Study of the *bldG* locus suggests that an anti-anti-sigma factor and an anti-sigma factor may be involved in *Streptomyces coelicolor* antibiotic production and sporulation

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A cloned 2.5 kb DNA fragment that can restore antibiotic production and sporulation to a *bldG* mutant encodes a 113 aa protein showing similarity to a family of anti-anti-sigma factors from *Bacillus* and *Staphylococcus*; and the deduced product of a closely spaced downstream ORF, designated ORF3, shows similarity to cognate anti-sigma factors. The homologues in *Bacillus* regulate the activity of sporulation- and stress-response-specific sigma factors. However, there is no sigma factor gene near *bldG* and ORF3. *bldG* is transcribed both as a monocistronic and a polycistronic mRNA, the latter including the downstream ORF3 gene. The two transcripts were present at all time points during growth and both were upregulated when aerial mycelium and pigmented antibiotics were seen. At all time points, the monocistronic *bldG* transcript was two- to threefold more abundant than the polycistronic transcript. Mapping of the mRNA 5' ends indicated that *bldG* transcription is initiated from two transcription start sites located 82 and 123 bp upstream of the *bldG* translation start. A constructed *bldG* null mutant had the same phenotype as previously isolated *bldG* point mutations, some of which were shown to have potentially significant base changes within *bldG*. When compared to the wild-type strain, the null mutant showed no differences in the levels of transcription from the two *bldG* promoters. These results suggest that *bldG* is not involved in autoregulation.

Keywords: *Streptomyces*, differentiation, antibiotic production, anti-sigma factor antagonist, anti-sigma factor

INTRODUCTION

Members of the genus *Streptomyces* are soil bacteria best known for their ability to produce a wide variety of secondary metabolites including many of the antibiotics used in human and veterinary medicine. In solid-phase laboratory culture, the production of these compounds coincides with the onset of morphological differentiation that leads to sporulation: aerial hyphae grow from the older parts of the substrate mycelium, then proceed through a series of changes that include nucleoid partitioning coupled to the formation of sporulation septa. The end result of this complex process is the

formation of long chains of unigenomic spores (reviewed by Chater, 1998; Kelemen & Buttner, 1998). Differentiation is under the control of an elaborate regulatory system that coordinates gene-expression pathways to bring about the gross morphological changes in both the substrate and aerial hyphae. In addition, the onset of antibiotic production (physiological differentiation) is intimately associated with the onset of morphological differentiation, and these two processes are now known to share common elements in genetic regulation.

In *Streptomyces coelicolor* A3(2), the genetically most-studied streptomycete, many genes required for morphological and physiological differentiation have been identified. As a result of such studies we know that the regulatory mechanisms involve both pathway-specific and global regulatory genes (Champness, 2000). Of

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particular note are the *bld* (bald) genes, mutations in which often result in the failure to form both aerial hyphae and secondary metabolites. Thus the *bld* genes provide a key to understanding how the physiological and structural changes are initiated in the ageing substrate mycelium.

Several *bld* genes have been cloned, sequenced and characterized. This, together with functional analysis of several other genes, in which mutations give rise to a bald mutant phenotype but which had previously been given other gene designations (Chakraborty & Bibb, 1997; Ma & Kendall, 1994; Susstrunk *et al.*, 1998), is beginning to paint a picture whereby the onset of physiological and morphological differentiation is governed by extracellular and metabolic signals, and is regulated at the level of transcription, translation and post-translational modification. When *S. coelicolor* is grown on rich media, Willey *et al.* (1991) showed that structural differentiation to form aerial mycelium depends on an extracellular protein, SapB. Although SapB production was impaired in all of the *bld* mutants tested, juxtaposition of pairs of *bld* mutants on the surface of agar plates resulted in the unidirectional restoration of both SapB and aerial-hyphae formation, suggesting that differentiation is governed by a hierarchical cascade of intercellular signals, with the *bld* genes themselves directly or indirectly responsible for the production of those signals (Willey *et al.*, 1993). Recent studies by Nodwell *et al.* (1996) have provided biochemical evidence in support of this extracellular signalling model; the *bldK* locus encodes the subunits for an ATP-binding cassette family, oligopeptide permease that appears to be the importer of a *bldJ* (formerly *bld261*)-dependent, extracellular factor (Nodwell & Losick, 1998). However, acceptance of this model is complicated by the observation that not all of the *bld* genes fit into the proposed hierarchy (Nodwell *et al.*, 1999; Willey *et al.*, 1993). Of particular note is *bldB*, which encodes a putative DNA-binding regulatory protein and mutations in which, along with many of the other *bld* mutations (*bldA*, -B, -C, -D, -G and -H), causes a deregulation of carbon utilization (Pope *et al.*, 1996). That these mutants activate the *gal* operon promoter under conditions where it is normally repressed, is consistent with the idea that a change in metabolic state is associated with the onset of aerial mycelium formation (Karandikar *et al.*, 1997; Nodwell *et al.*, 1999). This idea is further supported by the finding by Susstrunk *et al.* (1998) that mutations in the adenylate cyclase gene, *cya*, which result in a classic bald phenotype, also cause a medium pH decrease, suggesting that the onset of differentiation involves a switch in metabolism to utilize organic acids released to the medium.

To formulate a model for the regulation of differentiation that takes into account all of these observations, it would be valuable to understand the function of all of the *bld* gene products. To this end we have cloned and sequenced the *S. coelicolor bldG* gene. *bldG* was first identified in a study in which mutagenized colonies were screened for blocks in the formation of aerial hyphae

and antibiotic biosynthesis (Champness, 1988). Three of the mutants identified, C103, C107 and C101J, mapped to the same region of the chromosome and defined the *bldG* locus. Here we show that *bldG* encodes a protein product showing similarity to anti-sigma factor antagonists from *Bacillus* and *Staphylococcus*. Transcriptional analyses have also revealed that *bldG* and the ORF immediately downstream are co-transcribed. The deduced product of the downstream ORF shows some similarity to anti-sigma factors, suggesting that it and BldG might function as a regulatory pair governing the activity of an unknown sigma factor(s).

METHODS

Strains and media. *S. coelicolor* strains used in this study include J1501 [*hisA1 uraA1 strA1 pgl* SCP1⁻ SCP2⁻ (Chater *et al.*, 1982)] and its derivatives C103 [*bldG103* (Champness, 1988)], C107 [*bldG107* (Champness, 1988)] and C536 (*bldG536*; W. Champness, personal communication); C101J [*cysD18 mthB2 NF, bldG101J*] (Champness, 1988)] and M145 [prototrophic, SCP1⁻ SCP2⁻ Pgl⁺ (Hopwood *et al.*, 1985) provided by M. Bibb, John Innes Centre]. C103, C107, C536 and C101J were all provided by W. Champness, Michigan State University. *Streptomyces lividans* 66 (John Innes strain 1326) was the host for ϕ C31 propagation and for the transfection of protoplasts. Media, culture conditions and protoplast transformation and transfection were as described by Hopwood *et al.* (1985).

