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# The *bldC* Developmental Locus of *Streptomyces coelicolor* Encodes a Member of a Family of Small DNA-Binding Proteins Related to the DNA-Binding Domains of the MerR Family

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The *bldC* locus, required for formation of aerial hyphae in *Streptomyces coelicolor*, was localized by map-based cloning to the overlap between cosmids D17 and D25 of a minimal ordered library. Subcloning and sequencing showed that *bldC* encodes a member of a previously unrecognized family of small (58- to 78-residue) DNA-binding proteins, related to the DNA-binding domains of the MerR family of transcriptional activators. BldC family members are found in a wide range of gram-positive and gram-negative bacteria. Constructed Δ*bldC* mutants were defective in differentiation and antibiotic production. They failed to form an aerial mycelium on minimal medium and showed severe delays in aerial mycelium formation on rich medium. In addition, they failed to produce the polyketide antibiotic actinorhodin, and *bldC* was shown to be required for normal and sustained transcription of the pathway-specific activator gene *actII-orf4*. Although Δ*bldC* mutants produced the tripyrrole antibiotic undecylprodigiosin, transcripts of the pathway-specific activator gene (*redD*) were reduced to almost undetectable levels after 48 h in the *bldC* mutant, in contrast to the *bldC*⁺ parent strain in which *redD* transcription continued during aerial mycelium formation and sporulation. This suggests that *bldC* may be required for maintenance of *redD* transcription during differentiation. *bldC* is expressed from a single promoter. S1 nuclease protection assays and immunoblotting showed that *bldC* is constitutively expressed and that transcription of *bldC* does not depend on any of the other known *bld* genes. The *bldC18* mutation that originally defined the locus causes a Y49C substitution that results in instability of the protein.

A special feature of the developmental cycle of the filamentous bacteria *Streptomyces* is the formation at the start of differentiation of an aerial mycelium. This structure consists of hyphae that grow out of the aqueous environment of the substrate mycelium into the air, giving the developing colonies their characteristic fuzzy appearance. Subsequently, each multigenomic aerial hypha undergoes a synchronous septation event, giving rise to ~50 to 100 unigenomic prespore compartments that ultimately develop into mature exospores (10, 16, 26, 52).

Genetic analysis of differentiation in *Streptomyces coelicolor* has revealed at least three classes of genes required for the formation of an aerial mycelium: the *ram*, *chp*, and *bld* genes. The *ram* and *chp* genes specify hydrophobic structural components required for aerial hyphae to escape surface tension and grow into the air, while the majority of *bld* genes encode regulatory proteins (16).

The *ram* genes were discovered by virtue of their ability to induce rapid aerial mycelium formation when overexpressed in the wild type (32), and they have recently been shown to specify production of the morphogenetic peptide SapB (29). SapB is a 21-amino-acid peptide that plays a vital role in aerial mycelium formation during growth on rich medium (16, 26, 51, 52). It functions as a surfactant, releasing surface tension at the air-water interface to allow nascent aerial hyphae to escape into the air (47). The structure of SapB has recently been determined, revealing it to be a lantibiotic-like peptide containing two eight-residue loops formed by lanthionine bridges between Cys and dehydroalanine residues (29). Aerial mycelium formation on minimal medium is SapB independent, and SapB is not produced (51). The *ram* cluster consists of five genes, the SapB biosynthetic operon itself (*ramCSAB*) and the divergently encoded response regulator, RamR, which activates transcription of the *ramCSAB* operon on rich medium (25, 38, 40). SapB is derived from the 42-amino-acid primary translation product of the *ramS* gene through extensive posttranslational modification (29). RamC is likely to be the SapB synthetase involved in this posttranslational processing, and the *ramAB* genes encode components of an ABC transporter that may function in SapB export (29). A *ramR* mutant is blocked in SapB production and cannot erect aerial hyphae on rich medium (38, 40). Conversely, overexpression of *ramR* results in SapB overproduction and the biosynthesis of SapB by wild-type strains under conditions when its production is normally repressed (38). The *ram* genes are not transcribed during growth on minimal medium (25).

The chaplins are a family of eight hydrophobic cell wall-associated proteins that confer hydrophobicity on aerial hyphae and spores, and constructed strains lacking most or all of the chaplin (*chp*) genes fail to form aerial hyphae on all media

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tested (12, 13, 19). The chaplins can self assemble to form amyloid-like fibers and are believed to heteropolymerise into filaments on the surface of the aerial hyphae (12, 13, 19). Like SapB, the chaplins are very surface active and can reduce surface tension from 72 to 26 mJ/m$^2$ (12), an ability likely to facilitate the escape of aerial hyphae into the air and to prevent them from collapsing back through surface tension.

*bld* mutants are a class of developmental mutants that cannot erect aerial hyphae and therefore appear bald, lacking the characteristic fuzzy morphology of the wild type (10, 16, 26, 52). Many of the characterized *bld* genes encode proteins with regulatory functions; *bldB*, *bldD*, and *bldM* encode known or putative DNA-binding proteins (15, 17, 18, 27, 35, 42), *bldN* encodes a sigma factor (1, 2), and *bldG* specifies an anti-anti-sigma factor (4). Expression of the chaplin genes is developmentally regulated, and their transcription is blocked in all the *bld* mutants tested, including *bldC* (19). Expression of the *ram* genes is also developmentally regulated, and *ramR* and *ramCSAB* transcription is blocked in *bldA*, *bldB*, *bldD*, and *bldH* mutants (*bldC* has not been tested) (25). Further, almost all *bld* mutants regain the ability to form aerial structures when purified SapB is applied to the colony surface (51). However, these aerial filaments fail to metamorphose into chains of spores, implying that SapB plays a purely structural role and that the *bld* genes control other functions vital for differentiation in addition to SapB production (47). Similarly, the engineered overexpression of *ramR* in *bld* mutant strains induces SapB biosynthesis and restores aerial hyphae formation (38).

In addition to their morphological complexity, streptomycetes are also renowned for their ability to produce a diverse range of secondary metabolites, many of medical and veterinary importance. As well as causing loss of aerial mycelium formation, mutations in some *bld* loci have pleiotropic effects on antibiotic production. Certain *S. coelicolor bld* mutants are deficient in the production of actinorhodin, undecylprodigiosin, methylenomycin, and calcium-dependent antibiotic (7, 9, 34).

*bldC* was one of four loci identified in the first genetic screen for *S. coelicolor* mutants defective in aerial mycelium formation (34) and it is the only remaining uncharacterized locus from that study. Only one mutant allele of *bldC* (*bldC18*, carried by strain J660) was identified. Phenotypic characterization of *bldC* strains showed that, as with many of the *bld* mutants, aerial mycelium formation depended on the medium; although bald on many media, aerial hyphae formation and sporulation were restored on minimal medium containing mannitol, galactose, or maltose (34). Here, we use a map-based cloning strategy to isolate *bldC* and find that it encodes a member of a family of small DNA-binding proteins related to the DNA-binding domain found in members of the MerR family of transcriptional activators. We show that the *bldC18* allele carries a point mutation that results in loss of the *bldC* product, causing the same phenotype as constructed null mutants. We also show that *bldC* is required for expression of the pathway-specific activators of the actinorhodin and undecylprodigiosin biosynthetic genes during differentiation.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and growth conditions.** *S. coelicolor* strains used are listed in Table 1 and were cultured on R2YE, SMMS, MS, or DNA plates and in yeast extract-malt extract (YEME) or tryptic soy broth liquid media (28). *Escherichia coli* BL21λDE3(pLysS) (46) was used to overproduce BldC, and BW25113 (14) was used for PCR-targeted disruptions (21). Plasmids used were pSET152 (3), pIJ2925 (24), and pET15b (Novagen).

**Protoplast transformation and conjugation from *E. coli* into *S. coelicolor*.** To bypass the methyl-specific restriction system of *S. coelicolor*, cosmids and plasmids were passed through the *dam dcm hsdS E. coli* strain ET12567 (33) prior to protoplast transformation or conjugation. *E. coli* ET12567 carrying the nontransmissible, *oriT*-mobilizing "driver" plasmid pUZ8002 (41) was used for conjugation. *Streptomyces* protoplast transformation and conjugation from *E. coli* to *Streptomyces* were carried out as described by Kieser et al. (28).

