



DIGITAL ACCESS TO SCHOLARSHIP AT HARVARD

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

The Harvard community has made this article openly available. [Please share](#) how this access benefits you. Your story matters.

Citation	Makitrynsky, Roman, Bohdan Ostash, Olga Tsypik, Yuriy Rebets, Emma Doud, Timothy Meredith, Andriy Luzhetskyy, Andreas Bechthold, Suzanne Walker, and Victor Fedorenko. 2013. "Pleiotropic regulatory genes <i>bldA</i> , <i>adpA</i> and <i>absB</i> are implicated in production of phosphoglycolipid antibiotic moenomycin." <i>Open Biology</i> 3 (10): 130121. doi:10.1098/rsob.130121. http://dx.doi.org/10.1098/rsob.130121 .
Published Version	doi:10.1098/rsob.130121
Accessed	April 17, 2018 4:37:41 PM EDT
Citable Link	http://nrs.harvard.edu/urn-3:HUL.InstRepos:11878981
Terms of Use	This article was downloaded from Harvard University's DASH repository, and is made available under the terms and conditions applicable to Other Posted Material, as set forth at http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#LAA

(Article begins on next page)



Cite this article: Makitrynsky R, Ostash B, Tsyplik O, Rebets Y, Doud E, Meredith T, Luzhetskyy A, Bechthold A, Walker S, Fedorenko V. 2013 Pleiotropic regulatory genes *bldA*, *adpA* and *absB* are implicated in production of phosphoglycolipid antibiotic moenomycin. *Open Biol* 3: 130121. <http://dx.doi.org/10.1098/rsob.130121>

Received: 30 July 2013

Accepted: 26 September 2013

Subject Area:

genetics/microbiology

Keywords:

pleiotropic regulators, *Streptomyces*, moenomycin A, *adpA*, *bldA*, *absB*

Author for correspondence:

Victor Fedorenko

e-mail: v_fedorenko@franko.lviv.ua

[†]Present address: Proteomics Center of Excellence, Northwestern University, Evanston, IL 60201, USA.

[‡]Present address: Infectious Diseases Area, Novartis Institutes for BioMedical Research, Cambridge, MA 02139, USA.

Electronic supplementary material is available at <http://dx.doi.org/10.1098/rsob.130121>.

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

Roman Makitrynsky^{1,2}, Bohdan Ostash^{1,3}, Olga Tsyplik¹, Yuriy Rebets^{1,3}, Emma Doud^{3,†}, Timothy Meredith^{3,‡}, Andriy Luzhetskyy^{2,4}, Andreas Bechthold², Suzanne Walker³ and Victor Fedorenko¹

¹Department of Genetics and Biotechnology, Ivan Franko National University of Lviv, Hrushevskoho st. 4, Lviv 79005, Ukraine

²Albert-Ludwigs-University of Freiburg, Pharmazeutische Biologie, Stefan-Meier-Strasse 19, 79104 Freiburg, Germany

³Department of Microbiology and Immunobiology, Harvard Medical School, 4 Blackfan Circle, Boston, MA 02115, USA

⁴Helmholtz Institute for Pharmaceutical Research, Saarland Campus, Building C2.3, 66123 Saarbrücken, Germany

1. Summary

Unlike the majority of actinomycete secondary metabolic pathways, the biosynthesis of peptidoglycan glycosyltransferase inhibitor moenomycin in *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 for *Streptomyces* morphogenesis and antibiotic production—*bldA*, *adpA* and *absB*—exert multi-layered control over moenomycin biosynthesis in native and heterologous producers. The *bldA* gene for tRNA^{Leu}_{UAA} is required for the translation of rare UUA codons within two key moenomycin biosynthetic genes (*moe*), *moeO5* and *moeE5*. It also indirectly influences moenomycin production by controlling the translation of the UUA-containing *adpA* and, probably, other as-yet-unknown repressor gene(s). AdpA binds key *moe* promoters and activates them. Furthermore, AdpA interacts with the *bldA* promoter, thus impacting translation of *bldA*-dependent mRNAs—that of *adpA* and several *moe* genes. Both *adpA* expression and moenomycin production are increased in an *absB*-deficient background, most probably because AbsB normally limits *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* 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^{Leu}_{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_{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*_{gh} mRNA is dependent on *bldA*-encoded tRNA, although this dependence is not absolute. Finally, we provide circumstantial evidence that AdpA_{gh} expression is regulated at the posttranscriptional level through the action of the *absB*_{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^{Leu}_{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*_{gh} belongs to the transcriptional unit which comprises three genes: *SSFG_02131.1*, *SSFG_02130.1* and *SSFG_02129.1* (*absB*_{gh}) (figure 1).

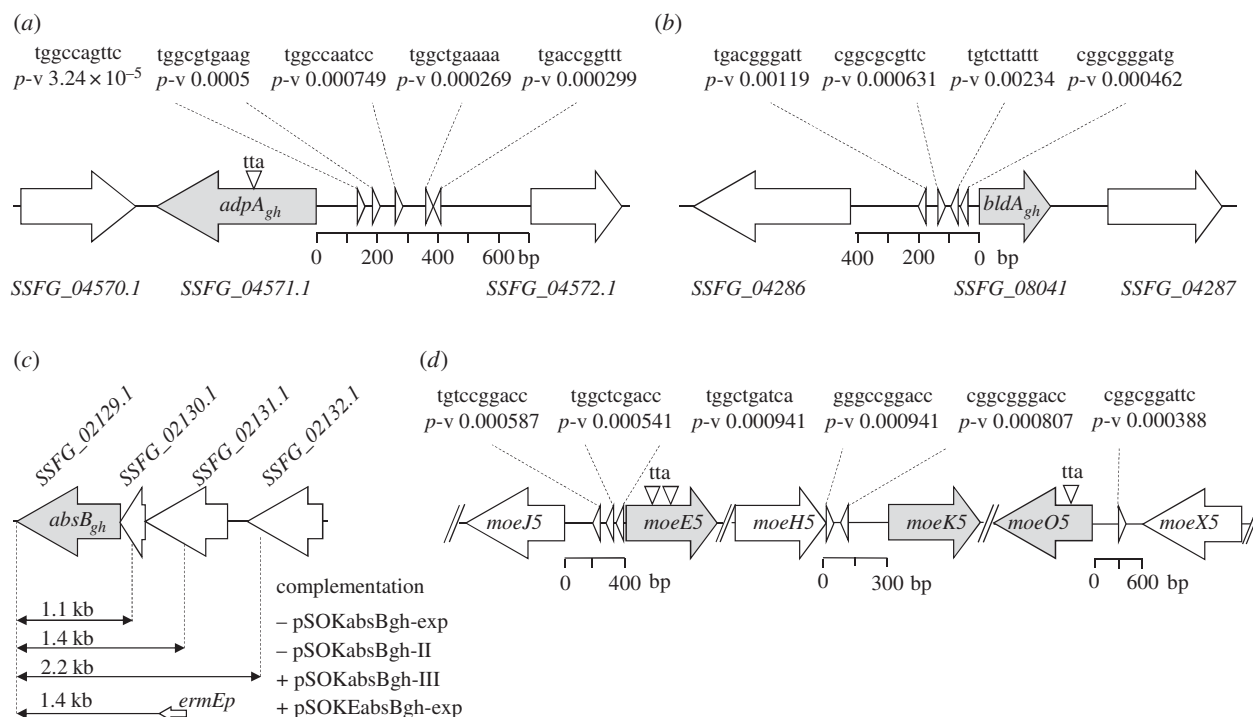


Figure 1. Fragments of *S. ghanensis* genome relevant to this study. Triangles indicate position of AdpA-binding sites as predicted *in silico*. Respective score values are given near the binding sites. (a) The *adpA_{gh}*-containing region with the adjacent genes. The distance between start and stop codons is shown. (b) Gene *bldA_{gh}* with its promoter region. The putative start of mature tRNA is shown. (c) Operon containing *absB_{gh}* and constructs used for complementation of *S. ghanensis* Δ *absB_{gh}* mutant. (d) Positions of high-scoring AdpA_{gh}-binding sites within intergenic regions of *moe* cluster 1 studied in this work. The distance between start and stop codons is shown.

