide complementary evidence for the importance of AdpA control over the secondary metabolome.

The severe morphological defects of S. ghananensis ΔbldAΔgh mutant could account for this observation, which, to our knowledge, would be the first case for the AdpA family of proteins. However in the same S. ghananensis ΔbldAΔgh strain, we failed to detect epimerase MoeE5, which contains two TTA codons, indicating that mis-translation of TTC codons does not occur 100% of the time in the bldAΔgh-minus background.

The increased transcription of adpAΔgh from constitutive promoter ermE improved moenomycin production 2.5-fold in spite of the fact that (as our work shows) it is the translation efficiency of UUA-containing adpAΔgh mRNA that should determine the degree of activation of moe genes. At the moment, we cannot fully explain our results although several possible scenarios can be outlined. First, once the charged tRNA^Leu^UAU is available, it might eventually lead to increased moenomycin production in the cells.
overexpressing adpA<sub>gh</sub> compared with the wild-type cells (note that we determine moenomycin production in the one time-point, which represents the total moenomycin produced over 72 h of growth). Second, if adpA<sub>gh</sub> mRNA is increased it might increase the probability of its mistranslation; this may also trigger moenomycin overproduction. Whatever the real mechanism is, it is practically useful because antibiotic titre improvement is a key requirement for the industry and it was one of the motivations for this work. In the case of CSR-free gene clusters, random mutagenesis and screening remain the only practical means to improve secondary metabolite production [59]. Recombinant DNA technology has yet to prove its utility for many industrial needs. Here, we demonstrate that the regulatory network bldA→adpA→absB is a cross-organism and large-effect system that can be harnessed to generate improved moenomycin producers. Upon combining absB<sub>gh</sub> deletion and adpA<sub>gh</sub> overexpression in <i>S. ghanaensis</i>, we observe, on average, a sevenfold increase in moenomycin production (data not shown). We anticipate that moenomycin titres can be further improved by bypassing bldA<sub>gh</sub> regulation, through the elimination of TTA codons from moe genes and adpA<sub>gh</sub>. Hence, genetic manipulations of the genes studied here could be a component of rational improvement of moenomycin producers. Recent studies [60,61] and several lines of evidence discussed above point to the fact that regulatory effects of adpA and bldA on SM are widespread and this could be exploited in other biosynthetic pathways. The amenability of SM to rational manipulations is also highlighted by a recent genome-wide study of the clavulanic acid overproducer, in which it was found that a small number of genetic changes, including AdpA overexpression, appeared to be associated with the desired phenotype [62].

5. Material and methods

5.1. Bacterial strains, plasmids and culture conditions

Strains and plasmids used in this study are described in the electronic supplementary material, table S2. <i>Escherichia coli</i> strains were grown in Luria-Bertani medium. <i>Streptomyces</i> strains were grown on SM and oatmeal agar media and in TSB and R2YE liquid media. Unless otherwise stated, <i>S. ghanaensis</i> was grown at 37°C and other streptomycetes at 30°C, with shaking at 200 r.p.m. All constructs were transferred into <i>Streptomyces</i> conjugally. The presence and stability of inheritance of qC31-based constructs in streptomycetes was checked as described previously [63,64].

5.2. Procedures for DNA manipulation

Oligonucleotides used in this work are listed in the electronic supplementary material, table S3. Standard procedures were used for plasmid/chromosomal DNA isolation, subcloning and analysis [65]. Polymerase chain reactions (PCRs) were performed using recombinant Pfu DNA polymerase (Fermentas) and all PCR products were sequenced. RedET-mediated gene replacements in cosmids and plasmids were carried out with the help of REDIRECT system [66]. All constructs were verified by sequencing, PCR or restriction mapping.