**PCR-based site-directed mutagenesis.** The oligonucleotides BLDC1 and BLDC2 were used to amplify *bldC* and its promoter region from M600. The PCR product was cloned into the SmaI site of pIJ2925 to create pIJ6839. A single base-pair substitution was engineered by PCR-based site-directed mutagenesis. Two abutting oligonucleotides, Y49F FOR and Y49F REV, were used to amplify the whole of the plasmid, introducing a change from TAC to TTC into the Y49 codon. The PCR program was 95°C for 5 min; then 10 cycles of 95°C for 1 min, 60°C for 30 s, and 72°C for 8 min 30 s; 10 cycles of 95°C for 1 min, 60°C for 30 s, and 72°C for 12 min 30 s; followed by a final elongation step of 72°C for 15 min. The resulting PCR product was circularized by self ligation to create pIJ6840 and sequenced to ensure that only the intended mutation had been introduced. The *bldC* alleles were subcloned as BamHI-EcoRI fragments into the conjugative vector pSET152 to create pIJ6842 (encoding wild-type BldC) and pIJ6844 (encoding BldCY49F). The *bldC18* allele was amplified from the chromosome of J660 with the oligonucleotides BLDC1 and BLDC2 and cloned into the *Eco*RV site of pSET152 to create pIJ6843 (encoding BldCY49C). The constructs were introduced into J660 by conjugation.

**Overexpression of His-tagged BldC and production of a BldC polyclonal antibody.** A BldC overexpression construct was produced by amplifying the *bldC*-coding region with M600 chromosomal DNA as a template and the oligonucleotides BLDC5 and BLDC6. These oligonucleotides introduced an NdeI site overlapping the ATG start codon and changed the fourth, sixth, seventh, and eighth codons to synonymous codons commonly associated with highly expressed genes in *E. coli*. The resulting PCR fragment was cloned into the SmaI site of pIJ2925 to create pIJ6837 and sequenced. The *bldC* coding region was removed from pIJ6837 as a NdeI-BamHI fragment and ligated to pET15b (Novagen) that had been digested with NdeI and BamHI to produce pIJ6838. pIJ6838 was introduced into *E. coli* BL21λDE3(pLysS) (46), and overexpression of BldC was induced in exponentially growing cells (optical density at 600 nm, 0.5) by addition of 0.1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 4 h at 30°C. The cell pellet was resuspended in 10 ml of binding buffer (20 mM Tris-HCl [pH 7.5], 0.5 M NaCl, 5 mM imidazole) before lysis was completed by four 15-s cycles of sonication at 1-min intervals on ice. The cell lysate was centrifuged at 18,000 rpm for 30 min at 4°C in a Sorvall SS34 rotor. His-tagged BldC was purified on a HiTrap Chelating HP column (Amersham Pharmacia Biotech) with a Biocad Sprint perfusion chromatography system. A total of 2 mg of His-tagged BldC was used to raise a polyclonal antiserum in rabbit (Genosys).

**Crude cell extract preparation and immunoblot analysis.** Surface-grown *Streptomyces* cultures were harvested from cellophane-covered R2YE plates into 1 ml of complete protease inhibitor buffer (Roche) in 0.1 M HEPES (pH 7.2). Samples were sonicated at half power for three cycles of 10 s at 1-min intervals on ice. Cell debris was removed by centrifugation at 14,000 rpm for 10 min at 4°C, and the protein concentration of the supernatant was determined with Bradford reagent (Bio-Rad). Tricine sodium dodecyl sulfate-polyacrylamide gels (45) were used to enhance resolution of proteins in the range of 5 to 20 kDa. Samples (each, 20 μg) were separated by electrophoresis, transferred to a Hybond-C Extra nylon membrane (Amersham Pharmacia Biotech), and probed with a 1:1,000 dilution of rabbit anti-BldC antibody. Horseradish peroxidase-coupled secondary antibody (Amersham Pharmacia Biotech) was used at a 1:5,000 dilution and detected by chemiluminescence with ECL Western blotting detection reagents (Amersham Pharmacia Biotech).

**Construction of *bldC* null mutants and genetic manipulations with cosmid SCD25.** Null mutants were constructed by replacing the entire *bldC* coding sequence with a cassette carrying the apramycin resistance gene (*apr*) and *oriT* of RK2 by PCR targeting (21). Cosmid SCD25 was introduced into *E. coli* BW25113 (14) carrying plasmid pIJ790 (21), and *bldC* was disrupted by electroporation of the cells with the PCR-amplified *apr-oriT* cassette, generated with the primers BLDC K-OUT FOR and BLDC K-OUT REV (Table 1). The resulting cosmid (SCD25Δ*bldC*::*apr*) was introduced into *E. coli* ET12567 carrying pUZ8002 (41) and transferred into *S. coelicolor* M600, M145, or J660 by conjugation; apramycin-resistant (Apr$^r$), kanamycin-sensitive (Kan$^s$) exconjugants were identified and purified (J2161, J2167, and J2162, respectively) (Table 1).

TABLE 1. Strains and oligonucleotides used in this study

| Strain or primer | Genotype or sequence | Reference or source |
|---|---|---|
| **Strains** | | |
| *S. coelicolor* | | |
| M600 | SCP1⁻ SCP2⁻ | 28 |
| M145 | SCP1⁻ SCP2⁻ | 28 |
| J660 | *bldC18 mthB2 cysD18 agaA7* SCP1ᴺᶠ SCP2* | 34 |
| J2161 | Δ*bldC*::*apr* derivative of M600 | This work |
| J2162 | Δ*bldC*::*apr* derivative of J660 | This work |
| J2166 | Δ*bldC* derivative of M600 | This work |
| J2167 | Δ*bldC*::*apr* derivative of M145 | This work |
| J2168 | Δ*bldC* derivative of M145 | This work |
| J2169 | *bldC⁺* derivative of J660 | This work |
| J3210 | *bldC18* derivative of M600 | This work |
| J3211 | *bldC18* derivative of M145 | This work |
| J1700 | *bldA39 hisA1 uraA1 strA1* SCP1⁻ SCP2⁻ | 30 |
| J669 | *bldB43 mthB2 cysD18 agaA7* SCP1ᴺᶠ SCP2* | 34 |
| J774 | *bldD53 cysA15 pheA1 mthB2 strA1* SCP1ᴺᶠ SCP2* | 34 |
| 166 | *bldF hisD3 pheA1 strA1* SCP1⁺ SCP2⁺ | 43 |
| WC103 | *bldG103 hisA1 uraA1 strA1* Pgl⁻ SCP1⁻ SCP2⁻ | 7 |
| WC109 | *bldH109 hisA1 uraA1 strA1* Pgl⁻ SCP1⁻ SCP2⁻ | 7 |
| HU261 | *bldJ261 hisA1 uraA1 strA1* Pgl⁻ SCP1ᴺᶠ SCP2* | 50 |
| NS17 | *bldK*::*aadA* derivative of M145 | 39 |
| J2151 | Δ*glkA119 bldM*::*hyg* SCP1⁻ SCP2⁻ | 35 |
| J2177 | Δ*glkA119 bldN*::*hyg* SCP1⁻ SCP2⁻ | 2 |
| **Primers** | | |
| Cloning and mutagenesis primers | | |
| BLDC1 | CGTCGCTTGGAGCTGTGGTTCC | |
| BLDC2 | TGCCGACAAGGGACGTAGGTCC | |
| BLDC5 | CCGCATATGACCGCTCGTACCCCGGACGCTGAGCCGCTGCTGACC | |
| BLDC6 | CCCGGTTATTCAGTTGTTCAGG | |
| Y49F FOR | TTCCGCGAAGCCGAGGTCCG | |
| Y49F REV | GCGGCGATGCCCGCCGAGCG | |
| Gene disruption primers | | |
| BLDC K-OUT FOR | ATGAACCGAGAAGGTTCGGTTCTCCCGAGGAGGCCGCTCATTCCGGGGATCGTCGACC | |
| BLDC-K-OUT REV | GGTTGGGGGACCCGCCGTTCTGCCCGGTTATTCAGTTGTTGTAGGCTGGAGCTGCTTC | |
| S1 nuclease mapping and runoff primers | | |
| BLDC2 | See cloning and mutagenesis primers | |
| BLDC3[a] | GAACATGGTGGCGACCTCAGCC | |
| BLDC4 | TATATACTGTGACTGATCGTCACAGAGG | |
| BLDC17 | GAGCGGCCTCCTCGGGAG | |
| BLDC25[a] | GCACCTTGACCCGCGTTGCC | |
| ACTIIORF4.1[a] | GTACGTCTGCAGCGTCGTCATGGCGCT | |
| ACTIIORF4.2 | ATCGGAGATCGCTTGTGACGGCA | |
| HRDB FOR | CGCAAGGTACGAGTTGATGACC | |
| HRDB REV[a] | CCATGACAGAGACGGACTCGG | |
| REDD.1[a] | GATCGATACGGGTCCCAATA | |
| REDD.JW2 | CATGGATCCTGCTTCGTTTGCGTCGTTCAGTTC | |
| REDZ.1[a] | CTCCAGCAATGCGCGAAT | |
| REDZ.2 | CGTGCACGTGTCCTTCTG | |