In our *in silico* analysis [10] of *S. ghanensis*, we identified an AdpA orthologue in *S. ghanensis* 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. ghanensis* genome (see the electronic supplementary material, table S1). Additionally, a single copy of the tRNA^{Leu}_{UAA} gene was identified in the *S. ghanensis* 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_{gh}* and *bldA_{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. ghanensis* 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 ghanensis* *adpA* and *bldA* mutants, and increased in the *absB* mutant

Deletion of *adpA_{gh}* in the *S. ghanensis* 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. ghanensis*. On solid media,

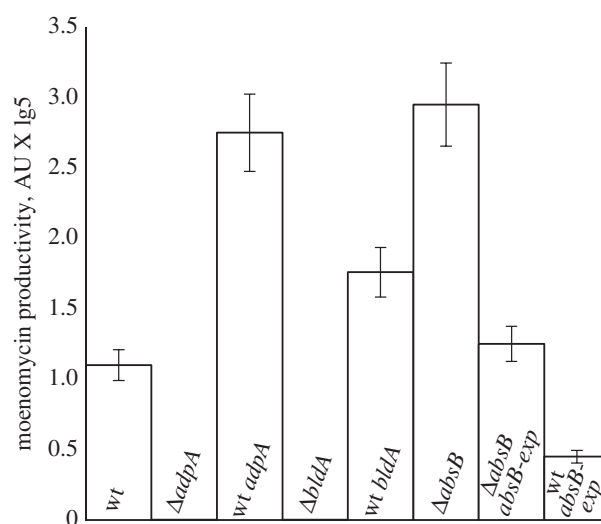


Figure 2. Levels of moenomycin production by various *S. ghanensis* strains. Column labels: wt, Δ *adpA*—wild-type and *adpA_{gh}* null mutant, respectively; wt *adpA*—wild-type strain overexpressing *adpA_{gh}*; Δ *bldA*—*bldA_{gh}*-minus mutant; wt *bldA*—wild-type strain overexpressing *bldA_{gh}*; Δ *absB*—*absB_{gh}*-minus mutant; Δ *absB* *absB*-exp—*absB_{gh}*-minus mutant expressing plasmid for complementation pSOKeabsBgh-exp; wt *absB*-exp—wild-type strain overexpressing *absB_{gh}*.

a phenotype of *S. ghanensis* Δ *adpA_{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 [26,32,35].

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),

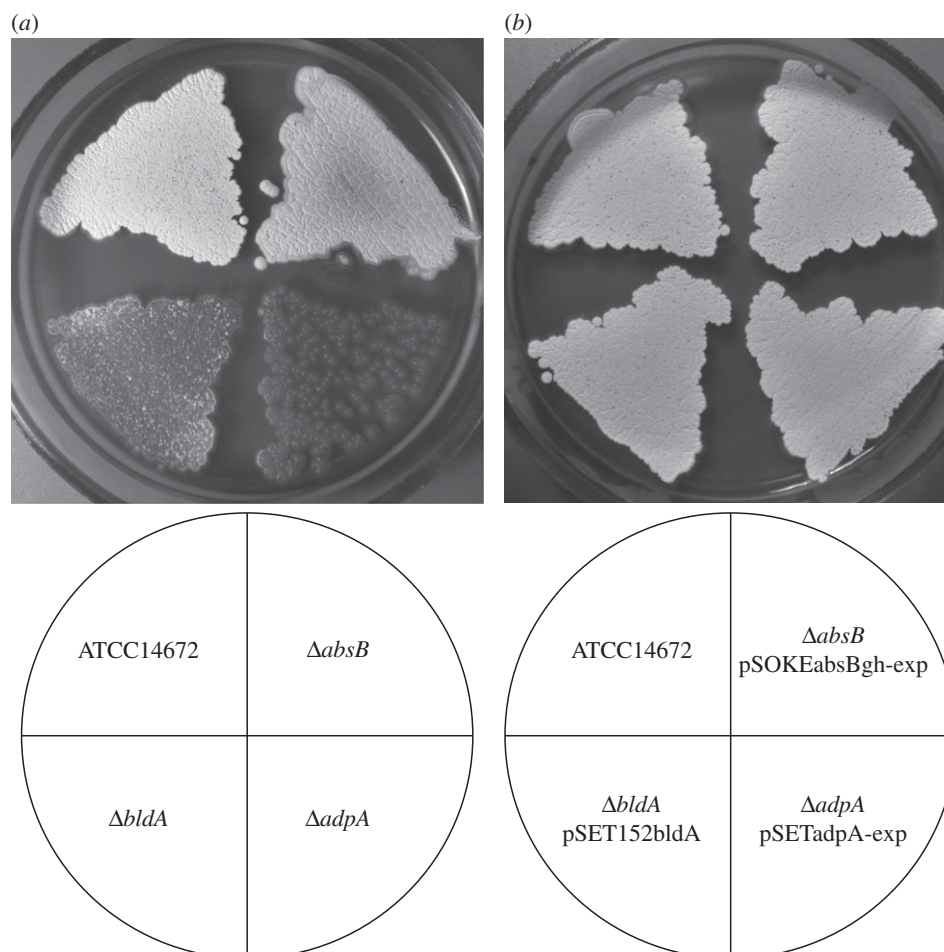


Figure 3. Lawns of *S. ghanaensis* mutants described in this study. (a) Wild-type *S. ghanaensis* ATCC14672, *S. ghanaensis* $\Delta absB_{gh}$, *S. ghanaensis* $\Delta bldA_{gh}$ and *S. ghanaensis* $\Delta adpA_{gh}$ (clockwise from the top left) were grown on TSB agar medium. (b) Respective mutants carrying plasmids for complementation, pSOKEabsBgh-exp, pSET152bldA and pSETadpA-exp.

caused a 2.5-fold increase in moenomycin production compared with the control strain (figure 2).

Like the $\Delta adpA_{gh}$, *S. ghanaensis* $\Delta bldA_{gh}$ did not produce MmA or any of its intermediates (figure 2). Deletion of *bldA_{gh}* 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 *bldA_{gh}* into $\Delta bldA_{gh}$ (plasmid pSETbldA) restored the moenomycin production and normal timing of morphogenesis, implying that the $\Delta bldA_{gh}$ phenotype is solely due to the absence of tRNA^{Leu}_{UAA}. The introduction of a second copy of *bldA* (pSET152bldA) into the wild-type strain led to a slight (1.6-fold on average) but reproducible increase in moenomycin production (figure 2).

The transcription and translation of several *moe* genes was analysed in further detail to determine whether the *bldA* mutation affected moenomycin production directly (by blocking the translation of UUA-containing *moeO5* and *moeE5* mRNAs) or indirectly (by arresting *adpA_{gh}* expression). Semi-quantitative RT-PCR analysis of *moeO5*, *moeE5* and *moeGT4* showed that their transcription was not decreased in $\Delta bldA_{gh}$; 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 $\Delta bldA_{gh}$ (figure 4), indicating a direct regulatory influence on the expression of TTA-containing *moe* genes by tRNA^{Leu}_{UAA}.

The RNase III-deficient mutant ($\Delta absB_{gh}$) produced on average 2.7 times more moenomycin compared with the

parental strain (figure 2). On solid media, $\Delta absB_{gh}$ 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 $\Delta bldA_{gh}$ and $\Delta adpA_{gh}$, and one peak disappeared in $\Delta adpA_{gh}$ extracts; detailed characterization of these peaks was not pursued.

Bioinformatic analysis indicated that *absB_{gh}* 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* $\Delta absB_{gh}$, a series of integrative plasmids with different portions of this putative operon were constructed (for details, see §5). Only the plasmid containing *absB_{gh}* in cis with the two upstream genes (pSOKabsBgh-III; figure 1) decreased moenomycin production to the wild-type level, suggesting that the *absB_{gh}* is the last gene in a tricistronic message. Additional complementation experiments were designed to confirm that *absB_{gh}* alone is sufficient to restore the wild-type phenotype. *absB_{gh}* under the control of *ermEp* (pSOKEabsBgh-exp) was integrated into the *S. ghanaensis* $\Delta absB_{gh}$ 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).

