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\textsuperscript{UAA} is required for the translation of rare UUA codons within two key moenomycin biosynthetic genes (\textit{moe}), \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.

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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 CSRs 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, moeO5 and moeE5. 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^{UAA}_\text{Loo}_{\text{A}2}\text{UAA} (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 postranscriptional 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\textsubscript{gh}–adpA\textsubscript{gh}–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

In 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^{UAA}_\text{Loo}_{\text{A}2}\text{UAA} (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\textsubscript{02131.1}, SSFG\textsubscript{02130.1} and SSFG\textsubscript{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\textsubscript{gh}. The coding sequence of adpA\textsubscript{gh} contains one TTA codon (figure 1), at the same position as other AdpA\textsubscript{gh} paralogues present in the S. ghanaensis genome (see the electronic supplementary material, table S1). Additionally, a single copy of the tRNA\textsubscript{Leu}\textsuperscript{UAA} gene was identified in the S. ghanaensis genome (see the electronic supplementary material). We mined the promoter regions of adpA\textsubscript{gh}, bldA\textsubscript{gh}, absB\textsubscript{gh}, and moe clusters for the presence of AdpA operator sequences [33]. As expected, such sequences were revealed within adpA\textsubscript{gh} and bldA\textsubscript{gh} (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 operon 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\textsubscript{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\textsubscript{gh} mutant (ΔadpA\textsubscript{gh}), showing that moenomycin production was blocked at the initial first steps. Knockout of adpA\textsubscript{gh} had a significant influence on the morphological development S. ghanaensis. On solid media, a phenotype of S. ghanaensis ΔadpA\textsubscript{gh} resembled that of the ‘bald’ (absB) mutants 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 ΔadpA\textsubscript{gh} were restored to the wild-type state upon introduction of an intact copy of adpA\textsubscript{gh} (plasmid pSETadpA-exp). Insertion of an extra copy of adpA\textsubscript{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 \( \text{D} \text{adpAgh} \), \( \text{S. ghanaensis} \ \text{D} \text{bldAgh} \) did not produce \( \text{MmA} \) or any of its intermediates (figure 2). Deletion of \( \text{bldAgh} \) impaired morphological development of \( \text{S. ghanaensis} \) (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 \( \text{bldAgh} \) into \( \text{D} \text{bldAgh} \) (plasmid \( \text{pSETbldA} \)) restored the moenomycin production and normal timing of morphogenesis, implying that the \( \text{D} \text{bldAgh} \) phenotype is solely due to the absence of tRNALeu\_UAA. The introduction of a second copy of \( \text{bldA} \) (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 \( \text{moe} \) genes was analysed in further detail to determine whether the \( \text{bldA} \) mutation affected moenomycin production directly (by blocking the translation of UUA-containing \( \text{moeO5} \) and \( \text{moeE5} \) mRNAs) or indirectly (by arresting \( \text{adpAgh} \) expression). Semi-quantitative RT-PCR analysis of \( \text{moeO5} \), \( \text{moeE5} \) and \( \text{moeGT4} \) showed that their transcription was not decreased in \( \text{D} \text{bldAgh} \); 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 \( \text{D} \text{bldAgh} \) (figure 4), indicating a direct regulatory influence on the expression of TTA-containing \( \text{moe} \) genes by tRNALeu\_UAA.

The RNase III-deficient mutant (\( \text{D} \text{absBgh} \)) produced on average 2.7 times more moenomycin compared with the parental strain (figure 2). On solid media, \( \text{D} \text{absBgh} \) 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 \( \text{D} \text{bldAgh} \) and \( \text{D} \text{adpAgh} \) extracts; detailed characterization of these peaks was not pursued.

