JOURNAL OF BACTERIOLOGY, Nov. 2011, p. 6358–6365 Vol. 193, No. 22
0021-9193/11/$12.00 doi:10.1128/JB.05734-11

The Level of AdpA Directly Affects Expression of Developmental Genes in *Streptomyces coelicolor*▽†

Marcin Wolański,[1] Rafał Donczew,[2] Agnieszka Kois-Ostrowska,[1] Paweł Masiewicz,[2] Dagmara Jakimowicz,[1,2] and Jolanta Zakrzewska-Czerwińska[1,2]*

Faculty of Biotechnology, University of Wrocław, ul. Tamka 2, 50-137 Wrocław, Poland,[1] and Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, ul. Weigla 12, 53-114 Wrocław, Poland[2]

Received 3 July 2011/Accepted 7 September 2011

AdpA is a key regulator of morphological differentiation in *Streptomyces*. In contrast to *Streptomyces griseus*, relatively little is known about AdpA protein functions in *Streptomyces coelicolor*. Here, we report for the first time the translation accumulation profile of the *S. coelicolor adpA* (*adpA*$_{Sc}$) gene; the level of *S. coelicolor* AdpA (AdpA$_{Sc}$) increased, reaching a maximum in the early stage of aerial mycelium formation (after 36 h), and remained relatively stable for the next several hours (48 to 60 h), and then the signal intensity decreased considerably. AdpA$_{Sc}$ specifically binds the *adpA*$_{Sc}$ promoter region *in vitro* and *in vivo*, suggesting that its expression is autoregulated; surprisingly, in contrast to *S. griseus*, the protein presumably acts as a transcriptional activator. We also demonstrate a direct influence of AdpA$_{Sc}$ on the expression of several genes whose products play key roles in the differentiation of *S. coelicolor*: STI, a protease inhibitor; RamR, an atypical response regulator that itself activates expression of the genes for a small modified peptide that is required for aerial growth; and ClpP1, an ATP-dependent protease. The diverse influence of AdpA$_{Sc}$ protein on the expression of the analyzed genes presumably results mainly from different affinities of AdpA$_{Sc}$ protein to individual promoters.

Streptomycetes, GC-rich Gram-positive soil bacteria known for their ability to produce many valuable antibiotics and other secondary metabolites, undergo complex morphological differentiation (4, 10). The genome of the model species *Streptomyces coelicolor* A3(2) was the first among the *Streptomycetes* to be completely sequenced (2).

Streptomyces bacteria grow by tip extension and hyphal branching to form a dense mycelial network of vegetative hyphae. In response to nutrient depletion and other signals, the vegetative mycelium is partially self-cannibalized by a nuclease(s) and protease(s) to supply nutrients for the growth of aerial hyphae, which subsequently transform into long chains of spores (3, 27). This morphological differentiation, which is usually accompanied by the production of secondary metabolite(s), is controlled by multilevel regulatory mechanisms. A key coordinating role in the regulation of morphological differentiation is played by the protein AdpA, which was originally discovered in *Streptomyces griseus* (37, 38). In all *Streptomyces* genomes sequenced so far, translation of *adpA* mRNA depends on a leucyl-tRNA for a rarely used TTA codon; the $tRNA^{Leu}_{UAA}$ is encoded by the *bldA* gene required for aerial mycelium formation. AdpA belongs to the AraC/XylS family of transcription regulators, whose members contain a dual helix-turn-helix (HTH) motif in the C-terminal DNA binding domain. In *S. griseus*, AdpA activates a number of genes whose products are required for morphological development and for secondary metabolites synthesis (e.g., streptomycin) (12, 28). During vegetative growth, the transcription of *S. griseus adpA* (*adpA*$_{Sg}$) is repressed by ArpA, the receptor protein for the signaling γ-butyrolactone molecule A-factor; accumulation of A-factor and its interaction with ArpA cause derepression of *adpA*$_{Sg}$ expression and the subsequent activation of genes involved in differentiation and synthesis of secondary metabolites (12, 16) (for clarity, we used the subscripts *Sg* [for *S. griseus*] and *Sc* [for *S. coelicolor*] to distinguish between the *adpA* genes and AdpA proteins of these two organisms).

In *Streptomyces coelicolor*, somewhat less is known about AdpA$_{Sc}$ protein and its contribution to the regulatory events during differentiation. In contrast to *S. griseus*, a comparable γ-butyrolactone signaling system seems to have no marked effect on *adpA*$_{Sc}$ expression in *S. coelicolor*, and AdpA$_{Sc}$ does not appear to directly regulate any of the antibiotic biosynthetic gene sets analyzed so far (7, 40). In this paper, we characterize the AdpA$_{Sc}$ protein from *S. coelicolor* and its interaction with DNA. We also demonstrate a direct influence of AdpA$_{Sc}$ on the expression of several genes whose products play a key role in the differentiation of *S. coelicolor*: STI, a protease inhibitor (20); RamR, an atypical response regulator that itself activates expression of the genes for a small modified peptide that is required for aerial growth (17); and ClpP1, an ATP-dependent protease (9).

MATERIALS AND METHODS

DNA manipulation and bacterial strain growth conditions. DNA manipulations were carried out by standard protocols (34). Enzymes were supplied by Fermentas, Promega, or Roche, and oligonucleotides were from Genomed (Poland). The *Streptomyces coelicolor* and *Escherichia coli* strains and plasmids used in this study are listed in Table 1. All the PCR-derived clones were analyzed by DNA sequencing to check their fidelity. Culture conditions, media, antibiotic

* Corresponding author. Mailing address: Faculty of Biotechnology, University of Wrocław, ul. Tamka 2, 50-137 Wrocław, Poland. Phone: 48 71 3752502. Fax: 48 71 3752608. E-mail: jolanta.zakrzewska@uni.wroc.pl.

† Supplemental material for this article may be found at http://jb.asm.org/.

▽ Published ahead of print on 16 September 2011.

TABLE 1. Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Relevant genotype or phenotype or description	Source or reference(s)
E. coli strains		
DH5α	ϕ80d*lacZ*ΔM15 *recA1 endA1 gyrAB thi-1 hsdR17*(r_K^- m_K^+) *supE44 relA1 deoR* Δ(*lacZYA-argF*)*U169 phoA*	Promega
BL21(DE3)	F^- *ompT* $hsdS_B$(r_B^- m_B^-) *dcm gal* λ(DE3)	Promega
BW25113/pIJ790	Δ(*araD-araB*)*567* Δ*lacZ4787*(::*rrnB4*) *lacIp-40000*(*lacI*^{q}) λ^- *rpoS369*(Am) *rph-1* Δ(*rhaD rhaB*)*568 hsdR514* on the bacterial chromosome; *oriR101 repA1001*(Ts) *araBp-gam-be-exo* on the pIJ790 plasmid	11
ET12567/pUZ8002	*dam dcm hsdS* Cm^r Tet^r on the bacterial chromosome; *tra* Kan^r *RP4 23* on pUZ8002	29
US0	Δ*pyrF* Δ*hisB* F′	25
S. coelicolor strains		
M145	$SCP1^-$ $SCP2^-$	2
M851	M145 Δ$adpA_{Sc}$	36
M145+pIJ6902-*hyg*	M145+pIJ6902-*hyg*	This study
M851+*adpA(TTG)*	M851+pIJ6902/2528-*hyg*	This study
M851+pIJ6902-*hyg*	M851+pIJ6902-*hyg*	This study
M851+*LadpA(TTA)*	M851+pHL71	This study
M851+*LadpA(TTG)*	M851+pHL72	This study
Plasmids		
2StC13	Cosmid carrying $adpA_{Sc}$ gene	33; http://strepdb.streptomyces.org.uk
pHZ2528	pSET152 with 2.4-kb fragment harboring the $adpA_{Sc}$ gene (with TTG codon)	36
pHL71	pSET152 with 3.2-kb fragment harboring the $adpA_{Sc}$ gene (with TTA codon) and surrounding genes SCO2791 and SCO2793	36
pHL72	pSET152 with 3.2-kb fragment harboring the $adpA_{Sc}$ gene (with TTG codon) and surounding genes SCO2791 and SCO2793	36
pIJ6902/2528-*hyg*	pIJ6902-*hyg* containing $adpA_{Sc}$ under the control of the thiostrepton-inducible *PtipA* promoter	This study
pIJ6902-*hyg*	pIJ6902 derivative containing Hyg^r instead of $Apra^r$	This study
pET-21a(+)adpAHis_6	pET-21a(+) harboring $adpA_{Sc}$ gene cloned into BamHI and XhoI sites	This study
pET-28a(+)His_6-nuc-adpA(51)	pET28a(+) expressing His_6-Nuc-AdpA(51) fusion protein cloned into BamHI and NotI restriction sites	This study
pB1H1-adpA_{Sc}	pB1H1 vector harboring the $adpA_{Sc}$ gene cloned into restriction sites NotI and AvrII	This study
pH3U3-18random	pH3U3 vector harboring 18-bp randomized library cloned into NotI and SgsI restriction sites	This study