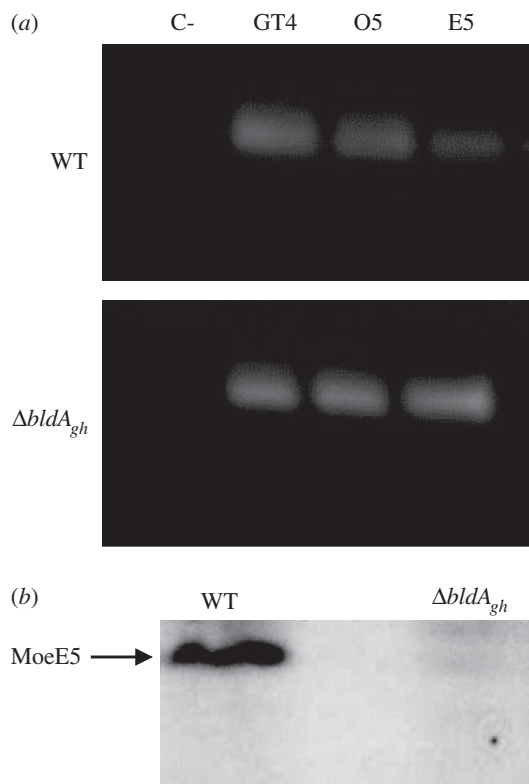


Figure 4. The *bldA_{gh}* gene directly affects translation of *moeE5*. (a) RT-PCR analysis of *moeE5*, *moeO5* and *moeGT4* transcription in *S. ghanaensis* wild-type (WT) and *bldA*-deficient ($\Delta bldA_{gh}$) strains. Lane C-, negative control (*rna* amplification from RNA sample in absence of RT). (b) Western blot analysis of cell-free lysates from WT and $\Delta bldA_{gh}$ strains. The lysates were obtained from mycelium harvested in moenomycin 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 $\Delta adpA_{gh}$ strain (figure 5). The *moeE5* transcription was increased more than twofold and threefold from wild-type levels in *S. ghanaensis* $\Delta absB_{gh}$ and $\Delta bldA_{gh}$ strains, respectively (figure 5), in agreement with RT-PCR data (figure 4). While the pattern of *moeEp* activity in $\Delta adpA_{gh}$ and $\Delta absB_{gh}$ is as anticipated [25], increased levels of *moeE5p* transcript in the $\Delta bldA_{gh}$ are somewhat unexpected. A plausible explanation is that *moeE5p* might be a target of an as-yet-unknown repressor(s) positively regulated by BldA, in which case the deletion of *bldA* would remove the repressive signal. To further delineate the involvement of *bldA_{gh}* in the translational regulation of moenomycin production, we analysed GusA activity of translational fusions of *gusA* to *moeE5* (plasmid pmoeE5transl) and *adpA_{gh}* in a $\Delta bldA_{gh}$ background. We found no GusA activity in $\Delta bldA_{gh}$ carrying *moeE5-gusA* fusion (figure 6), underscoring the essentiality of *bldA_{gh}* for

translation of the two UUA codons in *moeE5* mRNA. Surprisingly, GusA activity was detected in the $\Delta bldA_{gh}$ strain carrying *adpA_{gh}-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 *adpA_{gh}* UUA codon in the absence of tRNA^{Leu}_{UAA} [37,38]. As the expression of AdpA in other cases has been shown to be strictly dependent on BldA [23,32,39], our data set a precedent for this important group of pleiotropic activators.

Next, we analysed *adpA_{gh}p* transcription. In comparison to the wild-type strain, *adpA_{gh}p* levels (figure 5) increased 2.3-fold in *S. ghanaensis* $\Delta absB_{gh}$ and were almost undetectable in the $\Delta adpA_{gh}$ strain (figure 5). We also measured the level of translation when *adpA_{gh}p* and the entire *adpA_{gh}* genes were fused to *gusA* (*padpAtransl*; see §5) and found it in $\Delta absB_{gh}$ to be double that of wild-type (figure 6). Thus, AdpA_{gh} acts as a positive activator of its own expression and its activity is increased in the absence of ribonucleolytic activity of AbsB_{gh}. This conclusion is supported by observations in other streptomycetes [25,40]. Similar to our *moeE5p* data, *adpA_{gh}p* activity was also significantly increased in the $\Delta bldA_{gh}$ strain (figure 5), suggesting the existence of an unidentified *bldA*-dependent repressor(s) of AdpA_{gh}-regulated promoters.

There was no difference between *absB_{gh}p* transcriptional activity in $\Delta adpA_{gh}$ and wild-type strains, indicating that AdpA_{gh} does not influence the transcription of *absB_{gh}*.

At the same time, we revealed almost complete cessation of *bldA_{gh}* transcription in the $\Delta adpA_{gh}$ strain (figure 5).

3.4. AdpA_{gh} interacts with promoters of *bldA_{gh}*, *adpA_{gh}* and key *moe* genes

The GusA reporter data suggested that AdpA_{gh} is a transcriptional activator that regulates its own expression as well as that of *bldA_{gh}* and *moe* genes. To test this, we set out to demonstrate AdpA_{gh} binding to *moeO5*, *moeK5*, *moeE5*, *bldA_{gh}* and *adpA_{gh}* promoter regions using electrophoretic mobility shift assay (EMSA). A C-terminally His₆-tagged derivative of AdpA_{gh} was overexpressed in *Escherichia coli* and purified to homogeneity (see the electronic supplementary material, figure S2). Increasing amounts of AdpA_{gh}-His were incubated with radiolabelled DNA probes corresponding to the promoter regions of interest, and the complexes were separated by native gel electrophoresis. Purified AdpA_{gh}-His was bound to the promoter regions of *moeO5*, *moeK5*, *moeE5*, *bldA_{gh}* and *adpA_{gh}* in quantities as low as 1.1–11.0 pmol. Increasing concentrations of AdpA_{gh} resulted in more than one protein–DNA complex for *moeO5p* and *moeK5p* (figure 7), in agreement with multiple AdpA-binding sites predicted for these promoters *in silico*. While bioinformatics analysis predicted three putative AdpA-binding sites within *moeE5p*, only one shifted band was presented in the case of *moeE5p*. Unlabelled *adpA_{gh}* promoter competed with the radiolabelled one for AdpA_{gh} (see the electronic supplementary material, figure S3), while a non-specific DNA fragment (the *SCO3812* promoter region) was not recognized by AdpA_{gh} (see the electronic supplementary material, figure S4).

AdpA_{gh} formed three different *bldA_{gh}p*–AdpA_{gh} complexes suggesting the presence of multiple AdpA-binding motifs within *bldA_{gh}p*. Increasing concentrations of AdpA_{gh} caused a transition from multiple bands to a single retarded band, suggesting that above a certain AdpA_{gh} concentration,

