Pleiotropic regulatory genes bldA, adpA and absB are implicated in production of phosphoglycolipid antibiotic moenomycin

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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 ghanaensis} 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\_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 the UUA-containing \textit{adpA} and probably, other as-yet-unknown repressor gene(s). AdpA binds key \textit{moe} promoters and activates them. Furthermore, 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 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.
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 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\textsubscript{UAA} \textsuperscript{mol} (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 ipomicin 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 supra). 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\textsubscript{gh}, 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\textsubscript{gh} mRNA is dependent on bldA-encoded tRNA, although this dependence is not absolute. Finally, we provide circumstantial evidence that AdpA\textsubscript{gh} expression is regulated at the posttranscriptional level through the action of the absB\textsubscript{gh} 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

genome 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\textsubscript{UAA} \textsuperscript{mol} (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\textsubscript{gh} belongs to the transcriptional unit which comprises three genes: SSFG_02131.1, SSFG_02130.1 and SSFG_02129.1 (absB\textsubscript{gh}) (figure 1).
In our *in silico* analysis [10] of *S. ghanaensis*, we identified an AdpA orthologue in *S. ghanaensis* and designated it as *adpA*$_{gh}$. The coding sequence of *adpA*$_{gh}$ contains one TTA codon (figure 1), at the same position as other *adpA*$_{gh}$ orthologues [23,30–32]. Genes for several *AdpA*$_{gh}$ paralogues are present in the *S. ghanaensis* genome (see the electronic supplementary material, table S1). Additionally, a single copy of the tRNA$_{Leu,UAA}$ gene was identified in the *S. ghanaensis* genome (designated as *bldA*$_{gh}$; figure 1).

We mined the promoter regions of *adpA*$_{gh}$, *bldA*$_{gh}$, *absB*$_{gh}$ and *moe* clusters for the presence of AdpA operator sequences [33]. As expected, such sequences were revealed within *adpA*$_{adp}$ and *bldA*$_{adp}$ (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*$_{gh}$ 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*$_{gh}$ mutant (Δ*adpA*$_{gh}$), showing that moenomycin production was blocked at the initial first steps. Knockout of *adpA*$_{gh}$ had a significant influence on the morphological development *S. ghanaensis*. On solid media, a phenotypic expression of *S. ghanaensis* Δ*adpA*$_{gh}$ resembled that of the ‘bald’ (mutant) strains described for streptomycetes (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 Δ*adpA*$_{gh}$ were restored to the wild-type state upon introduction of an intact copy of *adpA*$_{gh}$ (plasmid pSETadpA-exp). Insertion of an extra copy of *adpA*$_{gh}$, under the control of a strong constitutive promoter *ermEp* (plasmid pTESadpA-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 into ΔbldAgh (plasmid pSETbldA) restored the moenomycin production and normal timing of morphogenesis, implying that the ΔbldAgh phenotype is solely due to the absence of tRNALeuUAA. 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 tRNALeuUAA.

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 (pSOKEabsBgh-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 (pSOKEabsBgh-exp) into the wild-type strain resulted in significantly decreased antibiotic biosynthesis (figure 2).
translation of the two UUA codons in moeE5 mRNA. Surprisingly, GusA activity was detected in the ΔbldAΔgh strain carrying adpAΔgh–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 adpAΔgh UAA codon in the absence of tRNA^{UAU}{_{ULC}} [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 adpAΔgh transcription. In comparison to the wild-type strain, adpAΔgh levels (figure 5) increased 2.3-fold in S. ghanaensis ΔabsBΔgh and were almost undetectable in the ΔadpAΔgh strain (figure 5). We also measured the level of translation when adpAΔgh and the entire adpAΔgh genes were fused to gusA (padpAΔtrans; see §5) and found it in ΔabsBΔgh to be double that of wild-type (figure 6). Thus, AdpAΔgh acts as a positive activator of its own expression and its activity is increased in the absence of ribonucleolytic activity of AbsBΔgh. This conclusion is supported by observations in other streptomycetes [25,40]. Similar to our moeEp data, adpAΔgh activity was also significantly increased in the ΔbldAΔgh strain (figure 5), suggesting the existence of an unidentified bldA-dependent repressor(s) of AdpAΔgh-regulated promoters.

