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BldG and SCO3548 Interact Antagonistically To Control Key Developmental Processes in *Streptomyces coelicolor*▽

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The similarity of BldG and the downstream coexpressed protein SCO3548 to anti-anti-sigma and anti-sigma factors, respectively, together with the phenotype of a *bldG* mutant, suggests that BldG and SCO3548 interact as part of a regulatory system to control both antibiotic production and morphological differentiation in *Streptomyces coelicolor*. A combination of bacterial two-hybrid, affinity purification, and far-Western analyses demonstrated that there was self-interaction of both BldG and SCO3548, as well as a direct interaction between the two proteins. Furthermore, a genetic complementation experiment demonstrated that SCO3548 antagonizes the function of BldG, similar to other anti-anti-sigma/anti-sigma factor pairs. It is therefore proposed that BldG and SCO3548 form a partner-switching pair that regulates the function of one or more sigma factors in *S. coelicolor*. The conservation of *bldG* and sco3548 in other streptomycetes demonstrates that this system is likely a key regulatory switch controlling developmental processes throughout the genus *Streptomyces*.

Streptomyces coelicolor A3(2), the well-studied model organism for processes of bacterial multicellular development and antibiotic production, possesses a large genome (8.67 Mbp) with a high degree of regulatory complexity (6). A large proportion of the coding sequence (12.3%) is predicted to encode the multitude of regulatory factors required to support a complex life cycle, involving the formation of sporulating aerial hyphae, that responds to the changing soil environment. Of particular note is the presence of 64 sigma factors, which are thought to play a critical role in the modulation of gene expression; this group is comprised of 4 housekeeping sigma factors, as well as 50 extracytoplasmic function sigma factors and 9 group 3 subfamily sigma factors (6, 23). The activity of alternative sigma factors is typically regulated by a number of mechanisms, including phosphorylation-dependent partner switching by antagonistic proteins. The best-studied examples of this regulatory mechanism, which is active against the group 3 sigma factors, are found in *Bacillus subtilis*, where partner switching controls the activity of both the sporulation-specific factor σ^F and the general stress response factor σ^B (1, 17, 18, 44, 50, 57, 59). In these systems, an anti-sigma factor protein (SpoIIAB and RsbW, respectively) sequesters the cognate sigma factor, preventing the expression of target genes. Upon sensing some activating signal, an anti-sigma factor antagonist (or anti-anti-sigma factor; SpoIIAA and RsbV, respectively) binds to the anti-sigma factor, mediating release of the active sigma factor to direct regulon transcription.

Partner-switching systems are also thought to play a critical role in sigma factor regulation in *S. coelicolor*. The first characterized example in this organism is the RsbV-RsbA partner-switching pair that controls the activity of the osmotic-stress-responsive factor σ^B (36). Many genes encoding additional putative paralogues of these regulatory factors are present in the *S. coelicolor* genome, including genes encoding 45 RsbW orthologues and 18 RsbV orthologues (45). One cluster of such genes is found at the *bldG* locus, which was originally identified as one of a group of key pleiotropic regulators, collectively termed the *bld* genes, that control both antibiotic production and aerial hypha formation in *S. coelicolor* (10, 11, 43). The *bldG* gene encodes an orthologue of the RsbV and SpoIIAA anti-anti-sigma factors (9). Immediately downstream of *bldG* is the open reading frame (ORF) sco3548 (http://strepdb.streptomyces.org.uk/) (previously referred to as *orf3* [9]), which encodes an orthologue of the RsbW and SpoIIAB anti-sigma factors. Much like the genes in the *B. subtilis* systems, the *bldG* and sco3548 genes are cotranscribed, although, unlike the equimolar expression of the *B. subtilis* systems, *bldG* transcripts are always expressed in excess of sco3548 transcripts in *S. coelicolor* (9). Also unlike the *B. subtilis* operons, no cognate sigma factor is encoded at the *bldG* locus, and therefore the biochemical target of BldG regulation is unknown.

The high level of similarity between BldG and its *B. subtilis* orthologues suggests, however, that BldG functions in a similar partner-switching mechanism. This hypothesis is supported by the presence of a sulfate transporter and anti-sigma factor antagonist (STAS) domain in BldG, which is known to form a key surface for the interaction of anti-sigma factor antagonists with their cognate anti-sigma factors (3). Contiguous with this STAS domain in the *B. subtilis* SpoIIAA anti-anti-sigma factor is a phosphorylated serine residue known to be essential for the posttranslational control of the interaction with its cognate anti-sigma factor; the phosphorylation event drives the partner-switching mechanism (2, 41). This serine residue is conserved not only among related *Bacillus* anti-anti-sigma factors but also in BldG. Furthermore, BldG has been shown to be reversibly phosphorylated on its conserved serine, and this phosphorylation is essential for the regulation of morphological differentiation and antibiotic production (7). On the basis

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TABLE 1. Strains used in this study

Strain	Description	Relevant genotype or description	Source and/or reference
E. coli strains			
DH5α	Host for plasmid cloning and propagation	F$^-$ ϕ80Δ*lacZ*ΔM15 *recA1 endA1 gyrA96 thi-1* λ$^-$ *hsdR17*(r_R^- m_R^+) *supE44 relA1* Δ(*lacZYA-argF*)*U169 phoA*	Invitrogen
BL21(DE3)	Host for expression of recombinant proteins	F$^-$ *ompT hsdS*(r_B^- m_B^-) *dcm*$^+$ *gal met* λ(DE3)	Stratagene
BTH101	Reporter strain for bacterial two-hybrid analysis	F$^-$ *cya-99 araD139 galE15 galK16 rpsL1* (Str^r) *hsdR2 mcrA1 mcrB1*	29
ET12567	Nonmethylating host strain	F$^-$ *dam13*::Tn*9 dcm6 hsdR recF143 zjj-202*::Tn*10 galK2 galT22 ara14 lacY1 xyl5 leuB6 thi-1 tonA31 rpsL136 hisG4 tsx78 mtl-1 glnV44*	D. MacNeil, Merck Sharp and Dohme Research Laboratories (39)
S. coelicolor strains			
M145	Wild type	Prototrophic, SCP1$^-$ SCP2$^-$ Pgl$^+$	John Innes Centre (6)
Δ*bldG* 1DB	*bldG* null mutant	M145 derivative with an in-frame deletion in *bldG*	7

of these similarities, it is predicted that BldG is involved in a phosphorylation-dependent partner-switching interaction. Because of the proximity and coexpression of *bldG* and sco3548, it was predicted that SCO3548 is the antagonistic partner of BldG.