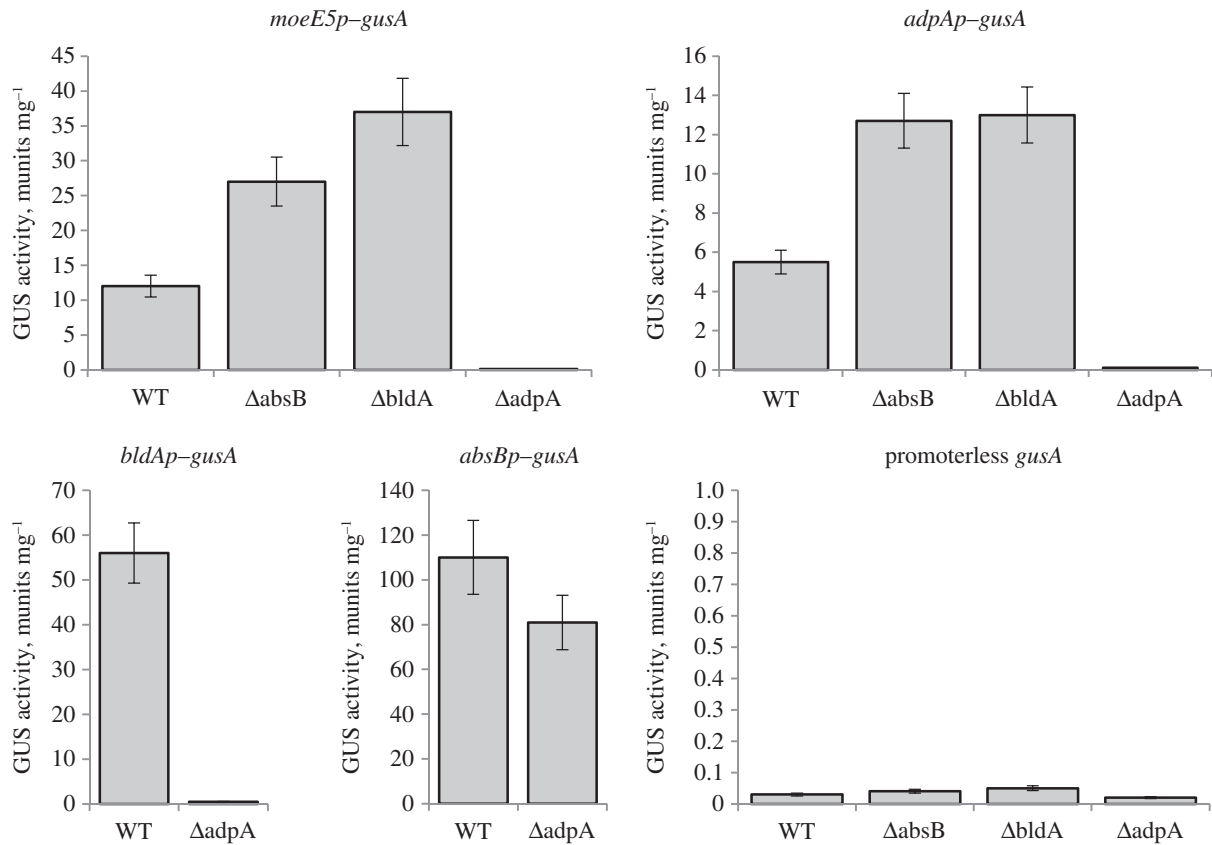


Figure 5. Transcriptional activity of selected promoters in *S. ghanaensis* $\Delta absB$, $\Delta bldA$ and $\Delta adpA$ strains. WT, $\Delta absB$, $\Delta bldA$ and $\Delta adpA$ correspond to wild-type, *absB_{ghr}*, *bldA_{ghr}* and *adpA_{ghr}* null mutant strains, respectively, of *S. ghanaensis* expressing *gusA* from different promoters. The *moeE5p*, *adpAp*, *absBp* and *bldAp* correspond to promoters of *moeE5*, *adpA_{ghr}*, *absB_{ghr}* and *bldA_{ghr}*, respectively.

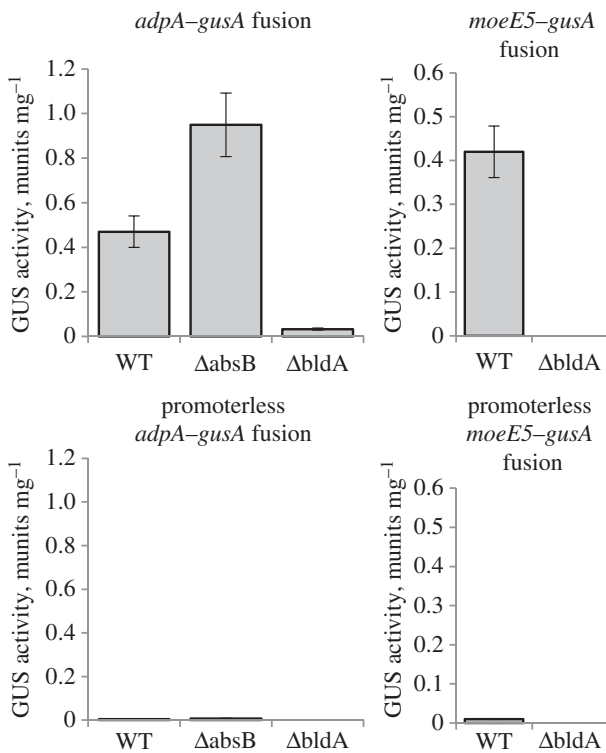


Figure 6. Translation of AdpA_{gh} and MoeE5 is strongly affected on *bldA_{gh}*-minus background. WT, $\Delta absB$ and $\Delta bldA$ correspond to wild-type, *absB_{ghr}* and *bldA_{ghr}* null mutant strains, respectively, of *S. ghanaensis* expressing *gusA* fused to tested genes along with their promoters. *adpA* and *moeE5* correspond to genes *adpA_{ghr}* 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.

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. 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 $\Delta absB$ -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* $\Delta absB$ strain J3410 [41]. *S. coelicolor* J3410 moeno38-5⁺ was grown in parallel with a control strain *S. coelicolor* M145 moeno38-5⁺ and NoB₂ was quantified. On average, J3410 moeno38-5⁺ 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 $\Delta absB$ strain compared with a control M145 strain (data not shown). Our results suggest that the AbsB RNase III-mediated

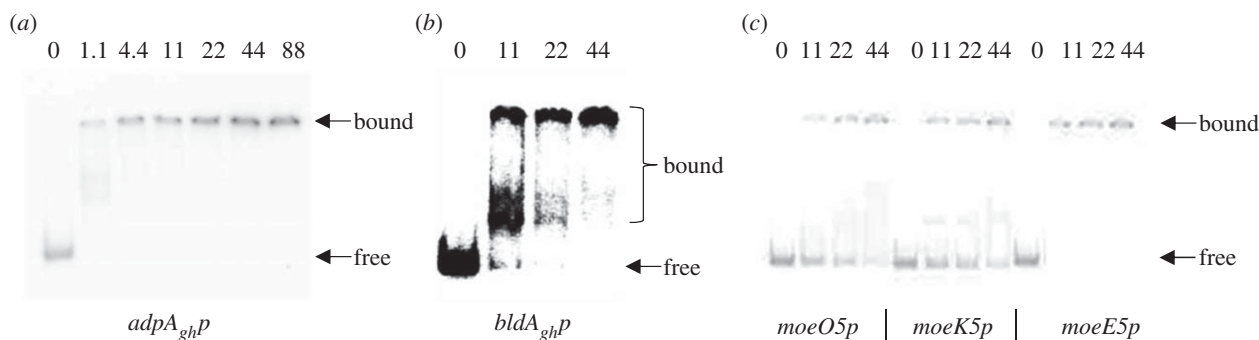


Figure 7. AdpA_{gh} interacts with promoter of its own gene (a), *bldA_{gh}* (b) and several *moe* genes (c). EMSA showing binding of purified AdpA_{gh} to various promoter regions comprising *in silico*-predicted AdpA-binding sites. *moeO5p*, *moeK5p*, *moeE5p*, *adpA_{ghp}* and *bldA_{ghp}* correspond to promoter regions of *moeO5*, *moeK5*, *moeE5*, *adpA_{gh}* and *bldA_{gh}* genes, respectively. Bands corresponding to protein–DNA complexes (bound) and free DNA (free) are indicated. The final amount of AdpA_{gh} (pmol) is indicated above each line.

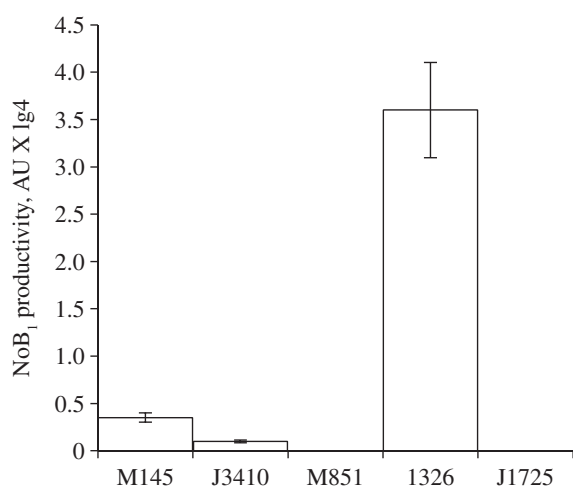


Figure 8. Levels of nosokomicin B₁ production by various streptomycetes expressing cosmid moeno38-5. Column labels: M145, J3410 and M851—wild-type, *rnc* (*absB*)-minus and *bldH* (*adpA*)-minus mutants of *S. coelicolor*, respectively; 1326 and J1725—wild-type and *bldA* null mutant of *S. lividans*, respectively.

regulatory pathway is important for moenomycin production even in other streptomycete heterologous hosts.