concentrations, transformation and conjugation methods followed the general procedures for *E. coli* (34) and *Streptomyces* (18). *S. coelicolor* was cultivated in 79 liquid medium (32). For expression studies, the strains were cultivated on plates containing R2 or MM agar supplemented with 1% mannitol (18).

qPCR. For total RNA preparation, *S. coelicolor* mycelium grown on cellophane-covered MM agar containing 1% mannitol was harvested at different time points as described previously (18). RNA was prepared using TRI reagent (Sigma) according to the instructions of the manufacturer. RNA concentration was quantified using a Nanodrop ND-1000 spectrophotometer, and the quality of the RNA was analyzed on an agarose gel. cDNA was obtained after reverse transcription of 4 μg of DNase I-treated total RNA with MultiScribe murine leukemia virus (MuLV) reverse transcriptase and random hexamer primers (high-capacity cDNA reverse transcription kit; Applied Biosystems) following the procedures recommended by the manufacturer. Control reactions were carried out without reverse transcriptase. Expression of genes was analyzed by quantitative real-time PCR (qPCR) using ABI (StepOnePlus) and a real-time 2× PCR master Mix SYBR kit (A&A Biotechnology). The primers used for real-time PCR are listed in Table S1 in the supplemental material. All primers were designed using the PrimerExpress v. 3.0 program. The specificity of the PCR products was verified by performing melting curve analysis and running the DNA fragment on a gel at the end of each PCR. The reaction mixtures were heated at 95°C for 10 min before PCR cycling for 40 cycles, with 1 cycle consisting of 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C. Fifty-picogram samples of cDNA from the wild type (WT) and mutants were used for analysis. The target gene transcript levels were normalized internally to the level of the *hrdB* gene. Each experiment was performed in duplicate, and each transcript level was measured in triplicate. With each set of primers, negative-control experiments, performed in the absence of reverse transcriptase, confirmed that PCR products were amplified on cDNA template (i.e., there was no significant contamination with chromosomal DNA).

Construction of mutant strains. (i) Construction of a strain overproducing AdpA$_{Sc}$ protein. To construct a strain overproducing AdpA$_{Sc}$ protein, we used plasmid pHZ2528, which carries intact $adpA_{Sc}$ with a TTA→TTG mutation (36), and the *PtipA* (thiostrepton-inducible promoter) expression vector pIJ6902-*hyg* (hygromycin resistance instead of the apramycin resistance cassette; M. Wolanski, unpublished data), which integrates into the *S. coelicolor* chromosome at the ϕC31 attachment site *attB* (21). The PCR-amplified tetracycline resistance cassette fragment ($p_{BR\text{-}tetF}$ and $p_{BR\text{-}tetR}$ primers and pBR322 template) was used in the PCR targeting strategy to replace the original $adpA_{Sc}$ promoter in the pHZ2528 vector with the *tetR* gene and to create an NdeI restriction site in front of the start codon of the $adpA_{Sc}$ gene. The $adpA_{Sc}$ gene was subsequently cut out of the vector using NdeI and EcoRI and cloned into pIJ6902-*hyg* to give pIJ6902/2528-*hyg*. This construct was integrated into the *attB* site on the chromosome of *S. coelicolor* M851, giving M851+*adpA(TTG)*.

(ii) Complementation of $adpA_{Sc}$ deletion mutant. For complementation of the *adpA* deletion mutant, plasmid pHL71 or pHL72 (36) carrying the wild-type $adpA_{Sc}$ gene or a TTA-free version of the $adpA_{Sc}$ gene (TTA was replaced by TTG), respectively, was introduced into the M851 strain by conjugation selecting for apramycin-resistant colonies, yielding the M851+*LadpA(TTA)* or M851+*LadpA(TTG)* strain, respectively (Table 1). The presence of the TTA or TTG codon was confirmed by sequencing of PCR-amplified fragment of the complemented *adpA* gene.

SDS-PAGE and Western blotting. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10% acrylamide) by the method of Laemmli (22) and transferred to a nitrocellulose membrane. The membrane was blocked for 1 h at room temperature with Tris-buffered saline with Tween 20 (TBST) (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.05% Tween 20) containing 3% bovine serum albumin (BSA) and subsequently incubated with anti-AdpA$_{Sc}$ polyclonal antibodies. The membrane was then washed and incubated with a goat anti-rabbit secondary antibody conjugated with alkaline phosphatase. The membrane was stained with 5-bromo-4-chloro-3-indolylphosphate (BCIP) and nitroblue tetrazolium (NBT).

EMSA. Electrophoretic mobility shift assay (EMSA) was carried out as described previously (42). Briefly, the ^{32}P-labeled DNA fragment (or nonradioactive fragment) was incubated with increasing amounts of purified AdpA$_{Sc}$ protein with a six-His (His$_6$) tag (AdpA$_{Sc}$His$_6$) protein in the presence of a nonspecific competitor [poly(dI-dC) · poly(dI-dC)] in 1× Marians' buffer (1× Marians' buffer is 20 mM HEPES-KOH [pH 8.0], 5 mM magnesium acetate, 1 mM EDTA, 4 mM dithiothreitol, 0.2% Triton X-100, 3 mM ATP, 5 g liter^{-1} BSA, and 5% glycerol) for 30 min at room temperature. The nucleoprotein complexes were resolved in a 4 or 5% polyacrylamide gel in 0.25× Tris-borate-EDTA (TBE) buffer at 5 to 10 V cm^{-1}. For the nonradioactive fragment, the gel was stained with ethidium bromide. The complexes were analyzed by a Typhoon 8600 variable-mode imager and ImageQuant software.

DNase I footprinting. For DNase I footprinting experiments, a 289-bp-long PCR product obtained with primers p$_{adpA\text{-}pf}$ and p$_{adpA\text{-}pr}$ and encompassing the *adpA*$_{Sc}$ promoter region was used. The 5′-end-radiolabeled DNA fragments (~10 fmol) were incubated with different amounts of AdpA$_{Sc}$His$_6$ protein in 1× Marians' buffer at 25°C for 30 min. After DNase I digestion (24), the cleavage products were separated in an 8% polyacrylamide-urea sequencing gel. The gel was analyzed by a Typhoon 8600 variable-mode imager and ImageQuant software.

