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Citation

Published Version
doi:10.1098/rsob.130121

Accessed
June 28, 2017 3:15:26 PM EDT

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

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1. Summary

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

Moenomycins (Mms) are a small family of secondary metabolites of actinomycete origin that display a number of remarkable traits in terms of their chemistry and biology [1]. Classified as phosphoglycolipids, they result from a unique assembly of glycoside- and isoprene-derived moieties bridged by 3-phosphoglyceric acid—an unprecedented building block in secondary metabolism (SM). Moenomycins strongly interfere with the growth of mainly Gram-positive bacteria, including VRE and MRSA pathogens, through direct inhibition of peptidoglycan glycosyltransferases (PGTs). High potency of these antibiotics and their unique mode of action explain much of the industrial and academic interest in them. We have recently identified genes for moenomycin production (moe 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 gene 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 seven 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<sub>Leu<sup>UAA</sup></sub> (specified by bloA gene) is only formed during late stationary growth, defining the onset of hyphae and antibiotic production [18,19]. *bloA* regulation occurs via the presence of UUA codons within CSR genes [20]. Recent work on ipomicin biosynthesis has provided initial evidence that *bloA* also regulates the translation of structural SM genes [21]. We hypothesize that *bloA* regulates moenomycin production at the level of translation of *moe* genes. However, it is unlikely that *bloA* 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<sub>gh</sub>, an *S. ghanaensis* orthologue of well-known *S. coelicolor* and *Streptomyces griseus* master regulator AdpA [22–24], is an important direct activator of *moe* gene expression. The translation of UUA-containing *adpA<sub>gh</sub>* mRNA is dependent on *bloA*-encoded tRNA, although this dependence is not absolute. Finally, we provide circumstantial evidence that AdpA<sub>gh</sub> expression is regulated at the posttranscriptional level through the action of the *absB<sub>gh</sub>* gene, encoding an orthologue of *S. coelicolor* RNase III [25]. Together these data outline the involvement of three interacting global regulatory genes, *absB<sub>b</sub>*–*adpA<sub>b</sub>*–*bloA<sub>b</sub>, in control of a CSR-free secondary metabolic pathway. The first gene, *absB*, directly regulates *adpA* expression, *bloA* 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 BloA 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* genomic 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<sub>Leu<sup>UAA</sup></sub> (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).
In our *in silico* analysis [10] of *S. ghanaensis*, we identified an AdpA orthologue in *S. ghanaensis* and designated it as *adpA*<sub>gh</sub>. The coding sequence of *adpA*<sub>gh</sub> contains one TTA codon (figure 1), at the same position as other AdpA orthologues [23,30–32]. Genes for several AdpA orthologues are present in the *S. ghanaensis* genome (see the electronic supplementary material, table S1). Additionally, a single copy of the tRNA<sub>Leu</sub><sup>*UAU*</sup> gene was identified in the *S. ghanaensis* genome (designated as *absB*<sub>gh</sub>; figure 1).

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

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

Deletion of *adpA*<sub>gh</sub> in the *S. ghanaensis* chromosome completely abolished moenomycin production, as determined by LC-MS (figure 2) and bioassays. No mass peaks corresponding to the earliest known moenomycin precursors [2] were found in the extracts of *adpA*<sub>gh</sub> mutant (Δ*adpA*<sub>gh</sub>), showing that moenomycin production was blocked at the initial first steps. Knockout of *adpA*<sub>gh</sub> had a significant influence on the morphological development *S. ghanaensis*. On solid media, a phenotype of *S. ghanaensis* Δ*adpA*<sub>gh</sub> 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*<sub>gh</sub> were restored to the wild-type state upon introduction of an intact copy of *adpA*<sub>gh</sub> (plasmid pSETadpA-exp). Insertion of an extra copy of *adpA*<sub>gh</sub>, under the control of a strong constitutive promoter *erm*<sub>Ep</sub> (plasmid pTESadpA-exp),
caused a 2.5-fold increase in moenomycin production compared with the control strain (figure 2).