Next, we tested NoB₂ production in *adpA*-deficient *S. coelicolor* M851 and *bldA*-deficient *S. lividans* J1725 strains. Mutant and parental strains carrying cosmid moeno38-5 did not differ in growth rate, but NoB₂ production was completely abolished (figure 8). No *moeE5p* activity was revealed in *S. coelicolor* M851. NoB₂ 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 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^{Leu}_{UAA}, 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,

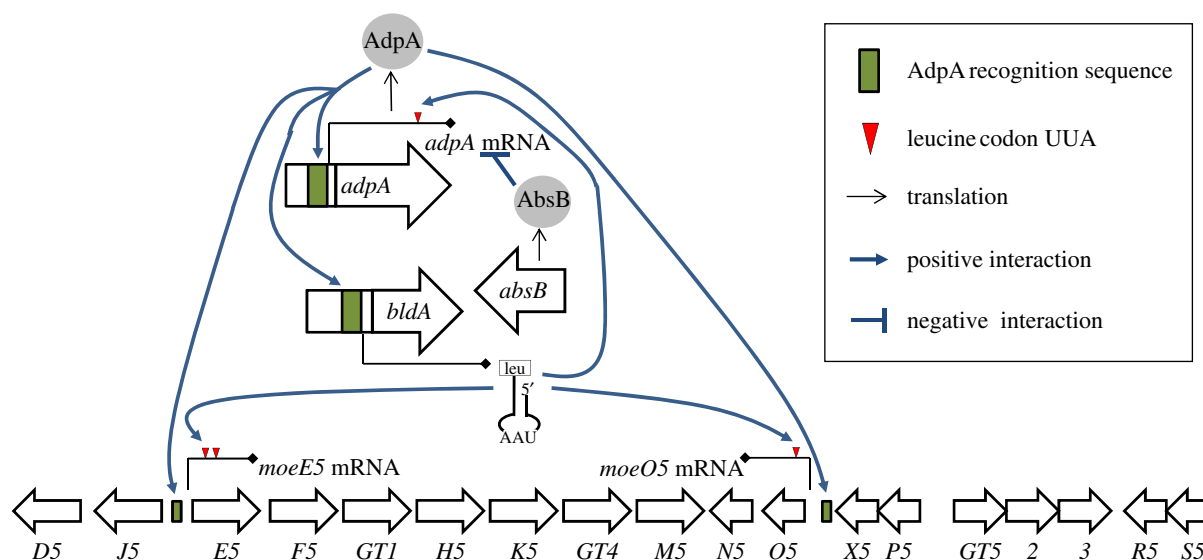


Figure 9. A model of the regulatory pathway that governs moenomycin biosynthesis in *S. ghanensis*.

pacidamycin, albonoursin, acarbose and puromycin all contain putative AdpA operator sequences within certain intergenic regions. Some of these clusters include structural genes containing TTA codons as well (tunicamycin, albonoursin, erythromycin and puromycin clusters). Echoing the idea put forward by Higo *et al.* [24], we think that the low DNA-binding specificity of AdpA may be the key to the evolution of its control over CSR-free antibiotic biosynthetic gene clusters. In fact, AdpA was shown to form the largest bacterial regulon known to date with over 500 genes under its direct control. AdpA, like no other pleiotropic transcriptional factor of *Streptomyces*, would therefore be capable of putting laterally acquired antibiotic biosynthesis gene clusters under growth phase-dependent control. We note that another moenomycin biosynthetic gene cluster, located within the giant plasmid pSCL4 of *Streptomyces clavuligerus* ATCC27064 [52], 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* (B. Ostash 2012, unpublished data). The *moe* gene clusters of *S. ghanensis* and *S. clavuligerus* are syntenous and respective (homologous) gene products share 71–94% similarity [1]; yet, intergenic regions of *S. clavuligerus* *moe* cluster do not contain AdpA-binding sites (data not shown). It will be interesting to determine the distribution of AdpA operators within silent gene clusters of streptomycetes to further elucidate the role of AdpA in control of the secondary metabolome.

The promoter region of tRNA^{Leu}_{UAA}-encoding gene *bldA_{gh}* is under the transcriptional control of AdpA_{gh}. However, *adpA_{gh}* contains one UUA codon in the middle of the coding sequence, putting it under BldA_{gh} translational control. In *S. coelicolor*, *S. griseus* and *S. clavuligerus* [23,30,32,39] this control is very strict, while in *S. ghanensis* Δ *bldA_{gh}* AdpA_{gh} is still detected, albeit at a considerably decreased level. AdpA_{gh} mistranslation in the Δ *bldA_{gh}* mutant could account for this observation, which, to our knowledge, would be the first case for the AdpA family of proteins. However in the same *S. ghanensis* Δ *bldA_{gh}* strain, we failed to detect epimerase MoeE5, which contains two TTA codons, indicating that mistranslation of TTA codons does not occur 100% of the time in the *bldA_{gh}*-minus background.

The severe morphological defects of *S. ghanensis* Δ *bldA_{gh}* vary under different growth conditions, which may contribute

to AdpA_{gh} expression. The complexity and conditionality of the *bldA_{gh}* 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 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 tRNA, absence of which indeed creates a regulatory event in the form of infinite delay of the translation of UUA⁺ 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_{gh} in a Δ *bldA_{gh}* background provides some clues about the early stages of moenomycin biosynthesis as well as morphological differentiation in *S. ghanensis*, when there would be little or no mature tRNA^{Leu}_{UAA} in the cells [18,39]. Just a small amount of AdpA_{gh} available during early stages of growth in the absence of BldA_{gh} could be sufficient to activate transcription from *bldA_{gh}p* leading to an avalanche-like increase in *bldA_{gh}* expression. Once available, charged tRNA^{Leu}_{UAA} could then amplify the translation of *adpA_{gh}* and other, as-yet-unknown, UUA⁺ 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. ghanensis*, 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}_{UAA} is available, it might eventually lead to increased moenomycin production in the cells

overexpressing *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 *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 *bldA*–*adpA*–*absB* is a cross-organism and large-effect system that can be harnessed to generate improved moenomycin producers. Upon combining *absB_{gh}* deletion and *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 *bldA_{gh}* regulation, through the elimination of TTA codons from *moe* genes and *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 *adpA* and *bldA* on SM are widespread and this could be exploited in other biosynthetic pathways. The amenability of SM to rational manipulations is also highlighted by a recent genome-wide study of the clavulanic acid overproducer, in which it was found that a small number of genetic changes, including AdpA overexpression, appeared to be associated with the desired phenotype [62].

5. Material and methods

5.1. Bacterial strains, plasmids and culture conditions

Strains and plasmids used in this study are described in the electronic supplementary material, table S2. *Escherichia coli* strains were grown in Luria-Bertani medium. *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 streptomycetes at 30°C, with shaking at 200 r.p.m. All constructs were transferred into *Streptomyces* conjugally. The presence and stability of inheritance of ϕ C31-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 Makitrynsky *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 Da) and nosokomycin B (NoB; [M-H][−] = 1484.6 Da). The mixture of these two equidominant compounds [64] is referred to as moenomycin in this work. Cosmid moeno38-5 directs the biosynthesis of nosokomycin B₁ (NoB₁; [M-H][−] = 1500.6 Da) and its production was followed in the extracts of heterologous hosts (*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 *moe* clusters, *bldA_{gh}* and *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 *Streptomyces ghanaensis*