SPR analysis. For the standard surface plasmon resonance (SPR) analysis, a promoter region of *adpA*$_{Sc}$ was PCR amplified with biotinylated oligonucleotide p$_{b\text{-}AdpAEmr}$ and nonbiotinylated oligonucleotide p$_{AdpA\text{-}Emf}$ and then immobilized on the chip surface (Sensor Chip SA; GE Healthcare) of the BIAcore 3000 apparatus; approximately 100 response units (RUs) of DNA were immobilized. A non-AdpA box DNA fragment was used as a negative control. DNA loosely attached to the surface of the chip was removed with a 20-μl pulse of 0.1% SDS (15 μl min^{-1}). To exclude the effects of mass transport on the kinetics of the protein-DNA interactions, the measurements were performed at various AdpA$_{Sc}$His$_6$ protein concentrations (2 to 100 nM) and at a continuous flow rate (15 μl min^{-1}) for 120 s. The measurements were performed in HBS-200 buffer (10 mM HEPES [pH 7.4], 10 mM magnesium acetate, 200 mM NaCl, 3.4 mM EDTA, and 0.05% Tween 20). At the end of each cycle, bound AdpA$_{Sc}$His$_6$ protein was removed by washing with a 20-μl pulse of 0.1% SDS (15 μl min^{-1}). The results were plotted as sensograms after subtraction of the background response signal obtained in a control experiment. The BIAevaluation v. 4.1 program (Pharmacia Biosensor AB) was used for data analysis.

***In vivo* immunoprecipitation.** The immunoprecipitation assay was performed as described earlier (13, 35). Briefly, *S. coelicolor* strains cultivated for 24 h in 79 liquid medium were cross-linked by the addition of formaldehyde, and then the nucleoprotein complexes were immunoprecipitated with anti-AdpA$_{Sc}$ antibodies. Cells not subjected to cross-linking and the *adpA*$_{Sc}$ deletion mutant served as negative controls and were treated subsequently in the same way as the experimental samples. PCRs were carried out with primers flanking the promoter region of *adpA*$_{Sc}$.

RESULTS AND DISCUSSION

AdpA reaches a maximum concentration at an early stage of aerial mycelium formation in *S. coelicolor*. Until now, in both *S. coelicolor* and *S. griseus*, the expression of the *adpA* gene has been analyzed only on the transcript level. However, the level of the AdpA protein depends not only on transcriptional regulation but also on the availability of leucyl-tRNA corresponding to the rare TTA triplet in the *adpA* gene. In order to examine the expression profiles of the *adpA* gene during the differentiation of *S. coelicolor*, we performed quantitative real-time PCR (qPCR) (see Materials and Methods) and Western blotting. The profile of the *adpA*$_{Sc}$ mRNA level was similar to that obtained by Takano et al. (36); the transcript was at its highest levels before the formation of aerial hyphae (18 to 24 h), slightly diminished (36 h), and then returned to its high levels with the onset of sporulation (see Fig. S2 in the supplemental material). The abundance of AdpA in the *S. coelicolor* cell extracts was examined using purified rabbit antibodies raised against the protein (as described in Materials and Methods; see Fig. S1 in the supplemental material). A weak signal had already appeared at 18 h, while the maximum concentration of AdpA was reached at 36 h, i.e., in the early stage of aerial mycelium formation (Fig. 1). The level of AdpA protein dropped slightly and remained stable for the next several hours (48 to 60 h)—during aerial mycelium formation. The signal intensity then decreased significantly (72 h) and remained low until the end of the time course experiment. Surprisingly, the protein expression profile of the *adpA* mutant in which a rare TTA codon was replaced with TTG was similar to the protein expression profile of bacteria with the wild-type *adpA* gene (Fig. 1 and Fig. S1C). This is in agreement with the expression profiles of corresponding tRNAs; recently, Pettersson and Kirsebom (31) demonstrated that tRNA$^{Leu}_{UAA}$ and tRNA$^{Leu}_{CAA}$ exhibited similar accumulation profiles and the levels of their accumulation increased after the onset of formation of aerial hyphae. Thus, our data showed that translation of the *adpA* gene is not limited by a rare tRNA$^{Leu}_{UAA}$ —the *bldA* tRNA. However, it has to be noted that AdpA is not produced in the *bldA* deletion mutant (11a, 22a, 26, 36).

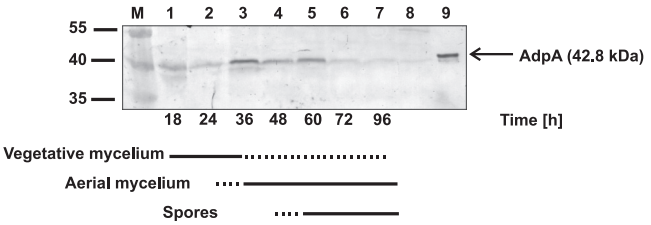

FIG. 1. AdpA level in *S. coelicolor* during growth of hyphae. Western blot analysis of total *S. coelicolor* cell proteins using rabbit antibodies raised against the C terminus of recombinant AdpA$_{Sc}$ protein. Protein extracts were prepared from cultures growing on R2 medium at the corresponding time points (in hours) indicated below the figure. Lanes: M, molecular mass markers; 1 to 7, cell extracts of *S. coelicolor* M145, plate cultures (50 μg of total protein); 8, cell extract of strain M851 (*adpA* deletion mutant), 50-h plate culture (50 μg of total protein); 9, purified recombinant protein AdpA$_{Sc}$His$_6$ (50 ng). The positions of molecular mass markers (in kilodaltons) are indicated to the left of the gel.

***S. coelicolor* AdpA protein binds *in vitro* and *in vivo* to its own promoter region.** So far, extensive studies regarding the interaction of AdpA protein with DNA have been focused on *S. griseus* AdpA. However, there are some known differences between *S. griseus* and the model organism *S. coelicolor* in the regulation and targets of AdpA; therefore, it was desirable to evaluate the details of the binding properties of AdpA$_{Sc}$ and target promoters in *S. coelicolor* (5, 36).

To elucidate the binding specificity of AdpA$_{Sc}$, we applied the bacterial one-hybrid (B1H) system (for details, see Materials and Methods in the supplemental material). After screening a library of 18-bp randomized DNA sequences (encompassing putative AdpA binding sites), 10 different DNA

fragments were selected. Sequence alignment of these fragments using the MEME algorithm (1) allowed us to determine the consensus binding site for the $AdpA_{Sc}$ protein (see Fig. S3 in the supplemental material), which as could be expected, is consistent with the consensus sequence for *S. griseus* AdpA protein (41) (both proteins exhibited high similarity, particularly within the DNA binding domain, which showed 100% identity). In particular, G, C, and A/T are highly conserved at positions 2, 4, and 8, respectively (Fig. S3).