There was no difference between absBΔgp transcriptional activity in ΔadpAΔgh and wild-type strains, indicating that AdpAΔgh does not influence the transcription of absBΔgh.

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

### 3.4. AdpAΔgh interacts with promoters of bldAΔgh, adpAΔgh and key moe genes

The GusA reporter data suggested that AdpAΔgh is a transcriptional activator that regulates its own expression as well as that of bldAΔgh and moe genes. To test this, we set out to demonstrate AdpAΔgh binding to moeO5, moeK5, moeE5, bldAΔgh, and adpAΔgh promoter regions using electrophoretic mobility shift assay (EMSA). A C-terminally His-tagged derivative of AdpAΔgh was overexpressed in *Escherichia coli* and purified to homogeneity (see the electronic supplementary material, figure S2). Increasing amounts of AdpAΔgh-His were incubated with radiolabelled DNA probes corresponding to the promoter regions of interest, and the complexes were separated by native gel electrophoresis. Purified AdpAΔgh-His was bound to the promoter regions of moeO5, moeK5, moeE5, bldAΔgh, and adpAΔgh in quantities as low as 1.1–11.0 pmol. Increasing concentrations of AdpAΔgh resulted in more than one protein–DNA complex for moeO5p and moeK5p (figure 7), in agreement with multiple AdpAΔgh-binding sites predicted for these promoters in silico. While bioinformatics analysis predicted three putative AdpAΔgh-binding sites within moeEp, only one shifted band was presented in the case of moeEp. Unlabelled adpAΔgh competed with the radiolabelled one for AdpAΔgh (see the electronic supplementary material, figure S3), while a non-specific DNA fragment (the SCO3812 promoter region) was not recognized by AdpAΔgh (see the electronic supplementary material, figure S4).

AdpAΔgh formed three different bldAΔghp–AdpAΔgh complexes suggesting the presence of multiple AdpAΔgh-binding motifs within bldAΔgh. Increasing concentrations of AdpAΔgh caused a transition from multiple bands to a single retarded band, suggesting that above a certain AdpAΔgh concentration,
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 ΔabsB-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 ΔabsB strain J3410 [41]. S. coelicolor J3410 moeno38-5- ΔabsB was grown in parallel with a control strain S. coelicolor M145 moeno38-5 and NoB2 was quantified. On average, J3410 moeno38-5 ΔabsB accumulated 20% less biomass than M145 moeno38-5- 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 ΔabsBgh strain compared with a control M145 strain (data not shown). Our results suggest that the AbsB RNase III-mediated
respectively; 1326 and J1725—wild-type and \( \text{bldA} \) respectively. In silico regions comprising \( \text{moeE}5 \), \( \text{adpA} \), and \( \text{rnc} \) wild-type, \( \text{S. coelicolor} \) M851 and revealed in respectively (data not shown). To M851 and J1725 upon introduction of biosynthetic cluster in the early 1990s provided the first elucidation of the genetic organization of the erythromycin component of actinomycete secondary metabolic pathways. That cluster-situated layers of regulation are not an obligatory synthetic genes [3,4,43]. However, a growing body of data suggest that CSR-free pathways 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, 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, albonoursin, 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 tRNAs, the absence of which limits the translation of both \( \text{adpA} \) and \( \text{moe} \) structural genes. Finally, \( \text{absB} \)-encoded RNase III 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, \( \text{S. coelicolor} \) and \( \text{S. griseus} \) [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, \( \text{absB} \), \( \text{adpA} \) and \( \text{bldA} \) orthologues are omnipresent in \( \text{Streptomyces} \) 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,

### 4. Discussion

The vast majority of natural product biosynthetic gene clusters do 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 regulatory pathway is important for moenomycin production even in other streptomycete heterologous hosts.