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

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

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

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

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

Figure 3. Lawns of S. ghanaensis mutants described in this study. (a) Wild-type S. ghanaensis ATCC14672, S. ghanaensis ΔabsBgh, S. ghanaensis ΔbldAgh and S. ghanaensis ΔadpAgh (clockwise from the top left) were grown on TSB agar medium. (b) Respective mutants carrying plasmids for complementation, pSOKEabsBgh-exp, pSET152bldA and pSETadpA-exp.
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 ΔmoeA5p strain (figure 5). The moeE5 transcription was increased more than twofold and threefold from wild-type levels in moeO5p and moeE5p strains, respectively (figure 5), in agreement with RT-PCR data (figure 4). While the pattern of moeEp activity in ΔmoeA5p and ΔabsB5p is as anticipated [25], increased levels of moeEp transcript in the ΔmoeA5p are somewhat unexpected. A plausible explanation is that moeEp might be a target of an as-yet-unknown repressor(s) positively regulated by BldA, in which case the deletion of bldA would remove the repressive signal. To further delineate the involvement of bldA in the translational regulation of moemonycin production, we analysed GusA activity of translational fusions of gusA to moeE5 (plasmid pmoE5trans) and adpA5p in a ΔbldA5p background. We found no GusA activity in ΔbldA5p carrying moeE5–gusA fusion (figure 6), underscoring the essentiality of bldA for translation of the two UUA codons in moeE5 mRNA. Surprisingly, GusA activity was detected in the ΔbldA5p strain carrying adpA5p–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 adpA5p UUA codon in the absence of tRNA^{UUA} [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 adpA5p transcription. In comparison to the wild-type strain, adpA5p levels (figure 5) increased 2.3-fold in *S. ghanaensis* ΔabsB5p and were almost undetectable in the ΔmoeA5p strain (figure 5). We also measured the level of translation when adpA5p and the entire adpA5 genes were fused to gusA (padpAtransl; see §5) and found it in ΔabsB5p to be double that of wild-type (figure 6). Thus, AdpA acts as a positive activator of its own expression and its activity is increased in the absence of ribonucleolytic activity of AbsB. This conclusion is supported by observations in other streptomycetes [25,40]. Similar to our moeEp data, adpA5p activity was also significantly increased in the ΔmoeA5p strain (figure 5), suggesting the existence of an unidentified bldA-dependent repressor(s) of AdpA5p-regulated promoters.

There was no difference between absB5p transcriptional activity in ΔmoeA5p and wild-type strains, indicating that AdpA5p does not influence the transcription of absB5p.

At the same time, we revealed almost complete cessation of bldA5p transcription in the ΔmoeA5p strain (figure 5).

3.4. AdpA5p interacts with promoters of bldA5p, adpA5p and key moe genes

The GusA reporter data suggested that AdpA5p is a transcriptional activator that regulates its own expression as well as that of bldA5p and moe genes. To test this, we set out to demonstrate AdpA5p binding to moeO5, moeK5, moeE5, bldA5p, and adpA5p promoter regions using electrophoretic mobility shift assay (EMSA). A C-terminally His-tagged derivative of AdpA5p was overexpressed in *Escherichia coli* and purified to homogeneity (see the electronic supplementary material, figure S2). Increasing amounts of AdpA5p-His were incubated with radiolabelled DNA probes corresponding to the promoter regions of interest, and the complexes were separated by native gel electrophoresis. Purified AdpA5p-His was bound to the promoter regions of moeO5, moeK5, moeE5, bldA5p, and adpA5p in quantities as low as 1.1–11.0 pmol. Increasing concentrations of AdpA5p resulted in more than one protein–DNA complex (padpAtransl; see §5) and found it in ΔmoeA5p to be double that of wild-type (figure 6). Thus, AdpA acts as a positive activator of its own expression and its activity is increased in the absence of ribonucleolytic activity of AbsB. This conclusion is supported by observations in other streptomycetes [25,40]. Similar to our moeEp data, adpA5p activity was also significantly increased in the ΔmoeA5p strain (figure 5), suggesting the existence of an unidentified bldA-dependent repressor(s) of AdpA5p-regulated promoters.

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At the same time, we revealed almost complete cessation of bldA5p transcription in the ΔmoeA5p strain (figure 5).
all AdpA operators will be occupied by the recombinant protein. Finally, we confirmed that AdpA gh binds to its own promoter (figure 7). Low concentrations of AdpA gh (4.4 pmol) caused the appearance of intermediate nucleoprotein complexes, whereas saturation of the reaction mixture with AdpAgh resulted in the formation of single band.

