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 $\sigma^B$ and the general stress response factor $\sigma^D$ (1, 17, 18, 44, 50, 57, 59). In these systems, an anti-sigma factor protein (SpoIAB 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; SpoIIA 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 $\sigma^B$ (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 SpoIIA 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 SpoIIB 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*. 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* SpoIIA 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...
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 tetracycline (Sigma), 50 μg/ml ampicillin (Sigma), 50 μg/ml apramycin (Provel), and 50 μg/ml kanamycin (Sigma), and 50 μg/ml nalidixic acid (Sigma). For induction of gene expression from the ptipA promoter, strains were supplemented as required with an antibiotic(s) as follows: 50 μg/ml kanamycin, and 50 μg/ml apramycin, 200 μg/ml tetacycline, and 50 μg/ml kanamycin, 25 μg/ml chloramphenicol (Sigma), and 25 μg/ml nalidixic acid (Sigma). For induction of gene expression from the ptpA promoter on recombinant plasmids, 30 μg/ml threonostetin (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 2. Recombinant plasmids were routinely introduced into E. coli ET1027(pUA28002) by electroporation and subsequently transferred to S. coelicolor ΔbldG 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 cellobiase plates (75-mm 325P disks; Courtald 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).

TABLE 1. Strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Relevant genotype or description</th>
<th>Source and/or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli strains</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5α</td>
<td>Host for plasmid cloning and propagation</td>
<td>F- d80lacZΔM15 recA1 endA1 gyr96 thi-1 λ- hsdR17 (F′ rK- mK+)/supE44 relA1 Δ(lacZYA-argF)U169 phaA</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>BL21(DE3)</td>
<td>Host for expression of recombinant proteins</td>
<td>F- ompT hsdS dcm (F′ mK+)/dcm galE44 galK16 rpsL1 (St++) hsdR2 mcrA1 mcrB1</td>
<td>Stratagene</td>
</tr>
<tr>
<td>BTH101</td>
<td>Reporter strain for bacterial two-hybrid analysis</td>
<td>F- cya-99 araD139 galE15 galK16 rpsL1 (St++) hsdR2 mcrA1 mcrB1</td>
<td>D. MacNeil, Merck Sharp &amp; Dohme Research Laboratories (39)</td>
</tr>
<tr>
<td>ET12567</td>
<td>Nonmethylation host strain</td>
<td>F- dam13::Tn9 dcm6 hsdR143 zj-202::Tn10 galK2 galT22 ara14 lacY1 xyl1 leuB6 thi-1 tonA31 rpsL36 hisG4 txy78 mitB1 gltV44</td>
<td></td>
</tr>
<tr>
<td>S. coelicolor strains</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M145</td>
<td>Wild type</td>
<td>Prototrophic, SCP1 SCP2 Pgl+</td>
<td>John Innes Centre (6)</td>
</tr>
<tr>
<td>ΔbldG 1DB</td>
<td>bldG null mutant</td>
<td>M145 derivative with an in-frame deletion in bldG</td>
<td></td>
</tr>
</tbody>
</table>

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 Ndel and XbaI and were cloned into similarly digested pJL9602. 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 ptpA 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 BK62 primers and was similarly digested with BglII. The resulting KC61-BK62 fragment was then ligated into the prepared pAU316 vector containing the proximal end of the bldG ORF under control of the ptpA 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 Ndel-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 His6-tagged fusion protein, the sco3548 coding region was isolated from plasmid pU317 and an Ndel-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 or AP13 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...
plasmid pAU381 pUT18 harboring sco3548; for expression of SCO3548-T18 in BTH101 Ampicillin This study

pAU382 pUT18 harboring sco3548; for expression of SCO3548-T18 in BTH101 Ampicillin This study

pAU377 pKT25 harboring bldG; for expression of T25-BldG in BTH101 Kanamycin This study

pAU378 pKT25 harboring bldG; for expression of BldG-T18 in BTH101 Kanamycin 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 SCO3548-3548 in BTH101 Kanamycin This study

pAU382 pUT18C harboring sco3548; for expression of T18-SCO3548 in BTH101 Kanamycin This study

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Relevant characteristics and/or use</th>
<th>Resistance</th>
<th>Source and/or reference(s)</th>
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<td>pAU376</td>
<td>pGEX-2TK harboring sco3548; for overexpression of GST-SCO3548 in S. coelicolor</td>
<td>Apramycin Kanamycin</td>
<td>This study Amersham Biosciences</td>
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</tbody>
</table>