Escherichia coli host strains were DH5 α (Gibco-BRL) and ET12567 (MacNeil *et al.*, 1992) (a gift from D. MacNeil, Merck Sharp & Dohme Research Laboratories). Media and culture conditions were as described by Sambrook *et al.* (1989).

Plasmid and bacteriophage vectors. *Streptomyces* vector KC304 is a derivative of bacteriophage ϕ C31 and contains the *tsr* (thiostrepton resistance) gene for vector selection; the *vph* (viomycin resistance) gene, flanked by *Bam*HI sites, as a 'stuffer' fragment for replacement by up to 6 kb of insert DNA; and the *att-int* region to allow efficient integration at single copy number into the chromosomal *att* site for ϕ C31. KC304 derivatives were manipulated as described by Hopwood *et al.* (1987). Cosmid H5 (Redenbach *et al.*, 1996) was obtained from Helen Kieser, John Innes Centre, Norwich, UK. Prior to their use to transform *S. coelicolor*, the *E. coli-Streptomyces* bifunctional vectors pSET152 (Bierman *et al.*, 1992) (NRRL B-14792) (containing a multiple-cloning site and replicon of pUC plasmids, the *att-int* region of ϕ C31 and the apramycin-resistance gene for vector selection in either *E. coli* or *Streptomyces*) and pKC1218 (Bierman *et al.*, 1992) (NRRL B-14790) (containing a multiple-cloning site and replicon of pUC plasmids, the SCP2* replicon and the apramycin-resistance gene for vector selection in either *E. coli* or *Streptomyces*) were replicated in the *dam dcm* host *E. coli* ET12567 using standard procedures (Sambrook *et al.*, 1989). *Streptomyces* plasmids were maintained by selection for resistance to thiostrepton (50 μ g ml⁻¹, a gift from S. Lucania, Bristol-Myers Squibb, Princeton, NJ, USA) or Apralan [50 μ g ml⁻¹ and containing 50%, w/w, apramycin; Provel (Division of Eli Lilly Canada)]. *E. coli* plasmids pAU5 (Giebelhaus *et al.*, 1996) and pBluescript II SK/KS (Stratagene) were manipulated as described by Sambrook *et al.* (1989).

Isolation of a *bldG103*-complementing clone from an *S. coelicolor* phage library. A library (generously provided by R. Passantino, Instituto di Biologia dello Sviluppo del Consiglio

Fig. 1. Restriction map of the 2.5 kb *Hind*III insert in pAU63. The thin black line represents *S. coelicolor* M145 DNA and the thick black line represents the flanking øC31 DNA. The hatched area indicates the region of non-contiguous M145 DNA sequence. The location and orientation of *bldG* and the two partial ORFs (ORF1 and ORF3) are shown below the line diagram (not to scale), as are the distances between some restriction sites in kb.

Nazionale dello Ricerche, Palermo, Italy) of *S. coelicolor* M145 DNA fragments in the *Streptomyces* phage vector KC304 was screened as previously described by Elliot *et al.* (1998). In brief, the library screening involved spotting 5 × 20 µl aliquots of the phage-library suspension onto a lawn of *S. coelicolor* C103 mycelial fragments on the surface of each of two R2YE (Hopwood *et al.*, 1985) agar plates. The lawns were allowed to grow for 2 weeks and then the mycelium in and around the spotted areas was scraped from the surface of the plates, pooled and resuspended in sterile Milli-Q water. The mycelia were then homogenized to break the hyphae into small fragments, diluted and plated to give about 100 single colonies per plate on minimal medium (MM; Hopwood *et al.*, 1985) containing glucose as the carbon source and thiostrepton (50 µg ml⁻¹) to select for phage-containing lysogens. Colonies showing aerial mycelium and pigmentation typical of the antibiotic-producing wild-type strain were sought as evidence of *bldG* complementation. Recombinant phages, containing the cloned *bldG* gene, were recovered from sporulating lysogens after CHCl₃ fuming (to prevent transfer of viable spores) and replicated onto Difco nutrient agar plates with SNA (Soft Nutrient Agar) overlays containing *S. lividans* 1326 spores (Hopwood *et al.*, 1985) where free phages released from the lysogens resulted in plaque formation in the *S. lividans* lawn.

Subcloning and sequencing. The ~5.5 kb *bldG*-complementing fragment from KC741 (see Results) was removed from the øC31 vector as a ~6.5 kb *EcoRV* fragment containing 1 kb of flanking øC31 vector DNA. The 6.5 kb blunt-ended fragment was ligated into *EcoRV*-digested pSET152 and the ligation mixture was used to transform *E. coli* DH5α. Digestion of the recombinant plasmid, designated pAU61, with *Hind*III allowed the subcloning of a ~2.5 kb fragment that extended from the unique *Hind*III site in the cloned *bldG*-containing DNA rightwards to the *Hind*III site located in the vector polylinker (see Fig. 1). The fragment was ligated into the *Hind*III site of pKC1218 and the recombinant plasmid, designated pAU63, was isolated after transformation of *E. coli* DH5α and selection for Apralan resistance. pAU63 was then passaged through *E. coli* ET12567 and used to transform protoplasts of *S. coelicolor* C103. Apralan-resistant transformants were selected and visually scored for their phenotype. The 2.5 kb complementing fragment was subcloned in both orientations into the *Hind*III site of pBluescript SK+ and recombinant plasmids were designated pAU64 and pAU65. Each of the pBluescript plasmids was used to generate a series of exonuclease III deletion derivatives by the method of Henikoff (1984) using the Erase-a-Base system (Promega). Additional DNA sequence for the region downstream of the right-hand end of the clone was obtained by first subcloning an overlapping 2.3 kb *Bam*HI fragment extending rightwards from the predicted *bldG* ribosome-binding site (see Fig. 1). The *Bam*HI fragment from the *S. coelicolor* cosmid H5 was shotgun cloned in both orientations into pBluescript KS+ and

recombinant plasmids were identified by hybridization using an ORF3-specific oligonucleotide, JWA9 (5'-CGTCGACGAGCTGGAGG-3'), as the probe. As described above, the pBluescript plasmids, designated pAU66 and pAU67, were then used to generate a series of exonuclease III deletion derivatives. The DNA sequence for the entire insert was determined both by manual and automated (Applied Biosystems model 373A) sequence analysis. The Universal sequencing primer and all other specific primers were obtained from the Department of Biological Sciences Synthesis Service, University of Alberta.