[a] Labeled primers.

The disruptions were confirmed by PCR and Southern analysis. An in-frame deletion allele of *bldC* was also constructed; cosmid SCD25Δ*bldC*::*apr* was introduced into *E. coli* BT340 (11), and deletion of the *apr-oriT* cassette (which is flanked by FRT sites) was obtained after induction of FLP recombinase (21). The mutant cosmid thus obtained, SCD25Δ*bldC*, was introduced into J2161 and J2167 by protoplast transformation, and Kanʳ transformants were selected. After growth in the absence of antibiotic selection, colonies that had lost both apramycin and kanamycin resistance were selected and purified (J2166 and J2168, respectively) (Table 1) and analyzed by PCR to confirm replacement of *apr-oriT* cassette by the nonpolar "scar" sequence (21).

A *bldC⁺* derivative of J660 was constructed by introducing the SCD25 cosmid into J2162 (the Δ*bldC*::*apr* derivative of J660) (Table 1) by protoplast transformation and selecting for Kanʳ transformants. After nonselective growth, a strain sensitive to both apramycin and kanamycin was obtained and purified (J2169) (Table 1).

*bldC18* derivatives of M600 and M145 were obtained by first rescuing the *bldC18* allele from the chromosome of J660 into the SCD25 cosmid. This was done by introducing the SCD25Δ*bldC*::*apr* cosmid into J660 by conjugation and selection for Aprʳ Kanʳ exconjugants; these were pooled and grown in 100 ml of liquid YEME medium without antibiotics until late exponential phase and used
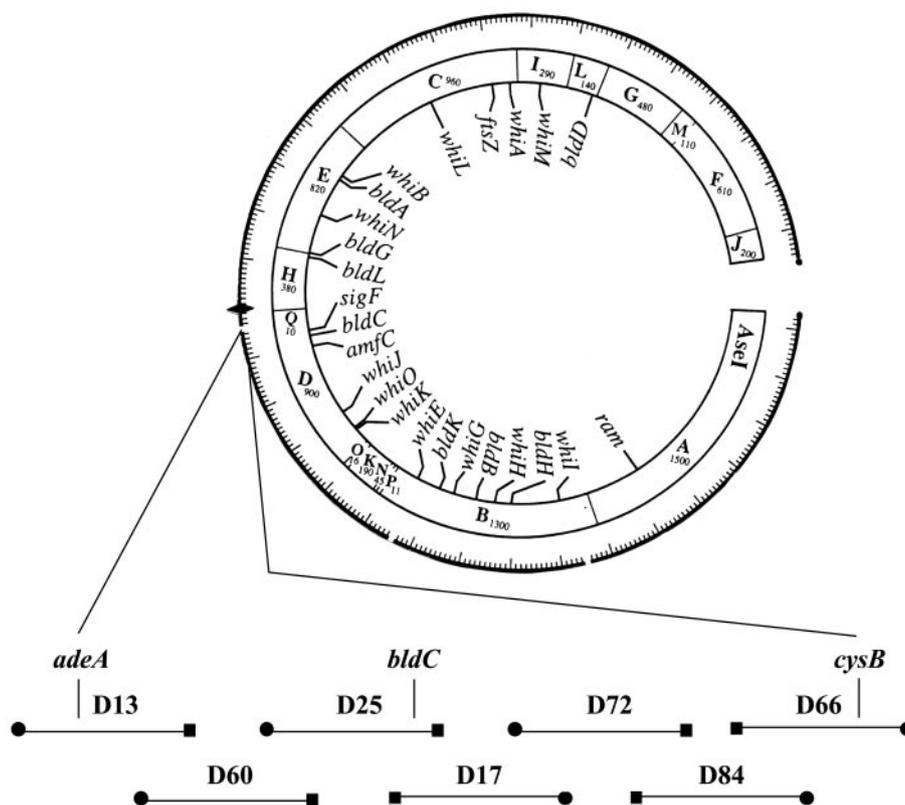
FIG. 1. A simplified version of the combined physical and genetic map of the *S. coelicolor* chromosome, showing the locations of developmental genes and the seven ordered cosmids that span the *adeA* to *cysB* interval in the 8 o'clock region. The location of *bldC* is shown on the overlap between cosmids D17 and D25, as determined by complementation. The locations of other developmental genes on the cosmid contig are shown on the inside of the circle. The cosmids and their overlaps are arbitrarily shown to be of equal length. The sizes of the *Ase*I fragments are given in kilobases. ●, T3 end; ■, T7 end; ◆, *oriC*.

to purify covalently closed circular DNA (28). The DNA was concentrated in a small volume and used to transform *E. coli* DH5α to kanamycin resistance; about 20% of all Kan$^r$ colonies were Apr$^s$ and carried cosmids whose restriction pattern was indistinguishable from that of the wild-type SCD25 cosmid. One of these cosmids (SCD25*bldC18*) was purified; DNA sequencing confirmed that it carried the *bldC18* allele. SCD25*bldC18* was introduced into J2161 (the Δ*bldC::apr* derivative of M600) (Table 1) and J2167 (the Δ*bldC::apr* derivative of M145) (Table 1) by protoplast transformation, and Kan$^r$ colonies were selected. After growth in the absence of antibiotics, Kan$^s$ Apr$^s$ colonies were selected and purified, resulting in strains J3210 and J3211, respectively (Table 1). Amplification and sequencing of *bldC* from the chromosome of these strains confirmed that they carried the *bldC18* allele.

**RNA isolation, S1 nuclease protection analysis, and in vitro transcription.** Mycelium was harvested from *S. coelicolor* strains grown on cellophane-covered R2YE plates, and RNA was extracted as described by Kieser et al. (28). Quantitation of RNA was carried out by UV spectroscopy and agarose gel electrophoresis. For each S1 nuclease reaction mixture, 30 μg of RNA was hybridized to a 5′ end-labeled probe at 45°C for 4 to 15 h following denaturation at 65°C for 10 min. S1 nuclease (Amersham Pharmacia Biotech) digestions were performed as described by Kieser et al. (28), and the reaction products were separated on 6% polyacrylamide denaturing sequencing gels. Uniquely end-labeled probes were generated by PCRs with a 5′ end-labeled primer internal to the coding sequence and an upstream, unlabeled primer. Primers were 5′ end labeled with [γ-$^{32}$P]ATP and T4 polynucleotide kinase (Transgenomic or Invitrogen).

In vitro transcription was carried out as described by Buttner et al. (6). Runoff templates were generated by PCR with oligonucleotide BLDC4 as the upstream primer and oligonucleotide BLDC3 (template 1; 327 bp) or BLDC17 (template 2; 264 bp) as the downstream primer. RNA polymerase was purified as described by Kieser et al. (28) from strain M600 and grown to late exponential phase in YEME medium.