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

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

To determine the level of moenomycins biosynthesis on a ΔabsB- background, a cosmid moeno38-5 [10] carrying the main part of moe cluster 1 and directing the production of nosokomycin B2 (NoB2) was introduced into S. coelicolor ΔabsB strain J3410 [41]. S. coelicolor J3410 moeno38-5 ΔabsB was grown in parallel with a control strain S. coelicolor M145 moeno38-5 ΔabsB and NoB2 was quantified. On average, J3410 moeno38-5 ΔabsB accumulated 20% less biomass than M145 moeno38-5 ΔabsB and produced three times less NoB2 compared with the control strain (figure 8). These data correlate with the results of reporter experiments, where we observed a 1.5-fold decrease in moeE5 transcription in a ΔabsBgh strain compared with a control M145 strain (data not shown). Our results suggest that the AbsB RNase III-mediated
respectively; 1326 and J1725—wild-type and wild-type, expressing cosmid moeno38-5. Column labels: M145, J3410 and M851—S. coelicolor M851 and J1725 strains. NoB2 production was restored to M851 and J1725 upon introduction of the latter is indicated above each line.

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

**Figure 8.** Levels of nosokomycin B1 production by various streptomycetes expressing cosmid moeno38-5. Column labels: M145, J3410 and M851—wild-type, mo (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 adpAgh and bldAgh, 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,
The translation of TTA codons does not occur 100% of the time in MoeE5, which contains two TTA codons, indicating that mis-

same the first case for the AdpA family of proteins. However in the

for this observation, which, to our knowledge, would be

trl-very strict, while in

S. clavuligerus moe

streptomycetes to further elucidate the role of AdpA in control

bution of AdpA operators within silent gene clusters of

S. clavuligerus

production of moenomycins by

lome. Despite numerous attempts, we failed to detect the

for the importance of AdpA control over the secondary metabo-

gerus

located within the giant plasmid pSCL4 of

We note that another moenomycin biosynthetic gene cluster,

therefore be capable of putting laterally acquired antibiotic bio-

y vary under different growth conditions, which may contribute
to AdpA expression. The complexity and conditionality of the

bldA 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 tran-

scripts 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+-

transcripts. Recent work showed that, compared with the more abundant RNAs, accumulation of primary bldA tran-

script 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 fac-

tors, which might be manifested in the form of leaky translation of UUA codons in the absence of cognate tRNA. The leaky translation of AdpA in a ΔbldA background provides some clues about the early stages of moenomycin biosynthesis as well as morphological differentiation in

S. ghancaensis, when there would be little or no mature

tRNALeuUAA in the cells [18,39]. Just a small amount of

AdpA, available during early stages of growth in the absence of BldA, could be sufficient to activate transcription from

bldA leading to an avalanche-like increase in

bldA expression. Once available, charged tRNALeuUAA could then

amplify the translation of AdpA, and other, as-yet-unknown, UUA+-

genes that lead to the downregulation of AdpA and

moeE5 promoters. Our data confirm the presence of a regulat-

ory feedback loop that amplifies a signal in dual regulation of

BldA–AdpA in S. ghancaensis, as was previously shown in

S. griseus [39].

The increased transcription of AdpA from constitutive promoter

ermE improved moenomycin production 2.5-fold in spite of the fact that (as our work shows) it is the translation efficiency of UUA-containing AdpA mRNA that should determine the degree of activation of moe genes. At the moment, we cannot fully explain our results although several possible scenarios can be outlined. First, once the charged tRNALeuUAA is available, it might eventually lead to increased moenomycin production in the cells

pacidamycin, albonoursin, acarbose and puromycin all contain

putative AdpA operator sequences within certain interge-

ric 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

over CSR-free antibiotic biosynthetic gene clusters. In fact, AdpA

is a unique tRNA, absence of which indeed creates a 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 trans-

scripts 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+-

transcripts. Recent work showed that, compared with the more abundant RNAs, accumulation of primary bldA tran-

script 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 fac-

tors, which might be manifested in the form of leaky translation of UUA codons in the absence of cognate tRNA. The leaky translation of AdpA in a ΔbldA background provides some clues about the early stages of moenomycin biosynthesis as well as morphological differentiation in