Sequencing of *bldG* mutant alleles. The mutant *bldG* gene was amplified from the chromosome of the *bldG* mutant strains *S. coelicolor* C103, C101J, C107 and C536 using the oligonucleotide primers BKL65 (5'-CCGGTCCAGGAGCATGC-3'; spanning nt -241 to -225) or JWA5 (5'-CACCGAGTCTGTCACTG-3'; spanning nt -160 to -144), located in the 5' flanking region of *bldG*, and JWA6 (5'-GTTCGACGGTGGCCATG-3'; complementary to nt 451-467), located downstream of the *bldG* ORF (nucleotide positions are relative to the *bldG* translation start; see Fig. 3). PCR amplification was carried out with 1 µg *S. coelicolor* genomic DNA as the template and 40 pmol each primer in 100 µl reaction volumes. Expand *Taq* DNA polymerase (1.25 U; Roche) was used in each reaction. The reaction mixtures were denatured at 95 °C and then subjected to 30 cycles of 95 °C for 30 s, 52 °C for 30 s and 72 °C for 1 min. The major 708 bp (BKL65 and JWA6) or 628 bp (JWA5 and JWA6) amplification product was purified from a 5% polyacrylamide gel by crushing and soaking (Sambrook *et al.*, 1989), and sequenced directly (DNA Sequencing Service, Department of Biological Sciences, University of Alberta) using as primers the oligonucleotides BKL65 (or JWA5) and JWA6 (described above); DBG4 (5'-GACCTGTCCCTGTGTCGAC-3'; nt 4-20); BKL81 [5'-CGCGGAATTCGGTTCGAGTCGTGAACG-3'; nt 102-118 and containing a 10 nt non-homologous extension (underlined)]; BKL92 (5'-CGCCACCGCTTCCTCGA-3'; complementary to nt 314-330); and JWA1 (5'-TCAGTCCACCCACCAGC-3'; complementary to nt 186-202) (nucleotide positions are relative to the *bldG* translation start; see Fig. 3) were also used.

Creation of a *bldG* disruption mutant. The *bldG* disruption construct pAU68 was made as follows. First, the 1.4 kb apramycin-resistance gene was cloned as an *EcoRI*-*Bam*HI fragment from pBSApR (a pBluescript derivative containing the apramycin-resistance gene cassette; a gift from S. E. Jensen, Department of Biological Sciences, University of Alberta) into similarly digested pUC119 to give pUC119ApR. The 2.5 kb *Hind*III fragment containing *bldG* (see above) was purified from pAU64 and ligated into the *Hind*III site of pUC119ApR. Disruption of *bldG* was achieved by insertion of a blunted 1 kb *tsr* fragment (isolated from pAU5 using *Sma*I and *Xba*I) into the blunted *Bgl*II site located in the *bldG*

coding region (see Fig. 3). The resulting recombinant plasmid was designated pAU68 and was passaged through *E. coli* ET12567 before being used to transform *S. coelicolor* J1501 and M145 protoplasts. Thiostrepton-resistant and Apralan-sensitive double-crossover mutants of each strain were selected and further tested by Southern blotting to confirm the double-crossover genotype. The *S. coelicolor* J1501 and M145 *bldG* disruption strains were designated *S. coelicolor* C3b (*bldG3b*) and *S. coelicolor* C1a (*bldG1a*), respectively.

Complementation of *bldG* mutants. A 917 bp fragment containing the *bldG* ORF and upstream promoter was generated by PCR using pAU64 as template and the oligonucleotides DBG12 [5'-GCGCGAATTCGTGCCGGTGCGGAC-3'; nt -450 to -434 and containing a non-homologous tail (underlined) with an *EcoRI* site] and DBG3 [5'-GCGCTCTAGAGTTCGACGGTGGCCATG-3'; complementary to nt 451-467 and containing a non-homologous tail (underlined) with an *XbaI* site] as primers (nucleotide positions are relative to the *bldG* translation start). The amplified DNA was gel purified by the trough-purification method (Zhen & Swank, 1993), digested with *EcoRI/XbaI* and ligated into *EcoRI/XbaI*-digested pSET152. The resulting recombinant plasmid, designated pAU69, was isolated after transformation of *E. coli* DH5 α and selection for Apralan resistance. After passage through *E. coli* ET12567, pAU69 was introduced into protoplasts of *S. coelicolor bldG* mutants C536, C107, C101J and C3b by protoplast transformation. Apralan-resistant transformants were scored for their morphological and pigmentation phenotype.

RNA isolation. *Streptomyces* cultures were grown on cellophane discs (75 mm, 325P discs; Courtaulds Films) on the surface of R2YE agar as previously described (Leskiw *et al.*, 1993). RNA was extracted essentially as described elsewhere (Hopwood *et al.*, 1985) except that mycelia were scraped directly from the cellophane discs into modified Kirby mix. The RNA was isolated at various time points as described in Results.

Northern blot analysis. This was performed according to Williams & Mason (1985). RNA (40 μ g) was denatured with glyoxal and DMSO, and size fractionated by electrophoresis at 4 V cm⁻¹ on a 1.25% agarose gel using a 10 mM Na₂HPO₄/NaH₂PO₄ (pH 7.0) recirculating buffer system. DNA molecular mass marker III (625 ng; Boehringer), treated in the same way, served as the size marker. Capillary transfer to a Hybond-N (Amersham) membrane was as described by Sambrook *et al.* (1989). For detection of *bldG* transcripts, the probe was an [α^{32} P]dCTP random-primer-labelled 207 bp PCR product internal to *bldG* (the probe was generated by PCR using primers BKL63, 5'-GTGAGGCCGGTGATACG-3', and BKL64, 5'-GCCCAAGCTGCGTGAGC-3'). For detection of transcripts extending through the intergenic region and into the ORF located downstream of *bldG*, the probe was an [α^{32} P]dCTP random-primer-labelled 428 bp PCR product internal to the downstream ORF [the probe was generated by PCR using primers DBG8, 5'-GCGCAAGCTTGTCGGTACCGCCCGTCT-3', and BKL83, 5'-GCGCAAGCTTGGGTGAATGCGGCGGTC-3', both containing 10 nt non-homologous extensions (underlined)]. Hybridization was performed overnight at 65 °C in a solution containing 50% formamide (Hopwood *et al.*, 1985) and 4 $\times$ 10⁶ c.p.m. probe. After hybridization, the nylon filter was washed at the same temperature for 2 $\times$ 30 min in a solution containing 2 $\times$ SSC (0.3 M NaCl, 0.03 M sodium citrate), 0.1% SDS and then 2 $\times$ 30 min in 0.2 $\times$ SSC, 0.1% SDS. The signals were detected using a PhosphorImager (Molecular Dynamics model 445 SI). The molecular mass marker was visualized by hybridizing

[α^{32} P]dCTP random-primer-labelled marker DNA to the filter at 44 °C. As a control for RNA loading levels, a probe for 16S rRNA was hybridized to the same blot. The 16S rRNA probe was an oligonucleotide, 5'-CCGCCTTCGCCACCGGT-3', corresponding to a conserved region in *Streptomyces* 16S rRNA sequences. Hybridization and washing were performed at 55 °C without formamide according to Procedure B described by Hopwood *et al.* (1985).