**Microscopy.** Scanning electron microscopy of *Streptomyces* colonies was performed as described previously (36).

**DNA sequence analysis.** BLAST and PSI-BLAST searches were done online at the National Center for Biotechnology Information (NCBI) site (http://www.ncbi.nlm.nih.gov). Sequence comparison and alignment were also done online with ClustalW and EMBOSS at the European Bioinformatics Institute sites (http://www.ebi.ac.uk). BLAST searches on draft actinomycete genome sequences were done at the respective web sites (http://genome.ornl.gov/microbial/tfus for *Thermobifida fusca*, http://www.bcgsc.ca/gc/rhodococcus for *Rhodococcus* sp. RHA1, and http://nocardia.nih.go.jp/for *Nocardia farcinica*).

## RESULTS

**Identification of the *bldC* gene by complementation.** The *bldC18* mutation was previously mapped genetically to a position between *adeA* and *cysB* in the 8 o'clock region of the *S. coelicolor* chromosome (34). This region is spanned by seven cosmids of the ordered library of Redenbach et al. (44), as illustrated in Fig. 1. Although these cosmids cannot replicate autonomously in *S. coelicolor*, they can integrate into the chromosome via insert-directed homologous recombination. Each of the seven cosmids shown in Fig. 1 was individually introduced into *bldC18* strain J660 by protoplast transformation, selecting for kanamycin resistance. Transformants were patched onto R5 medium and analyzed for restoration of aerial hyphae formation, sporulation, and pigmented antibiotic pro-
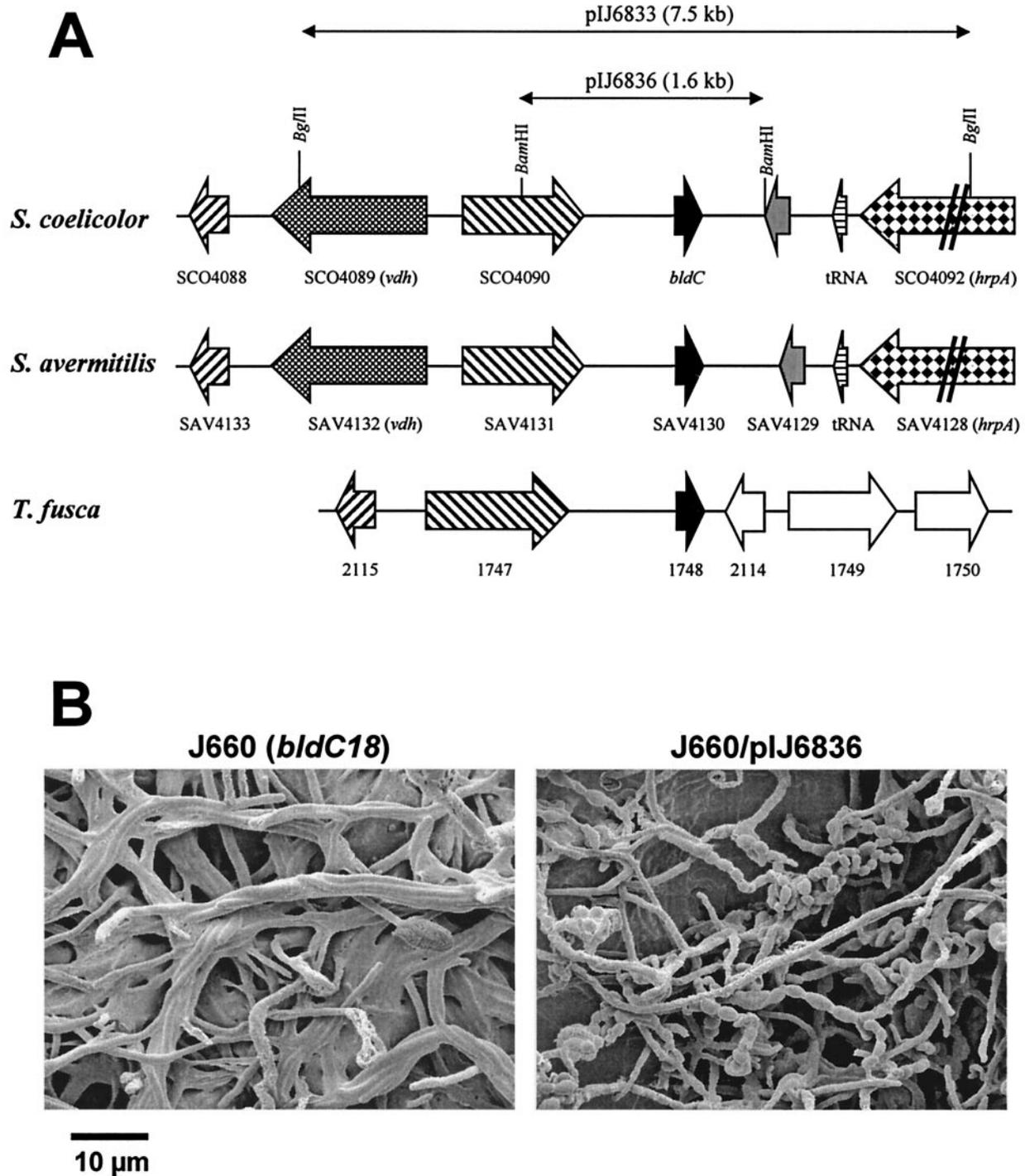
FIG. 2. (A) Conservation of genetic organization of the *bldC* region between *S. coelicolor*, *S. avermitilis*, and *T. fusca*. The positions of protein-encoding genes and tRNA genes are indicated by arrows, and restriction sites referred to in the text are marked. Equivalent shading of arrows indicates homology. The extent of the pIJ6833 and pIJ6836 subclones described in the text is shown above the *S. coelicolor* map. (B) Scanning electron micrographs showing the developmental phenotype of J660 (*bldC18*) and its complementation by *bldC* carried on pIJ6836. Strains were grown on R2YE plates.

duction. Two overlapping cosmids, D25 and D17, complemented the *bldC18* mutation, indicating that *bldC* resides in the 27.8-kb overlap between these two cosmids that extends from SCO4075 to SCO4097 and encompasses 23 protein-en-

coding genes and five tRNA genes. Subcloning identified a 7.5-kb *Bgl*II fragment that complemented the *bldC18* mutation when cloned into the integrating vector pSET152 and introduced into J660 (pIJ6833) (Fig. 2A). Further rounds of sub-

FIG. 3. Amino acid sequence alignments. (A) Alignment of BldC with the N-terminal DNA-binding domains of MerR family proteins. Note that the complete BldC sequence is shown but that the MerR family proteins are arbitrarily truncated halfway through the dimerization helix. Proteins and their corresponding NCBI accession numbers are as follows: *S. coelicolor* BldC (CAB56371), *E. coli* ZntR (BAB37580), *Ralstonia metallidurans* PbrR (CAC28872), *E. coli* CueR (AAC73589), *Bacillus subtilis* MtaN (CAB15677), *S. lividans* TipAL (CAB42766), *Pseudomonas aeruginosa* MerR (CAA83897), *E. coli* SoxR (CAA42162), *B. subtilis* BltR (CAB14599), and *B. subtilis* BmrR (CAB14333). (B) Alignment of BldC with other members of the BldC family of small DNA-binding proteins. The complete sequence of each protein is shown. Proteins and their corresponding NCBI accession numbers are as follows: *S. coelicolor* BldC (CAB56371), *S. avermitilis* BldC (= SAV4130) (BAC71842), *T. fusca* (ZP_00293028), *N. farcinica* (partial sequence), *S. coelicolor* SCO3328 (CAB45362), *C. diphtheriae* (CAE48899), *M. tuberculosis* (CAE55290), *P. gingivalis* (AAQ66571), *C. violaceum* (AAQ61522), *B. cereus* (AAP12345), *B. thuringiensis* bacteriophage Bam35c (CAD59942), and *S. meliloti* (CAC41930). H T H, helix-turn-helix motif. The position of the BldC Y49C amino acid substitution caused by the *bldC18* point mutation is shown.

cloning localized the complementing region to a 1.6-kb BamHI fragment, which contained only one complete open reading frame (ORF), SCO4091 (pIJ6836) (Fig. 2A). Scanning electron micrographs of J660 with and without pIJ6836 confirmed that this plasmid complemented the *bldC18* mutation, restoring the ability to produce aerial hyphae, spores (Fig. 2B), and pigmented antibiotics. SCO4091 was therefore designated *bldC*.