S. ghancaensis, when there would be little or no mature

tRNALeuUAA in the cells [18,39]. Just a small amount of

AdpA, available during early stages of growth in the absence of BldA, could be sufficient to activate transcription from

bldA leading to an avalanche-like increase in

bldA expression. Once available, charged tRNALeuUAA could then

amplify the translation of AdpA, and other, as-yet-unknown, UUA+-

genes that lead to the downregulation of AdpA and

moeE5 promoters. Our data confirm the presence of a regulat-

ory feedback loop that amplifies a signal in dual regulation of

BldA–AdpA in S. ghancaensis, as was previously shown in

S. griseus [39].

The increased transcription of AdpA from constitutive promoter

ermE improved moenomycin production 2.5-fold in spite of the fact that (as our work shows) it is the translation efficiency of UUA-containing AdpA mRNA that should determine the degree of activation of moe genes. At the moment, we cannot fully explain our results although several possible scenarios can be outlined. First, once the charged tRNALeuUAA is available, it might eventually lead to increased moenomycin production in the cells

Figure 9. A model of the regulatory pathway that governs moenomycin biosynthesis in S. ghancaensis.
overexpressing \( \text{adpA}_{\text{gh}} \) 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 \( \text{adpA}_{\text{gh}} \) mRNA is increased it might increase the probability of its mistranslation; this may also trigger moenomycin overproduction. Whatever the real mechanism is, it is practically useful because antibiotic titre improvement is a key requirement for the industry and it was one of the motivations for this work. In the case of CSR-free gene clusters, random mutagenesis and screening remain the only practical means to improve secondary metabolite production [59]. Recombinant DNA technology has yet to prove its utility for many industrial needs. Here, we demonstrate that the regulatory network \( \text{blda}–\text{adpA}–\text{absB} \) is a cross-organism and large-effect system that can be harnessed to generate improved moenomycin producers. Upon combining \( \text{absB}_{\text{gh}} \) deletion and \( \text{adpA}_{\text{gh}} \) overexpression in \( S. \text{ghanaensis} \), we observe, on average, a sevenfold increase in moenomycin production (data not shown). We anticipate that moenomycin titres can be further improved by bypassing \( \text{blda} \) regulation, through the elimination of TTA codons from \( \text{moe} \) genes and \( \text{adpA}_{\text{gh}} \). 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 \( \text{SM} \) are widespread and this could be exploited in other biosynthetic pathways. The amenability of \( \text{SM} \) to rational manipulations is also highlighted by a recent genome-wide study of the clavulanic acid overproducer, in which it was found that a small number of genetic changes, including AdpA overexpression, appeared to be associated with the desired phenotype [62].

5. Material and methods

5.1. Bacterial strains, plasmids and culture conditions

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

5.2. Procedures for DNA manipulation

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

5.3. Quantitative analysis of moenomycins production

Growth of the strains, moenomycin extraction, conditions of LC-MS and quantitative analysis of the data are described by Ostash et al. [2] and 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 \( S. \text{ghanaensis} \) extracts: \( \text{MmA} \) ([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 (\( S. \text{lividans} \) and \( S. \text{coelicolor} \)). 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 \( S. \text{ghanaensis} \), known AdpAbs sequences were collected from GenBank. This dataset was used as input for the MEME software tool [67] to search for the consensus motif. Search for the occurrence of the identified motif within \( \text{moe} \) clusters, \( \text{blda} \) and \( \text{adpA} \) promoter regions was performed using FIMO software suite [68].