The purpose of this study was to test the hypothesis that BldG and SCO3548 are involved in an antagonistic protein interaction. To this end, a variety of biochemical and genetic experimental approaches were used to identify potential BldG-containing protein complexes, to characterize partners interacting with BldG, and to examine the antagonistic nature of the interactions in *S. coelicolor* development.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Escherichia coli* and *S. coelicolor* strains used in this study are listed in Table 1. The growth conditions and media used for *E. coli* cultures have been described previously (52). Plasmid-containing cultures were supplemented as required with an antibiotic(s) as follows: 100 μg/ml ampicillin (Sigma), 50 μg/ml kanamycin (Sigma), and 50 μg/ml apramycin (Provel). *S. coelicolor* strains were grown in R2YE liquid medium or on R2YE agar as described previously (31). Plasmid-containing cultures were supplemented with antibiotic(s) as follows: 50 μg/ml apramycin, 200 μg/ml kanamycin, 25 μg/ml chloramphenicol (Sigma), and 25 μg/ml nalidixic acid (Sigma). For induction of gene expression from the *ptipA* promoter on recombinant plasmids, 30 μg/ml thiostrepton (Sigma) was used unless otherwise indicated.

DNA manipulations. *E. coli* and *S. coelicolor* plasmids used in this study are listed in Table 2. The standard protocols used for in vitro DNA manipulation have been described previously (52). PCR was performed using the Expand high-fidelity PCR system (Roche) and DNA sequencing was performed using the DYEnamic ET system (Amersham), both using oligonucleotide primers listed in Table 3. Recombinant plasmids were routinely introduced into *E. coli* ET12567(pUZ8002) by electroporation and subsequently transferred to *S. coelicolor* Δ*bldG* 1DB by intergeneric conjugation (31).

Preparation of *S. coelicolor* cell lysates. For preparation of *S. coelicolor* crude cell lysates, strains were grown in liquid cultures to mid-exponential phase and were harvested by centrifugation. Cell pellets were washed with double-distilled water and resuspended in Tris sonication buffer (50 mM Tris-Cl [pH 8.5], 300 mM NaCl, 1× Complete EDTA-free protease inhibitor cocktail [Roche]). Alternatively, strains were grown on R2YE agar overlaid with cellophane disks (75-mm 325P disks; Courtalds Films), harvested by scraping them directly from plates, and resuspended in HEPES sonication buffer (50 mM HEPES [pH 7.2], 1× Complete EDTA-free protease inhibitor cocktail). Mycelia were lysed by sonication, the resulting lysate was clarified by centrifugation at 4°C, and the protein content of the lysate was quantified using Bio-Rad protein assay dye reagent concentrate according to the manufacturer's instructions. Western analysis of both crude cell lysates and purified proteins was performed as described previously (7).

Construction of BldG and SCO3548 expression plasmids. For construction of *bldG* and sco3548 expression plasmids, the corresponding ORFs were amplified by PCR using primers DBG35 and DBG3 (*bldG*) or DBG34 and DBG33 (sco3548). The resulting PCR products were digested with NdeI and XbaI and were cloned into similarly digested pIJ6902. The resulting plasmid constructs were verified by DNA sequencing and were designated pAU316 for *bldG* expression and pAU317 for sco3548 expression.

Construction of a BldG-SCO3548 coexpression plasmid. In order to clone the entire *bldG* locus under control of the *ptipA* promoter, pAU316 was digested with BglII to remove and discard the distal end of the *bldG* ORF. Separately, the distal end of the *bldG* gene linked to the entire sco3548 ORF by the native intergenic region was PCR amplified from the H5 cosmid (51) using the KC61 and BKL62 primers and was similarly digested with BglII. The digested KC61-BKL62 fragment was then ligated into the prepared pAU316 vector containing the proximal end of the *bldG* ORF under control of the *ptipA* promoter. The sequence integrity and insert orientation of the resulting recombinant plasmid were verified by DNA sequencing, and the plasmid was designated pAU365.

Overexpression and purification of recombinant proteins from *E. coli*. In order to express BldG and SCO3548 as glutathione *S*-transferase (GST) fusions, the *bldG* and sco3548 coding regions were isolated from plasmids pAU316 and pAU317, respectively, as NdeI-EcoRI fragments and were cloned into similarly digested vector pGEX-2TK (Amersham Biosciences). The resulting recombinant plasmids, designated pAU375 (GST-BldG) and pAU376 (GST-SCO3548), were transformed into *E. coli* BL21(DE3), and expression of the fusion proteins was induced in mid-logarithmic-phase cultures by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. The resulting soluble protein was released by sonication in lysis buffer (20 mM Tris-HCl [pH 8.0], 100 mM NaCl, 1 mM EDTA, 0.5% NP-40, 1× Complete EDTA-free protease inhibitor cocktail), and the GST fusion proteins were purified by glutathione-Sepharose affinity chromatography (Amersham Biosciences) according to the manufacturer's recommendations. Eluted fusion protein samples were desalted (PD-10 column; Amersham Biosciences), and the protein was quantified using the Bio-Rad protein assay dye reagent concentrate. As a control, GST was expressed from unmodified pGEX-2TK and was similarly purified from *E. coli* BL21(DE3).

In order to express SCO3548 as a His_{10}-tagged fusion protein, the sco3548 coding region was isolated from the pAU317 plasmid as an NdeI-EcoRI fragment and was cloned into similarly digested vector pET30(a)$^+$. The resulting recombinant plasmid, designated pAU318, and the pET30(a)$^+$ control were transformed into *E. coli* BL21(DE3).

Bacterial two-hybrid analysis of protein interactions. Bacterial two-hybrid analysis of protein interactions was performed as described by Karimova et al. (27, 29, 30). To construct recombinant plasmids for use in bacterial two-hybrid analysis, the *bldG* and sco3548 ORFs were amplified by PCR from the pAU316 and pAU317 plasmids, respectively, using the corresponding oligonucleotide primers (AP3 and AP4 or AP3 and AP11 for *bldG* and AP12 and AP13 or AP12 and AP14 for sco3548). To create in-frame fusions to the T25 and T18 fragments of the adenylate cyclase gene, all amplified fragments were digested with BamHI and KpnI; AP3-4 and AP12-13 fragments were cloned into similarly digested vectors pKT25 and pUT18C, and AP3-11 and AP12-14 fragments were cloned