Bioinformatic analysis indicated that \( \text{absBgh} \) and two upstream genes (\( \text{SSFG}_02131.1 \) and \( \text{SSFG}_02130.1 \)) are separated by 2 and 19 bp, indicative of transcriptional operon organization (figure 1). For complementation of \( \text{S. ghanaensis} \ \text{D} \text{absBgh} \), a series of integrative plasmids with different portions of this putative operon were constructed (for details, see §5). Only the plasmid containing \( \text{absBgh} \) in cis with the two upstream genes (pSOKabsBgh-III; figure 1) decreased moenomycin production to the wild-type level, suggesting that the \( \text{absBgh} \) is the last gene in a tricistronic message. Additional complementation experiments were designed to confirm that \( \text{absBgh} \) alone is sufficient to restore the wild-type phenotype. \( \text{absBgh} \) under the control of \( \text{ermEp} \) (pSOKEabsBgh-exp) was integrated into the \( \text{S. ghanaensis} \ \text{D} \text{absBgh} \) 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).

Figure 3. Lawns of \( \text{S. ghanaensis} \) mutants described in this study. (a) Wild-type \( \text{S. ghanaensis} \ \text{ATCC14672} \), \( \text{S. ghanaensis} \ \text{D} \text{absBgh} \), \( \text{S. ghanaensis} \ \text{D} \text{bldAgh} \) and \( \text{S. ghanaensis} \ \text{D} \text{adpAgh} \) (clockwise from the top left) were grown on TSB agar medium. (b) Respective mutants carrying plasmids for complementation, pSOKabsBgh-exp, pSET152bldA and pSETadpA-exp.
obtained from mycelium harvested in muenomycin production phase (TSB, from wild-type levels in D analysis of cell-free lysates from WT and transcript in the of key structural gene moe genes. First, we measured transcription from the promoter tion between the aforementioned pleiotropic regulators and system [36] was applied to investigate the functional connec-

Figure 4. The bldaGh gene directly affects translation of moeE5. (a) RT-PCR analysis of moeE5, moeO5 and moeGT4 transcription in S. ghanaensis wild-type (WT) and bldaGh-deficient (ΔbldaGh) strains. Lane C, negative control (rrna amplification from RNA sample in absence of RT). (b) Western blot analysis of cell-free lysates from WT and ΔbldaGh strains. The lysates were obtained from mycelium harvested in monomycin production phase (TSB, 72 h) and probed with anti-MoeE5 rabbit serum (raised as described in §5).

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. ghanaensis 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 ΔadpAgh strain (figure 5). The moeE5 tran-
scription was increased more than twofold and threefold from wild-type levels in S. ghanaensis ΔabsBgh and ΔbldaGh strains, respectively (figure 5), in agreement with RT-PCR data (figure 4). While the pattern of moeEp activity in ΔadpAgh and ΔabsBgh is as anticipated [25], increased levels of moeEp transcript in the ΔbldaGh are somewhat unexpected. A plausible explanation is that moeEp 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 bldaGh in the translational regulation of monomycin 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 bldaGh for translation of the two UUA codons in moeE5 mRNA. Surpris-
ingly, 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 rrna [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–gusA transcription. In comparison to the wild-type strain, adpAgh–gusA levels (figure 5) increased 2.3-fold in S. ghanaensis ΔabsBgh and were almost undetectable in the ΔadpAgh strain (figure 5). We also measured the level of translation when adpAgh–gusA and the entire adpA gene fusions were fused to gusA (padpAtrans; 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–gusA 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–gusA 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, 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, moeE5, bldaGh, and adpAgh in quantities as low as 1.1–11.0 pmol. Increasing concentrations of AdpAgh resulted in more than one protein–DNA complex (see the electronic supplementary material, figure S3), while a non-

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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 AdpA$_{gh}$ 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. ghanensis 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 B$_2$ (NoB$_2$) was introduced into S. coelicolor ΔabsB strain J3410 [41]. S. coelicolor J3410 moeno38-5$_+^+$ was grown in parallel with a control strain S. coelicolor M145 moeno38-5$_-^-$ and NoB$_2$ was quantified. On average, J3410 moeno38-5$_+^+$ accumulated 20% less biomass than M145 moeno38-5$_-^-$ and produced three times less NoB$_2$ 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 moe5 transcription in a ΔabsB$_{gh}$ strain compared with a control M145 strain (data not shown). Our results suggest that the AbsB RNase III-mediated
respective; 1326 and J1725—wild-type and respectively.

regions comprising in silico moeE5, adpAgh wild-type, rnc expressing cosmid moeno38-5. Column labels: M145, J3410 and M851—M851 and S. coelicolor revealed in respectively (data not shown).