S1 nuclease mapping of the *bldG* transcription-start site. The probe for S1 nuclease mapping of *bldG* was generated by PCR amplification of a 264 bp fragment using pAU64 as the template. The primers were an 18-mer synthetic oligonucleotide, DBG14 (5'-CGTCAATTCGCCACCGA-3'), corresponding to a sequence internal to the *bldG* ORF and a 27-mer synthetic oligonucleotide, JWA20 [5'-GCGCAA-GCTTGGGATCGATCGGGTCGG-3', corresponding to a region 194 nt upstream of the *bldG* start codon and containing a 10 nt non-homologous extension (underlined)]. The probes for S1 nuclease protection of the intergenic region between *bldG* and the downstream ORF were generated by PCR amplification of a 172 bp and a 401 bp fragment using pAU64 as template. The primers were a 17-mer synthetic oligonucleotide, JWA17 (5'-GGTACGGACGTGCTCGG-3'), corresponding to a sequence internal to the downstream ORF, and either a 27-mer synthetic oligonucleotide, JWA18 (5'-GCGCAAGCTTGCCACCGACTGACGACC-3') or a 27-mer oligonucleotide, BKL81 (5'-GCGGGAATTCGGTCG-AGCTCGTGAACG-3'), corresponding to regions 121 nt and 350 nt upstream of the start codon for the downstream ORF and containing 10 nt non-homologous extensions (underlined). The amplified DNA was purified from a 2% agarose gel by the trough-purification method (Zhen & Swank, 1993). The 5' ends of the amplified DNA (about 2 pmol) were labelled with [γ^{32} P]ATP using T4 polynucleotide kinase. The probes, labelled at both ends, were used without treatment since the non-homologous extensions would be removed by the S1 nuclease treatment and would not result in the appearance of labelled, protected fragments (Leskiw *et al.*, 1993). The sequencing ladders for the *bldG* and intergenic region S1 nuclease mapping were generated by the dideoxy chain-termination method (Sanger *et al.*, 1977) using DBG14 and JWA17 as primers, and pAU64 as the template. For each S1 nuclease protection reaction, 40 μ g RNA was hybridized to 50000 Cerenkov c.p.m. probe in formamide buffer as described by Hopwood *et al.* (1985) except that glycogen (Roche) replaced the carrier tRNA. To control for RNA loading levels, the RNA samples were first subjected to Northern blot analysis using the 16S rRNA probe (see above) and aliquots showing equivalent signals were subsequently used for S1 nuclease mapping. The samples were run under standard conditions on a 6% polyacrylamide sequencing gel.

Primer-extension mapping of the *bldG* transcription-start site. The primer was a 17-mer synthetic oligonucleotide, DBG15 (5'-GTCGACAGGGACAGGTC-3'), that was designed to hybridize immediately downstream from the *bldG* start codon and approximately 100 bp away from the proposed transcription-start point. Primer (50 pmol) was end-labelled with [γ^{32} P]ATP as described above. Approximately 5 pmol of 32 P-labelled primer and 40 μ g RNA were dissolved in 1 $\times$ SB buffer (60 mM NH₄Cl, 10 mM Tris-acetate, pH 7.4, 6 mM 2-mercaptoethanol) (Hartz *et al.*, 1988), denatured by heating to 90 °C for 5 min and annealed by transferring to 75 °C, slow cooling to 55 °C and then incubating at 55 °C for 1 h. The primer-annealed RNA was ethanol precipitated, washed with 80% ethanol and air-dried for 10 min. Extension of the primer was performed at 45 °C for 1 h in a solution of

1×SB buffer, 15 mM magnesium acetate, 3 mM dNTPs, 17·5 U RNAGuard (Amersham) and 12·5 U AMV reverse transcriptase (Roche). Loading dye (98% deionized formamide, 10 mM EDTA, pH 8·0, 0·025% xylene cyanol, 0·025% bromophenol blue) was added and the reaction was evaporated at 80 °C for 20 min. The entire reaction mixture was loaded onto a 6% polyacrylamide sequencing gel. A sequencing ladder was generated as described above using the same oligonucleotide as for the primer-extension reactions.

Computer-assisted sequence analysis. General raw sequence handling was done using the GeneTool 1.0 program (BioTools). A version of the FRAME (Bibb *et al.*, 1984) program modified to run on an Apple Macintosh (obtained from S. E. Jensen, University of Alberta, Canada) was used to detect putative ORFs. Similarities between deduced protein products and known proteins in the databases were detected using BLAST at the internet site <http://ncbi.nlm.nih.gov>. Multiple sequence alignments were generated using the PILEUP program of the Genetics Computer Group. Potential RNA secondary structures, together with ΔG values, were determined using the Mfold 2.3 program with the folding temperature set to 30 °C, the standard growth temperature used for these studies. The Mfold programs are available at the internet site <http://www.ibc.wustl.edu/~zucker/rna/>. Analysis of the ORF3 protein sequence for conserved bacterial histidine kinase domains was done using the Prosite ProfileScan server located at the internet site http://www.isrec.isb-sib.ch/software/PFSCAN_form.html.

RESULTS

Cloning of the *bldG* gene

Before the *S. coelicolor* genome project (see http://www.sanger.ac.uk/Projects/S_coelicolor/) began, we had cloned *bldG* by complementation of the *S. coelicolor bldG103* mutation. *bldG*-containing recombinant KC304 phage derivatives were identified from a library of *S. coelicolor* M145 DNA fragments by their ability to restore antibiotic production and aerial hyphae formation to the *S. coelicolor* C103 mutant strain. The phage library was introduced into the *bldG* mutant, and two thiostrepton-resistant lysogens that were able to sporulate and produce the pigmented antibiotics actinorhodin and undecylprodiosin were selected for further study out of ~4000 colonies screened. The phages released from the lysogens were respooned on a *S. coelicolor* C103 lawn to verify that the cloned DNA was responsible for restoring antibiotic production and sporulation. It was not determined whether the production of calcium-dependent antibiotic, the other antibiotic produced by *S. coelicolor* J1501, was restored. Both lysogens appeared to have identical inserts and therefore only one, designated KC741, was chosen for further study.