TABLE 2. Plasmids used in this study

Plasmid	Relevant characteristics and/or use	Resistance	Source and/or reference(s)
pUZ8002	Encodes transfer functions for mobilization of *oriT*-containing vectors from *E. coli* to *Streptomyces* by conjugative transfer	Kanamycin	M. Buttner, John Innes Centre (31)
pIJ6902	Expression vector that contains thiostrepton-inducible *ptipA* promoter; integrates into the ϕC31 *attB* site in *S. coelicolor*	Apramycin	John Innes Centre (24)
pAU316	pIJ6902 harboring *bldG*; for expression of BldG in *S. coelicolor*	Apramycin	This study
pAU317	pIJ6902 harboring sco3548; for expression of SCO3548 in *S. coelicolor*	Apramycin	This study
pAU318	pET30(a)$^+$ derivative containing sco3548 cloned downstream of a 10-histidine tag; for overexpression of His_{10}-SCO3548 in *E. coli*	Kanamycin	This study
pAU365	pIJ6902 derivative containing the entire *bldG* and sco3548 coding regions and their native intergenic region; for expression of BldG and SCO3548 in *S. coelicolor*	Apramycin	This study
pGEX-2TK	Expression vector that contains IPTG-inducible *tac* promoter; for overexpression of protein with an N-terminal GST tag in *E. coli*	Ampicillin	Amersham Biosciences
pAU375	pGEX-2TK harboring *bldG*; for overexpression of GST-BldG in *E. coli*	Ampicillin	This study
pAU376	pGEX-2TK harboring sco3548; for overexpression of GST-SCO3548 in *E. coli*	Ampicillin	This study
pKT25	pSU40 derivative that carries the T25 fragment of *B. pertussis* adenylate cyclase (residues 1 to 224) with a multicloning sequence at the 3′ end of T25	Kanamycin	28, 30
pUT18	pUC19 derivative that carries the T18 fragment of *B. pertussis* adenylate cyclase (residues 225 to 399) with a multicloning sequence at the 5′ end of T18	Ampicillin	28
pUT18C	pUC19 derivative that carries the T18 fragment of *B. pertussis* adenylate cyclase with a multicloning sequence at the 3′ end of T18	Ampicillin	28
pKT25Zip	pKT25 harboring the T25 fragment fused in frame to the 35-codon leucine zipper domain of the yeast GNC4 activator	Kanamycin	30
pUT18CZip	pUT18C harboring the 35-codon leucine zipper domain of the yeast GNC4 activator fused in frame to the T18 fragment	Ampicillin	28
pAU377	pKT25 harboring *bldG*; for expression of T25-BldG in BTH101	Kanamycin	This study
pAU378	pUT18 harboring *bldG*; for expression of BldG-T18 in BTH101	Ampicillin	This study
pAU379	pUT18C harboring *bldG*; for expression of T18-BldG in BTH101	Ampicillin	This study
pAU380	pKT25 harboring sco3548; for expression of T25-SCO3548 in BTH101	Kanamycin	This study
pAU381	pUT18 harboring sco3548; for expression of SCO3548-T18 in BTH101	Ampicillin	This study
pAU382	pUT18C harboring sco3548; for expression of T18-SCO3548 in BTH101	Ampicillin	This study

into similarly digested pUT18. The resultant recombinant plasmids (pAU377 to pAU382 [Table 2]) were verified by DNA sequencing using vector-based primers AP7 and AP8 for pKT25 constructs, AP9 and AP10 for pUT18 constructs, and AP30 and AP31 for pUT18C constructs.

For analysis of protein interactions, pKT25-derived plasmids and pUT18 or pUT18C-derived plasmids, as well as empty vector controls, were cotransformed into the adenylate cyclase-deficient strain *E. coli* BTH101 in all possible combinations. Leucine zipper fragments cloned into pKT25 and pUT18C were used as a positive control for protein interaction (28). The primary assay for protein interactions was conducted by plating cotransformants either on LB agar containing 0.5 mM IPTG and 40 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) or on MacConkey-maltose agar; both media contained ampicillin and kanamycin to select for plasmid maintenance. The plates were then incubated at 30°C for a maximum of 36 h.

The secondary assay for protein interactions involved quantitative determination of β-galactosidase activity (55). Five individually isolated replicate cotransformants were grown overnight at 37°C in LB medium containing ampicillin and kanamycin. The cultures were then diluted 1:50 in fresh LB medium containing antibiotics and grown until an optical density at 600 nm of 0.5 was reached. Cells were harvested by centrifugation and permeabilized using chloroform and sodium dodecyl sulfate (SDS), and β-galactosidase activity was measured with a microtiter plate assay using *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as a substrate.

Affinity purification of interacting protein complexes. To examine the interaction between BldG and SCO3548, *E. coli* BL21(DE3) strains containing the recombinant plasmid pAU375 (GST-BldG) or pAU318 (His_{10}-SCO3548) were grown to mid-logarithmic phase, and fusion protein expression was induced by addition of IPTG to a final concentration of 1 mM. After incubation for 3 h, cells were harvested by centrifugation, and the pellets were resuspended in GST extraction buffer (50 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA; pH 8.5) and lysed by sonication. Equal volumes of the two cell lysates (one containing GST-BldG and one containing His_{10}-SCO3548) were mixed together and were incubated at 4°C for 1 h. The mixture was subsequently combined with a 50% slurry of glutathione-Sepharose 4B in GST extraction buffer and was incubated at 4°C for 2 h before application to a gravity flow Poly-Prep chromatography column (Bio-Rad). Columns were washed sequentially with 10 bed volumes of GST extraction buffer containing 0.5% Triton X-100, 10 bed volumes of GST extraction buffer containing 0.1% Triton X-100, and 5 bed volumes of GST extraction buffer alone. Bound proteins were eluted with GST extraction buffer containing 20 mM reduced glutathione and were examined by Western analysis using anti-His antibody (Amersham Biosciences).

To examine self-interaction of SCO3548, *E. coli* BL21(DE3) strains containing recombinant plasmid pAU318 (His_{10}-SCO3548) or pAU376 (GST-SCO3548) were grown, and fusion protein expression was induced as described above. After incubation for 3 h, cells were harvested by centrifugation, and the pellets were resuspended in Ni-nitrilotriacetic acid (NTA) buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole [pH 8.0], 1× Complete EDTA-free protease inhibitor cocktail) and lysed by sonication. Equal volumes of the two cell lysates (one containing His_{10}-SCO3548 and one containing GST-SCO3548) were mixed together and were incubated at 4°C for 1 h. The mixture was subsequently combined with a 50% slurry of Ni-NTA agarose (Qiagen) in Ni-NTA buffer and was incubated at 4°C for 2 h before application to a gravity flow Poly-Prep chromatography column. The columns were washed with 10 bed volumes of Ni-NTA buffer containing 50 mM imidazole, and bound proteins were eluted with Ni-NTA buffer containing 250 mM imidazole (pH 8.0) and were examined by Western analysis using anti-GST antibody (Amersham Biosciences).