5.3. Quantitative analysis of moenomycins production

Growth of the strains, moenomycin extraction, conditions of LC-MS and quantitative analysis of the data are described by Ostash et al. [2] and Makitrynskyy et al. [10]. The levels of moenomycin production were calculated from at least three independent experiments and referred back to equal amounts of dry biomass (10 mg) in different strains. The cells were exhaustively extracted three times; the fourth extraction did not contain any measurable amounts of moenomycins confirming that all moenomycin had already been recovered (data not shown). The following compounds were monitored via LC/MS in <i>S. ghanaensis</i> extracts: MmA ([M-H]⁻ = 1580.6 Da) and nosokomycin B (NoB; [M-H]⁻ = 1484.6 Da). The mixture of these two equidominant compounds [64] is referred to as moenomycin in this work. Cosmid moeno38-5 directs the biosynthesis of nosokomycin B1 (NoB1; [M-H]⁻ = 1500.6 Da) and its production was followed in the extracts of heterologous hosts (<i>S. lividans</i> and <i>S. coelicolor</i>). LC/MS data were acquired on Agilent 1110 LC/MSD and Bruker Esquire 3000 ESI-MS spectrometers.

5.4. Identification of AdpA<sub>gh</sub>-binding sites

To identify conserved AdpA-binding sites (AdpAbs) in <i>S. ghanaensis</i>, known AdpA sequences were collected from GenBank. This dataset was used as input for the MEME software tool [67] to search for the consensus motif. Search for the occurrence of the identified motif within <i>moe</i> clusters, bldA<sub>gh</sub> and adpA<sub>gh</sub> promoter regions was performed using FIMO software suite [68].

5.5. Semiquantitative RT-PCR

Mycelia of <i>S. ghanaensis</i> were harvested in moenomycin production phase (72 h) and processed as described previously [10].

5.6. Construction of the <i>Streptomyces ghanaensis</i> ΔabsB<sub>gh</sub> and plasmids for complementation experiments

A construct for absB<sub>gh</sub> knockout was prepared as follows. A 2.5 kb DNA fragment containing absB<sub>gh</sub> and its flanking regions were amplified from <i>S. ghanaensis</i> genomic DNA by PCR using primers absBgh_kn_for and absBgh_kn_rev. The PCR product was ligated to Smal-digested pBluescriptKS+ to yield pBlabsBgh-kn. The loxP site-flanked apramycin resistance cassette (aac(3)IV) from plasmid pLERE<sub>C</sub> was amplified with primers red_absBgh_kn_for and red_absBgh_kn_rev. The resulting amplicon was used to replace the coding sequence of absB<sub>gh</sub> in pBlabsBgh-kn via recombinering, giving pBlabsBgh-kn:aac(3)IV. The latter was digested with BamH1 and EcoRI and the fragment containing the absB<sub>gh</sub>:aac(3)IV mutant allele was cloned into the same sites of plasmid pKC11398 to yield pKCabsB-kn::aac(3)IV. <i>Streptomyces ghanaensis</i> transconjugants carrying the latter were selected for resistance to apramycin (25 μg ml⁻¹). To generate <i>S. ghanaensis</i> single-crossover Am<sup>R</sup> mutants, initial transconjugants were incubated at 40°C for 5 days, and then screened for apramycin resistance and kanamycin sensitivity (an indicative of vector loss and double crossover). Replacement of absB<sub>gh</sub> with aac(3)IV in <i>S. ghanaensis</i> ΔabsB<sub>gh</sub>:aac(3)IV was confirmed by PCR (primers absBgh_ex_for and absBgh_ex_rev; data not shown). The
Cre-expressing helper plasmid pUWLCre was then introduced into S. ghanaensis ΔabsBgh::ermEp to evict Δ(3)IV from its genome. The pUWLCreΔ transconjugants resistant to tetracycline were incubated on oatmeal agar plates and selected for apramycin sensitivity. The helper plasmid was lost after two subsequent passages of selected AmR clone in the absence of thiostrepton. Excision of Δ(3)IV from the S. ghanaensis ΔabsBgh genome was confirmed by PCR (primers absBgh_ex_for and absBgh_ex_rev; data not shown).