5.5. Semiquantitative RT-PCR

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

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

A construct for \( \text{absB}_{\text{gh}} \) knockout was prepared as follows. A 2.5 kb DNA fragment containing \( \text{absB}_{\text{gh}} \) and its flanking regions were amplified from \( S. \text{ghanaensis} \) genomic DNA by PCR using primers \( \text{absBgh}_{\text{kn}} \_\text{for} \) and \( \text{absBgh}_{\text{kn}} \_\text{rev} \). The PCR product was digested with SmaI-digested pBluescriptKS+ to yield pBlabsBgh-kn. The \( \text{loxP} \) site-flanked apramycin resistance cassette (aac(3)IV) from plasmid pLERECJ was amplified with primers \( \text{red}_{\text{absBgh}} \_\text{kn} \_\text{for} \) and \( \text{red}_{\text{absBgh}} \_\text{kn} \_\text{rev} \). The resulting amplicon was used to replace the coding sequence of \( \text{absB}_{\text{gh}} \) in pBlabsBgh-kn via recombineering, giving pBlabsBgh-kn::aac(3)IV. The latter was digested with BamHI and EcoRI and the fragment containing the \( \text{absB}_{\text{gh}}::\text{aac(3)IV} \) mutant allele was cloned into the same sites of pKCabsB-kn via recombineering, giving pBlabsBgh-kn::aac(3)IV. The resulting amplicon was used to replace the coding sequence of \( \text{absB}_{\text{gh}} \) in pBlabsBgh-kn via recombineering, giving pBlabsBgh-kn::aac(3)IV. The latter was digested with BamHI and EcoRI and the fragment containing the \( \text{absB}_{\text{gh}}::\text{aac(3)IV} \) mutant allele was cloned into the same sites of pKC1139Kn to yield pKCabsB-kn::aac(3)IV. The \( S. \text{streptomyces ghanaensis} \) transconjugants carrying the latter were selected for resistance to apramycin (25 µg ml\(^{-1}\)). To generate \( S. \text{ghanaensis} \) single-crossover Am\(^{Km}\) 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 \( \text{absB}_{\text{gh}} \) with \( \text{aac(3)IV} \) in \( S. \text{ghanaensis} \) \( \Delta \text{absB}_{\text{gh}}::\text{aac(3)IV} \) was confirmed by PCR (primers \( \text{absBgh}_{\text{ex}} \_\text{for} \) and \( \text{absBgh}_{\text{ex}} \_\text{rev} \); data not shown). The
Cre-expressing helper plasmid pUWLCre was then introduced into *S. ghanaensis ΔabsB$_{gh}$-aac(3)IV to evict aac(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 aac(3)IV from the *S. ghanaensis ΔabsB$_{gh}$ genome was confirmed by PCR (primers absB$_{gh}$ex_for and absB$_{gh}$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 pSOKabsBgh-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 absB$_{gh}$ex_for and absB$_{gh}$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-II is based on pSOK804 and carries a 2.2 kb DNA fragment containing three genes, SSFG$_{02131.1}$, SSFG$_{02130.1}$ and SSFG$_{02129.1}$ (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* ΔadpA$_{gh}$ 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$_{gh}$kn_for and adpA$_{gh}$kn_rev. The resulting amplicon was ligated to EcoRV-digested pBluescriptKS+ to yield pBladpAkn. To replace adpA$_{gh}$ the aac(3)IV cassette from pLERECl was amplified using primers adpA$_{gh}$red_for and adpA$_{gh}$red_rev, and the resulting amplicon was used for recombineering-mediated replacement of adpA$_{gh}$ within pBladpAkn to give pBladpAkn::aac(3)IV. The latter was further used as a template in PCR for amplification (primers adpA$_{gh}$kn_for and adpA$_{gh}$kn_rev) of a 3.4 kb DNA fragment harbouring ΔadpA$_{gh}$-aac(3)IV. The obtained amplicon was cloned into EcoRV-digested vector pKC0702. The final adpA$_{gh}$ knockout plasmid was labelled pKCHandpAkn::aac(3)IV. Generation of ΔadpA$_{gh}$ mutant was carried out as described above. Mutant phenotype of *S. ghanaensis* ΔadpA$_{gh}$-aac(3)IV was confirmed by PCR using primers adpA$_{gh}$exp_for and adpA$_{gh}$exp_rev. Generation and verification of aac(3)IV-evicted strain ΔadpA$_{gh}$ was carried out as described for ΔabsB$_{gh}$ strain (primers adpA$_{for}$ and adpA$_{rev}$; data not shown).

For the complementation of *S. ghanaensis* ΔadpA$_{gh}$, 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 ΔblbA$_{gh}$ strain and plasmid for complementation experiment

The 2.0 kb *S. ghanaensis* genomic regions flanking blbA$_{gh}$ were amplified with primers blbA-left-up plus blbA-left-rp (‘left’ homology arm) and blbA-right-up plus blbA-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 blbA$_{gh}$ knockout plasmid pKC1139blbA$_{gh}$-del contains markerless deletion of the 87 bp blbA$_{gh}$ coding sequence. Manipulations of pKC1139blbA$_{gh}$-del$^+$ transconjugants to generate the blbA$_{gh}$ 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 blbAXbaIup and blbA-diagn-rp and sequencing confirmed the deletion of the 87 bp blbA$_{gh}$ sequence from the genome of ΔblbA$_{gh}$. For complementation and expression experiments, the blbA$_{gh}$ coding region along with the 320 bp upstream segment was amplified with primers blbAXbaIup and blbAEcoRirp and cloned into respective sites of pSET152 to yield pSET152blbA.