Sequencing and analysis of ORFs

The ~5·5 kb DNA fragment containing *bldG* was subcloned from KC741 as a 6·5 kb *EcoRV* fragment containing 1 kb of flanking ϕ C31 vector DNA. The fragment was introduced into pSET152, which integrates site specifically into the *S. coelicolor* chromosome at the ϕ C31 *attB* site, generating pAU61. Further

subcloning of a 2·5 kb *HindIII* fragment from pAU61 into pKC1218, a low-copy-number *E. coli*–*Streptomyces* shuttle vector, generated pAU63. Introduction of pAU63 into the *S. coelicolor* C103 mutant restored apparently wild-type levels of both pigmented-antibiotic production and aerial-mycelium formation. Nucleotide sequencing of the 2·5 kb *HindIII* fragment revealed two partial (ORF1 and ORF3) and one complete ORF (ORF2) (Fig. 1). BLAST analysis indicated that the predicted product of ORF2 resembles a group of anti-anti-sigma factor proteins that include RsbV from *Staphylococcus aureus* (Wu *et al.*, 1996) and SpoIIAA and RsbV of *Bacillus subtilis* (Kalman *et al.*, 1990; Dufour & Haldenwang, 1994; Duncan *et al.*, 1996). Since ORF2 was the only complete ORF present on the subcloned 2·5 kb DNA fragment that was able to complement the *bldG103* mutation, this ORF was designated *bldG* (GenBank accession no. AF134889). Since genes encoding anti-anti-sigma factors are typically encoded as the first gene in an operon also encoding a cognate anti-sigma factor and sigma factor (Kalman *et al.*, 1990), the remainder of the ORF3 sequence, as well as an additional ~1 kb of downstream sequence was determined. Comparison with sequences in the databases revealed that the ORF3 protein product resembles anti-sigma factor proteins of *B. subtilis*, including SpoIIAB (Duncan & Losick, 1993; Min *et al.*, 1993) and RsbW (Benson & Haldenwang, 1993a); anti-sigma F and anti-sigma B proteins, respectively. Surprisingly, a sigma factor was not encoded in the sequence downstream of the putative anti-sigma factor. Instead, a partial ORF showing similarity to pyrophosphate synthases was located 339 nt downstream of the ORF3 stop codon. The long intergenic region, together with the existence downstream of the ORF3 stop codon of a sequence capable of forming, in RNA, a stable stem-loop structure [$\Delta G = -21·3 \text{ kcal mol}^{-1}$ (-89 kJ mol^{-1})] that might serve as a transcription-termination signal, suggested that the putative pyrophosphate synthase gene is not part of a *bldG* operon. The partial sequence of the divergently expressed ORF1 suggests that the protein product belongs to a group of bacterial RNA helicases. The sequences for *bldG* and the surrounding ORFs on cosmid H5 have been now been determined as part of the *S. coelicolor* genome sequencing project (http://www.sanger.ac.uk/Projects/S_coelicolor/) and have been deposited under accession no. AL035636. It is clear from the sequence analysis that the 2·5 kb *HindIII* fragment present in our clone does not arise from contiguous chromosomal sequences and has come from the ligation of two non-contiguous *Sau3AI* fragments during library preparation. The non-contiguous DNA is shown as a hatched box in Fig. 1.

Alignment of *BldG* and the ORF3 protein products with known proteins from the databases

The *bldG* gene encodes a 113 aa protein with end-to-end similarity to a number of anti-anti-sigma factors that regulate the activity of sigma factors responsible for

Fig. 2. Multiple protein sequence alignment of *S. coelicolor* (*S.coel*) BldG with RsbV and SpoIIAA homologues (a), and of ORF3 with RsbW, SpoIIAB and other anti-sigma B homologues (b) from *B. licheniformis* (*B.lich*), *B. subtilis* (*B.sub*, *B.s*), *S. aureus* (*S.aur*) and *L. monocytogenes* (*L.mono*). B.lichASF refers to the putative anti-sigma B regulator of *B. licheniformis*. Alignments were generated using the PILEUP program of the Genetics Computer Group. Black shading indicates identical or similar amino acid residues in all six sequences, dark grey shading with white lettering indicates identical or similar amino acid residues in five of the six sequences and light grey shading with black lettering indicates identical or similar amino acid residues in four of the six sequences. (a) The serine residue of SpoIIAA that is phosphorylated by SpoIIAB is indicated by the arrowhead above the alignment. (b) Conserved residues of bacterial histidine kinase proteins that are thought to be important for ATP and magnesium binding in *B. subtilis* SpoIIAB and RsbW are indicated by asterisks above the alignment.

stress-induced or growth-stage-specific transcription. BldG is most closely related to the *Sta. aureus* and *B. subtilis* RsbV proteins (38% and 40% identity, and 61% and 60% similarity, respectively), and to *B. subtilis* SpoIIAA (26% identity, 56% similarity). Alignment of BldG with RsbV and SpoIIAA proteins (Fig. 2a) reveals a highly conserved region of sequence surrounding the serine residue known to be phosphorylated on the *B. subtilis* SpoIIAA (Najafi *et al.*, 1995). Likewise, alignment of the ORF3 product with other anti-sigma factors revealed about the same degree of similarity to RsbW from *B. subtilis* and *Listeria monocytogenes* (28% identity, 43% similarity and 25% identity, 44% similarity, respectively), and to SpoIIAB from *B. subtilis* (26% identity, 42% similarity). These anti-sigma factors are encoded along with, and interact directly with, RsbV and SpoIIAA, respectively. Since both RsbW

and SpoIIAB in *B. subtilis* have been shown to have kinase activity that regulates their interaction with either their cognate sigma factor or anti-anti-sigma factor by phosphorylation of the anti-sigma factor antagonist (Dufour & Haldenwang, 1994; Min *et al.*, 1993), the ORF3 amino acid sequence was aligned with RsbW and SpoIIAB sequences to look for conserved kinase domains. As shown in Fig. 2b, the ORF3 protein product contains only two of the five conserved amino acid residues that are thought to be important for ATP and magnesium binding in RsbW (Kang *et al.*, 1996) and SpoIIAB (Min *et al.*, 1993), and in bacterial histidine kinases in general (Stock *et al.*, 1995). Also, analysis of the ORF3 protein sequence using the Prosite ProfileScan Server did not reveal any conserved histidine kinase domains within the sequence. These findings suggest that the ORF3 protein product lacks the kinase activity

Fig. 3. Nucleotide sequence of the *bldG* promoter and coding region. The deduced amino acid sequence of *bldG* and the partial amino acid sequences of ORF1 and ORF3 are shown below in single letter code. Nucleotide positions are indicated relative to the first nucleotide of the *bldG* start codon (designated +1). The proposed transcription start sites (P1, P2; ○→) and putative -10 and -35 sequences for *bldG* are shown, as well as potential ribosome-binding sites (RBS) for *bldG* and ORF3. The two inverted repeats at the end of the *bldG* coding region are indicated by the arrows. Point mutations identified in the *bldG* mutant strains are shown in bold, with the base changes indicated above. Oligonucleotide primers used for sequencing of the *bldG* mutations are indicated by (□) above the sequence. The unique *Bgl*II site used to construct the *bldG* disruption vector is underlined.

that has been shown in *B. subtilis* to be important for the regulation of anti-anti-sigma factor and anti-sigma factor interactions.

