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\text{UAA} is required for the translation of rare UUA codons within two key moenomycin bio-
synthetic 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 \textit{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\textsubscript{UA} (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{UA} (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. griseus 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*$_{ab}$ orthologues [23,30–32]. Genes for several *AdpA*$_{ab}$ 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 relevant to this study. Triangles indicate position of AdpA-binding sites as predicted *in silico*.

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*$_{ab}$ and *bldA*$_{ab}$ (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 (*ΔadpA*$_{gh}$), 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 phenotype of *S. ghanaensis* *ΔadpA*$_{gh}$ resembled that of the ‘bald’ (*bld*) 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 [26,32,33].

The moenomycin production and morphology in the *ΔadpA*$_{ab}$ were restored to the wild-type state upon introduction of an intact copy of *adpA*$_{ab}$ (plasmid pSETadpA-exp). Insertion of an extra copy of *adpA*$_{ab}$, 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 \textit{D}adpAgh \textit{S. ghanaensis} \textit{D}bldAgh, did not produce MmA or any of its intermediates (figure 2). Deletion of \textit{bldAgh} impaired morphological development of \textit{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 \textit{bldAgh} into \textit{D}bldAgh (plasmid pSETbldA) restored the moenomycin production and normal timing of morphogenesis, implying that the \textit{D}bldAgh phenotype is solely due to the absence of tRN\text{ALeu}_\text{UAA}. The introduction of a second copy of \textit{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 \textit{moe} genes was analysed in further detail to determine whether the \textit{bldA} mutation affected moenomycin production directly (by blocking the translation of UUA-containing \textit{moeO5} and \textit{moeE5} mRNAs) or indirectly (by arresting \textit{adpAgh} expression). Semi-quantitative RT-PCR analysis of \textit{moeO5}, \textit{moeE5} and \textit{moeGT4} showed that their transcription was not decreased in \textit{D}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 \textit{D}bldAgh (figure 4), indicating a direct regulatory influence on the expression of TTA-containing \textit{moe} genes by tRN\text{ALeu}_\text{UAA}.

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

Bioinformatic analysis indicated that \textit{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 \textit{S. ghanaensis} \textit{D}absBgh, a series of integrative plasmids with different portions of this putative operon were constructed (for details, see §5). Only the plasmid containing \textit{absBgh} in \textit{cis} with the two upstream genes (pSOKabsBgh-III; figure 1) decreased moenomycin production to the wild-type level, suggesting that the \textit{absBgh} is the last gene in a tricistronic message. Additional complementation experiments were designed to confirm that \textit{absBgh} alone is sufficient to restore the wild-type phenotype. \textit{absBgh} under the control of \textit{ermEp} (pSOKEabsBgh-exp) was integrated into the \textit{S. ghanaensis} \textit{D}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).
translation of the two UUA codons in moeE5 mRNA. Surprisingly, GusA activity was detected in the Δblda
gene directly affects translation of moeE5. (a) RT-PCR analysis of moeE5, moeO5 and moeE4T4 transcription in S. ghanaensis wild-type (WT) and blda-deficient (Δblda
gene promoters. To test this, we set out to demonstrate AdpA
genes were fused to gusA (padpAtransl; see §5) and found it in ΔabsB
to be double that of wild-type (figure 6). Thus, AdpA
to be strictly dependent on BldA [23,32,39], our data set a precedent for this important group of pleiotropic activators.

Next, we analysed adpA
to be double that of wild-type (figure 6). Thus, AdpA
activity was also detected in the Δblda
gene (plasmid pmoeE5transl) and adpA in a Δblda
background. We found no GusA activity in Δblda
carrying moeE5–gusA fusion (figure 6), underscoring the essentiality of blda for

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 genes moeE5 (moeEp) in all of the S. ghanaensis mutants. The wild-type strain had relatively high levels of transcription from moeEp (see, for comparison, the activity of other SM gene promoters [36]), but we failed to detect transcription in the ΔadpA
gene (WT) and Δblda
csequences as low as 1.1–11.0 pmol. Increasing concentrations of AdpA
class is supported by observations in other streptomycetes [25,40]. Similar to our moeEp data, adpA
to AdpA
activity was also significantly increased in the Δblda
in the absence of ribonucleolytic activity of AbsB. This conclusion is supported by observations in other streptomycetes [25,40]. Similar to our moeEp data, adpA
At the same time, we revealed almost complete cessation of blda
to transcription of absB.