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 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 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 AP30 and AP31 for pUT18C constructs.
TABLE 3. Oligonucleotide primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Restriction site</th>
<th>Sequence (5′–3′)*</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP3</td>
<td>BamHI</td>
<td>ATATAGGATCCCGTGACCTGTCCCTG</td>
<td>Forward primer for creation of bldG-containing pKT25-, pUT18C-, and pUT18-based constructs</td>
</tr>
<tr>
<td>AP4</td>
<td>KpnI</td>
<td>ATATAGGTACCCGTCAGTCGTTGGCCGC</td>
<td>Reverse primer for creation of bldG-containing pKT25- and pUT18C-based constructs</td>
</tr>
<tr>
<td>AP11</td>
<td>KpnI</td>
<td>ATATAGGTACCCGTCGTTGGCCGCAC</td>
<td>Reverse primer for creation of bldG-containing pUT18-based constructs</td>
</tr>
<tr>
<td>AP12</td>
<td>BamHI</td>
<td>ATATAGGATCCCAGCCACGGTGCAACTCCGT</td>
<td>Forward primer for creation of sco3548-containing pKT25-, pUT18C-, and pUT18-based constructs</td>
</tr>
<tr>
<td>AP13</td>
<td>KpnI</td>
<td>ATATAGGTACCCGTCAGTCGTTGGCCGC</td>
<td>Reverse primer for creation of sco3548-containing pKT25- and pUT18C-based constructs</td>
</tr>
<tr>
<td>AP14</td>
<td>KpnI</td>
<td>ATATAGGTACCCGTCAGTCGTTGGCCGC</td>
<td>Reverse primer for creation of sco3548-containing pUT18-based constructs</td>
</tr>
<tr>
<td>AP7</td>
<td></td>
<td>GGGGCGCGAGTCCTGCCACGGGCGC</td>
<td>Forward primer for sequencing pKT25 inserts</td>
</tr>
<tr>
<td>AP8</td>
<td></td>
<td>GGGATGTGCTGCAAGGCACCTAAG</td>
<td>Reverse primer for sequencing pKT25 inserts</td>
</tr>
<tr>
<td>AP9</td>
<td></td>
<td>TTTATGCTTCGGGCTCTGATTTT</td>
<td>Forward primer for sequencing pUT18 inserts</td>
</tr>
<tr>
<td>AP10</td>
<td></td>
<td>CAATGCGATGGCTTTCGGAT</td>
<td>Reverse primer for sequencing pUT18 inserts</td>
</tr>
<tr>
<td>AP30</td>
<td></td>
<td>CTCGCCGAGATGACTGGAAC</td>
<td>Reverse primer for sequencing pUT18C inserts</td>
</tr>
<tr>
<td>AP31</td>
<td></td>
<td>CGGCGCTGCTTAACTATGC</td>
<td>Reverse primer for pAU365 construction</td>
</tr>
<tr>
<td>BKL62</td>
<td>EcoRI</td>
<td>CGCGAAATTCTGAGACGGACGCG</td>
<td>Reverse primer for pAU316 construction</td>
</tr>
<tr>
<td>DBG3</td>
<td>XbaI</td>
<td>GGGTCTAGATGCGTTGGCCAT</td>
<td>Reverse primer for pAU317 construction</td>
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<tr>
<td>DBG33</td>
<td>XbaI</td>
<td>GGGTCTAGATGCGTTGGCCAT</td>
<td>Reverse primer for pAU365 construction</td>
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<tr>
<td>DBG34</td>
<td>NdeI</td>
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<td>Reverse primer for pAU316 construction</td>
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<tr>
<td>DBG35</td>
<td>NdeI</td>
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<td>Reverse primer for pAU365 construction</td>
</tr>
<tr>
<td>KC61</td>
<td>XbaI</td>
<td>CGCGAGATGCTGCGACGACGGTCGAGCG</td>
<td>Reverse primer for pAU365 construction</td>
</tr>
</tbody>
</table>

* 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 Borrelia 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 *lacZ*-containing plasmids pKT25-, pUT18C-, and pUT18-based constructs indicated that there was 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 His10-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. His10-SCO3548 was not detected in a control experiment in which native GST was expressed from the parent plasmid pGEX-2TK (Fig. 2A), indicating that His10-SCO3548 does not interact with GST alone and does not associate nonspecifically with glutathione-Sepharose 4B. Affinity purification of His10-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* BldG 1DB containing either pAU316 or pLU6902 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...
Reduction

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* /H9004 /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* /H9004 /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 /H9252 -galactosidase activity was expressed as (units per unit of optical density at 600 nm × ml of cell suspension × 10^9), 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 by bacterial two-hybrid analysis. The *bldG* (aro) and *sco3548* (aro) 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 × 10^9), where 1 U was defined as 1 μmol of o-nitrophenol formed per min. The error bars indicate the standard deviation from the mean.
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 ß-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 ΔbldG 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 ß-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 His6-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 His6-Sco3548 therefore confirmed SCO3548 self-interaction (Fig. 5B).
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 SpoIIA 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.
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-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, $\sigma^F$ 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 $\sigma^F$ 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-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, 37, 49, 54) 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 orthologs of BldG and ApgA have been found in syntetic 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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REFERENCES


44. Min, K. T., C. M. Hilditch, B. Diederich, J. Errington, and M. D. Yudkin. 1993. σB, the first compartment-specific transcription factor of B. subtilis, is regulated by an anti-anti-sigma factor that is also a protein kinase. Cell 74:735–742.