A constructed *bldG* null mutant has the same phenotype as the strains with *bldG* point mutations

A *bldG* null mutant allele was constructed by inserting the thiostrepton-resistance gene (*tsr*) at a unique *Bgl*II restriction site internal to *bldG* (Fig. 3). The mutant allele was used to replace the wild-type allele in the chromosomes of *S. coelicolor* J1501 and M145 as described in Methods. Disruption of *bldG* in the chromosome of representative J1501 and M145 *bldG* mutants was confirmed by Southern blotting (data not shown) and the strains were designated *S. coelicolor* C3b (*bldG3b*) and *S. coelicolor* C1a (*bldG1a*), respectively. Both phenotypically resembled the *bldG* mutants of Champness (1988) in being devoid of blue or red pigments and failing to make detectable aerial mycelium on R2YE or MM+glucose (but sporulating aerial mycelium was formed, as with other *bldG* mutants, on MM+mannitol). The M145 *bldG* mutant was not studied further.

To confirm that the constructed J1501 *bldG* null mutant did not contain any other defects, pAU69, a pSET152-derivative containing a copy of *bldG* together with its promoter region, was introduced into *bldG3b*. The same complementation plasmid was also introduced into the *bldG* point mutants used in this study. pAU69 restored the wild-type phenotype to *bldG3b* as well as the C103, C107 and C536 strains; however, introduction of the plasmid had no effect on the phenotype of C101J.

Sequencing of *bldG* mutations

To map the location of the *bldG* mutation in C103, C107 and C536, as well as to confirm the absence of a mutation in the *bldG* coding region of C101J, the *bldG* ORF from the four *bldG* mutants was amplified by PCR and the resulting products were sequenced. As expected on the basis of the complementation studies, *bldG101J* did not have a mutation in the *bldG* coding or promoter region. Since the mutation in this strain maps to the same region of the chromosome as the mutations in C103, C107 and C536, it may reside in one of the nearby ORFs. Alternatively, the C101J strain may be a double mutant, making the genetic mapping data difficult to interpret and possibly misleading. As shown in Fig. 3, the *bldG103* and *bldG536* mutations both involve an A→T substitution at the same base, introducing a stop codon that would generate a truncated 87 aa BldG protein. The *bldG107* mutation, the substitution of an adjacent CT for AG, leads to an amino acid change of aspartate to glutamate at position 56, and of a serine to alanine at position 57 of BldG. The latter residue corresponds to one known to be phosphorylated in SpoIIAA by the SpoIIAB protein kinase. These data suggest that the C-terminal 26 aa of BldG, as well as the conserved region corresponding to the phosphorylation

Fig. 4. Northern blot analysis of *bldG* and ORF3 transcripts. RNA (40 µg) was isolated from *S. coelicolor* J1501 and the *bldG* disruption mutant, *bldG3b*, at various times post-inoculation (h) as indicated. The probe for *bldG* was a random-primer-labelled 207 bp PCR product internal to *bldG*, and a 428 bp random-primer-labelled PCR product was used to probe for ORF3 transcripts. Molecular mass marker III (Boehringer) was used for size determination. RNA integrity and loading levels were controlled by hybridizing the same blots with an oligonucleotide probe specific for 16S rRNA.

site on the *B. subtilis* SpoIIAA, are important for BldG function.

Although some *bldG* transcripts are monocistronic, some also include ORF3

Northern blotting was performed using RNA isolated from *S. coelicolor* J1501 to determine the size of the *bldG* transcript as well as the timing of expression. The experiments were performed at least twice using RNA from three different time courses, and representative results are shown in Fig. 4. The probe, a 207 bp fragment internal to *bldG*, hybridized to two RNA species with sizes of 600–700 nt and 1100–1200 nt. The size of the smaller transcript is consistent with a monocistronic *bldG* transcript terminating in the intergenic region between *bldG* and the start of ORF3, whereas the larger transcript has a size comparable to that expected for a polycistronic transcript including both *bldG* and the downstream ORF3 gene. When a second blot, prepared at the same time and in the same manner as the first, was probed with a PCR-amplified probe internal to the putative anti-sigma factor-encoding ORF3 (Fig. 4), only a band corresponding to the larger transcript was observed, and the same results were observed when a single blot was first hybridized with the *bldG*-specific probe, stripped and reprobed with the ORF3-specific probe (data not shown). The two transcripts were present at low levels during vegetative growth and at higher levels from 24 h post-inoculation, the time point

Fig. 5. High resolution S1 nuclease mapping of the intergenic region between *bldG* and ORF3. RNA (40 µg) was isolated from surface-grown *S. coelicolor* J1501 at 24 and 36 h post-inoculation. The probe was an end-labelled 182 bp PCR product containing a non-homologous extension to distinguish between probe reannealing and full-length protection. The sequencing ladder was generated with the oligonucleotide (JWA17) used to synthesize the probe. Probe+S1, control lane containing probe that went through the S1 procedure; Probe-S1, control lane containing probe that did not go through the S1 procedure.

corresponding to the appearance of aerial hyphae and pigmented antibiotics. Quantitative analysis of the two bands indicated that the smaller transcript is present at a two- to three-fold higher concentration than the larger transcript.