A set of plasmids containing absBgh gene along with its upstream region of different lengths (figure 1) was constructed for complementation analysis. To create a plasmid pSOC-KabsBgh-exp, a 1.1 kb fragment carrying entire absBgh with its 150 bp 5′-region was amplified from S. ghanaensis genomic DNA using primers absBgh_ex_for and absBgh_ex_rev. The obtained amplicon was cloned into integrative VWB-based vector pSOK804 digested with EcoRV to give pSOCabsBgh-exp.

To construct plasmid pSOKEabsBgh-exp, where transcription of absBgh is under ermEp control, the above 1.1 kb PCR fragment was first cloned into EcoRV-treated pKC1218YE, yielding pKCEabsBgh-exp. Then pKCEabsBgh-exp was digested with HindIII and EcoRI and 1.4 kb DNA fragment harbouring absBgh plus ermEp was ligated to pSOK804, digested with respective endonucleases, to generate pSOKEabsBgh-exp.

To create a plasmid pSOKabsBgh-II encompassing two genes, SSFG_02130.1 and SSFG_02129.1 (absBgh), along with the 200 bp upstream region, a 1.4 kb DNA fragment was amplified using primers absB-gh-II-for and absB-gh-II-rev. The resulting amplicon was cloned into EcoRV-treated pSOK804 to give pSOCabsBgh-II.

Plasmid pSOCabsBgh-III is based on pSOK804 and carries a 2.2 kb DNA fragment containing three genes, SSFG_02131.1, SSFG_02130.1 and SSFG_02129.1 (absBgh), along with the 250 bp upstream region. It was constructed by cloning an amplicon generated with primers absB-gh-III-for and absB-gh-III-rev into EcoRV site of pSOK804.

5.7. Construction of the Streptomyces ghanaensis ΔadpAgh and plasmid for complementation experiment

A 3.5 kb DNA fragment containing adpAgh and its flanking regions was amplified from the chromosome of S. ghanaensis using primers adpA_for and adpA_rev. The resulting amplicon was ligated to EcoRV-digested pBluescriptKS+ to yield pBladpAkn. To replace adpAgh the nac(3)IV cassette from pLEREC] was amplified using primers adpA_red_for and adpA_red_rev, and the resulting amplicon was used for recombineering-mediated replacement of adpAgh within pBladpAkn to give pBladpA-kn::nac(3)IV. The latter was further used as a template in PCR for amplification (primers adpA_kn_for and adpA_kn_rev) of a 3.4 kb DNA fragment harbouring ΔadpAgh::nac(3)IV. The obtained amplicon was cloned into EcoRV-digested vector pKC0702. The final adpAgh knockout plasmid was labelled pKCHadpA-kn::nac(3)IV. Generation of ΔadpAgh mutant was carried out as described above. Mutant phenotype of S. ghanaensis ΔadpAgh::nac(3)IV was confirmed by PCR using primers adpA_exp_for and adpA_exp_rev. Generation and verification of nac(3)IV-evicted strain ΔadpAgh was carried out as described for ΔabsBgh strain (primers adpA_for and adpA_rev; data not shown).

For the complementation of S. ghanaensis ΔadpAgh, a 1.9 kb fragment carrying adpAgh with its promoter region was amplified with primers adpA_for and adpA_rev compl. The resulting amplicon was digested with XbaI and EcoRV and cloned into respective sites of pSET152, to give pSETadpA-exp.

For adpAgh expression under ermEp control, a 1.4 kb fragment containing only the coding sequence of adpAgh was amplified with primers adpA_exp_for and adpA_exp_rev. The amplicon was digested with EcoRV and EcoRI and ligated to EcoRV–EcoRI-linearized pTES to generate pTESadpA-exp.