Far-Western analysis. To examine BldG protein interactions, cell lysates of *S. coelicolor* Δ*bldG* 1DB containing either pAU316 or pIJ6902 were separated by 15% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. After equilibration in wash buffer (50 mM Tris [pH 7.5], 100 mM sodium acetate, 350 mM NaCl, 1 mM EDTA, 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.2% Tween 20), membranes were blocked with 3% skim milk in wash buffer. To examine the BldG-SCO3548 interaction, membranes were subsequently incubated with purified GST-SCO3548 fusion protein

TABLE 3. Oligonucleotide primers used in this study

Primer	Restriction site	Sequence (5′–3′)[a]	Application
AP3	BamHI	ATATAGGATCCCGTGGACCTGTCCCTG	Forward primer for creation of *bldG*-containing pKT25-, pUT18C-, and pUT18-based constructs
AP4	KpnI	ATATAGGTACCCGTCAGTCGGTGGCCGC	Reverse primer for creation of *bldG*-containing pKT25- and pUT18C-based constructs
AP11	KpnI	ATATAGGTACCCGGTCGGTGGCCGCCAC	Reverse primer for creation of *bldG*-containing pUT18-based constructs
AP12	BamHI	ATATAGGATCCCATGGCCACCGTCGAACTCCGT	Forward primer for creation of sco3548-containing pKT25-, pUT18C-, and pUT18-based constructs
AP13	KpnI	ATATAGGTACCCGTCAGATCGGTGGCAGCACCGC	Reverse primer for creation of sco3548-containing pKT25- and pUT18C-based constructs
AP14	KpnI	ATATAGGTACCCGGATCGGTGGCAGCACCGCCGA	Reverse primer for creation of sco3548-containing pUT18-based constructs
AP7		GGCGCGCAGTTCGGTGACCAGCGGC	Forward primer for sequencing pKT25 inserts
AP8		GGGATGTGCTGCAAGGCGATTAAG	Reverse primer for sequencing pKT25 inserts
AP9		TTTATGCTTCCGGCTCGTATGTT	Forward primer for sequencing pUT18 inserts
AP10		CAAGTCGATGCGTTCGCGAT	Reverse primer for sequencing pUT18 inserts
AP30		CTCGCCGGATGTACTGGAAAC	Forward primer for sequencing pUT18C inserts
AP31		CGGGGCTGGCTTAACTATGC	Reverse primer for sequencing pUT18C inserts
BKL62	EcoRI	CGGCGAATTCGTCTGCAACCAGGAGCGC	Forward primer for pAU365 construction
DBG3	XbaI	GCGCTCTAGAGTTCGACGGTGGCCATG	Reverse primer for pAU316 construction
DBG33	XbaI	GCGCTCTAGATCCCATGCCATTGATCGTGAA	Reverse primer for pAU317 construction
DBG34	NdeI	GCGCCATATGGCCACCGTCGAACTCCGT	Forward primer for pAU317 construction
DBG35	NdeI	GCGCCATATGGACCTGTCCCTGTCGAC	Forward primer for pAU316 construction
KC61	XbaI	CGCGAGATCTGTCCGCACGGCACCAATG	Reverse primer for pAU365 construction

[a] Restriction enzyme sites are underlined.

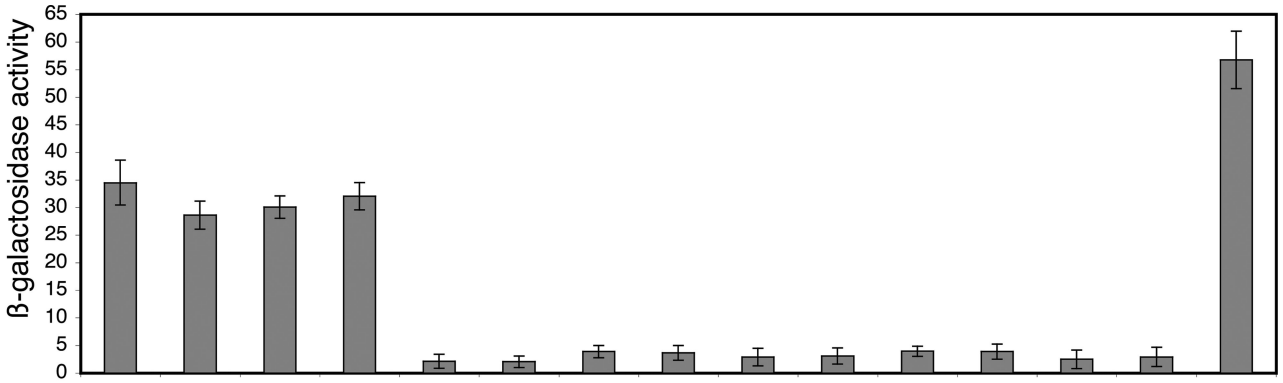
(final concentration, 2.5 μg/ml) for 2 h at room temperature. To examine BldG self-interaction, membranes were similarly incubated with purified GST-BldG fusion protein. In both cases, unbound protein was removed by gentle agitation with wash buffer, the membranes were examined by Western analysis using anti-GST antibody (Amersham Biosciences) (7).

RESULTS

BldG interacts with SCO3548. Orthologues of BldG typically interact with antagonistic protein partners; therefore, experiments to assess the ability of BldG to form protein complexes were performed. Preliminary chemical cross-linking experiments performed with BldG-containing *S. coelicolor* cell extracts demonstrated that BldG was present in several higher-molecular-weight complexes (major bands at ~30 and 70 kDa), as well as in its monomeric form (predicted molecular mass, 12.3 kDa) (not shown), indicating that BldG indeed forms protein complexes. Given that in orthologous *B. subtilis* systems the cotranscribed gene encodes the antagonistic protein partner (5, 26, 53, 58), SCO3548 was therefore the most likely candidate to form a complex with BldG. To assess this potential interaction, we first used a bacterial two-hybrid system based on interaction-mediated reconstitution of the *Bordetella pertussis* adenylate cyclase enzyme in adenylate cyclase-deficient *E. coli* strain BTH101 (27, 28). Full-length *bldG* and sco3548 coding sequences were cloned into plasmids pKT25, pUT18, and pUT18C, and the resulting recombinant constructs were cotransformed into BTH101 in all possible combinations. Primary screening by color development of cotransformants on LB (with IPTG and X-Gal) and MacConkey-maltose media suggested that there was an interaction between BldG and SCO3548 (not shown). A secondary assay of the protein interaction was performed by quantitatively determining β-galactosidase activity. Coexpression of BldG and SCO3548 resulted in β-galactosidase activity comparable to that observed for the well-established leucine zipper interaction and at least sevenfold higher than that observed for cotransformants containing either two empty vectors or one empty vector and one recombinant construct, confirming that there is an interaction between BldG and SCO3548 (Fig. 1).