The elucidation of the genetic organization of the erythromycin biosynthetic cluster in the early 1990s provided the first component of actinomycete secondary metabolic pathways.

That cluster-situated layers of regulation are not an obligatory biosynthetic genes [3,4,43]. However, a growing body of data suggest proteins directly activate the transcription of structural biosynthetic genes, for example AdpA [26,42]. Once produced, CSR shown in many cases to be dependent on global pleiotropic regulators, for example AdpA [26,42]. Once produced, CSR shown in many cases to be dependent on global pleiotropic regulators, for example AdpA [26,42].

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 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 tRNA\textsubscript{A\textsubscript{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 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 *Streptomyces* to further elucidate the role of AdpA in control of CSR-free antibiotic biosynthetic gene clusters under growth phase-dependent control.

The increased transcription of *adpA* from constitutive promoter *ermE* improved moenomycin production 2.5-fold in spite of the fact that (as our work shows) it is the transcription efficiency of UUA-containing *adpA* 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<sub>Leu<sup>UUA</sup></sub> is available, it might eventually lead to increased moenomycin production in the cells.
overexpressing adpAgh 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 adpAgh 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 absBgh deletion and adpAgh overexpression in S. ghanaensis, we observe, on average, a sevenfold increase (note that we determine moenomycin production in the one codons from earlier [63,64].

5.5. Identification of AdpAgh-‐binding sites

To identify conserved AdpA-‐binding sites (AdpAbs) in S. 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 moe clusters, blda and adpAgh promoter regions was performed using FIMO software suite [68].

5.6. Construction of the Streptomyces ghanaensis ΔabsBgh and plasmids for complementation experiments

A construct for absBgh knockout was prepared as follows. A 2.5 kb DNA fragment containing absBgh and its flanking regions were amplified from S. ghanaensis genomic DNA by PCR using primers absBgh_for and absBgh_rev. The PCR product was ligated to SmaI-digested pBluescriptKS-1 to yield pBlabsBgh-Δ. The loxP site-flanked apramycin resistance cassette (aac(3)IV) from plasmid pRE1 was amplified with primers red_absBgh_for and red_absBgh_rev. The resulting amplicon was used to replace the coding sequence of absBgh in pBlabsBgh via recombineering, giving pBlabsBgh-Δaac(3)IV. The latter was digested with BamHI and EcoRI and the fragment containing the absBgh::aac(3)IV mutant allele was cloned into the same sites of S. lividans kn::aac(3)IV. The resulting amplicon was used to replace the coding sequence of absBgh in pBlabsBgh-Δ via recombineering, giving pBlabsBgh::aac(3)IV. The resulting amplicon was used to replace the coding sequence of absBgh in pBlabsBgh-Δ via recombineering, giving pBlabsBgh::aac(3)IV. The latter was digested with BamHI and EcoRI and the fragment containing the absBgh::aac(3)IV mutant allele was cloned into the same sites of pKC1395Km to yield pKCabsB::aac(3)IV. Streptomyces ghanaensis transconjugants carrying the latter were selected for resistance to apramycin (25 μg ml⁻¹). To generate S. ghanaensis single-crossover 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 absBgh with aac(3)IV in S. ghanaensis ΔabsBgh::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::aac(3)IV to evict aac(3)IV from its genome. The pUWLCreCre- transconjugants resistant to ticlopre- ton were incubated on oatmeal agar plates and selected for apramycin sensitivity. The helper plasmid was lost after two subsequent passages of selected Am’ clone in the absence of thiostrepton. Excision of aac(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 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_ex_for and absBgh_ex_rev. The obtained amplicon was cloned into integrative WB-based vector pSOK804 digested with EcoRV to give pSOKabsBgh-exp.