5.8. Construction of the ΔbldAgh strain and plasmid for complementation experiment

The 2.0 kb S. ghanaensis genomic regions flanking bldAgh were amplified with primers bldA-left-up plus bldA-left-rp (‘left’ homology arm) and bldA-right-up plus bldA-right-rp (‘right’ arm). ‘Left’ and ‘right’ amplicons were digested with HindIII + XbaI and XbaI + EcoRI, respectively, and cloned into HindIII–EcoRI-digested pKC1139. The resulting bldAgh knockout plasmid pKC1139bldA-del contains markerless deletion of the 87 bp bldAgh coding sequence. Manipulations of pKC1139bldA-del transconjugants to generate the bldAgh knockout strain were essentially the same as described above, except that double crossover clones were screened among those displaying impaired sporulation, as no antibiotic selection was possible. Diagnostic PCR with primers bldAXbaIup and bldA-diagn-rp and sequencing confirmed the deletion of the 87 bp bldAgh sequence from the genome of ΔbldAgh. For complementation and expression experiments, the bldAgh coding region along with the 320 bp upstream segment was amplified with primers bldAXbaIup and bldAECori and cloned into respective sites of pSET152 to yield pSET152bldA.

5.9. Construction of GusA reporter plasmids and β-glucuronidase activity measurements

To probe the activities of moeE5, absBgh, adpAgh and adpAgh promoters, DNA fragments containing putative promoter regions (500 bp upstream of the translation start codons) were amplified by PCR using upstream primers carrying an XbaI site and downstream primers carrying a KpnI site (primers moeE5_for and moeE5_script_rev for moeE5p; absB_for and absB_script_rev for absBgph; adpA_for and adpA_script_rev for adpAghp; bldA_for and bldA_script_rev for bldAghp) respectively. The moeE5p, absBgph, adpAghp and bldAghp fragments were cloned into XbaI–KpnI-digested pGUS, to give plasmids pmoeE5script, pabsBscript, padpAscript and pbldAscript, respectively.

To investigate the expression of moeE5 and adpAgh on the translational level, DNA fragments containing the entire stop codon-free genes with putative promoter (500 bp upstream of the translation start codons) were amplified by PCR using upstream primers carrying an XbaI site and downstream primers carrying a KpnI site (primers moeE5_for and moeE5_script_rev for moeE5p; absB_for and absB_script_rev for absBgph; adpA_for and adpA_script_rev for adpAghp; bldA_for and bldA_script_rev for bldAghp) respectively.
carrying XbaI site and downstream primers carrying EcoRV site (primers moeE5_for_contr and moeE5_rev for moeE5; adpA_for_contr and adpA_rev for adpAgh) and cloned in XbaI–EcoRV-treated pGUSHL4aadA, giving proE5Contr and padpAcontr, respectively.

The spore suspensions (2 × 10⁵ cfu) of streptomycetes reporter plasmid-bearing strains were inoculated in 300 ml flasks with 100 ml of TSB, and grown for 30 h. One millilitre of the preculture was inoculated into fresh TSBB medium (100 ml) and grown for 24–28 h (depending on experiment). Mycelium was harvested, washed twice with distilled water, then resuspended in lysis buffer (50 mM phosphate buffer (pH 7.0), 0.1% Triton X-100, 5 mM DTT, 4 mg ml⁻¹ lysozyme) and incubated for 30 min at 37°C. Lysates were centrifuged for 10 min at 5000 r.p.m. 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-β-D-glucuronide and used for measuring optical density at λ = 415 nm every minute during 20 min of incubation at 37°C. As a reference, a 1 : 1 mixture of lysate and dilution buffer was used.

5.10. Expression and purification of His-tagged AdpAgh

For the production of C-terminal hexahistidine-tagged AdpAgh, the coding region of gene adpAgh was amplified with primers AdpA_pr_for and AdpA_pr_rev from S. gha

5.11. Gel electrophoretic mobility shift assay

The 500 bp promoter regions of the targeted genes (adpAgh, bldAgh, moeE5, moeK5, moeO5) were used in EMSAs. These probes were amplified from chromosomal DNA of S. gha

Reference


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