The putative anti-sigma factor encoded downstream of *bldG* does not have its own promoter

To further investigate whether the putative anti-sigma factor gene is co-transcribed with *bldG* rather than being transcribed from its own promoter, S1 nuclease protection studies were performed using a probe corresponding to the intergenic region between the two ORFs. RNA samples isolated from three independent time courses were tested and representative results are shown in Fig. 5. The major S1 product observed at all the time points tested has a size of 172 nt, which is equivalent to the size of the full-length probe excluding the 10 nt non-homologous extension. Full-length protection was also observed with a 401 bp probe generated using the same anti-sigma factor gene-specific internal primer (JWA17) and the oligonucleotide BKL81 (corresponding to nt 102–118 relative to the *bldG* start codon), which anneals to a region within the *bldG* ORF (data not shown). These data confirm that an anti-sigma factor gene-specific promoter is not located either in the intergenic region or within 3' sequence of the BldG

Fig. 6. Detection of *bldG* mRNA 5' ends by primer-extension analysis and high-resolution S1 nuclease mapping. RNA (40 µg) was isolated from surface-grown *S. coelicolor* J1501 and *bldG3b* at various times (h post-inoculation) as indicated. For the primer-extension reactions, an end-labelled oligonucleotide (DBG15) complementary to the sequence immediately downstream of the *bldG* translation start codon was hybridized to the RNA. The probe for S1 mapping was an end-labelled 264 bp PCR product. Sequencing ladders for primer extension and S1 mapping were generated using the oligonucleotides DBG15 and DBG14 (used for probe synthesis), respectively. *, most probable transcription-start point; Probe+S1, control lane containing probe that went through the S1 procedure; Probe-S1, control lane containing probe that did not go through the S1 procedure.

coding region. In the light of these data, the question was raised as to why disruption of *bldG* did not lead to polar effects on expression of the downstream gene. Analysis of the sequence of the disruption plasmid revealed that *tsr* was transcribed in the same direction as *bldG* and ORF3. Since the *tsr* terminator was not present on the cloned *tsr*-containing fragment, it is possible that ORF3 could be expressed by readthrough transcription from either the *bldG* promoter (located upstream of the inserted *tsr* gene) or from the *tsr* gene promoter. Both the ability to complement the *bldG3b* strain with a plasmid containing only the *bldG* ORF, as well as a Northern blot analysis using an ORF3-specific probe, confirmed the existence of such transcripts (Fig. 4).

S1 nuclease mapping and primer-extension analysis reveal two *bldG* mRNA 5' ends

To determine the transcription-initiation site for the *bldG* transcripts, high-resolution S1 nuclease mapping studies were performed using a 264 bp PCR-amplified probe labelled at the 5' ends. As above, RNA samples isolated from three independent time courses were tested and representative results are shown in Fig. 6.

Interestingly, two major S1 nuclease-protected products of 152 nt and 193 nt were detected when the hybridization was carried out at 5 °C and at 12 °C (not shown) above the predicted probe melting temperature. If these products correspond to transcription-start points, the +1 positions would be situated 82 and 123 nt upstream of the *bldG* translation-start codon. The same mRNA 5' ends were identified by primer-extension analysis of the same RNA samples using an end-labelled 17 nt oligonucleotide primer (Fig. 6), showing that the shorter protected species (see Fig. 3) was not an artefact caused by an S1 nuclease hypersensitive site. The transcription-start site proximal to the translation-start codon is located just downstream of a putative -10 sequence similar to those found in *E. coli*-like *Streptomyces* promoters (Strohl, 1992). At an atypically short spacing of 16 bp, a -35-like sequence was also present in this region. Similarly, a putative -10 sequence was found upstream of the distal transcription-start point. In this case, it was not possible to identify appropriately spaced -35 sequences. As was seen in the Northern analysis, both transcripts are expressed at low levels during vegetative growth and are upregulated when aerial mycelium and pigmented antibiotics become visible. The relative intensities of the signals for transcripts initiating at both promoters correlate well with the relative intensities of the long and short transcripts seen by Northern analysis. These results may suggest that initiation from the distal promoter gives rise to the polycistronic transcript and that transcription initiation at the proximal promoter gives rise to the monocistronic transcript. However, the low resolution of the Northern blots did not permit us to address this directly.

The *bldG* gene product does not appear to be involved in an autoregulatory circuit

The well-studied *bldG* homologue *spoIIAA* plays an indirect part in its own transcriptional regulation. Like *bldG*, *spoIIAA* is transcribed from two promoters. One of these is dependent on the σ^F factor encoded by *spoIIAC*, the third gene in the *spoIIA* operon. Inactivation of *spoIIAA* leaves σ^F locked in a complex with SpoIIAB, preventing use of the σ^F -dependent promoter (Schuch & Piggot, 1994). To find out whether *bldG* is likewise important for transcription of one or both of its promoters, S1 nuclease protection studies using RNA samples isolated from the constructed *bldG* null mutant, *bldG3b*, were performed (Fig. 6). The same two mRNA 5' ends that were identified in *bldG*⁺ strain were detected at comparable relative abundance in the mutant, indicating that the loss of *bldG* activity has no effect on *bldG* transcript initiation.

DISCUSSION

The simplest interpretation of the extracellular-signalling model for regulation of differentiation in *S. coelicolor* is that all of the *bld* genes might directly

encode the intercellular signals, or the components for sensing or uptake of those signals. On the basis of what we know about the *bld* gene products characterized to date, this simple interpretation is not supported. So far, only *bldK* and *bldJ* appear to encode direct components of the hierarchical signalling cascade (Nodwell *et al.*, 1996) and all of the other *bld* genes appear to encode products that must have indirect roles in signal synthesis, sensing or uptake. *bldA* encodes a leucyl tRNA that is the only means for efficient translation of rare UUA codons (Leskiw *et al.*, 1991, 1993) found, for example, in the messages for the pathway-specific activators of pigmented-antibiotic production (Fernández-Moreno *et al.*, 1991; White & Bibb, 1997); *bldD* encodes a small transcription factor that appears to regulate gene expression by its DNA-binding ability (Elliot *et al.*, 1998; Elliot & Leskiw, 1999); *bldB* encodes a small protein with some similarity to BldD in that it also contains a putative DNA-binding helix–turn–helix motif near its C terminus (Harasym *et al.*, 1990; Pope *et al.*, 1998); and now here we propose that *bldG* encodes a putative anti-anti-sigma factor that might control developmentally regulated transcription by regulating the activity of a specific sigma factor(s).