**BldC is a member of a previously unrecognized family of small DNA-binding proteins related to the DNA-binding domains of the MerR family.** *bldC* encodes a protein only 68 amino acids long. BLAST searches of the NCBI databases showed that BldC is most similar to the DNA-binding domains of members of the MerR family of transcriptional activators (Fig. 3A). MerR family members function as homodimers. Each subunit consists of a conserved N-terminal DNA-binding domain and a nonconserved C-terminal effector recognition domain, and transcriptional activation is triggered by effector binding (5, 8, 20, 37, 53). Between the N-terminal and C-terminal domains lies a long α-helix that interacts with the same helix in the other subunit, forming a coiled coil responsible for dimerization (5, 8, 20, 37, 53). However, BldC apparently lacks this dimerization helix and a C-terminal effector

recognition domain, with sequence similarity limited to the N-terminal DNA-binding domain of the MerR family (Fig. 3A).

A more thorough PSI-BLAST search was carried out to look for BldC-like proteins consisting exclusively of a MerR-like DNA-binding domain. This search revealed several such proteins from both gram-positive and gram-negative bacteria (Fig. 3B). The most similar proteins of this kind were found in other actinomycetes. The product of *Streptomyces avermitilis* gene SAV4130 is 100% identical to BldC and is the predicted orthologue in this species (see below). The *Thermobifida fusca* genes TFU627 and TFU1748 encode proteins with 60 and 91% identity to BldC, respectively, the *Rhodococcus* sp. RHA1 ORF 19459 encodes a protein with 50% identity, and BLAST searches on the draft genome sequence of *N. farcinica* revealed a partial protein sequence with 50% identity to BldC. A second BldC-like protein, SCO3328, is present in *S. coelicolor* and shows 42% identity to BldC; this also has an orthologue in *S. avermitilis*. Other proteins consisting exclusively of a MerR-like DNA-binding domain, but with lower levels of identity to BldC (usually around 30%), were identified in a wide range of bacteria, including actinomycetes (e.g., *Mycobacterium tuberculosis*, *Mycobacterium leprae*, *Corynebacterium diphtheriae*, *Coryne-*
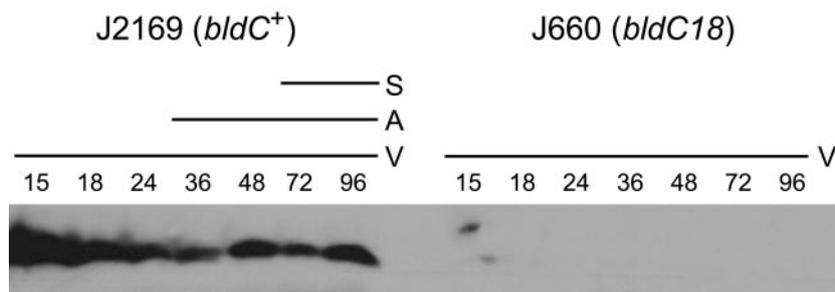
FIG. 4. Immunoblot analysis of BldC expression during developmental time courses of *S. coelicolor* J2169 (*bldC*⁺) and J660 (*bldC18*), grown on R2YE solid medium. The time points in hours at which mycelium was harvested and the presence of vegetative mycelium (V), aerial mycelium (A), and spores (S) are shown, as judged by microscopic examination.

*bacterium glutamicum*, and *Corynebacterium efficiens*), other gram-positive bacteria (e.g., *Bacillus cereus* and *Bacillus thuringiensis*), and gram-negative bacteria (e.g., *Sinorhizobium meliloti*, *Porphyromonas gingivalis*, *Chromobacterium violaceum*, and *Leptospirillum ferrooxidans*) (Fig. 3B and data not shown). One member of the BldC family was also identified in the genome of the *B. thuringiensis* bacteriophage, Bam35c (Fig. 3B).

**bldC gene organization is conserved in some other actinomycetes.** Analysis of the SAV4130 locus showed that the genetic organization in the region surrounding *bldC* is conserved between *S. coelicolor* and *S. avermitilis* (Fig. 2A). In *S. coelicolor*, there appears to be a large (901-bp) noncoding region downstream of *bldC*, whereas in *S. avermitilis* there is an ORF for an additional hypothetical protein (SAV4129) annotated in this region. This additional ORF is also conserved in the equivalent locus of "*Streptomyces diversa*" (Mervyn Bibb, Diversa Corporation, personal communication), and closer examination of the *S. coelicolor* sequence suggests that there is indeed an ORF downstream of *bldC*, not annotated in the genome sequence, that would encode a protein of unknown function similar to SAV4129 and the "*S. diversa*" protein (Fig. 2A and data not shown). A comparison with the *T. fusca* genome sequence shows some degree of conservation in gene organization between the *bldC* locus from *S. coelicolor* (and *S. avermitilis*) and the *T. fusca* region surrounding TFU1748, which encodes the protein showing 91% identity to BldC (Fig. 2A). Gene 1748 in *T. fusca* is preceded by homologues of two genes found upstream of *bldC*, although the intervening *vdh* gene for valine dehydrogenase is absent. There is, however, no conservation of gene organization downstream of gene 1748 in *T. fusca*. The regions surrounding gene TFU627 in *T. fusca* and ORF 19459 in *Rhodococcus* sp. RHA1 do not show any conservation of gene organization with the *bldC* locus.

**The bldC18 mutation causes a Y49C change that results in BldC instability.** To determine the nature of the *bldC18* mutation, a 750-bp region encompassing *bldC* was amplified by PCR from J660 and sequenced. A single-nucleotide difference from the wild-type sequence was identified: *bldC18* has an AT-to-GC transition that results in a tyrosine-to-cysteine substitution at position 49 in the primary amino acid sequence of BldC. This tyrosine is very highly conserved among the N-terminal DNA-binding domains of MerR family members (Fig. 3A). The loss of BldC activity resulting from substitution of a tyrosine raised the possibility that BldC might be regulated

by phosphorylation of this residue. To examine this possibility, we constructed a *bldC* allele encoding Y49F, a conservative substitution that should eliminate the potential for phosphorylation of the residue without grossly affecting protein structure. The integrative vector pSET152 was used to introduce the wild-type *bldC* allele (pIJ6842), the Y49C *bldC18* allele (pIJ6843), and the Y49F allele (pIJ6844) into J660 (*bldC18*) by conjugation, and the phenotype of exconjugants was analyzed on SMMS. As expected, the wild-type *bldC* allele restored sporulation to J660, whereas the *bldC18* allele did not. J660 carrying pIJ6844 (Y49F) sporulated, although at a slightly lower level compared to J660 carrying pIJ6842 (wild-type *bldC* allele) (data not shown). Thus, Y49 is not essential for BldC function, making regulation of BldC activity through phosphorylation of this residue extremely unlikely.

To determine whether the *bldC18* mutation affects the stability of BldC, immunoblots were performed. His-tagged BldC was overexpressed in *E. coli*, purified, and used to generate a polyclonal anti-BldC antiserum. For valid comparison, congenic strains were required. Unfortunately, J650, the *bldC*⁺ parent strain from which J660 was derived by *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine mutagenesis (34), has been lost. Therefore, we used the SCD25 cosmid to create J2169, a new congenic *bldC*⁺ parent of J660, as described in Materials and Methods. Crude cell extracts prepared from R2YE surface-grown cultures of the J2169 and J660 (*bldC18*) strains were subjected to immunoblot analysis with the anti-BldC antibodies (Fig. 4). The BldC protein was readily detected throughout development in the wild-type strain, but no BldC protein could be detected at any time point in the *bldC18* mutant. Thus, it appears that the *bldC18* Y49C mutation results in instability of the BldC protein.