A genome search for the AdpA consensus sequence (5′ TGGCSNGWWY 3′ [41]) allowed us to identify 157 intergenic regions containing at least one consensus AdpA binding sequence in the *S. coelicolor* chromosome; usually the perfect binding site (0 mismatch) was accompanied by sequences with one or two mismatches. The $adpA_{Sc}$ promoter was found among the identified promoter regions; this region contains a single perfect sequence and five weak sequences (one with a single mismatch and four with two mismatches [Fig. 2A]). To examine whether $AdpA_{Sc}$ interacts with its own promoter, the PCR-amplified $adpA_{Sc}$ gene was cloned into pET-21a(+) and then overexpressed as a C-terminal His-tagged protein in *E. coli* BL21 strain. The purified $AdpA_{Sc}His_6$ protein was more than 95% pure (as judged by SDS-PAGE analysis [data not shown]) and formed a dimer in solution (per gel filtration column chromatography [data not shown]), similar to *S. griseus* AdpA [$AdpA_{Sg}$] and other proteins belonging to the AraC/XylS protein family (41). To elucidate the interaction of the purified $AdpA_{Sc}His_6$ protein with the $adpA_{Sc}$ promoter ($PadpA_{Sc}$), we applied three independent methods: electrophoretic mobility shift assays (EMSAs), surface plasmon resonance (SPR), and DNase I footprinting. The EMSA analysis (Fig. 2B) demonstrated that the $AdpA_{Sc}His_6$ protein binds specifically to its own promoter region: at low $AdpA_{Sc}His_6$ concentrations, one nucleoprotein complex was observed, while increasing protein concentrations resulted in the formation of additional (one or two) higher-molecular-weight complexes. This suggests that at higher protein concentrations, $AdpA_{Sc}$ binds a weaker site(s). This is consistent with the DNase I footprint analysis; $AdpA_{Sc}His_6$ protein did not protect the region with weak $AdpA_{Sc}$ sites at low protein concentrations (light-gray and medium-gray arrows in Fig. 2A and C), but the sites were not efficiently digested by the DNase I at high $AdpA_{Sc}His_6$ concentrations. The weak sequences were bound by the $AdpA_{Sc}His_6$ protein only if they were located in the vicinity of the strong $AdpA_{Sc}$ binding site(s); they did not form complexes with $AdpA_{Sc}His_6$ protein when present alone even at a higher protein concentration (data not shown). Thus, $AdpA_{Sc}$ presumably interacts with weak target sequences via protein molecules anchored to the strong binding sequence. Binding to multiple sequences located in the $adpA_{Sc}$ promoter region leads to the formation of a large nucleoprotein complex; a long stretch of DNA was protected not only along AdpA boxes (see black and medium-gray arrows in Fig. 2C) but also in the region lacking in AdpA sequences (see the solid black vertical line in Fig. 2C). Binding of $AdpA_{Sc}His_6$ also promoted many distortion points that were preferentially accessible to DNase I (see DNase I-hypersensitive sites in Fig. 2C). Moreover, in addition to the concentration-dependent association of the protein with the $adpA_{Sc}$ promoter, the SPR analysis (Fig. 2D) revealed the slow dissociation of the $AdpA_{Sc}His_6$, suggesting that the complexes formed by $AdpA_{Sc}$-$PadpA_{Sc}$ are fairly stable.

To address the question of whether $AdpA_{Sc}$-$PadpA_{Sc}$ complexes exist *in vivo* as well, we performed formaldehyde cross-linking of proteins to DNA in intact cells followed by selective immunoprecipitation of protein-DNA complexes with antibodies raised against the $AdpA_{Sc}$ protein, and then the immunoprecipitated DNA fragments bound to $AdpA_{Sc}$ were detected by PCR (Fig. 2E). Since we had problems with the cross-linking reaction of cultures grown on solid medium, we decided to use the *S. coelicolor* M851+*adpA(TTG)* strain, which was able to produce the $AdpA_{Sc}$ protein (upon induction with thiostrepton) in liquid culture. The $AdpA_{Sc}$-$PadpA_{Sc}$ complexes were indeed detected in the M851+*adpA(TTG)* strain, while no signal was observed in the M851+pIJ6902-*hyg* strain, which served as a negative control (Fig. 2E).

In summary, $AdpA_{Sc}$ specifically binds the $adpA_{Sc}$ promoter region *in vitro* and *in vivo*. We speculated that as in *S. griseus*, the transcription of $adpA_{Sc}$ is self-regulated (16). However, unlike the data presented for *S. griseus*, $AdpA_{Sc}$ acts as an activator rather than a repressor on its own gene transcription. Transcription analysis of the $adpA_{Sc}$ gene promoter indicates higher transcription activity for the M145 strain (wild-type strain) than for the M851 strain ($adpA_{Sc}$ deletion strain) (Fig. 2F). It has to be noted that Xu et al. (40) recently discovered an additional mechanism involved in the regulation of $adpA_{Sc}$ expression; they showed that RNase III (AbsB), which was initially discovered as a global regulator of antibiotic production, could cleave the $adpA_{Sc}$ mRNA. Thus, the expression of the $adpA_{Sc}$ gene appears to be regulated at three levels: transcription (autoactivation), posttranscription (RNase III), and translation (via the TTA codon). Moreover, both proteins, AdpA and RNase III, take part in a novel feedback control loop that reciprocally regulates their cellular levels (40).

AdpA directly switches on genes required for the morphological differentiation of *S. coelicolor*. We found that strong $AdpA_{Sc}$ binding sites are present in promoters of several genes known to be implicated in the formation of aerial mycelium and spores, including *sti1* (encoding *Streptomyces* trypsin inhibitor) (as previously reported [6, 20]), *ramR* (regulator of production of aerial mycelium-promoting peptide SapB [17, 23, 39]), and *clpP1* (encoding ClpP1, an ATP-dependent protease that affects morphological and physiological differentiation [9]). We therefore examined the interaction of $AdpA_{Sc}$ with the promoters of these genes and the expression of *sti1*, *ramR*, and *clpP1* in the $adpA_{Sc}$ deletion mutant (see Fig. 4) and in the strain overexpressing $AdpA_{Sc}$ protein in relation to the parental strain. For comparative expression studies, we constructed a strain, *S. coelicolor* M851+*adpA(TTG)* (Table 1) that upon induction with thiostrepton overexpresses the $AdpA_{Sc}$ protein (Fig. 3). The overexpression of $AdpA_{Sc}$ caused an acceleration in the formation of aerial hyphae which is consistent with the earlier observation reported by Nguyen et al. (26) (Fig. 3).

In EMSA experiments (Fig. 4), each of the PCR-amplified promoter regions was bound by $AdpA_{Sc}$ in a concentration-dependent manner. The protein showed the highest affinity toward the *sti1* promoter region, presumably due to the presence of two closely spaced inverted $AdpA_{Sc}$ sequences: one perfect and the other containing a single mismatch. Moreover, the distance between the binding sites (2 bp) is supposed to be