As a further test of the direct interaction between BldG and SCO3548, affinity purification of BldG protein complexes was performed. Recombinant plasmids pAU375 and pAU318, encoding GST-BldG and His_{10}-SCO3548 fusion proteins, respectively, were introduced separately into *E. coli* BL21(DE3). Protein expression was induced in the resulting strains, and equal volumes of cell lysates of the strains were combined and incubated to allow protein interaction. Protein complexes were then captured by addition of glutathione-Sepharose 4B, were eluted with glutathione-containing buffer, and were examined by Western analysis with anti-His antibody. His_{10}-SCO3548 was not detected in a control experiment in which native GST was expressed from the parent plasmid pGEX-2TK (Fig. 2A), indicating that His_{10}-SCO3548 does not interact with GST alone and does not associate nonspecifically with glutathione-Sepharose 4B. Affinity purification of His_{10}-SCO3548 in complex with GST-BldG, therefore, confirmed that there was an interaction between the two proteins (Fig. 2A).

Lastly, direct interaction between BldG and SCO3548 was investigated by far-Western analysis. Cell lysates of *S. coelicolor* Δ*bldG* 1DB containing either pAU316 or pIJ6902 were separated by SDS-PAGE and immobilized on a PVDF membrane. The membrane was probed first with purified GST-SCO3548 and then with anti-GST antibody to detect protein interactions. In the lane containing cell lysate from the *bldG*-expressing strain, GST-SCO3548 was detected on the membrane at a position corresponding to the apparent molecular weight of BldG (Fig. 2B). This interaction was not observed in



pKT25	αασ	ασ	αασ	ασ	v	v	v	v	v	v	αασ	αασ	ασ	ασ	zip
pUT18	ασ	αασ			v		αασ		ασ		v		v		
pUT18C			ασ	αασ		v		αασ		ασ		v		v	zip

FIG. 1. BldG-SCO3548 interaction was detected by bacterial two-hybrid analysis. The *bldG* (ααα) and sco3548 (ασ) ORFs were cloned into plasmids pKT25, pUT18, and pUT18C and were cotransformed into *E. coli* BTH101 in all possible combinations. Either two empty vectors (v) or one empty vector and one recombinant plasmid were used as negative controls. Plasmids containing leucine zipper fragments (zip) were used as the positive controls. The β-galactosidase activities of five individually isolated replicate liquid cultures of each cotransformant were determined by a microtiter plate assay using ONPG as a substrate. β-Galactosidase activity was expressed as (units per unit of optical density at 600 nm × ml of cell suspension × 10^3), where 1 U was defined as 1 μmol of *o*-nitrophenol formed per min. The error bars indicate the standard deviation from the mean.

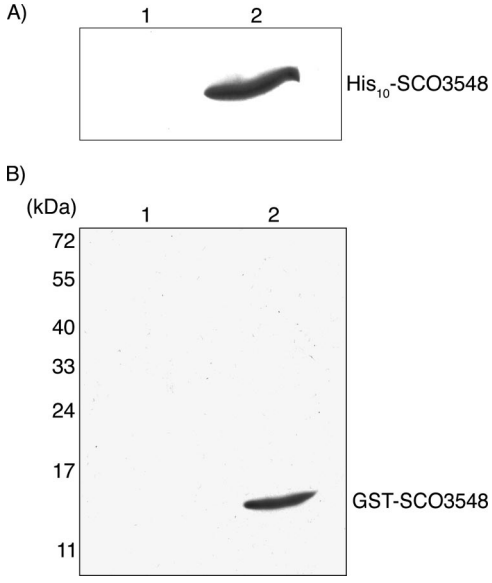


FIG. 2. BldG-SCO3548 interaction was detected in vitro by affinity purification and far-Western analysis. (A) Cell lysate from *E. coli* BL21(DE3) containing pGEX-2TK (GST control protein) (lane 1) or containing pAU375 (GST-BldG) (lane 2) was mixed with cell lysate from *E. coli* BL21(DE3) containing pAU318 (His_{10}-SCO3548) at a ratio of 1:1 (vol/vol). Glutathione-Sepharose 4B beads were added to capture GST-containing protein complexes, which were then eluted with 20 mM reduced glutathione. Eluted proteins were analyzed by SDS-PAGE and Western analysis using anti-His antibody. (B) Cell lysates from *S. coelicolor* Δ*bldG* 1DB containing pIJ6902 (vector control) (lane 1) or pAU316 (*bldG* expression) (lane 2) were subjected to SDS-PAGE, and the separated proteins were transferred to a PVDF membrane. For far-Western analysis, the membrane was incubated with purified GST-SCO3548, washed thoroughly, and probed with anti-GST antibody. The positions of molecular mass markers are indicated on the left.

the lane containing cell lysate from the vector control strain (Fig. 2B) or in a control experiment in which the membrane was probed with native GST (not shown). These results further confirm the finding described above that BldG and SCO3548 form a complex both in vitro and in *E. coli*.

SCO3548 antagonizes BldG function in *S. coelicolor*. Based on analogy to the corresponding systems in *B. subtilis*, the BldG-SCO3548 interacting pair was predicted to be functionally antagonistic in vivo. In order to examine this prediction, the pAU365 coexpression construct was created such that the entire *bldG* locus (*bldG* ORF, native intergenic region, and sco3548 ORF) was expressed from the thiostrepton-inducible *ptipA* promoter. The pAU365 construct was then introduced into *S. coelicolor* Δ*bldG* 1DB, which lacks a functional *bldG* gene but expresses the wild-type sco3548 gene from the native *bldG* promoter, producing a recombinant strain that expresses an abnormally high proportion of SCO3548 relative to BldG. *S. coelicolor* Δ*bldG* 1DB strains containing the pAU365 coexpression construct, the pAU316 *bldG* expression construct, or the pIJ6902 vector control were grown in the presence or absence of thiostrepton, and crude cell lysates were examined by Western analysis with anti-BldG antibody. The *bldG* expression and *bldG*-sco3548 coexpression strains were found to accumulate equivalent amounts of BldG protein upon thiostrepton induction (Fig. 3A), whereas no BldG protein was detected in the vector control strain or in the expression strains in the absence of induction.