**Construction and phenotypic characterization of bldC null mutants.** A series of *bldC* deletion mutants was constructed in morphologically wild-type strains of *S. coelicolor* using the PCR targeting method of Gust et al. (21), in order to compare their phenotypes to that of J660 (*bldC18*). The widely used strains M600 and M145 (plasmid-free derivatives of wild-type *S. coelicolor*) were chosen. These two strains, which until recently had been considered genetically similar, are in fact different, as the chromosome of M600 carries a 1.06-Mbp-long terminal inverted repeat absent from M145 (48). Analysis of strain J660 (*bldC18*) showed that it lacks the long terminal inverted repeats present in M600 (data not shown). The null mutations constructed in the M600 and M145 backgrounds
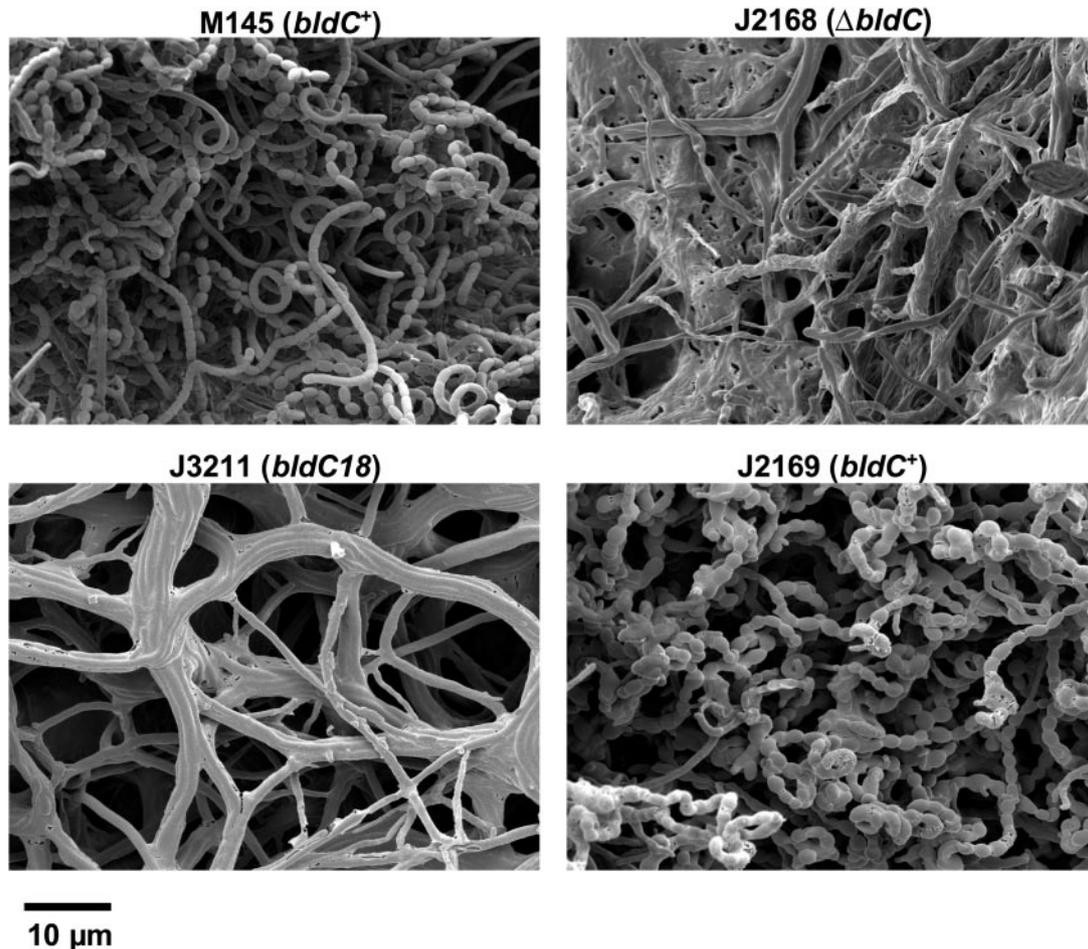
FIG. 5. Scanning electron micrographs showing the developmental phenotypes of M145 (a plasmid-free derivative of wild-type *S. coelicolor*), J2168 (M145Δ*bldC*), J3211 (M145*bldC18*), and J2169 (*bldC*⁺ derivative of J660). Strains were grown on R2YE plates.

replaced *bldC* either with an apramycin-resistance cassette (containing the *apr* gene and *oriT*) or with an in-frame deletion. No difference in phenotype could be detected between null mutants carrying the resistance cassette and those carrying the in-frame deletion, and so the in-frame deletion mutants were chosen for analysis. After 4 days of growth on minimal medium, SMMS, colonies of both M600 and M145 had produced a dense covering of aerial mycelium and grey-pigmented spores. In contrast, the Δ*bldC* null mutants derived from them (J2166 and J2168, respectively) had not differentiated to form aerial hyphae but had a bald phenotype after 4 days, and they remained bald even on prolonged incubation (data not shown). When the phenotypes of the same strains were analyzed on the rich medium, R2YE, M600, and M145 readily sporulated within 4 days, whereas the *bldC* deletion mutants did not (Fig. 5). However, upon prolonged incubation on R2YE (>1 week), both J2166 and J2168 did go on to produce aerial mycelium and spores (data not shown). The phenotype of these strains contrasted with that of J660 (*bldC18*), which, even on prolonged incubation, did not make an aerial mycelium on R2YE. pIJ6836 fully complemented the sporulation defects of the *bldC* null mutants J2166 and J2168 on both SMMS and R2YE;

no complementation was observed when only the vector pSET152 was introduced (data not shown).

These experiments showed that deletion of *bldC* in M600 or M145 resulted in a less severe phenotype than that of J660 (*bldC18*) when grown on R2YE, raising the possibility that the severe bald phenotype exhibited by J660 was allele specific. To assess this possibility, we deleted *bldC* from the chromosome of J660 by PCR targeting, resulting in strain J2162. The phenotype of J2162 was indistinguishable from that of J660, suggesting that the severe bald phenotype of J660 is not *bldC18* specific but is due to the genetic background of this strain. Confirmation of this conclusion was obtained by constructing *bldC18* derivatives of M600 (J3210) and M145 (J3211), as described in Materials and Methods; these *bldC18* derivatives had phenotypes indistinguishable from that of the *bldC* deletion mutants constructed in the same backgrounds, i.e., they were bald on SMMS and differentiation was delayed to the same extent on R2YE (Fig. 5). These results suggest that *bldC18* is a null allele, consistent with the lack of detectable BldC protein in a *bldC18* background (Fig. 4).

A consistent feature of the *bldC18* and Δ*bldC* null mutants when viewed under the scanning electron microscope was the
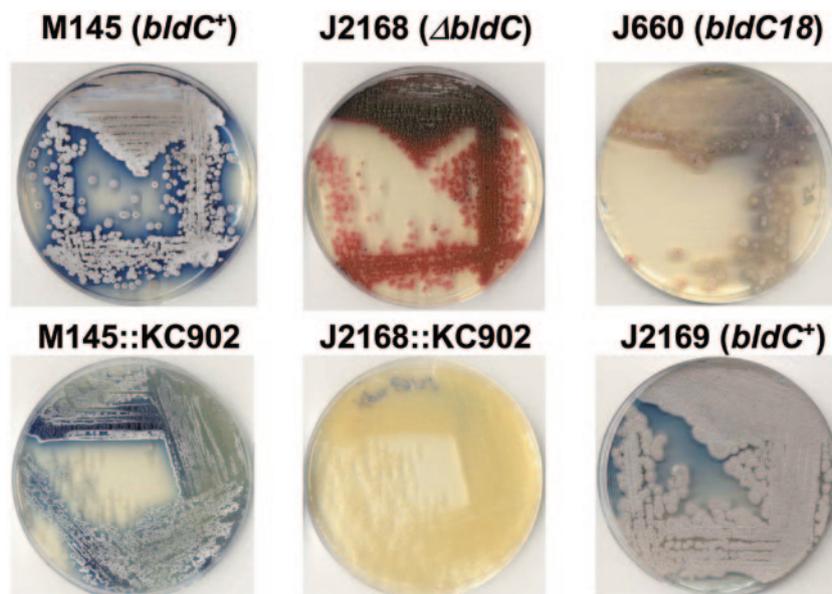
FIG. 6. Phenotypes of M145, J2168 (M145Δ*bldC*), J660 (*bldC18*), J2169 (*bldC+* derivative of J660), and KC902 lysogens of M145 and J2168 on R2YE. Note that J2168 and J660 cannot raise an aerial mycelium and that they fail to synthesize the blue-pigmented antibiotic actinorhodin. Lysogenization by KC902 disrupts the *redX* gene and therefore eliminates production of the red-pigmented antibiotic undecylprodigiosin (22).

aggregation of the hyphae into cable-like bundles (Fig. 2B and 5). It seems possible that this phenotype might be connected to the leather-like quality of *bldC* mycelium grown on plates: for example, when confluent lawns of *bldC* mutants are grown on cellophane disks for RNA isolation, the mycelium can be peeled off in one piece, a phenomenon not seen with wild-type strains or other *bld* mutants we have worked with extensively.