To construct plasmid pSOKEabsBgh-exp, where tran- scription of absBgh is under ermEp control, the above 1.1 kb PCR fragment was first cloned into EcoRV-treated pKC1218E, 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 pSOKabsBgh-II.

Plasmid pSOKabsBgh-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 adpA_g, 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 in pBladpAkn to give pBladpAkn::aac(3)IV. The latter was further used as a template in PCR for amplification (primers adpA_for and adpA_rev) of a 3.4 kb DNA fragment harbouring ΔadpA_g::aac(3)IV. The obtained amplicon was cloned into EcoRV-digested vector pKC0702. The final ΔadpA_g knock out plasmid was labelled pKChadpAkn::aac(3)IV. Generation of ΔadpA_g mutant was carried out as described above. The mutant phenotype of S. ghanaensis ΔadpA_g::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 ΔadpA_g was carried out as described for ΔabsBgh strain (primers adpA_for and adpA_rev; data not shown).

For the complementation of S. ghanaensis ΔadpA_g, a 1.9 kb fragment carrying adpA_g 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 adpA_g expression under ermEp control, a 1.4 kb fragment containing only the coding sequence of adpA_g 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 Δblda_g strain and plasmid for complementation experiment

The 2.0 kb S. ghanaensis genomic regions flanking blda_g 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 blda_g knockout plasmid pKC1139blda_g-del contains markerless deletion of the 87 bp blda_g coding sequence. Manipulations of pKC1139blda_g-del transconjugants to generate the blda_g 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 bldaXbalaup and blda-Diagn-rp and sequencing confirmed the deletion of the 87 bp blda_g sequence from the genome of Δblda_g. For complementation and expression experiments, the blda_g coding region along with the 320 bp upstream segment was amplified with primers bldaXbalup and bldaAEOriRip and cloned into respective sites of pSET152 to yield pSET152blda_g.

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

To probe the activities of moeE5, absB_g, adpA_g and blda_g promoters, DNA fragments containing putative promoter regions (500 bp upstream of the translation start codons) were amplified by PCR using upstream primers carrying an XhoI site and downstream primers carrying a KpnI site (primers moeE5_for and moeE5_script_rev for moeE5; absB_for and absB_script_rev for absBg; adpA_for and adpA_script_rev for adpA_g; blda_for and blda_script_rev for blda_g). The moeE5, absB_g, adpA_g and blda_g fragments were cloned into XbaI–KpnI-digested pGUS, to give plasmids pmoeE5script, pabsBscript, padpAscript and pbladscript, respectively.

To investigate the expression of moeE5 and adpA_g 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 XhoI site and downstream primers carrying a KpnI site (primers moeE5_for and moeE5_rev for moeE5; adpA_for and adpA_rev for adpA_g). For complementation and expression experiments, the blda_g coding region along with the 320 bp upstream segment was amplified with primers bldaXbalup and bldaAEOriRip and cloned into respective sites of pSET152 to yield pSET152blda_g.
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 pmoeE5contr 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 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 AdpA<sub>gh</sub>

For the production of C-terminal hexahistidine-tagged AdpA<sub>gh</sub>, the coding region of gene adpA<sub>gh</sub> 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 YTB medium containing 50 μg ml⁻¹ ampicillin and kanamycin until The OD₆₀₀ reached 0.8–1.0. The work was supported by grant Bg-98F from the Ministry of Education and Science (to V.F.), by NIH grants 2P01AI083214-04 (to S.W.) and R03TW009424 (to V.F. and S.W.). R.M. and B.O. were supported by DAAD fellowships.

5.11. Gel electrophoretic mobility shift assay

The 500 bp promoter regions of the targeted genes (adpA<sub>gh</sub>, bldA<sub>gh</sub>, 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 adpA<sub>gh</sub>, bldA_for and bldA_script_rev for bldA<sub>gh</sub>, 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 [γ-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 AdpA<sub>gh</sub> 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(dl-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


during exponential phase and is required for antibiotic production and for proper sporulation.