To determine the effect of increased levels of SCO3548 on the ability of plasmid-expressed BldG to complement the Δ*bldG* 1DB mutant phenotype, the strains described above were examined by growing them in the presence of increasing amounts of thiostrepton inducer (Fig. 3B). The pAU316 construct restored aerial hypha formation and pigmented antibiotic production to *S. coelicolor* Δ*bldG* 1DB as the amount of

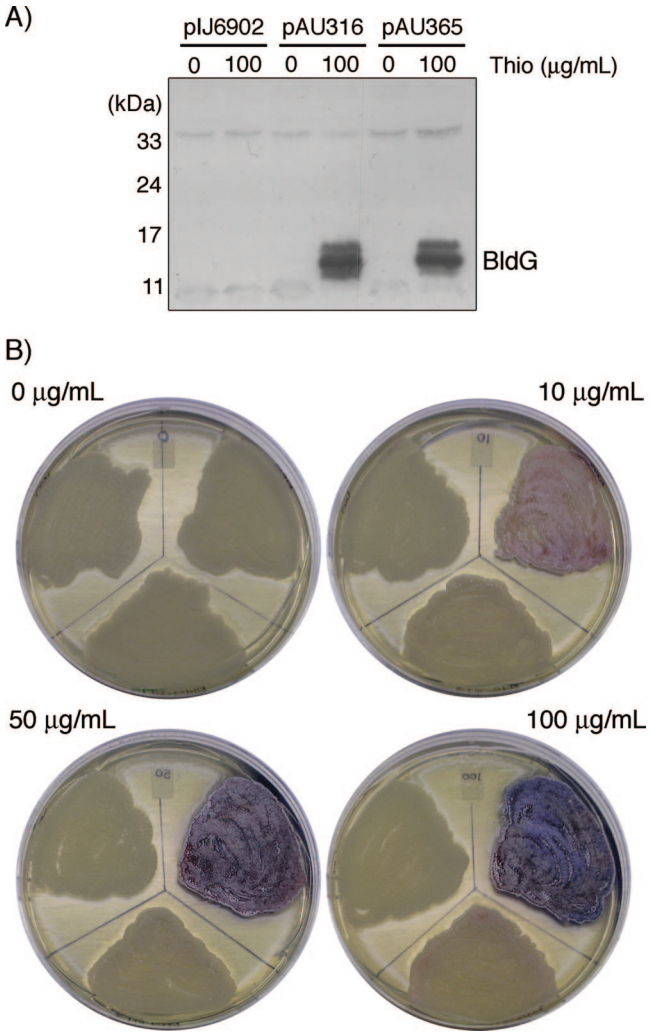


FIG. 3. Inducible coexpression of BldG and SCO3548 does not complement the *S. coelicolor bldG* mutant phenotype. (A) *S. coelicolor* Δ*bldG* 1DB containing pIJ6902 (vector control), pAU316 (*bldG* expression), or pAU365 (*bldG*-sco3548 coexpression) was grown on R2YE agar containing 0 or 100 μg/ml thiostrepton (Thio) at 30°C for 40 h. Crude cell lysates were prepared and examined by SDS-PAGE, followed by Western analysis with anti-BldG antibody. (B) *S. coelicolor* Δ*bldG* 1DB containing pIJ6902 (top left region of each plate), pAU316 (top right region), or pAU365 (bottom center region) was grown at 30°C on R2YE agar containing 0, 10, 50, or 100 μg/ml thiostrepton and photographed after 80 h.

thiostrepton inducer was increased; at the highest thiostrepton concentrations, the *bldG* mutant phenotype was completely complemented. In contrast, the pAU365 coexpression construct was unable to complement the *bldG* mutant phenotype at even the highest thiostrepton concentration, despite accumulation of the BldG protein to levels equivalent to that in the strain containing pAU316. The increased expression of SCO3548 in the coexpression strain therefore abrogated the ability of the BldG protein to complement the *bldG* mutant phenotype, indicating that these two proteins have antagonistic functions in vivo in the regulation of aerial hypha formation and secondary metabolism.

BldG exhibits self-interaction. The similarity of BldG to proteins known to form homodimers (22, 38, 48), together with preliminary identification by chemical cross-linking of a BldG-containing complex whose size was consistent with dimerization (not shown), led to examination of BldG self-interaction. First, bacterial two-hybrid analysis was performed using *bldG*-containing recombinant plasmids (pAU377 to pAU379). Primary screening of cotransformants showed that there was an interaction between the T25- and T18-BldG fusion proteins. Secondary assays confirmed this interaction, demonstrating that there was at least ninefold-higher β-galactosidase activity in the *bldG*-expressing cotransformants than in controls containing either two empty vectors or one empty vector and one recombinant construct (Fig. 4A).

BldG self-interaction was further examined in vitro by far-Western analysis. Cell lysates from *S. coelicolor* Δ*bldG* 1DB containing either pAU316 or pIJ6902 were separated by SDS-PAGE and immobilized on a PVDF membrane. The membrane was probed first with purified GST-BldG and then with anti-GST antibody to detect protein interactions. In the lane containing cell lysate from the *bldG*-expressing strain, GST-BldG was detected on the membrane at a position corresponding to the apparent molecular weight of BldG (Fig. 5A). This interaction did not occur in the lane containing cell lysate from the vector control strain (Fig. 5A) or in a control experiment in which the membrane was probed with native GST (not shown). These results, combined with those described above, demonstrate that BldG exhibits self-interaction.

SCO3548 exhibits self-interaction. Anti-sigma factors typically form homodimers (16, 21, 48, 57); therefore, based on similarity to this family of proteins, SCO3548 would be predicted to self-interact. Bacterial two-hybrid analysis, using the sco3548-containing recombinant plasmids pAU380 to pAU382, was employed to examine this interaction. Primary screening of cotransformants showed that there was interaction between the T25- and T18-SCO3548 fusion proteins. Secondary assays confirmed this interaction, demonstrating that there was at least 11-fold-higher β-galactosidase activity in the sco3548-expressing cotransformants than in controls containing either two empty vectors or one empty vector and one recombinant construct (Fig. 4B).

To further examine SCO3548 self-interaction, affinity purification of SCO3548 protein complexes was performed. Recombinant plasmids pAU376 and pAU318, encoding the GST-SCO3548 and His_{10}-SCO3548 fusion proteins, respectively, were introduced separately into *E. coli* BL21(DE3). Protein expression was induced in the resulting strains, and equal volumes of their cell lysates were combined and incubated to allow protein interaction. Protein complexes were then captured by addition of Ni-NTA, were eluted with imidazole-containing buffer, and were examined by Western analysis with anti-GST antibody. The possibility of a nonspecific interaction of GST-SCO3548 with Ni-NTA was ruled out by the results of a control experiment using crude cell lysate from a BL21(DE3) derivative containing the pET30(a)$^+$ vector control (Fig. 5B). Affinity purification of GST-SCO3548 in complex with His_{10}-SCO3548 therefore confirmed SCO3548 self-interaction (Fig. 5B).