Δ *absB_{gh}* and plasmids for complementation experiments

A construct for *absB_{gh}* knockout was prepared as follows. A 2.5 kb DNA fragment containing *absB_{gh}* and its flanking regions were amplified from *S. ghanaensis* genomic DNA by PCR using primers *absBgh_kn_for* and *absBgh_kn_rev*. The PCR product was ligated to SmaI-digested pBluescriptKS+ to yield pBlabsBgh-kn. The *loxP* site-flanked apramycin resistance cassette (*aac(3)IV*) from plasmid pLERCJ was amplified with primers *red_absBgh_kn_for* and *red_absBgh_kn_rev*. The resulting amplicon was used to replace the coding sequence of *absB_{gh}* in pBlabsBgh-kn via recombineering, giving pBlabsBgh-kn::*aac(3)IV*. The latter was digested with BamHI and EcoRI and the fragment containing the *absB_{gh}::aac(3)IV* mutant allele was cloned into the same sites of pKC1139Km to yield pKCabsB-kn::*aac3(IV)*. *Streptomyces ghanaensis* transconjugants carrying the latter were selected for resistance to apramycin (25 μ g ml^{−1}). To generate *S. ghanaensis* single-crossover Am^rKm^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 *absB_{gh}* with *aac(3)IV* in *S. ghanaensis* Δ *absB_{gh}::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* $\Delta absB_{gh}::aac(3)IV$ to evict *aac(3)IV* from its genome. The pUWLCre⁺ transconjugants resistant to tiosrepton were incubated on oatmeal agar plates and selected for apramycin sensitivity. The helper plasmid was lost after two subsequent passages of selected Am^s clone in the absence of thiostrepton. Excision of *aac(3)IV* from the *S. ghanaensis* $\Delta absB_{gh}$ genome was confirmed by PCR (primers *absBgh_ex_for* and *absBgh_ex_rev*; 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_ex_for* and *absBgh_ex_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 *absB_{gh}* 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 *absB_{gh}* 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* (*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 pBladpAkn. To replace *adpA_{gh}* the *aac(3)IV* cassette from pLRECEJ was amplified using primers *adpA_red_for* and *adpA_red_rev*, and the resulting amplicon was used for recombineering-mediated replacement of *adpA_{gh}* 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 $\Delta adpA_{gh}::aac(3)IV$. The obtained amplicon was cloned into EcoRV-digested vector pKC0702. The final *adpA_{gh}* knockout plasmid was labelled pKCHadpA-kn::*aac(3)IV*. Generation of $\Delta adpA_{gh}$ mutant was carried out as described above. Mutant phenotype of *S. ghanaensis* $\Delta adpA_{gh}::aac(3)IV$ was confirmed by PCR using primers *adpA_exp_for* and *adpA_exp_rev*. Generation and verification of *aac(3)IV*-evicted strain $\Delta adpA_{gh}$ was carried out as described for $\Delta absB_{gh}$ 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 *ermEp* 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 *bldAXbaIup* and *bldA-diagn-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 *bldAXbaIup* and *bldAEcoRIrp* and cloned into respective sites of pSET152 to yield pSET152bldA.

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

To probe the activities of *moeE5*, *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 *moeE5p*; *absB_for* and *absB_script_rev* for *absB_{gh}p*; *adpA_for* and *adpA_script_rev* for *adpA_{gh}p*; *bldA_for* and *bldA_script_rev* for *bldA_{gh}p*). 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 XbaI site and downstream primers carrying an EcoRV site (primers *moeE5_for* and *moeE5_rev* for *moeE5*; *adpA_for* and *adpA_rev* for *adpA_{gh}*). The *moeE5* and *adpA_{gh}* fragments were cloned into XbaI–EcoRV-digested pGUSHL4aadA [36], an integrative *Streptomyces* vector where the examined gene is fused to the *gusA* reporter gene through the helical linker HL4 [69], yielding *pmoeE5transl* and *padpAtransl*, 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 (2×10^5 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_{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. ghanaensis* chromosomal DNA. PCR product was purified and cloned into NcoI–XhoI cloning sites of expression vector pET24b, giving pETAdpA_{gh}.

Escherichia coli BL21-GOLD cells harbouring the pETAdpA_{gh} were grown in YTB medium containing 50 µg ml⁻¹ ampicillin and kanamycin until the OD₆₀₀ reached 0.8–1.0. Expression of AdpA_{gh} was induced with 0.4 mM IPTG at 20°C for 16–18 h. After incubation, cells were harvested by centrifugation and lysed in wash buffer (50 mM sodium phosphate, 300 mM sodium chloride) by French press. As AdpA_{gh} was expressed in both soluble and insoluble fractions, the lysate without centrifugation was directly mixed with Cobalt affinity resin. Purification of the protein was performed according to TALON Metal Affinity Resin manual (Clontech). The resin with attached protein was loaded on a column, washed with PBS. AdpA_{gh} was eluted with PBS containing 150 mM imidazole. Aliquots were examined using SDS-polyacrylamide gel electrophoresis and Coomassie blue staining. The eluted fraction was washed from imidazole by dialysis with a storage buffer (50 mM disodium hydrogen phosphate, 300 mM sodium chloride, 1 mM EDTA, 25% glycerol, pH = 7). Purified AdpA_{gh} samples were stored at

–20°C in storage buffer. Protein concentration was determined according to the Bio-Rad DC Protein Assay.

5.11. Gel electrophoretic mobility shift assay

The 500 bp promoter regions of the targeted genes (*adpA_{gh}*, *bldA_{gh}*, *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_{gh}*; *bldA_for* and *bldA_script_rev* for *bldA_{gh}*; *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 [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_{gh} 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.

Acknowledgements. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH. We are grateful to Eriko Takano for providing the *S. coelicolor* M851 strain and Jason Sello for *S. coelicolor* J3410; Nestor Zaburanny is thanked for help in screening of AdpAbs.

Funding statement. 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.

References

- Ostash B, Walker S. 2010 Moenomycin family antibiotics: chemical synthesis, biosynthesis, and biological activity. *Nat. Prod. Rep.* **27**, 1594–1617. (doi:10.1039/c001461n)
- Ostash B, Doud EH, Lin C, Ostash I, Perlstein DL, Fuse S, Wolpert M, Kahne D, Walker S. 2009 Complete characterization of the seventeen step moenomycin biosynthetic pathway. *Biochemistry* **48**, 8830–8841. (doi:10.1021/bi901018q)
- van Wezel GP, McDowall KJ. 2011 The regulation of the secondary metabolism of *Streptomyces*: new links and experimental advances. *Nat. Prod. Rep.* **28**, 1311–1333. (doi:10.1039/c1np00003a)
- Liu G, Chater KF, Chandra G, Niu G, Tan H. 2013 Molecular regulation of antibiotic biosynthesis in *Streptomyces*. *Microbiol. Mol. Biol. Rev.* **77**, 112–143. (doi:10.1128/MMBR.00054-12)
- McKenzie NL, Nodwell JR. 2007 Phosphorylated AbsA2 negatively regulates antibiotic production in *Streptomyces coelicolor* through interactions with pathway-specific regulatory gene promoters. *J. Bacteriol.* **189**, 5284–5292. (doi:10.1128/JB.00305-07)