Next, we tested NoB2 production in \( \text{adpA} \)-deficient \( \text{S. coelicolor} \) M851 and \( \text{bldA} \)-deficient \( \text{S. lividans} \) J1725 strains. Mutant and parental strains carrying cosmids \( \text{moe}38-5 \) did not differ in growth rate, but NoB2 production was completely abolished (figure 8). No \( \text{moeESP} \) activity was revealed in \( \text{S. coelicolor} \) M851. NoB2 production was restored to M851 and J1725 upon introduction of \( \text{adpA}_{\text{gh}} \) and \( \text{bldA}_{\text{gh}} \) null mutant of \( \text{S. lividans} \), respectively.

![Figure 7. AdpA<sub>gh</sub> interacts with promoter of its own gene (a), bldA<sub>gh</sub> (b) and several moe genes (c). EMSA showing binding of purified AdpA<sub>gh</sub> to various promoter regions comprising in silico-predicted AdpA-binding sites. moeO5p, moeK5p, moeESP, adpA<sub>gh</sub>p and bldA<sub>gh</sub>p correspond to promoter regions of moeO5, moeK5, moeESP, adpA<sub>gh</sub> and bldA<sub>gh</sub> genes, respectively. Bands corresponding to protein–DNA complexes (bound) and free DNA (free) are indicated. The final amount of AdpA<sub>gh</sub> (pmol) is indicated above each line.](image)

![Figure 8. Levels of nosokomycin B<sub>1</sub> production by various streptomycetes expressing cosmids moen38-5. Column labels: M145, J3410 and M851—wild-type, \( \text{mc} \) (\( \text{absB} \))-minus and \( \text{bldH} \) (\( \text{adpA} \))-minus mutants of \( \text{S. coelicolor} \), respectively; 1326 and J1725—wild-type and \( \text{bldA} \) null mutant of \( \text{S. lividans} \), respectively.](image)

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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 pSCLA of Streptomyces clavuligerus ATCC27064 [32], may provide complementary evidence for the importance of AdpA control over the secondary metabolome. Despite numerous attempts, we failed to detect the production of moenomycins by S. clavuligerus, S. griseus [23,30,32,39] this combination of TTA codons does not occur 100% of the time in MoeE5, which contains two TTA codons, indicating that mis-translation of UUA codons in the absence of cognate tRNA is likely to be more conditional than that of transcriptonal factors, which might be manifested in the form of leaky translation of UUA codons in the absence of cognate tRNA. The leaky translation of AdpA in a ΔbldAgh background provides some clues about the early stages of moenomycin biosynthesis as well as morphological differentiation in S. ghanaensis, when there would be little or no mature tRNA^Leu^available during early stages of growth in the absence of BldAgh, could be sufficient to activate transcription from bldA^gh^leading to an avalanche-like increase in bldA^gh^expression. Once available, charged tRNA^Leu^can then amplify the translation of adpA^gh^and other, as-yet-unknown, UUA^+^genes that lead to the downregulation of adpA^gh^and moeE5 promoters. Our data confirm the presence of a regulatory feedback loop that amplifies a signal in dual regulation of BldA–AdpA in S. ghanaensis, as was previously shown in S. griseus [39]. The increased transcription of adpA^gh^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 adpA^gh^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^is available, it might eventually lead to increased moenomycin production in the cells.