3.4. AdpA interacts with promoters of blda, adpA and key moe genes

The GusA reporter data suggested that AdpA is a transcriptional activator that regulates its own expression as well as that of blda and moe genes. To test this, we set out to demonstrate AdpA
activity was detected in the Δblda
increased more than twofold and threefold
examination of cell-free lysates from WT and Δblda
strains harvested in moenomycin production phase (TSB, 72 h) and probed with anti-MoeE rabbit serum (raised as described in §5).

Figure 4. The blda gene directly affects translation of moeE5. (a) RT-PCR analysis of moeE5, moeO5 and moeE4T4 transcription in S. ghanaensis wild-type (WT) and blda-deficient (Δblda) 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 Δblda strains. The lysates were obtained from mycelium harvested in moenomycin production phase (TSB, 72 h) and probed with anti-MoeE rabbit serum (raised as described in §5).
Figure 5. Transcriptional activity of selected promoters in *S. ghanaensis* ΔabsB, ΔbldA and ΔadpA strains. WT, ΔabsB, ΔbldA and ΔadpA correspond to wild-type, absBΔ, bldAΔ and adpAΔ null mutant strains, respectively, of *S. ghanaensis* expressing gusA from different promoters. The moeE5p, adpAp, absBp and bldAp correspond to promoters of moeE5, adpAΔ, absBΔ and bldAΔ, respectively.

Figure 6. Translation of AdpAΔ and MoeE5 is strongly affected on bldAΔ minus background. WT, ΔabsB and ΔbldA correspond to wild-type, absBΔ and bldAΔ null mutant strains, respectively, of *S. ghanaensis* expressing gusA fused to tested genes along with their promoters. adpA and moeE5 correspond to genes adpAΔ 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.

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 moeno38-5Δ was grown in parallel with a control strain *S. coelicolor* M145 moeno38-5Δ and NoB2 was quantified. On average, J3410 moeno38-5Δ accumulated 20% less biomass than M145 moeno38-5Δ 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 ΔabsBΔ strain compared with a control M145 strain (data not shown). Our results suggest that the AbsB RNase III-mediated
regulatory pathway is important for moenomycin production even in other streptomycete heterologous hosts.

Next, we tested NoB 2 production in adpA-deficient S. coelicolor M851 and bldA-deficient S. lividans J1725 strains. Mutant and parental strains carrying cosmids moeno38-5 did not differ in growth rate, but NoB 2 production was completely abolished (figure 8). No moeS 5 activity was revealed in S. coelicolor M851. NoB 2 production was restored to M851 and J1725 upon introduction of adpA gh and bldA gh, respectively (data not shown).