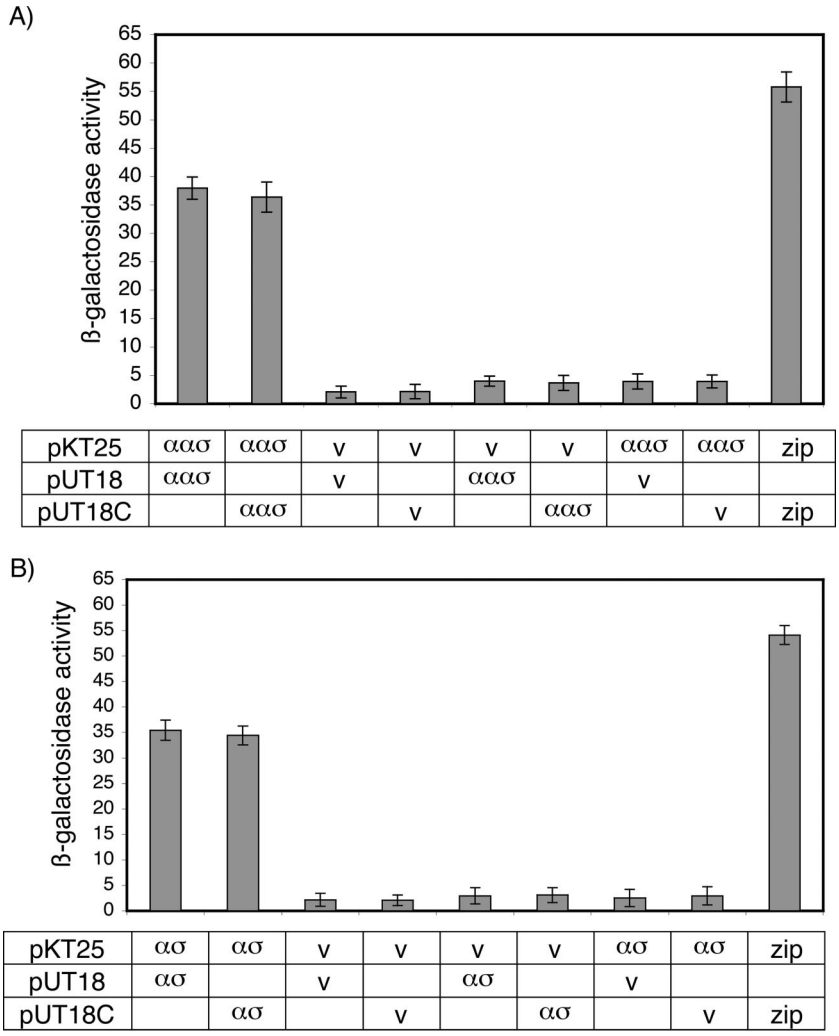


FIG. 4. BldG and SCO3548 self-interactions were detected by bacterial two-hybrid analyses. The *bldG* ORF (ααα) (A) or the sco3548 ORF (ασ) (B) was cloned into plasmids pKT25, pUT18, and pUT18C, which were cotransformed into *E. coli* BTH101 in all possible combinations. Either two empty vectors (v) or one empty vector and one recombinant plasmid were used as negative controls. Plasmids containing leucine zipper fragments (zip) were used as the positive control. The β-galactosidase activities of five individually isolated replicate liquid cultures of each cotransformant were determined by a microtiter plate assay using ONPG as a substrate, as described in the legend to Fig. 1.

DISCUSSION

On the basis of similarity to the prototypical *Bacillus* systems, BldG was predicted to exert its regulatory function through interaction with the downstream-encoded protein SCO3548. This prediction was experimentally supported by the results of two-hybrid, affinity purification, and far-Western analyses, which conclusively demonstrated that there is a direct interaction between BldG and SCO3548. Preliminary cross-linking analysis also revealed the presence of a BldG-containing complex consistent with the predicted size of a BldG dimer (not shown). A combination of two-hybrid and far-Western analyses was therefore employed to demonstrate the self-interaction of BldG. Although RsbV and SpoIIAA do not form homodimers in solution (16, 34, 35, 57), other homologous anti-anti-sigma factors, including TM1442 of *Thermotoga maritima* (22, 38) and several examples in *Mycobacterium tuberculosis* (48), exist in solution in a dimeric form. Based on our results, we therefore propose that BldG also forms homodimers.

Similarly, many anti-sigma factors, including RsbW and SpoIIAB (16, 21, 57) and several from *M. tuberculosis* (48), form homodimers in solution. Results of two-hybrid analysis and affinity purification demonstrated that SCO3548 also forms homomultimers, which we propose to be homodimers based on similarity to the examples mentioned above. In all examined partner-switching systems, the two protein components associate at a 1:1 ratio to form a heterotetrameric complex (14, 16, 19, 20, 40, 42). The identification of a high-molecular-weight BldG-containing complex by preliminary chemical cross-linking analysis (~70 kDa) (not shown) suggests that BldG and SCO3548 may form a similar heterotetrameric complex. These results are therefore consistent with the prediction that BldG and SCO3548 form a partner-switching regulatory complex with stoichiometry similar to that of previously characterized orthologous systems.