**Figure 9.** A model of the regulatory pathway that governs moenomycin biosynthesis in S. ghanaensis.
overexpressing \( \text{adpA}_{gh} \) 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 \( \text{adpA}_{gh} \) 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 \( \text{bldA}--\text{adpA}--\text{absB} \) is a cross-organism and large-effect system that can be harnessed to generate improved moenomycin producers. Upon combining \( \text{absB}_{gh} \) deletion and \( \text{adpA}_{gh} \) overexpression in \( S. \text{ghanaensis} \), we observe, on average, a sevenfold increase in moenomycin production (data not shown). We anticipate that moenomycin titres can be further improved by bypassing \( \text{bldA}_{gh} \) regulation, through the elimination of TTA codons from \( \text{moe} \) genes and \( \text{adpA}_{gh} \). 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 \( \text{adpA} \) and \( \text{bldA} \) on SM are widespread and this can 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. \( E. \text{coli} \) strains were grown in Luria-Bertani medium. \( S. \text{streptomyces} \) strains were grown on SM and oatmeal agar media and in TSB and R2YE liquid media. Unless otherwise stated, \( S. \text{ghanaensis} \) was grown at 37°C and other streptomycetes at 30°C, with shaking at 200 r.p.m. All constructs were transferred into \( S. \text{streptomyces} \) conjugally. The presence and stability of inheritance of \( qC31 \)-based constructs in streptomycetes was checked as described earlier [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 Makiritsynsky 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 \( S. \text{ghanaensis} \) extracts: \( \text{MmA} ([M-H]^- = 1580.6 \text{Da}) \) and nosokomycin B (\( \text{NoB}; [M-H]^- = 1484.6 \text{Da} \)). The mixture of these two equidominant compounds [64] is referred to as moenomycin in this work. Cosmid moeno38-5 directs the biosynthesis of nosokomycin B\(_2\) (\( \text{NoB}_2; [M-H]^- = 1500.6 \text{Da} \)) and its production was followed in the extracts of heterologous hosts (\( S. \text{lividans} \) and \( S. \text{coelicolor} \)). LC/MS data were acquired on Agilent 1110 LC/MS and Bruker Esquire 3000 ESI-MS spectrometers. Mycelia of \( S. \text{ghanaensis} \) were harvested in moenomycin production phase (72 h) and processed as described previously [10].

5.4. Identification of AdpA\(_{gh}\)-binding sites

To identify conserved AdpA-binding sites (AdpAbs) in \( S. \text{ghanaensis} \), known AdpAbs 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 \( \text{moe} \) clusters, \( \text{bldA}_{gh} \) and \( \text{adpA}_{gh} \) promoter regions was performed using FIMO software suite [68].

5.5. Semiquantitative RT-PCR

A construct for \( \text{absB}_{gh} \) knockout was prepared as follows. A 2.5 kb DNA fragment containing \( \text{absB}_{gh} \) and its flanking regions were amplified from \( S. \text{ghanaensis} \) genomic DNA by PCR using primers \( \text{absB}_{gh}\text{for} \) and \( \text{absB}_{gh}\text{rev} \). The PCR product was ligated to pBluescriptKS+ to yield pBlabsBgh-kn. The loxP site-flanked apramycin resistance cassette (\( \text{aac}(3)I\text{V} \)) from plasmid pLERIC was amplified with primers red\_absBgh\_kn\_for and red\_absBgh\_kn\_rev. The resulting amplicon was used to replace the coding sequence of \( \text{absB}_{gh} \) in pBlabsBgh-kn via recombineering, giving pBlabsBgh-kn::\( \text{aac}(3)\text{I}\text{V} \). The latter was digested with BamHI and EcoRI and the fragment containing the \( \text{absB}_{gh}\text{::\( \text{aac}(3)\text{I}\text{V} \)) was cloned into the same sites of pKC1139Km to yield pKCabsB-kn::\( \text{aac}(3)\text{I}\text{V} \). Streptomyces ghanaensis transconjugants carrying the latter were selected for resistance to apramycin (25 μg ml\(^{-1}\)). To generate \( S. \text{ghanaensis} \) single-crossover \( \text{AmKm}^+ \) 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 \( \text{absB}_{gh} \) with \( \text{aac}(3)\text{I}\text{V} \) in \( S. \text{ghanaensis} \) \( \Delta\text{absB}_{gh}\text{::\( \text{aac}(3)\text{I}\text{V} \)) was confirmed by PCR (primers \( \text{absB}_{gh}\text{ex}\text{for} \) and \( \text{absB}_{gh}\text{ex}\text{rev} \); data not shown). The
Cre-expressing helper plasmid pUWLCre was then introduced into *S. ghanaensis* ΔabsBgh::aac(3)IV to evict aac(3)IV from its genome. The pUWLCre**Δ** 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 Am*<sup>+</sup>* clone in the absence of thiostrepton. Excision of aac(3)IV from the *S. ghanaensis* ΔabsBgh genome was confirmed by PCR (primers absBgh_**for** and absBgh_**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 pSO-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_**for** and absBgh_**rev**. The obtained amplicon was cloned into integrative VWB-based vector pSOK804 digested with EcoRV to give pSOKabsBgh-exp.