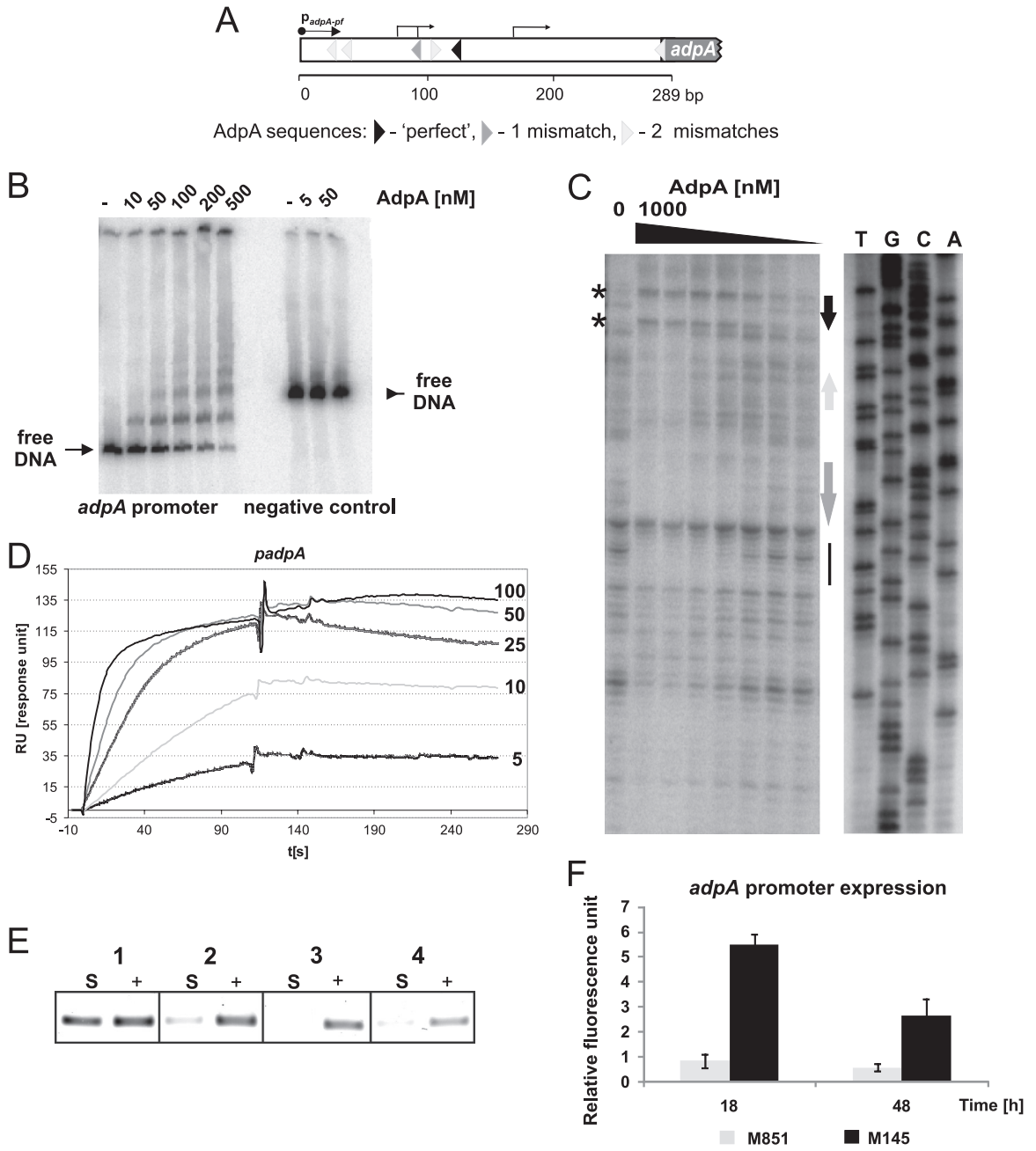

FIG. 2. Interaction of $AdpA_{Sc}$ with the promoter region of its own gene. (A) Structure of the $adpA_{Sc}$ promoter region. Transcription start sites (36) are indicated by the bent arrows. $p_{adpA\text{-}pf}$ is the primer that was labeled and used (together with the $p_{adpA\text{-}pr}$ primer) for amplification of the $adpA_{Sc}$ promoter region and for sequencing. (B) EMSA. A ^{32}P-labeled 289-bp DNA fragment (PCR amplified by the $p_{adpA\text{-}pf}$ and $p_{adpA\text{-}pr}$ primers [see Table S1 in the supplemental material]) was incubated with increasing amounts of $AdpA_{Sc}His_6$, and the nucleoprotein complexes were analyzed in a 5% polyacrylamide gel. A DNA fragment amplified using the primer pair $p_{H24parSfw}$ and $p_{H24parSrv}$ served as a negative control for $AdpA_{Sc}$ binding. (C) DNase I footprinting. A ^{32}P-labeled 289-bp DNA fragment was incubated with increasing amounts of $AdpA_{Sc}His_6$ and then subjected to DNase I digestion. Black, medium-gray, and light-gray arrows mark the positions of AdpA sequences (see panel A). The asterisks indicate DNase I-hypersensitive sites. The solid black vertical line corresponds to the region lacking in AdpA boxes and protected from DNase I digestion. Lanes T, G, C, and A are sequencing reactions. (D) Surface plasmon resonance (SPR). Sensograms obtained at different concentrations of the $AdpA_{Sc}His_6$ with biotinylated DNA immobilized on a streptavidin-coated chip of the BIAcore apparatus. $AdpA_{Sc}His_{6\ was\ used}$ at concentrations of 5, 10, 25, 50, and 100 nM. (E) *In vivo* binding and PCR amplification of immunoprecipitated DNA fragment bound to $AdpA_{Sc}$. Purified antibodies raised against $AdpA_{Sc}$ were used to immunoprecipitate $AdpA_{Sc}$-DNA complexes after formaldehyde cross-linking. PCR was carried out with primers flanking the $adpA_{Sc}$ promoter region (Table S1). Lanes: S, sample of immunoprecipitated DNA; +, input (not immunoprecipitated) DNA (positive control); 1, *S. coelicolor* M851+*adpA(TTG)* strain, cross-linked; 2, M851+pIJ6902-*hyg* (deletion mutant) strain, cross-linked; 3, M851+*adpA(TTG)* strain, not cross-linked; 4, M851+pIJ6902-*hyg* (deletion mutant), not cross-linked. (F) Influence of $AdpA_{Sc}$ on its own expression. The bars represent $adpA_{Sc}$ promoter expression in M145 (wild-type strain) and M851 (deletion mutant) at the corresponding time points indicated below.

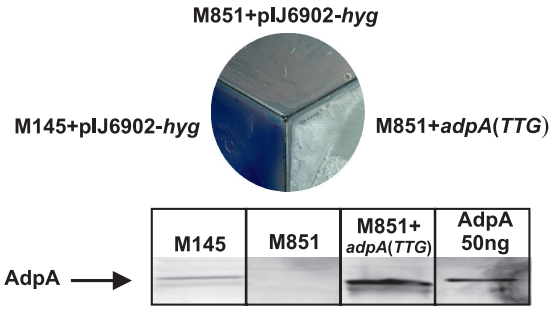

FIG. 3. Phenotypes of *S. coelicolor* M851+pIJ6902-*hyg* (*adpA* deletion mutant) and M851+*adpA(TTG)* compared to the wild-type *S. coelicolor* M145+pIJ6902-*hyg*. Cultures were grown on R2 medium (supplemented with 2 μg ml^{-1} thiostrepton) for 48 h. The levels of expression of AdpA protein in the analyzed *S. coelicolor* strains are shown in the blot at the bottom of the figure. Western blot analysis of total *S. coelicolor* cell proteins (40 μg protein per lane) was performed using rabbit antibodies raised against the C terminus of recombinant $AdpA_{Sc}$ protein.

optimal for $AdpA_{Sc}$ binding, as was previously determined for *S. griseus* AdpA (41). The expression studies were performed when $AdpA_{Sc}$ was at its high level (at 48 h), and the *S. coelicolor* life cycle is associated with the formation of aerial hyphae. qPCR expression analysis showed a spectacular increase in *sti1* transcript in the *S. coelicolor* M851+*adpA(TTG)* strain in relation to the parental strain (approximately 200 times), whereas the level of *sti1* mRNA in the $adpA_{Sc}$-null mutant was more than seven times lower than in the wild-type strain (Fig. 4). The data clearly demonstrated that the $AdpA_{Sc}$ protein is directly involved as a transcription activator in the expression of the *sti1* gene: the binding of the $AdpA_{Sc}$ protein to the *sti1* promoter region presumably recruits RNA polymerase and facilitates transcription initiation. Thus, the results confirm earlier suggestions regarding the involvement of $AdpA_{Sc}$ in *sti1* gene regulation in *S. coelicolor* (20). It has been found that overproduction of STI (*sti1* under the control of the strong constitutive *ermE*p promoter) has an inhibitory effect on the production of the blue polyketide antibiotic called actinorhodin (Act). We also found that the $AdpA_{Sc}$ overexpression resulted in significant inhibition of Act synthesis (Fig. 3). Interestingly, a red pigment antibiotic (undecylprodigiosin) is also not synthesized in the M851+*adpA(TTG)* strain (Fig. 3). STI is believed to act as an inhibitor of some extracellular trypsin-like protease(s) (15) and thus may play a role in aerial hyphal formation, since a relationship has been found between protease activity and the development of hyphae (19). The degradation of STI (presumably by a specific protease) releases trypsin-like protease(s), which then digests proteins of the old mycelium to supply nutrients for the growth of the reproductive aerial hyphae (3, 6).