**Extracellular complementation.** Previous studies have provided evidence for an extracellular signaling cascade between different *bld* mutants, based on the ability of some *bld* strains to induce aerial mycelium formation in certain other *bld* strains when they are grown in close proximity but without actual contact (35, 39, 50). These studies placed *bldC* between *bldG* and *bldD* in the extracellular complementation cascade, based on the behavior of J660 (*bldC18*), the only *bldC* mutant previously available (50). We therefore carried out experiments in an attempt to determine if the Δ*bldC* null mutant derivatives of M600 (J2166) and M145 (J2168) constructed in this work showed the same extracellular complementation properties as the original *bldC* strain, J660 (*bldC18*). Unfortunately, no clear-cut results could be obtained in these experiments, which were complicated in part by the fact that the constructed *bldC* null mutants eventually sporulate in R2YE, the rich medium on which extracellular complementation is tested.

**Antibiotic production in the *bldC* mutants.** In addition to the differences in sporulation between J660 (*bldC18*) and the *bldC* null mutants constructed in the M600 and M145 backgrounds, there appeared to be significant differences in production of the pigmented antibiotics actinorhodin and undecylprodigiosin. J660 remained largely unpigmented when grown on R2YE (Fig. 6), whereas the wild-type strains M600 and M145 produced significant levels of both antibiotics, as did J2169, the *bldC+* derivative of J660 (Fig. 6). Strains J2166 and J2168, in which the *bldC* gene had been deleted, produced significant amounts of red pigment when grown on R2YE, but produced

less blue pigment (Fig. 6); the same was true of the constructed *bldC18* derivatives of M600 (J3210) and M145 (J3211). Confirmation that the red pigment was indeed undecylprodigiosin was obtained by lysogenizing J2166 and J2168 with the *aHP* φC31 derivative KC902 (22), which disrupts the *redX* gene and therefore eliminates undecylprodigiosin production. These lysogens were unpigmented (Fig. 6), confirming that the red pigment observed in the *bldC* null mutant derivatives of M600 (J2166) and M145 (J2168) was indeed undecylprodigiosin and that these strains were strongly affected in actinorhodin production (KC902 lysogens of M600 and M145, by contrast, produced significant amounts of blue pigment) (Fig. 6). The defect in actinorhodin production shown by J2166 and J2168 was fully complemented by introduction of plasmid pIJ6836 (data not shown).

To further understand the effect of *bldC* deletion on antibiotic production, M145 and J2168 (Δ*bldC*) were grown on R2YE plates, and RNA was isolated at different time points. These RNA preparations were used to determine the level of transcription of *actII-orf4*, *redD*, and *redZ*, which encode pathway-specific activators of the actinorhodin and undecylprodigiosin biosynthetic gene clusters. The results shown in Fig. 7 indicate that deletion of *bldC* does indeed affect production of both antibiotics. In the case of actinorhodin, there is clearly a delay and a reduction in the levels of *actII-orf4* transcripts, disappearing after 48 h, when the highest levels are observed in the wild-type strain. Therefore, *bldC* is required for normal and sustained transcription of *actII-orf4* throughout development. In contrast, early transcription of *redD* was not affected by lack of *bldC*. However, *redD* transcripts were reduced to almost undetectable levels after 48 h in the *bldC* mutant, in contrast to M145 in which *redD* continued to be transcribed during aerial mycelium formation and sporulation (Fig. 7). Since transcription of *redD* depends in turn on RedZ (49), *redZ*
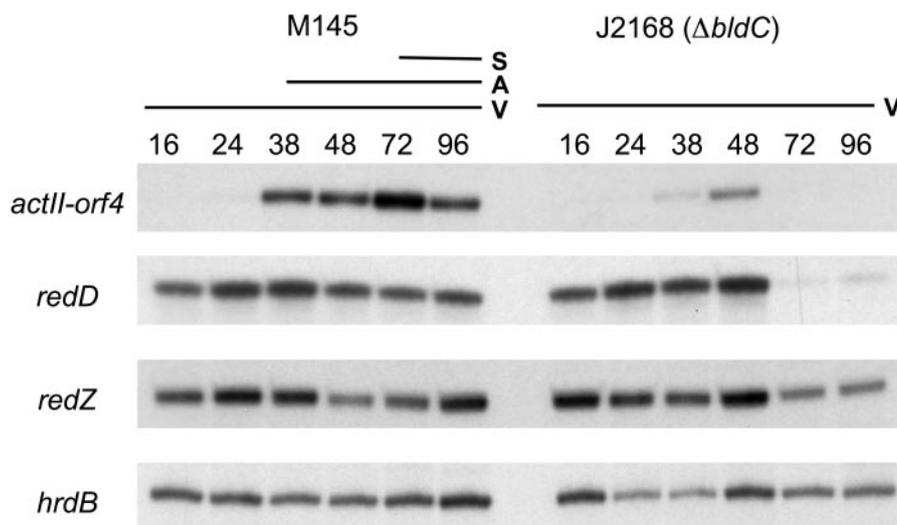
FIG. 7. S1 nuclease protection analysis of *actII-ORF4*, *redD*, *redZ*, and *hrdB* (positive control) transcription during development of *S. coelicolor* M145 and J2168 (M145Δ*bldC*) on R2YE solid medium. The time points in hours at which mycelium was harvested for RNA isolation and the presence of vegetative mycelium (V), aerial mycelium (A), and spores (S) are shown, as judged by microscopic examination.

transcription levels were also analyzed; *redZ* transcripts were present at all time points in both M145 and in J2168 (Fig. 7).

**Transcriptional analysis of bldC.** High-resolution S1 nuclease mapping of the *bldC* promoter region was performed with a PCR-generated probe and RNA isolated from either M600 or M145. A single *bldC* promoter (*bldCp*) was identified, initiating transcription 125 bp upstream from the ATG start codon (Fig. 8A). In vitro transcription experiments carried out with highly purified *S. coelicolor* RNA polymerase confirmed the location of the single *bldC* promoter (Fig. 8B).

Transcription of *bldC* was monitored by S1 nuclease mapping during development of *S. coelicolor* M145 on R2YE. *bldC* transcripts were detectable at all of the time points analyzed, representing growth from the early vegetative stage through to sporulation (Fig. 8A). The levels of *bldC* transcript did not change markedly during this time, showing that *bldC* is transcribed constitutively from a single promoter throughout development in wild-type *S. coelicolor*. Although the *bldC* transcript was approximately equally abundant at all time points, the possibility that *bldC* transcription may be spatially restricted, for example to the vegetative mycelium, is not excluded because no attempt was made to fractionate the harvested cell material used for RNA preparation. Transcription of *bldC* was analyzed in the *bldC18* point mutant, J660, and in the *bldC* null mutant, J2161. *bldC* transcripts were readily detectable throughout development and were present at the same level as in the corresponding congenic *bldC*⁺ parent strains (data not shown), showing that *bldC* is not autoregulated.