6. Huang J *et al.* 2005 Cross-regulation among disparate antibiotic biosynthetic pathways of *Streptomyces coelicolor*. *Mol. Microbiol.* **58**, 1276–1287. (doi:10.1111/j.1365-2958.2005.04879.x)
7. Rodríguez M, Núñez LE, Braña AF, Méndez C, Salas JA, Blanco G. 2008 Identification of transcriptional activators for thienamycin and cephamycin C biosynthetic genes within the thienamycin gene cluster from *Streptomyces cattleya*. *Mol. Microbiol.* **69**, 633–645. (doi:10.1111/j.1365-2958.2008.06312.x)
8. Baltz RH. 2011 Strain improvement in actinomycetes in the postgenomic era. *J. Ind. Microbiol. Biotechnol.* **38**, 657–666. (doi:10.1007/s10295-010-0934-z)
9. Ostash B, Saghatelian A, Walker S. 2007 A streamlined metabolic pathway for the biosynthesis of moenomycin A. *Chem. Biol.* **14**, 257–267. (doi:10.1016/j.chembiol.2007.01.008)
10. Makitrynsky R, Rebets Y, Ostash B, Zaburannyi N, Rabyk M, Walker S, Fedorenko V. 2010 Genetic factors that influence moenomycin production in streptomycetes. *J. Ind. Microbiol. Biotechnol.* **37**, 559–566. (doi:10.1007/s10295-010-0701-1)
11. Cortes J, Haydock SF, Roberts GA, Bevvitt DJ, Leadlay PF. 1990 An unusually large multifunctional polypeptide in the erythromycin-producing polyketide synthase of *Saccharopolyspora erythraea*. *Nature* **348**, 176–178. (doi:10.1038/348176a0)
12. Donadio S, Staver MJ, McAlpine JB, Swanson SJ, Katz L. 1991 Modular organization of genes required for complex polyketide biosynthesis. *Science* **252**, 675–679. (doi:10.1126/science.2024119)
13. Kelly WL, Pan L, Li C. 2009 Thiostrepton biosynthesis: prototype for a new family of bacteriocins. *J. Am. Chem. Soc.* **131**, 4327–4334. (doi:10.1021/ja807890a)
14. Wyszynski FJ, Hesketh AR, Bibb MJ, Davis BG. 2010 Dissecting tunicamycin biosynthesis by genome mining: cloning and heterologous expression of a minimal gene cluster. *Chem. Sci.* **1**, 581–589. (doi:10.1039/C0SC00325E)
15. Zhang W, Ostash B, Walsh CT. 2010 Identification of the biosynthetic gene cluster for the pacidamycin group of peptidyl nucleoside antibiotics. *Proc. Natl Acad. Sci. USA* **107**, 16 828–16 833. (doi:10.1073/pnas.1011557107)
16. Claesen J, Bibb MJ. 2011 Biosynthesis and regulation of grisemycin, a new member of the linaridin family of ribosomally synthesized peptides produced by *Streptomyces griseus* IFO 13350. *J. Bacteriol.* **193**, 2510–2516. (doi:10.1128/JB.00171-11)
17. Zaburannyi N, Ostash B, Fedorenko V. 2009 TTA Lynx: a web-based service for analysis of actinomycete genes containing rare TTA codon. *Bioinformatics* **25**, 2432–2433. (doi:10.1093/bioinformatics/btp402)
18. Leskiw BK, Mah R, Lawlor EJ, Chater KF. 1993 Accumulation of *bldA* specified tRNA is temporally regulated in *Streptomyces coelicolor* A3(2). *J. Bacteriol.* **175**, 1995–2005.
19. Chater KF, Chandra G. 2008 The use of the rare UUA codon to define ‘expression space’ for genes involved in secondary metabolism, development and environmental adaptation in streptomycetes. *J. Microbiol.* **46**, 1–11. (doi:10.1007/s12275-007-0233-1)
20. Chandra G, Chater KF. 2008 Evolutionary flux of potentially *bldA*-dependent *Streptomyces* genes containing the rare leucine codon TTA. *Antonie Van Leeuwenhoek* **94**, 111–126. (doi:10.1007/s10482-008-9231-5)
21. Wang J, Schully KL, Pettis GS. 2009 Growth-regulated expression of a bacteriocin, produced by the sweet potato pathogen *Streptomyces ipomoeae*, that exhibits interstrain inhibition. *Appl. Environ. Microbiol.* **75**, 1236–1242. (doi:10.1128/AEM.01598-08)
22. Ohnishi Y, Kameyama S, Onaka H, Horinouchi S. 1999 The A-factor regulatory cascade leading to streptomycin biosynthesis in *Streptomyces griseus*: identification of a target gene of the A-factor receptor. *Mol. Microbiol.* **34**, 102–111. (doi:10.1046/j.1365-2958.1999.01579.x)
23. Takano E *et al.* 2003 A rare leucine codon in *adpA* is implicated in the morphological defect of *bldA* mutants of *Streptomyces coelicolor*. *Mol. Microbiol.* **50**, 475–486. (doi:10.1046/j.1365-2958.2003.03728.x)
24. Higo A, Hara H, Horinouchi S, Ohnishi Y. 2012 Genome-wide distribution of AdpA, a global regulator for secondary metabolism and morphological differentiation in *Streptomyces*, revealed the extent and complexity of the AdpA regulatory network. *DNA Res.* **19**, 259–273. (doi:10.1093/dnares/dss010)
25. Xu W, Huang J, Lin R, Shi J, Cohen SN. 2010 Regulation of morphological differentiation in *S. coelicolor* by RNase III (AbsB) cleavage of mRNA encoding the AdpA transcription factor. *Mol. Microbiol.* **75**, 781–791. (doi:10.1111/j.1365-2958.2009.07023.x)
26. Ohnishi Y, Yamazaki H, Kato J-Y, Tomono A, Horinouchi S. 2005 AdpA, a central transcriptional regulator in the A-factor regulatory cascade that leads to morphological development and secondary metabolism in *Streptomyces griseus*. *Biosci. Biotechnol. Biochem.* **69**, 431–439. (doi:10.1271/bbb.69.431)
27. Horinouchi S. 2007 Mining and polishing of the treasure trove in the bacterial genus *Streptomyces*. *Biosci. Biotechnol. Biochem.* **71**, 283–299. (doi:10.1271/bbb.60627)
28. Akanuma G, Hara H, Ohnishi Y, Horinouchi S. 2009 Dynamic changes in the extracellular proteome caused by absence of a pleiotropic regulator AdpA in *Streptomyces griseus*. *Mol. Microbiol.* **73**, 898–912. (doi:10.1111/j.1365-2958.2009.06814.x)
29. Wolański M, Jakimowicz D, Zakrzewska-Czerwińska J. 2012 AdpA, key regulator for morphological differentiation regulates bacterial chromosome replication. *Open Biol.* **2**, 120097. (doi:10.1098/rsob.120097)
30. Nguyen KT, Tenor J, Stettler H, Nguyen LT, Nguyen LD, Thompson CJ. 2003 Colonial differentiation in *Streptomyces coelicolor* depends on translation of a specific codon within the *adpA* gene. *J. Bacteriol.* **185**, 7291–7296. (doi:10.1128/JB.185.24.7291-7296)
31. Zhu D, He X, Zhou X, Deng Z. 2005 Expression of the *melC* operon in several *Streptomyces* strains is positively regulated by AdpA, an AraC family transcriptional regulator involved in morphological development in *Streptomyces coelicolor*. *J. Bacteriol.* **187**, 3180–3187. (doi:10.1128/JB.187.9.3180-3187.2005)
32. López-García MT, Santamarta I, Liras P. 2010 Morphological differentiation and clavulanic acid formation are affected in a *S. clavuligerus adpA*-deleted mutant. *Microbiology* **156**, 2354–2365. (doi:10.1099/mic.0.035956-0)
33. Yamazaki H, Tomono A, Ohnishi Y, Horinouchi S. 2004 DNA-binding specificity of AdpA, a transcriptional activator in the A-factor regulatory cascade in *Streptomyces griseus*. *Mol. Microbiol.* **53**, 555–572. (doi:10.1111/j.1365-2958.2004.04153.x)
34. Hopwood DA, Wildermuth H, Palmer HM. 1970 Mutants of *Streptomyces coelicolor* defective in sporulation. *J. Gen. Microbiol.* **61**, 397–408. (doi:10.1099/00221287-61-3-397)
35. Du YL, Li SZ, Zhou Z, Chen SF, Fan WM, Li YQ. 2011 The pleiotropic regulator AdpA_{ch} is required for natamycin biosynthesis and morphological differentiation in *Streptomyces chattanoogaensis*. *Microbiology* **157**, 1300–1311. (doi:10.1099/mic.0.046607-0)
36. Myronovskiy M, Welle E, Fedorenko V, Luzhetskyy A. 2011 Beta-glucuronidase as a sensitive and versatile reporter in actinomycetes. *Appl. Environ. Microbiol.* **77**, 5370–5383. (doi:10.1128/AEM.00434-11)
37. Passantino R, Puglia A-M, Chater K. 1991 Additional copies of the *actII* regulatory gene induce actinorhodin production in pleiotropic *bld* mutants of *Streptomyces coelicolor* A3(2). *J. Gen. Microbiol.* **137**, 2059–2064. (doi:10.1099/00221287-137-9-2059)
38. Guthrie EP, Flaxman CS, White J, Hodgson DA, Bibb MJ, Chater KF. 1998 A response-regulator-like activator of antibiotic synthesis from *Streptomyces coelicolor* A3(2) with an amino-terminal domain that lacks a phosphorylation pocket. *Microbiology* **144**, 727–738. (doi:10.1099/00221287-144-3-727)
39. Higo A, Horinouchi S, Ohnishi Y. 2011 Strict regulation of morphological differentiation and secondary metabolism by a positive feedback loop between two global regulators AdpA and BldA in *Streptomyces griseus*. *Mol. Microbiol.* **81**, 1607–1622. (doi:10.1111/j.1365-2958.2011.07795.x)
40. Wolański M, Donczew R, Kois-Ostrowska A, Masiewicz P, Jakimowicz D, Zakrzewska-Czerwińska J. 2011 The level of AdpA directly affects expression of developmental genes in *Streptomyces coelicolor*. *J. Bacteriol.* **193**, 6358–6365. (doi:10.1128/JB.05734-11)
41. Sello JK, Buttner MJ. 2008 The gene encoding RNase III in *Streptomyces coelicolor* is transcribed