In the case of the *ramR* gene, knowing that $AdpA_{Sc}$ exhibited a lower affinity toward its promoter than toward the *sti1* promoter, we expected a weaker effect of $AdpA_{Sc}$ on *ramR* expression. Indeed, overexpression of $AdpA_{Sc}$ resulted in a slight increase in *ramR* mRNA level (nearly 2-fold) (Fig. 4). However, the expression of *ramR* in the $adpA_{Sc}$ deletion mutant was found to be considerably reduced relative to the wild-type strain (10-fold). These observations are consistent

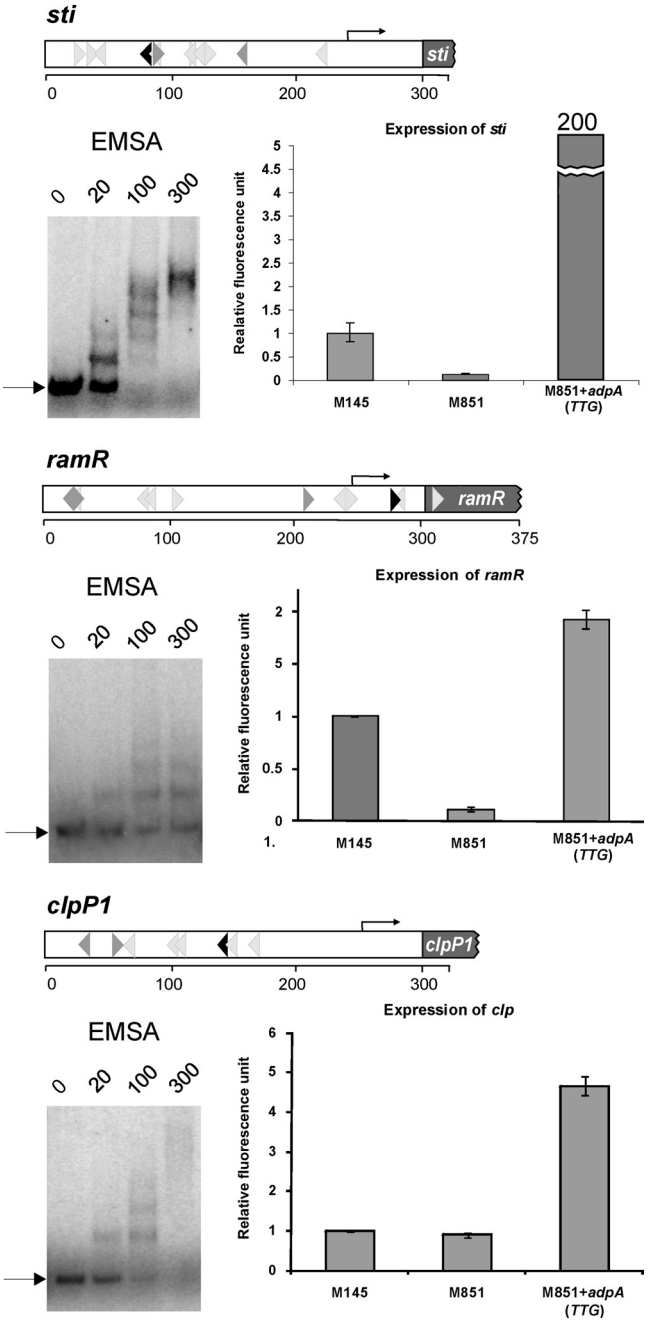

FIG. 4. Influence of $AdpA_{Sc}$ on the expression of *sti*, *ramR*, and *clpP1*. For the three genes, each panel contains the structure of the analyzed promoter region (for details, see the legend to Fig. 2C), the interaction of $AdpA_{Sc}His_6$ with the analyzed promoter region by EMSA (the arrow shows the position of free DNA; numbers indicate the nanomolar concentration of $AdpA_{Sc}His_6$ protein), and quantitative real-time PCR analysis of the abundance of the analyzed mRNA. Total RNA was extracted from a culture growing on MM agar plates supplemented with 1% mannitol for 48 h. The primer pairs for the analyzed genes used in qPCR are listed in Table S1 in the supplemental material. cDNA levels of the target genes were normalized internally to *hrdB* cDNA, which is the control for a constitutively expressed gene. The error bars indicate the standard deviations from triplicate samples.

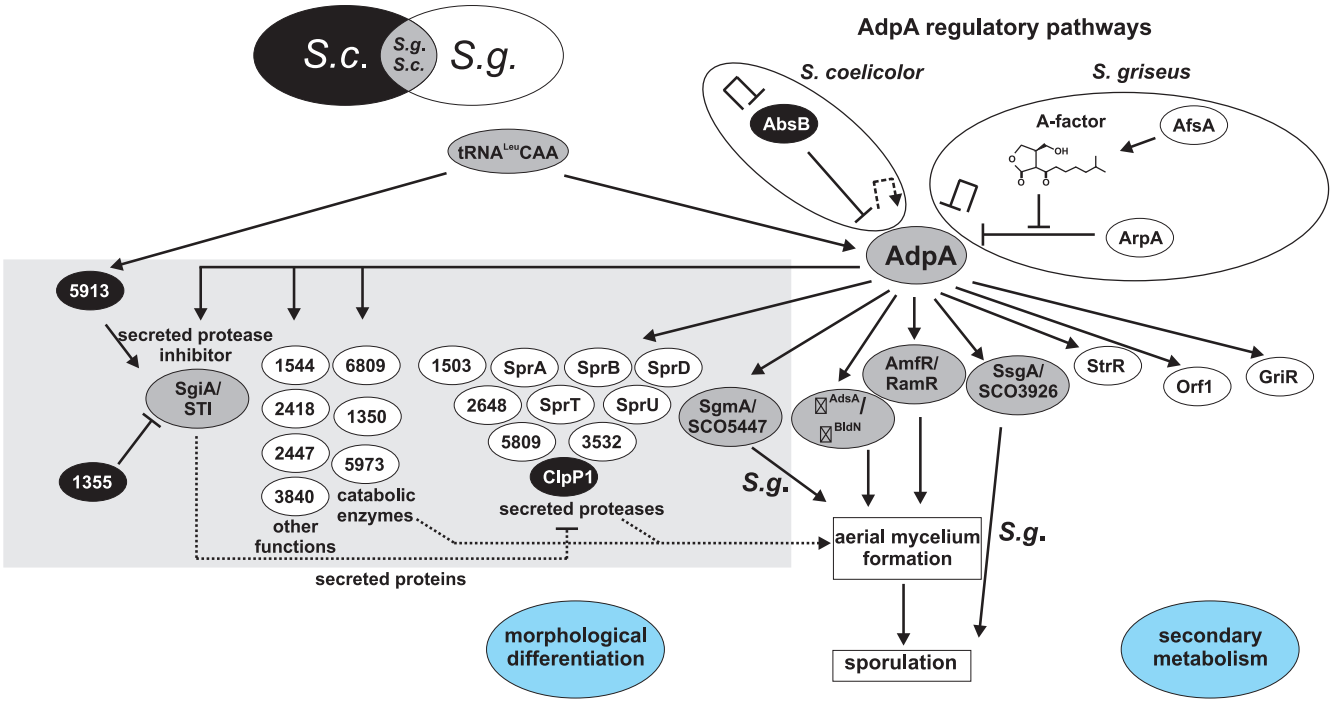

FIG. 5. Comparison of functions dependent on AdpA protein in *S. griseus* (some data taken from reference 28) and *S. coelicolor* (some data taken from reference 40). Arrows indicate positive control. Perpendicular lines indicate negative control. *S.c.*, *S. coelicolor*; *S.g.*, *S. griseus.*