To construct plasmid pSOKEabsBgh-exp, where transcription of absBgh is under ermE<sup>r</sup> control, the above 1.1 kb PCR fragment was first cloned into EcoRV-treated pKC1218YE, yielding pKEabsBgh-exp. Then pKEabsBgh-exp was digested with HindIII and EcoRI and 1.4 kb DNA fragment harbouring absBgh plus ermE<sup>r</sup> 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 pSOKabsBgh-II.

Plasmid pSOKabsBgh-II 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* Δ*adpA*<sub>gh</sub> and plasmid for complementation experiment

A 3.5 kb DNA fragment containing *adpA*<sub>gh</sub> and its flanking regions was amplified from the chromosome of *S. ghanaensis* using primers adpA_**kn_for** and adpA_**kn_rev**. The resulting amplicon was ligated to EcoRV-digested pBlueScriptKS+ to yield pBladpAkn. To replace *adpA*<sub>gh</sub> the *aac(3)IV* cassette from pLERECJ was amplified using primers adpA_**red_for** and adpA_**red_rev**, and the resulting amplicon was used for recombineering-mediated replacement of *adpA*<sub>gh</sub> within pBladpAkn to give pBladpA-kn::*aac(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 Δ*adpA*<sub>gh</sub>::*aac(3)IV*). The obtained amplicon was cloned into EcoRV-digested vector pKC0702. The final Δ*adpA*<sub>gh</sub> knockout plasmid was labelled pKC1hadpA-kn::*aac(3)IV*. Generation of Δ*adpA*<sub>gh</sub> mutant was carried out as described above. Mutant phenotype of *S. ghanaensis* Δ*adpA*<sub>gh</sub>::*aac(3)IV* was confirmed by PCR using primers adpA_**exp_for** and adpA_**exp_rev**. Generation and verification of *aac(3)IV*-evicted strain Δ*adpA*<sub>gh</sub> was carried out as described for ΔabsBgh strain (primers adpA_**for** and adpA_**rev**; data not shown).

For the complementation of *S. ghanaensis* Δ*adpA*<sub>gh</sub>, a 1.9 kb fragment carrying *adpA*<sub>gh</sub> with its promoter region was amplified with primers adpA_**for** and adpA_**rev**. The resulting amplicon was digested with Xbal and EcoRV and cloned into respective sites of pSET152, to give pSETadpA-exp.

For *adpA*<sub>gh</sub> expression under ermE<sup>r</sup> control, a 1.4 kb fragment containing only the coding sequence of *adpA*<sub>gh</sub> 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 Δ*bldA*<sub>gh</sub> strain and plasmid for complementation experiment

The 2.0 kb *S. ghanaensis* genomic regions flanking *bldA*<sub>gh</sub> 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 Xbal + EcoRI, respectively, and cloned into HindIII–EcoRI-digested pKC1139. The resulting *bldA*<sub>gh</sub> knockout plasmid pKC1139blda-del contains markerless deletion of the 87 bp *blda*<sub>gh</sub> coding sequence. Manipulations of pKC1139blda-del+ transconjugants to generate the *blda*<sub>gh</sub> 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 bldaXbaulp and blda-Diagn-rp and sequencing confirmed the deletion of the 87 bp *blda*<sub>gh</sub> sequence from the genome of Δ*blda*<sub>gh</sub>. For complementation and expression experiments, the *blda*<sub>gh</sub> coding region along with the 320 bp upstream segment was amplified with primers bldaXbaulp and bldaEcoRlup and cloned into respective sites of pSSET152 to yield pSSET152blda.

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

To probe the activities of *moeE5*, *absB*<sub>gh</sub>, *adpA*<sub>gh</sub> and *blda*<sub>gh</sub> 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 *moeE5*; absB_for and absB_script_rev for *absB*<sub>gh</sub>; adpA_for and adpA_script_rev for *adpA*<sub>gh</sub>; blda_for and blda_script_rev for *blda*<sub>gh</sub>). The *moeE5*, *absB*<sub>gh</sub>, *adpA*<sub>gh</sub> and *blda*<sub>gh</sub> fragments were cloned into XbaI–KpnI-digested pGUS, to give plasmids pmoeE5script, pabsBscript, padpAscript and pbldAscript, respectively.