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 nonribosomal 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 A154, 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,
The severe morphological defects of *S. ghanaensis* vary under different growth conditions, which may contribute to AdpA<sub>gh</sub> expression. The complexity and conditionality of the bldA<sub>gh</sub> phenotype is well known in *S. coelicolor* [53, 54]. It is the chief reason for ongoing debate as to whether bldA constitutes a ‘true regulatory device’ [55, 56] or just a ‘wiring’ of the other regulatory networks [57, 58]. Our data as well as that of Wang et al. [21] unequivocally demonstrate that a bldA deletion directly abrogates the translation of UUA-containing transcripts and, subsequently, antibiotic production. Hence, bldA is a unique RNA, absence of which indeed creates a regulatory event in the form of infinite delay of the translation of UUA<sup>+</sup> transcripts. Recent work showed that, compared with the more abundant tRNAs, accumulation of primary bldA transcript began at earlier stages, and BldA tRNA scaffold does not determine its regulatory role [56]. Availability of mature BldA may thus be regulated by posttranscriptional modification, but no evidence for that is available. Function of BldA is likely to be more conditional than that of transcriptional 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<sub>gh</sub> in a ∆bldA<sub>gh</sub> 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<sup>Leu<sub>UAA</sub></sup> in the cells [18, 39]. Just a small amount of AdpA<sub>gh</sub> available during early stages of growth in the absence of BldA<sub>gh</sub> could be sufficient to activate transcription from bldA<sub>gh</sub><sup>+</sup> leading to an avalanche-like increase in bldA<sub>gh</sub> expression. Once available, charged tRNA<sup>Leu<sub>UAA</sub></sup><sub>bldA<sub>gh</sub></sub> could then amplify the translation of adpA<sub>gh</sub> and other, as-yet-unknown, UUA<sup>+</sup> genes that lead to the downregulation of adpA 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<sub>gh</sub> from constitutive promoter ermE<sub>UAA</sub> 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<sub>gh</sub> 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<sup>Leu<sub>UAA</sub></sup> is available, it might eventually lead to increased moenomycin production in the cells.
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 \( \text{bldA}_{gh} - \text{adpA}_{gh} - \text{absB} \) 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 \( S. \ ghanaensis \), 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 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 \( \text{AdpA} \) 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. \( \text{Escherichia coli} \) strains were grown in Luria-Bertani medium. \( \text{Streptomyces} \) strains were grown on SM and oatmeal agar media and in TSB and R2YE liquid media. Unless otherwise stated, \( S. \ ghanaensis \) was grown at 37°C and other \( \text{Streptomyces} \) at 30°C and the fragment containing the \( \text{absB}_{gh} \) mutant allele was cloned into the same sites of pBlabsBgh-kn via recombineering, giving pBlabsBgh-kn::\( \text{aac}(3)\text{IV} \). The resulting ampiclon was used to replace the coding sequence of \( \text{absB}_{gh} \) in pBlabsBgh-kn via recombineering, giving pBlabsBgh-kn::\( \text{aac}(3)\text{IV} \). The latter was digested with BamHI and EcoRI and the fragment containing the \( \text{absB}_{gh}::\text{aac}(3)\text{IV} \) mutant allele was cloned into the same sites of pKC1139Km to yield pKCabsB-kn::\( \text{aac}(3)\text{IV} \). \( \text{Streptomyces} \) strains, \( S. \ ghanaensis \) transconjugants carrying the latter were selected for resistance to apramycin (25 µg ml\(^{-1}\)). To generate \( S. \ ghanaensis \) single-crossover Am\(^{R}\)Km\(^{R}\) 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 \( \text{aac}(3)\text{IV} \) in \( \text{S. ghanaensis} \) \( \text{absB}_{gh}::\text{aac}(3)\text{IV} \) was confirmed by PCR (primers \( \text{absBgh} \text{ex} \text{for} \) and \( \text{absBgh} \text{ex} \text{rev} \); data not shown). The

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 \( S. \ ghanaensis \) extracts: MmA (\( [M-H]^{+} = 1580.6 \text{ Da} \)) and nosokomycin B (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\(_{2} \) (NoB\(_{2} \); \( [M-H]^{+} = 1500.6 \text{ Da} \)) and its production was followed in the extracts of heterologous hosts (\( S. \ lividans \) and \( S. \ coelicolor \)). LC/MS data were acquired on Agilent 1110 LC/MSD and Bruker Esquire 3000 ESI-MS spectrometers.

5.4. Identification of AdpA\(_{gh}\)-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 \( \text{moe} \) clusters, \( \text{bldA}_{gh} \) and \( \text{adpA}_{gh} \) promoter regions was performed using FIMO software suite [68].

5.5. Semiquantitative RT-PCR

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

5.6. Construction of the \( \text{Streptomyces ghanaensis} \) \( \Delta \text{absB}_{gh} \) and plasmids for complementation experiments

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 \( S. \ ghanaensis \) genomic DNA by PCR using primers \( \text{absBgh} \text{kn for} \) and \( \text{absBgh} \text{kn rev} \). The PCR product was ligated to Smal-digested pBluescriptKS+ and transformed via electroporation into \( \text{S. lividans} \) devoid of any nptIII cassette. The resulting plasmid, pBlabsBgh-kn::\( \text{aac}(3)\text{IV} \), was introduced into \( \text{S. ghanaensis} \) via conjugation using \( \text{S. lividans} \) as helper. The resulting ampiclon was used to replace the coding sequence of \( \text{absB}_{gh} \) in pBlabsBgh-kn via recombineering, giving pBlabsBgh-kn::\( \text{aac}(3)\text{IV} \). The latter was digested with BamHI and EcoRI and the fragment containing the \( \text{absB}_{gh}::\text{aac}(3)\text{IV} \) mutant allele was cloned into the same sites of pKC1139Km to yield pKCabsB-kn::\( \text{aac}(3)\text{IV} \).
Cre-expressing helper plasmid pUWLCre was then introduced into *S. ghanaensis DabsB*gh-act(3)IV to evict act(3)IV from its genome. The pUWLCre” 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” clone in the absence of tetracycline. Excision of act(3)IV from the *S. ghanaensis DabsB*gh genome was confirmed by PCR (primers absBgh ExFor and absBgh ExRev; data not shown).