with other studies showing that *sti* and *ramR* expression are upregulated in strains that exhibit an elevated level of the $AdpA_{Sc}$ protein (26, 40). In the case of the *clpP1* gene, overexpression of the $AdpA_{Sc}$ protein caused a moderate elevation of *clpP1* mRNA level (4- to 5-fold), but the lack of $AdpA_{Sc}$ did not influence gene expression (Fig. 4).

Earlier studies suggested that in *S. griseus*, $AdpA_{Sg}$ protein regulates the expression of other genes essential for morphological differentiation: *ssgA*, which encodes a small acidic protein essential for sporulation (septum formation), and *sgmA*, which encodes a metalloendopeptidase probably involved in the apoptosis of substrate hyphae during the development of aerial hyphae. We decided to examine whether $AdpA_{Sc}$ can also regulate the expression of these genes in *S. coelicolor.* However, it has to be noted that in contrast to *S. griseus*, where each of these promoter regions contains two perfect AdpA binding sites (41), in *S. coelicolor*, neither *sgmA* nor *ssgA* possesses a perfect $AdpA_{Sc}$ binding site and their promoter regions were bound by $AdpA_{Sc}$ with rather weak affinity (data not shown) compared with the affinity of the protein to the *sti1* or $adpA_{Sc}$ promoter. The results of qPCR analysis revealed that the levels of expression of *ssgA* and *sgmA* in the $adpA_{Sc}$ deletion mutant were 2- and 8-fold lower, respectively, than in the wild-type strain. Kato et al. (14) showed that no *sgmA* transcript production occurred in the $adpA_{Sg}$ deletion mutant of *S. griseus*. Surprisingly, overexpression of $AdpA_{Sc}$ reduced the expression of these genes in *S. coelicolor* (3 and 8 times for *ssgA* and *sgmA*, respectively [data not shown]), suggesting that at a higher protein concentration, $AdpA_{Sc}$ may somehow hinder the access of RNA polymerase to these promoter regions.

In summary, the diverse influence of the $AdpA_{Sc}$ protein on the expression of the analyzed genes presumably results mainly from the different affinities of the $AdpA_{Sc}$ protein to individual promoters. However, the possibility that the spatial arrangement of AdpA sites and their location in respect to the promoter(s) may also influence gene expression cannot be excluded. In *E. coli*, DnaA protein (besides its primary function as an initiator) acts as a transcription factor that represses or activates several genes depending on the location and arrangement of DnaA boxes (25a).

Although the $AdpA_{Sg}$ and $AdpA_{Sc}$ proteins are highly conserved (84% identity) and *adpA* mutants are bald in both organisms (*S. griseus* and *S. coelicolor)*, the $AdpA_{Sg}$ protein in *S. griseus*, in addition to morphological differentiation, is responsible for the positive regulation of secondary metabolites (including streptomycin) synthesis. The *S. coelicolor adpA* deletion strain retains the ability to synthesize red pigment antibiotic (undecylprodigiosin) (we observed premature and enhanced synthesis of red pigment), while in the *S. coelicolor* strain that overproduced AdpA protein, none of the two pigmented antibiotics were synthesized (Fig. 3). Knowledge regarding the involvement of the AdpA protein in the regulation of antibiotic production in *S. coelicolor* is rather obscure; $AdpA_{Sc}$ presumably acts directly (as in the case of STI) and/or indirectly at different levels of regulation. Moreover, there are also differences in the mechanisms regulating the *adpA* gene expression in both organisms (for details, see Fig. 5). In contrast to *S. griseus*, $adpA_{Sc}$ is not under the control of γ-butyrolactones, and its expression is presumably positively regulated by the $AdpA_{Sc}$ protein. Taking all the data together, it could be concluded that the AdpA-dependent regulation pathways in both organisms, despite some similarities, exhibit diversity (Fig. 5). It has to be emphasized that *S. griseus* and *S. coelicolor* are not closely related streptomycetes and the diversity in the

AdpA-dependent pathways is presumably one of the important aspects of speciation which reflect differences in evolutionary adaptation to different soil microenvironments (5).

ACKNOWLEDGMENTS

We are grateful to Keith Chater for valuable comments on the manuscript, Zhi-Xiong Deng for providing the *S. coelicolor* M851 strain, Scott Wolfe for plasmids required for bacterial one-hybrid system, Pawel Mackiewicz for help searching for AdpA binding motifs, and Olivier Bertrand for helpful comments regarding antibody preparation.

This work was supported by the Ministry of Science and Higher Education (grant N301 523838). M.W. was supported by a scholarship of the President of Polish Academy of Sciences. A.K.-O., R.D., P.M., and J. Z.-C. gratefully acknowledge financial support received from the Foundation for Polish Science (Start and MISTRZ Programmes).

REFERENCES

1. **Bailey, T. L., and C. Elkan.** 1994. Fitting a mixture model by expectation maximization to discover motifs in biopolymers. Proc. Int. Conf. Intell. Syst. Mol. Biol. **2:**28–36.
2. **Bentley, S. D., et al.** 2002. Complete genome sequence of the model actinomycete *Streptomyces coelicolor* A3(2). Nature **417:**141-147.
3. **Chater, K. F., and R. Losick.** 1997. Mycelial life style of *Streptomyces coelicolor* A3(2) and its relatives, p. 149–182. *In* J. A. Shapiro and M. Dworkin (ed.), Bacteria as multicellular organisms. Oxford University Press, Oxford, England.
4. **Chater, K. F.** 2001. Regulation of sporulation in *Streptomyces coelicolor* A3(2): a checkpoint multiplex? Curr. Opin. Microbiol. **4:**667–673.
5. **Chater, K. F., and S. Horinouchi.** 2003. Signalling early developmental events in two highly diverged *Streptomyces* species. Mol. Microbiol. **48:**9–15.
6. **Chater, K. F.** 2006. *Streptomyces* inside-out: a new perspective on the bacteria that provide us with antibiotics. Philos. Trans. R. Soc. Lond. B Biol. Sci. **361:**761–768.
7. **Chater, K. F., and G. Chandra.** 2008. The use of the rare UUA codon to define "expression space" for genes involved in secondary metabolism, development and environmental adaptation in *Streptomyces*. J. Microbiol. **46:**1–11.
8. Reference deleted.
9. **de Crécy-Lagard, V., P. Servant-Moisson, J. Viala, C. Grandvalet, and P. Mazodier.** 1999. Alteration of the synthesis of the Clp ATP-dependent protease affects morphological and physiological differentiation in *Streptomyces*. Mol. Microbiol. **32:**505–517.
10. **Flärdh, K., and M. J. Buttner.** 2009. *Streptomyces* morphogenetics: dissecting differentiation in a filamentous bacterium. Nat. Rev. Microbiol. **7:**36–49.
11. **Gust, B., G. L. Challis, K. Fowler, T. Kieser, and K. F. Chater.** 2003. PCR-targeted *Streptomyces* gene replacement identifies a protein domain needed for biosynthesis of the sesquiterpene soil odor geosmin. Proc. Natl. Acad. Sci. U. S. A. **100:**1541–1546.