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

To probe the activities of moeE5, absB$_{gh}$, adpA$_{gh}$ and blbA$_{gh}$ 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 moE5_script.rev for moeE5p; absB$_{gh}$for and absB$_{gh}$script.rev for absB$_{gh}$p; adpA$_{for}$ and adpA$_{script.rev}$ for adpA$_{gh}$p; blbA$_{forward}$ and blbA$_{script.rev}$ for blbA$_{gh}$p). The moeE5p, absB$_{gh}$p, adpA$_{gh}$p and blbA$_{gh}$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 moE5_script.rev for moeE5p; absB$_{gh}$for and absB$_{gh}$script.rev for absB$_{gh}$p; adpA$_{for}$ and adpA$_{script.rev}$ for adpA$_{gh}$p; blbA$_{forward}$ and blbA$_{script.rev}$ for blbA$_{gh}$p). The moeE5p, absB$_{gh}$p, adpA$_{gh}$p and blbA$_{gh}$p fragments were cloned into XbaI–EcoRV-digested pGUSHL4adA [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) and cloned in XbaI–EcoRV-treated pGUSHL4aadA, giving pmoeE5constr and padpAcontr, respectively.

The spore suspensions (2 × 10⁵ cfu) of streptomycetes reporter plasmid-bearing strains were inoculated in 300 ml flasks with 100 ml of TS medium, and grown for 30 h. One millilitre of the preculture was inoculated into fresh TSB medium (100 ml) and grown for 24–28 h (depending on experiment). Mycelium was harvested, washed twice with distilled water, then resuspended in lysis buffer (50 mM phosphate buffer (pH 7.0), 0.1% Triton X-100, 5 mM DTT, 4 mg ml⁻¹ lysozyme) and incubated for 30 min at 37°C. Lysates were centrifuged for 10 min at 5000 r.p.m. Then, 0.5 ml of lysate was mixed with 0.5 ml of dilution buffer (50 mM phosphate buffer (pH 7.0), 5 mM DTT, 0.1% Triton X-100) supplemented with 5 μl 0.2 M p-nitrophenyl-β-D-glucuronide and used for measuring optical density at λ = 415 nm every minute during 20 min of incubation at 37°C. As a reference, a 1 : 1 mixture of lysate and dilution buffer was used.

5.10. Expression and purification of His-tagged AdpA<sub>gh</sub>

For the production of C-terminal hexahistidine-tagged AdpA<sub>gh</sub>, the coding region of gene adpA<sub>gh</sub> was amplified with primers AdpA_pr_for and AdpA_pr_rev from S. ghanaensis chromosomal DNA. PCR product was purified and cloned into NcoI–XhoI cloning sites of expression vector pET24b, giving pETAdpAgh.

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

5.11. Gel electrophoretic mobility shift assay

The 500 bp promoter regions of the targeted genes (adpA<sub>gh</sub>, bldA<sub>gh</sub>, moeE5, moeK5, moeO5) were used in EMSA. These probes were amplified from chromosomal DNA of *S. ghanaensis* by PCR using primers adpA_for and adpA_script_rev for adpA<sub>gh</sub>, bldA_for and bldA_script_rev for bldA<sub>gh</sub>, moeE5_for and moeE5_script_rev for moeE5; moeK5_for and moeK5_script_rev for moeK5; moeO5_for and moeO5_script_rev for moeO5 (see the electronic supplementary material, table S3). A total of 10 pmol of each probe was 5'-end labelled with 20 pmol [γ-32P] using T4 polynucleotide kinase according to established protocols (Fermentas). Unincorporated labelled dATP was removed using ProbeQuant G-50 Micro columns (GE Healthcare). A total of 20 fmol of labelled probe was incubated with purified 1.1, 4.4, 11, 22, 44, 88 pM His-tagged AdpA<sub>gh</sub>, at 25°C for 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<sub>gh</sub> was expressed in both soluble and insoluble fractions, the lysate without centrifugation was directly mixed with 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 Zubarananny 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.

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