- during exponential phase and is required for antibiotic production and for proper sporulation. *J. Bacteriol.* **190**, 4079–4083. (doi:10.1128/JB.01889-07)
42. Pan Y, Liu G, Yang H, Tian Y, Tan H. 2009 The pleiotropic regulator AdpA-L directly controls the pathway-specific activator of nikkomycin biosynthesis in *Streptomyces ansochromogenes*. *Mol. Microbiol.* **72**, 710–723. (doi:10.1111/j.1365-2958.2009.06681.x)
 43. Bibb MJ. 2005 Regulation of secondary metabolism in streptomycetes. *Curr. Opin. Microbiol.* **8**, 208–215. (doi:10.1016/j.mib.2005.02.016)
 44. Olynyk M, Samborskyy M, Lester JB, Mironenko T, Scott N, Dickens S, Haydock SF, Leadlay PF. 2007 Complete genome sequence of the erythromycin-producing bacterium *Saccharopolyspora erythraea* NRRL23338. *Nat. Biotechnol.* **25**, 447–453. (doi:10.1038/nbt1297)
 45. Lautru S, Gondry M, Genet R, Pernodet JL. 2002 The albonoursin gene cluster of *Streptomyces noursei* biosynthesis of diketopiperazine metabolites independent of nonribosomal peptide synthetases. *Chem. Biol.* **9**, 1355–1364. (doi:10.1016/S1074-5521(02)00285-5)
 46. Li C, Kelly WL. 2010 Recent advances in thiopeptide antibiotic biosynthesis. *Nat. Prod. Rep.* **27**, 153–164. (doi:10.1039/b922434c)
 47. Tercero JA, Espinosa JC, Jiménez A. 1998 Expression of the *Streptomyces alboniger pur* cluster in *Streptomyces lividans* is dependent on the *bldA*-encoded tRNA^{Leu}. *FEBS Lett.* **421**, 221–223. (doi:10.1016/S0014-5793(97)01564-0)
 48. Rackham EJ, Grüşchow S, Ragab AE, Dickens S, Goss RJ. 2010 Pacidamycin biosynthesis: identification and heterologous expression of the first uridyl peptide antibiotic gene cluster. *Chembiochem* **11**, 1700–1709. (doi:10.1002/cbic.201000200)
 49. Wehmeier UF, Piepersberg W. 2004 Biotechnology and molecular biology of the alpha-glucosidase inhibitor acarbose. *Appl. Microbiol. Biotechnol.* **63**, 613–625. (doi:10.1007/s00253-003-1477-2)
 50. Yu Y *et al.* 2005 Gene cluster responsible for validamycin biosynthesis in *Streptomyces hygroscopicus* subsp. *jinggangensis* 5008. *Appl. Environ. Microbiol.* **71**, 5066–5076. (doi:10.1128/AEM.71.9.5066-5076)
 51. Hara H, Ohnishi Y, Horinouchi S. 2009 DNA microarray analysis of global gene regulation by A-factor in *Streptomyces griseus*. *Microbiology* **155**, 2197–2210. (doi:10.1099/mic.0.027862-0)
 52. Medema MH *et al.* 2010 The sequence of a 1.8-mb bacterial linear plasmid reveals a rich evolutionary reservoir of secondary metabolic pathways. *Genome Biol. Evol.* **2**, 212–224. (doi:10.1093/gbe/evq013)
 53. Lawlor EJ, Baylis HA, Chater KF. 1987 Pleiotropic morphological and antibiotic deficiencies result from mutations in a gene encoding a tRNA-like product in *Streptomyces coelicolor* A3(2). *Genes Dev.* **1**, 1305–1310. (doi:10.1101/gad.1.10.1305)
 54. Leskiw BK, Lawlor EJ, Fernandez-Abalos JM, Chater KF. 1991 TTA codons in some genes prevent their expression in a class of developmental, antibiotic-negative, *Streptomyces* mutants. *Proc. Natl Acad. Sci. USA* **88**, 2461–2465. (doi:10.1073/pnas.88.6.2461)
 55. den Hengst CD, Tran NT, Bibb MJ, Chandra G, Leskiw BK, Buttner MJ. 2010 Genes essential for morphological development and antibiotic production in *Streptomyces coelicolor* are targets of BldD during vegetative growth. *Mol. Microbiol.* **78**, 361–379. (doi:10.1111/j.1365-2958.2010.07338.x)
 56. Pettersson BM, Kirsebom LA. 2011 tRNA accumulation and suppression of the *bldA* phenotype during development in *Streptomyces coelicolor*. *Mol. Microbiol.* **79**, 1602–1614. (doi:10.1111/j.1365-2958.2011.07543.x)
 57. Gramajo HC, Takano E, Bibb MJ. 1993 Stationary-phase production of the antibiotic actinorhodin in *Streptomyces coelicolor* A3(2) is transcriptionally regulated. *Mol. Microbiol.* **7**, 837–845. (doi:10.1111/j.1365-2958.1993.tb01174.x)
 58. Hodgson DA. 2000 Primary metabolism and its control in streptomycetes: a most unusual group of bacteria. *Adv. Microb. Physiol.* **42**, 47–238. (doi:10.1016/S0065-2911(00)42003-5)
 59. Endler K, Schuricht U, Hennig L, Welzel P, Holst U, Aretz W, Bottger D, Huber G. 1998 Exploratory investigations into the biosynthesis of the antibiotic moenomycin A. *Tetrahedron Lett.* **39**, 13–16. (doi:10.1016/S0040-4039(97)10456-7)
 60. Tan GY, Bai L, Zhong JJ. 2013 Exogenous 1,4-butyrolactone stimulates A-factor-like cascade and validamycin biosynthesis in *Streptomyces hygroscopicus* 5008. *Biotechnol. Bioeng.* **110**, 2984–2993. (doi:10.1002/bit.24965)
 61. Lee HN, Kim JS, Kim P, Lee HS, Kim ES. 2013 Repression of antibiotic down-regulator WbIA by AdpA in *Streptomyces coelicolor*. *Appl. Environ. Microbiol.* **79**, 4159–4163. (doi:10.1128/AEM.00546-13)
 62. Medema MH, Alam MT, Heijne WH, van den Berg MA, Müller U, Trefzer A, Bovenberg RA, Breitling R, Takano E. 2011 Genome-wide gene expression changes in an industrial clavulanic acid overproduction strain of *Streptomyces clavuligerus*. *Microb. Biotechnol.* **4**, 300–305. (doi:10.1111/j.1751-7915.2010.00226.x)
 63. Ostash B, Makitrinsky R, Walker S, Fedorenko V. 2009 Identification and characterization of *Streptomyces ghanaensis* ATCC14672 integration sites for three actinophage-based plasmids. *Plasmid* **61**, 171–175. (doi:10.1016/j.plasmid.2008.12.002)
 64. Ostash B, Doud E, Walker S. 2012 ABC transporter genes from *Streptomyces ghanaensis* moenomycin biosynthetic gene cluster: roles in antibiotic production and export. *Arch. Microbiol.* **194**, 915–922. (doi:10.1007/s00203-012-0827-9)
 65. Sambrook J, Fritsch EF, Maniatis T. 1989 *Molecular cloning. A laboratory manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
 66. Gust B. 2009 Cloning and analysis of natural product pathways. *Methods Enzymol.* **458**, 159–180. (doi:10.1016/S0076-6879(09)04807-1)
 67. Bailey TL, Elkan C. 1994 Fitting a mixture model by expectation maximization to discover motifs in biopolymers. *Proc. Int. Conf. Intell. Syst. Mol. Biol.* **2**, 28–36.
 68. Grant CE, Bailey TL, Noble WS. 2011 FIMO: Scanning for occurrences of a given motif. *Bioinformatics* **27**, 1017–1018. (doi:10.1093/bioinformatics/btr064)
 69. Arai R, Ueda H, Kitayama A, Kamiya N, Nagamune T. 2001 Design of the linkers which effectively separate domains of a bifunctional fusion protein. *Protein Eng.* **14**, 529–532. (doi:10.1093/protein/14.8.529)