11a. **Higo, A., S. Horinouchi, and Y. Ohnishi.** 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.

12. **Horinouchi, S.** 2007. Mining and polishing of the treasure trove in the bacterial genus *Streptomyces*. Biosci. Biotechnol. Biochem. **71:**283–299.
13. **Jakimowicz, D., K. Chater, and J. Zakrzewska-Czerwínska.** 2002. The ParB protein of *Streptomyces coelicolor* A3(2) recognizes a cluster of *parS* sequences within the origin-proximal region of the linear chromosome. Mol. Microbiol. **45:**1365–1377.
14. **Kato, J. Y., A. Suzuki, H. Yamazaki, Y. Ohnishi, and S. Horinouchi.** 2002. Control by A-factor of a metalloendopeptidase gene involved in aerial mycelium formation in *Streptomyces griseus*. J. Bacteriol. **184:**6016–6025.
15. **Kato, J. Y., S. Hirano, Y. Ohnishi, and S. Horinouchi.** 2005. The *Streptomyces* subtilisin inhibitor (SSI) gene in *Streptomyces coelicolor* A3(2). Biosci. Biotechnol. Biochem. **69:**1624–1629.
16. **Kato, J. Y., Y. Ohnishi, and S. Horinouchi.** 2005. Autorepression of AdpA of the AraC/XylS family, a key transcriptional activator in the A-factor regulatory cascade in *Streptomyces griseus*. J. Mol. Biol. **350:**12–26.
17. **Keijser, B. J., G. P. van Wezel, G. W. Canters, T. Kieser, and E. Vijgenboom.** 2000. The ram-dependence of *Streptomyces lividans* differentiation is bypassed by copper. J. Mol. Microbiol. Biotechnol. **2:**565–574.
18. **Kieser, T., M. J. Bibb, M. J. Buttner, K. F. Chater, and D. A. Hopwood.** 2000. Practical *Streptomyces* genetics. John Innes Foundation, Norwich, England.
19. **Kim, I. S., and K. J. Lee.** 1995. Physiological roles of leupeptin and extracellular proteases in mycelium development of *Streptomyces exfoliatus* SMF13. Microbiology **141:**1017–1025.
20. **Kim, D. W., K. Chater, K. J. Lee, and A. Hesketh.** 2005. Changes in the extracellular proteome caused by the absence of the *bldA* gene product, a developmentally significant tRNA, reveal a new target for the pleiotropic regulator AdpA in *Streptomyces coelicolor*. J. Bacteriol. **187:**2957–2966.
21. **Kuhstoss, S., and R. N. Rao.** 1991. Analysis of the integration function of the streptomycete bacteriophage ϕC31. J. Mol. Biol. **222:**897–908.
22. **Laemmli, U. K.** 1970. Cleavage of structural proteins during assembly of the head of the bacteriophage T4. Nature **227:**680–685.

22a. **Lopez-Garcia, M. T., I. Santamarta, and P. Liras.** 2010. Morphological differentiation and clavulanic acid formation are affected in a *Streptomyces clavuligerus adpA*-deleted mutant. Microbiology **156:**2354–2365.

23. **Ma, H., and K. Kendall.** 1994. Cloning and analysis of a gene cluster from *Streptomyces coelicolor* that causes accelerated aerial mycelium formation in *Streptomyces lividans*. J. Bacteriol. **176:**3800–3811.
24. **Majka, J., et al.** 1999. Interactions of the *Streptomyces lividans* initiator protein DnaA with its target. Eur. J. Biochem. **260:**325–335.
25. **Meng, X., M. H. Brodsky, and S. A. Wolfe.** 2005. A bacterial one-hybrid system for determining the DNA-binding specificity of transcription factors. Nat. Biotechnol. **23:**988–994.

25a. **Messer, W., and C. Weigel.** 1997. DnaA initiator–also a transcription factor. Mol. Microbiol. **24:**1–6.

26. **Nguyen, K. T., et al.** 2003. Colonial differentiation in *Streptomyces coelicolor* depends on translation of a specific codon within the *adpA* gene. J. Bacteriol. **185:**7291–7296.
27. **Nicieza, R. G., J. Huergo, B. A. Connolly, and J. Sanchez.** 1999. Purification, characterization, and role of nucleases and serine proteases in *Streptomyces* differentiation. Analogies with the biochemical processes described in late steps of eukaryotic apoptosis. J. Biol. Chem. **274:**20366–20375.
28. **Ohnishi, Y., H. Yamazaki, J. Y. Kato, A. Tomono, and S. Horinouchi.** 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.
29. **Paget, M. S., L. Chamberlin, A. Atrih, S. J. Foster, and M. J. Buttner.** 1999. Evidence that the extracytoplasmic function sigma factor σ^E is required for normal cell wall structure in *Streptomyces coelicolor* A3(2). J. Bacteriol. **181:**204–211.
30. Referee deleted.
31. **Pettersson, B. M., and L. A. Kirsebom.** 2011. tRNA accumulation and suppression of the bldA phenotype during development in *Streptomyces coelicolor*. Mol. Microbiol. **79:**1602–1614.
32. **Prauser, H., and R. Falta.** 1968. Phagensensibilitat, Zellwandzusammensetzung und Taxonomie von Actinomyceten. Z. Allg. Mikrobiol. **8:**39–46.
33. **Redenbach, M., et al.** 1996. A set of ordered cosmids and a detailed genetic and physical map for the 8 Mb *Streptomyces coelicolor* A3(2) chromosome. Mol. Microbiol. **21:**77–96.
34. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
35. **Solomon, M. J., and A. Varshavsky.** 1985. Formaldehyde-mediated DNA-protein crosslinking: a probe for in vivo chromatin structures. Proc. Natl. Acad. Sci. U. S. A. **82:**6470–6474.
36. **Takano, E., et al.** 2003. A rare leucine codon in *adpA* is implicated in the morphological defect of *bldA* mutants of *S. coelicolor*. Mol. Microbiol. **50:**475–486.
37. **Vujaklija, D., K. Ueda, S. K. Hong, T. Beppu, and S. Horinouchi.** 1991. Identification of an A-factor-dependent promoter in the streptomycin biosynthetic gene cluster of *Streptomyces griseus*. Mol. Gen. Genet. **229:**119–128.
38. **Vujaklija, D., S. Horinouchi, and T. Beppu.** 1993. Detection of an A-factor-responsive protein that binds to the upstream activation sequence of *strR*, a regulatory gene for streptomycin biosynthesis in *Streptomyces griseus*. J. Bacteriol. **175:**2652–2661.
39. **Willey, J., R. Santamaria, J. Guijarro, M. Geistlich, and R. Losick.** 1991. Extracellular complementation of a developmental mutation implicates a small sporulation protein in aerial mycelium formation by *S. coelicolor*. Cell **65:**641–650.
40. **Xu, W., J. Huang, R. Lin, J. Shi, and S. N. Cohen.** 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.
41. **Yamazaki, H., A. Tomono, Y. Ohnishi, and S. Horinouchi.** 2004. DNA-binding specificity of AdpA, a transcriptional activator in the A-factor regulatory cascade in *Streptomyces griseus*. Mol. Microbiol. **53:**555–572.
42. **Zawilak-Pawlik, A., et al.** 2005. Architecture of bacterial replication initiation complexes: orisomes from four unrelated bacteria. Biochem. J. **389:**471–481.