A set of plasmids containing absB*gh* 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 absB*gh* with its 150 bp 5’-region was amplified from *S. ghanaensis* genomic DNA using primers absBgh For and absBgh ExRev. The obtained amplicon was cloned into integrative VWB-based vector pSOK804 digested with EcoRV to give pSOKabsBgh-exp.

To construct plasmid pSOKabsBgh-Exp, where transcription of absB*gh* is under erm*Ep* control, the above 1.1 kb PCR fragment was first cloned into EcoRV-treated pKC1218E, yielding pKCabsBgh-Exp. Then pKCabsBgh-Exp was digested with HindIII and EcoRI and 1.4 kb DNA fragment harbouring absB*gh* plus erm*Ep* was ligated to pSOK804, digested with respective endonucleases, to generate pSOKabsBgh-Exp.

To create a plasmid pSOKabsBgh-II encompassing two genes, SSFG_02130.1 and SSFG_02129.1 (absB*gh*), 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 (absB*gh*), 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* \(\Delta adpA_{gh}\) and plasmid for complementation experiment

A 3.5 kb DNA fragment containing *adpA*gh* 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 pHladapAkn. To replace *adpA*gh* the *erm*Ep* 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*gh* with pHladapAkn to give pHladapA-Kn::act(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 \(\Delta adpA_{gh}:\text{act}(3)IV\). The obtained amplicon was cloned into EcoRV-digested vector pKC0702. The final \(\Delta adpA_{gh}\) knockout plasmid was labelled pKChadpAkn::act(3)IV. Generation of \(\Delta adpA_{gh}\) mutant was carried out as described above. Mutant phenotype of *S. ghanaensis \(\Delta adpA_{gh}:\text{act}(3)IV\) was confirmed by PCR using primers adpA Exp For and adpA Exp Rev. Generation and verification of act(3)IV-evicted strain \(\Delta adpA_{gh}\) was carried out as described for \(\Delta tsbsB_{gh}\) strain (primers adpA For and adpA Rev; data not shown).

For the complementation of *S. ghanaensis \(\Delta adpA_{gh}\), a 1.9 kb fragment carrying *adpA*gh* 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*gh* expression under erm*Ep* control, a 1.4 kb fragment containing only the coding sequence of *adpA*gh* 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 \(\Delta bldA_{gh}\) strain and plasmid for complementation experiment

The 2.0 kb *S. ghanaensis* genomic regions flanking *bldA*gh* 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*gh* knockout plasmid pKC1139bldA-del contains markerless deletion of the 87 bp *bldA*gh* coding sequence. Manipulations of pKC1139bldA-del+ transconjugants to generate the *bldA*gh* 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 bldAXbup and bldA-Diag-rp and sequencing confirmed the deletion of the 87 bp *bldA*gh* sequence from the genome of \(\Delta bldA_{gh}\). For complementation and expression experiments, the *bldA*gh* coding region along with the 320 bp upstream segment was amplified with primers bldAxbup and bldAEcoRiP and cloned into respective sites of pSET152 to yield pSET152bldA.

### 5.9. Construction of GusA reporter plasmids and \(\beta\)-glucuronidase activity measurements

To probe the activities of *moeE5*, *absB*gh*, *adpA*gh* and *bldA*gh* 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*gh*; adpA For and adpA Script Rev for adpA*gh*; bldA For and bldA Script Rev for bldA*gh*). The moeE5p, absB*gh*P, adpA*gh*P and bldA*gh*P 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*gh* 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 moeE5; absB For and absB Script Rev for absB*gh*; adpA For and adpA Script Rev for adpA*gh*). The moeE5 and *adpA*gh* fragments were cloned into XbaI–EcoRV-digested pGUSH64adA [36], an integrative *Streptomyces* vector where the examined gene is fused to the gusA reporter gene through the helical linker HLA [69], yielding pmoeE5trans and padpAtrans, respectively. In control experiments, promoterless *moeE5* and *adpA*gh* genes without stop codon were amplified by PCR using upstream primers
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 adpA gh) and cloned in XbaI–EcoRV-treated pGUSHL4aadA, giving pmoeE5contr and padpAcontr, respectively.

The spore suspensions (2 × 10^8 cfu) of streptomycetes reporter plasmid-bearing strains were inoculated in 300 ml flasks with 100 ml of TSB, and grown for 30 h. One milliliter 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 gh

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

References


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