To investigate the expression of *moeE5* and *adpA*<sub>gh</sub> 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 XbaI site and downstream primers carrying an EcoRV site (primers moeE5_for and moeE5_rev for *moeE5*; adpA_for and adpA_rev for *adpA*<sub>gh</sub>), and amplified into plasmids pmoeE5script and padpAscript, respectively.
carrying XbaI site and downstream primers carrying EcoRV site (primers moe5_for_contr and moe5_rev for moe5; adpA_for_contr and adpA_rev for adpAgh) and cloned in XbaI–EcoRV-treated pGUSHL4aadA, giving pmoeE5contr and padpAcontr, respectively.

The spore suspensions (2 × 10⁸ 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 TSB 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. ghanaensis chromosomal DNA. PCR product was purified and cloned into NcoI–XhoI cloning sites of expression vector pET24b, giving pETAdpAgh.

Escherichia coli BL21-GOLD cells harbouring the pETAdpAgh were grown in YT medium containing 50 μg ml⁻¹ ampicillin and kanamycin until The OD₆₀₀ reached 0.8–1.0. Expression of AdpAgh was induced with 0.4 mM IPTG at 25°C for 15 min in 15 μl binding buffer (20 mM Tris–HCl (pH 8.0), 1 mM EDTA, 1 mM DTT, 100 mM KCl, 10 mM MgCl₂, 10% glycerol) containing 1 μg of poly(dI–dC). The reactions products (protein-bound and free DNA) were separated on 4% non-denaturing polyacrylamide gel in TBE-buffer. The gels were visualized by phosphorimaging.

5.12. Western blotting MoeE5

Plasmid and conditions for expression and purification of N-terminal thioredoxin/His6-tagged MoeE5 protein in E. coli were previously described [2]. Purified recombinant MoeE5 protein was used as antigen to raise antibodies in a rabbit (as performed by Jackson ImmunoResearch laboratories (West Grove, PA, USA)). The same batch of S. ghanaensis mycelia was used for RT-PCR and Western blot analysis. Briefly, biomass samples were taken from ~80°C, thawed on ice and resuspended in small volume of PBS. The mixture was French-pressed three times, centrifuged and supernatant taken for further analysis. Twenty microgram protein samples were separated in 7.5% SDS-polyacrylamide gels and upon blotting were probed with a 1 : 1000 dilution of the primary antiserum.

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References


5.11. Gel electrophoretic mobility shift assay

The 500 bp promoter regions of the targeted genes (adpAgh, bldAgh, moeE5, moeK5, moeO5) were used in EMSA. These probes were amplified from chromosomal DNA of S. ghanaensis by PCR using primers adpA_for and adpA_script_rev for adpAgh, bldA_for and bldA_script_rev for bldAgh, moeE5_for and moeE5_script_rev for moeE5; moeK5_for and moeK5_script_rev for moeK5; moeO5_for and moeO5_script_rev for moeO5 (see the electronic supplementary material, table S3). A total of 10 pmol of each probe was 5'-end labelled with 20 pmol [y-32P] using T4 polynucleotide kinase according to established protocols (Fermentas). Unincorporated labelled dATP was removed using ProbeQuant G-50 Micro columns (GE Healthcare). A total of 20 fmol of labelled probe was incubated with purified 1.1, 4.4, 11, 22, 44, 88 pM His-tagged AdpAgh at 25°C for 15 min in 15 μl binding buffer (20 mM Tris–HCl (pH 8.0), 1 mM EDTA, 1 mM DTT, 100 mM KCl, 10 mM MgCl₂, 10% glycerol) containing 1 μg of poly(dI–dC). The reactions products (protein-bound and free DNA) were separated on 4% non-denaturing polyacrylamide gel in TBE-buffer. The gels were visualized by phosphorimaging.