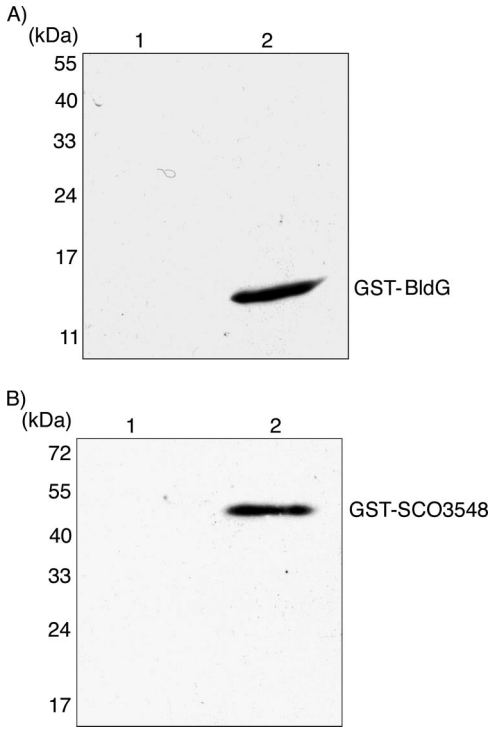


FIG. 5. BldG and SCO3548 self-interactions were detected by far-Western analysis and affinity purification, respectively. (A) For far-Western analysis, cell lysates from *S. coelicolor* Δ*bldG* 1DB containing pIJ6902 (vector control) (lane 1) or pAU316 (*bldG* expression) (lane 2) were subjected to SDS-PAGE, and the separated proteins were transferred to a PVDF membrane. The membrane was incubated with purified GST-BldG, washed thoroughly, and probed with anti-GST antibody. (B) For affinity purification, cell lysate from *E. coli* BL21(DE3) containing pET30(a)$^+$ (vector control) (lane 1) or containing pAU318 (His_{10}-SCO3548) (lane 2) was mixed with cell lysate from *E. coli* BL21(DE3) containing pAU376 (GST-SCO3548) at a ratio of 1:1 (vol/vol). Ni-NTA agarose was added to capture His_{10}-containing protein complexes, which were then eluted with 250 mM imidazole. Eluted proteins were analyzed by SDS-PAGE and Western analysis using anti-GST antibody. The positions of molecular mass markers are indicated on the left.

Without an identified sigma factor target for the BldG-SCO3548 pair, the existence of a partner-switching mechanism cannot be examined further biochemically. Furthermore, the apparently lethal nature of an sco3548 deletion (not shown) restricted our ability to address the in vivo role of SCO3548. Complementation analysis was therefore performed to examine the antagonistic relationship between BldG and SCO3548. The results of this analysis demonstrated that an increase in expression of SCO3548 abrogated the ability of BldG to complement the phenotype of a Δ*bldG* null mutant. This result confirms that the interaction between BldG and SCO3548 affects the regulation of morphological and physiological differentiation in vivo and supports our hypothesis that BldG and SCO3548 likely form an antagonistic partner-switching pair. Given these results, we propose that sco3548 should be given the more descriptive gene designation *apgA* (for *a*ntagonistic *p*artner of Bld*G*).

Furthermore, under wild-type conditions, the *bldG* monocistronic transcript is present at a two- to three-fold molar excess over the *bldG-apgA* polycistronic transcript (9). The fact that introduction of a single additional copy of *apgA* completely antagonizes BldG function therefore indicates that the regulatory function of this system requires an excess of BldG, suggesting that BldG may have additional anti-sigma factor interacting partners. Although a similar system, with an anti-anti-sigma factor regulating two or more anti-sigma factors, has not been identified so far, there are other complex systems which deviate from the *B. subtilis* paradigm. In *M. tuberculosis*, σ^F activity is regulated by a single coexpressed anti-sigma factor, which is in turn antagonized by two distantly encoded anti-anti-sigma factors (4). As well, the σ^F sporulation factor of *S. coelicolor* is regulated by an anti-sigma factor (RsfA) and at least two anti-anti-sigma factors, all encoded at separate loci (32). The presence of 45 additional RsbW orthologues in the *S. coelicolor* genome (45) provides a plethora of possible alternative binding partners for BldG. The possibility that the BldG system involves additional anti-sigma factors therefore must be examined further. As previously discussed (32), analysis of these interactions may prove to be difficult due to the apparent promiscuity of the interactions between the many anti-anti-sigma factors and anti-sigma factors in *S. coelicolor*.

The identity of the regulatory target(s) of the BldG-ApgA pair also remains to be determined. Similarity to the Rsb and SpoIIA systems suggests that the likely target is one of the nine group 3 subfamily sigma factors of *S. coelicolor*; however, current work (13, 15, 33, 37, 49, 54, 56) has not identified a single sigma factor whose mutant phenotype can solely account for the regulatory effect of BldG. It is therefore likely that more than one sigma factor is affected by BldG and/or ApgA. A similar situation was observed by Kim et al. (32), who found that the phenotypes of an *rsfA* anti-sigma factor mutant and the corresponding *sigF* sigma factor mutant were not completely complementary, suggesting that RsfA regulates additional sigma factor targets. Further characterization of the BldG-ApgA system is therefore necessary to identify its regulatory targets and to determine the roles that its interactions play in regulating morphological and physiological differentiation in *S. coelicolor*.

Finally, reversible phosphorylation of the conserved serine residue of BldG has been shown to be critical for regulating the onset of aerial hypha formation and antibiotic production (7) and therefore is predicted to play a critical role in the proposed partner-switching mechanism between BldG and ApgA. In *B. subtilis*, phosphorylation of the anti-anti-sigma factor is carried out by the cognate anti-sigma factor (RsbW and SpoIIAB), which possesses kinase activity (1, 17, 18, 46). A similar relationship does not exist between BldG and ApgA; sequence alignment of ApgA with the *B. subtilis* anti-sigma factors showed that ApgA lacks the conserved residues required for kinase function (9), and protein domain analysis did not reveal the presence of any kinase domain in ApgA (45). Moreover, a *bldG* in-frame deletion mutant does not possess BldG kinase activity (7), despite the fact that *apgA* is known to be expressed in *bldG* mutants. A similar situation has been observed in *M. tuberculosis*, in which the anti-sigma factor UsfX lacks both the conserved kinase domain and the ability to phosphorylate its cognate anti-anti-sigma factor RsfB (4). These observations clearly demonstrate that an additional unidentified kinase must be involved in the phosphorylation of BldG. Candidates

for this unknown kinase are plentiful; a plethora of proteins possessing the necessary HATPase_c kinase domain are present in the *S. coelicolor* genome (45). Identification of this BldG kinase is a critical avenue for future investigation, as the regulation of the phosphorylation state of BldG likely plays a critical role in controlling the antagonistic partner-switching mechanism of BldG and ApgA and thereby in controlling the activity of their unidentified direct regulatory target(s).

Close orthologues of BldG and ApgA have been found in syntenic regions of the genomes in all sequenced *Streptomyces* spp. examined to date (*Streptomyces avermitilis* [25], *Streptomyces griseus* [47], and *Streptomyces scabies* [http://www.sanger.ac.uk/Projects/S_scabies/]), as well as in the partially sequenced species *Streptomyces clavuligerus* (8). In addition, transcriptional regulation of the *bldG* locus is conserved in *S. clavuligerus* and is known to play a similar key role in regulating both morphological and physiological differentiation (8). This system therefore appears to be a key developmental regulatory switch common to members of the genus *Streptomyces*, and understanding it may increase our ability to understand and manipulate a variety of industrially important *Streptomyces* species. Furthermore, the conservation of *bldG* and *apgA* in *Thermobifida fusca* (12) suggests that the *bldG* locus may be important not only in *Streptomyces* but also in a wide range of other actinobacteria.

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