Despite the sequence similarities between BldG and the deduced protein product of its co-transcribed, downstream ORF3 with proteins known to regulate the activity of certain sigma factors in *Bacillus*, we see differences between the way that the genes are organized and regulated in the two organisms. In contrast to both the *spoIIA* and *rsb* operons from *Bacillus*, a sigma factor is not encoded at the *bldG* locus. So far, there are no reports in the literature of genes for an anti-sigma factor antagonist and its anti-sigma factor lying at a distant location from the gene for the cognate sigma factor. If *bldG* and ORF3 do in fact encode an anti-anti-sigma/anti-sigma factor pair, this raises the possibility that the regulatory pair might regulate the activity of more than one sigma factor, or that they may regulate a particular sigma factor only in a subset of the conditions in which that sigma factor directs gene expression. The antibiotic- and aerial-mycelium deficient phenotype of *bldG* mutants then presents us with two possibilities: the regulatory pair could control one or more globally acting sigma factors that serve to activate transcription of both antibiotic and sporulation-specific genes; or alternatively, the pair could regulate two sigma factors, one of which activates transcription of antibiotic biosynthetic genes and one which activates sporulation-specific gene expression. Interestingly, of two sporulation-specific sigma factors so far identified in *Streptomyces* (Kelemen *et al.*, 1996; Méndez & Chater, 1987), one (σ^F) is of the subfamily regulated by homologues of BldG/ORF3 in *Bacillus*. Sigma factors recognizing antibiotic-biosynthetic-gene promoters have not been definitively identified. It is attractive to suggest that immobilization of the putative anti-sigma factor on an affinity column might lead us to one such sigma factor. A search of the *S. coelicolor* database reveals that there is no shortage of potential sigma factor

targets. The *Bacillus* sigma factors that are regulated by anti-anti-sigma/anti-sigma factor pairs make up a subfamily of σ^{70} -like sigma factors (Lonetto *et al.*, 1992), and so far at least 9 sigma factors that would fall into this subfamily are found on the *S. coelicolor* chromosome; several of them (in addition to *sigF*) are not located next to *bldG*/ORF3-like gene pairs (Gabriella Kelemen, personal communication).

The C-terminal region of sigma factors in this subfamily shares homology with the DNA-binding region of a group of bacterial transcriptional-activator proteins (Kahn & Ditta, 1991; Lonetto *et al.*, 1992). Our analysis of the σ^F sequence using the Prosite ProfileScan server revealed similarity between region 4.2 near the σ^F C terminus and the helix–turn–helix domain of LuxR-related DNA-binding proteins. This raises the alternative possibility that the BldG and ORF3 regulatory pair could target a DNA-binding protein rather than a sigma factor. However, this possibility seems unlikely since the candidate σ^F contact residues for SpoIIAB are not located in region 4.2 of σ^F . SpoIIAB contacts σ^F in three areas corresponding to conserved regions 2.1, 3.1 and 4.1 of σ^{70} -like sigma factors (Decatur & Losick, 1996). So far, only two putative contact residues on SpoIIAB have been identified (Garsin *et al.*, 1998); and one of these two residues, R20, is conserved in the ORF3 gene product.

In addition to the absence of an operon-encoded sigma factor, the *bldG* operon differs from the *Bacillus* operons in the way that the expression of the genes is controlled. In the case of both the *spoIIA* and *rsb* operons, the genes for the anti-anti-sigma factor, the anti-sigma factor and the sigma factor are expressed as a single transcript, from either a σ^H -dependent promoter in the case of *spoIIA* (Wu *et al.*, 1991) or a σ^A -dependent promoter in the case of *rsb* (Wise & Price, 1995). Expression of the genes is also upregulated from a second promoter that is recognized by the operon-encoded sigma factor (Schuch & Piggot, 1994; Wise & Price, 1995). For the *spoIIA* operon, upregulation is dependent on the activity of a prespore-compartment-specific phosphatase, SpoIIE, that activates the SpoIIAA anti-anti-sigma factor by dephosphorylation, allowing the formation of SpoIIAA/SpoIIAB complexes and the liberation of σ^F (Duncan *et al.*, 1995; Wu *et al.*, 1998). Upregulation of σ^B occurs either in response to energy stress, where reduced ATP levels in the cell influence the phosphorylation state of the anti-anti-sigma factor, RsbV, or in response to environmental stress signals that activate a phosphatase that dephosphorylates RsbV and results in the liberation of σ^B (Voelker *et al.*, 1995).

For the *bldG* operon we also see a complex transcription pattern involving not only two different promoters, but also the generation of two differently sized *bldG*-containing transcripts. Based on the Northern analyses, the longer and less abundant of the two transcripts extends through *bldG* to include the downstream putative anti-sigma ORF3. The shorter, more strongly expressed transcript appears to terminate in the inter-

genic region, and includes only the *bldG* coding sequence, a situation not seen with either the *spoIIA* or *rsb* operons. Analysis of the intergenic sequence for potential RNA secondary structures revealed two inverted repeats that could give rise to stem-loop structures with ΔG values of -22.2 and -31.3 kcal mol $^{-1}$ (-93 and -131 kJ mol $^{-1}$) (see Fig. 3), respectively, and either of which might play a role in mRNA stabilization or as a transcription-termination signal. Although a string of U residues is not present downstream of either inverted repeat, it is well documented that inverted repeats without poly (U) tails can act as terminators in *Streptomyces* (Deng *et al.*, 1987; Ingham *et al.*, 1995). The existence of the shorter transcript that hybridizes only to the *bldG* probe might suggest that a terminator is functional; however, an alternative mechanism for the generation of the shorter transcript could be endo- or exo-nucleolytic removal of the 3' ORF3-containing end of the longer transcript. Similarly, although a comparison of transcript abundance as seen by Northern analysis, primer-extension and S1 nuclease protection studies may suggest that the longer transcript arises from initiation at the more upstream promoter, and that transcripts initiating from the proximal *bldG* promoter terminate in the intergenic region, our analyses do not allow a definitive conclusion.

For both the *Bacillus spoIIA* and *rsb* operons, the genes appear to be translationally coupled such that they are expressed in equimolar concentrations (Magnin *et al.*, 1997; Benson & Haldenwang, 1993b; Kalman *et al.*, 1990). The lack of a promoter in the intergenic region between *bldG* and the downstream ORF3 encoding the putative anti-sigma factor suggests that these two genes might also be translationally coupled. However, the very long intergenic region means that there cannot be a straightforward mechanism of coupling that involves either closely spaced or overlapping start and stop codons to ensure a 1:1 stoichiometry. This, together with the added complication of a *bldG* monocistronic transcript that might lead to an excess of BldG, highlights the need to address the levels of the BldG and ORF3 proteins throughout the life cycle. By analysing the protein levels we should be able to answer questions about how the equilibrium between BldG and the ORF3 product is shifted at different stages of growth. Certainly, appearance of a transcript does not mean that the RNA is being translated to protein, a fact that warrants further investigation because of the expression of a putative RNA helicase from a divergently expressed promoter that overlaps the -10 region of the most upstream *bldG* promoter (J. Stoehr & B. K. Leskiw, unpublished).

The availability of the purified proteins and antibodies to those proteins will also help us to explore the role, if any, of the conserved phosphorylation site on BldG. Although mutation at this site does abolish BldG activity in the same way that Ser58 mutation in SpoIIAA does, the existence of a second mutation in this region in *bldG107*, together with the lack in the putative anti-sigma factor of conserved residues found in bacterial

histidine protein kinases, makes it difficult to draw any conclusions.

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