To determine whether transcription of *bldC* depends on any of the other known *bld* genes, transcription from *bldCp* was examined in RNA samples isolated from *bldA*, *bldB*, *bldD*, *bldF*, *bldG*, *bldH*, *bldJ*, *bldK*, *bldM*, and *bldN* mutants grown for 36 h on R2YE. As these *bld* mutations exist in a complicated variety of genetic backgrounds (Table 1), only striking effects would be considered potentially significant. However, *bldC* transcripts were readily detected in all of the *bld* mutants

analyzed, showing that transcription of *bldC* does not depend on any of the other known *bld* genes (data not shown).

## DISCUSSION

The BldC family of small DNA-binding proteins is related to the N-terminal DNA-binding domain of transcriptional activators belonging to the MerR family. The basic structure of MerR-like proteins is a dimer consisting of two identical subunits, each composed of an N-terminal DNA-binding domain containing a winged helix-turn-helix motif, a C-terminal effector recognition domain, and an interconnecting linker region which consists of a long α-helix that interacts with the same helix in the other subunit, forming a coiled coil responsible for dimerization (5, 8, 20, 37, 53). MerR family proteins share similarity only within their DNA-binding domains; as different family members bind different effectors, their C-terminal domains are variable and show little, if any, similarity to one another. BldC consists exclusively of a MerR-like DNA-binding domain and lacks the dimerization and C-terminal effector recognition domains. These observations raise interesting questions about the evolution of BldC and the MerR family. Brown et al. (5) have suggested that MerR family members might have arisen from gene fusion events that brought together N-terminal DNA-binding domains with separate effector recognition domains. This suggestion was prompted in part by analysis of two members of the MerR family, TipA and NolA. Two separate proteins are synthesized from the *tipA* gene of *Streptomyces lividans*: TipA_L, the typical full-length MerR-like protein with an N-terminal DNA-binding domain and a C-terminal effector recognition domain, and TipA_S, a protein consisting only of the C-terminal effector recognition domain, translated from an in-frame initiation site 110 amino acids downstream from the TipA_L start codon (23). Similarly, three proteins are synthesized from alternative start codons of the *Bradyrhizobium japonicum nolA* gene, only one of which (NolA₁) contains the helix-turn-helix DNA-binding motif (31).
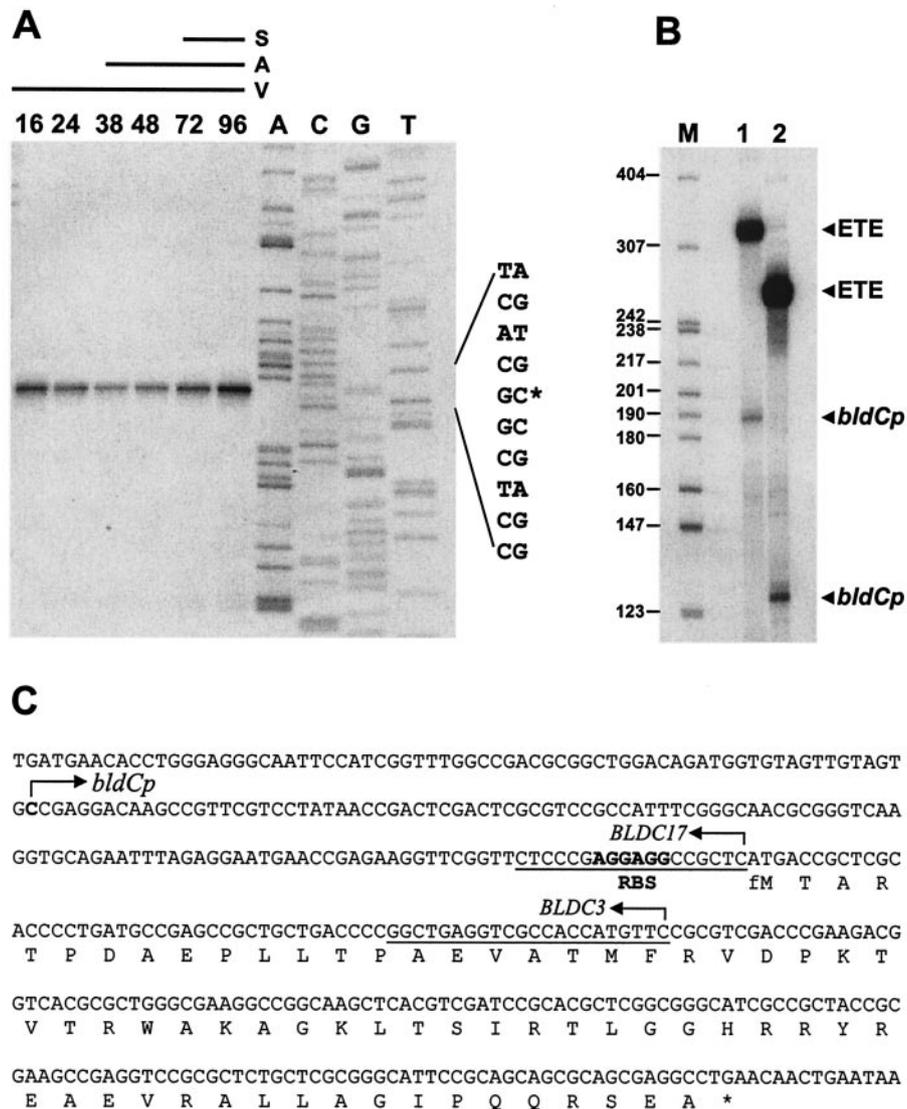
FIG. 8. Transcriptional analysis of *bldC*. (A) High-resolution mapping of the 5′ end of the *bldC* message and analysis of *bldC* transcription levels during development of *S. coelicolor* M145 on R2YE solid medium. The time points in hours at which mycelium was harvested for RNA isolation and the presence of vegetative mycelium (V), aerial mycelium (A), and spores (S) are shown, as judged by microscopic examination. Lanes labeled A, C, G, and T represent a dideoxy sequencing ladder generated with the same radiolabeled oligonucleotide that was used to make the S1 mapping probe. (B) In vitro transcription of the *bldCp* promoter by *S. coelicolor* RNA polymerase holoenzyme. Transcripts were generated from template 1 (327 bp) or template 2 (264 bp) (see Materials and Methods). The expected sizes of the runoff transcripts from the *bldCp* promoter were 188 nucleotides (template 1) and 125 nucleotides (template 2). The size markers (M) were a $^{32}$P-end-labeled HpaII digest of pBR322. ETE, end-to-end transcription of the linear template. (C) Nucleotide sequence of the *bldC* gene showing the *bldCp* transcription start point, the putative ribosome-binding site (RBS), the complete *bldC* encoding sequence, and the sequences of the BLDC3 and BLDC17 downstream oligonucleotide PCR primers used to generate the templates.

The characterization of BldC and the discovery of other members of the BldC family is the first evidence that proteins equivalent to the N-terminal DNA-binding domains of MerR family regulators can function independently of a C-terminal effector recognition domain. The presence of *bldC* family genes in many different bacterial genera might contribute significantly to the evolution of new members of the MerR family by allowing reshuffling of the DNA-binding region with different effector-binding domains, as proposed by Brown et al. (5).

Loss of *bldC* function results in a complex phenotype. When *bldC* was deleted in the M600 or M145 backgrounds, the re-

sulting strains exhibited a bald phenotype on SMMS minimal medium. However, on the widely used rich medium R2YE, *bldC* null mutants still managed to sporulate, although differentiation was severely delayed with respect to the corresponding wild-type strains. In contrast, the original *bldC18* point mutant, J660, failed to differentiate on R2YE, even with prolonged incubation. We showed that this phenotypic difference between the constructed null mutants and J660 (*bldC18*) was not due to allele specificity but was due to the genetic background. In fact, our results suggest that *bldC18* is a null allele. J660 (*bldC18*) failed to accumulate detectable levels of BldC,

presumably because Y49 is essential for proper folding and stability. This suggestion is supported by studies of three MerR-like proteins whose structures have recently been determined, BmrR, MtaN, and CueR (8, 20, 37, 53). BmrR, MtaN, and CueR contain three regions that contact their target promoters: the helix-turn-helix motif and two wing domains (20, 53). BmrR Y45, MtaN Y41, and CueR Y39, the tyrosine residues equivalent to Y49 in BldC, fall within wing 1, which consists of two β-sheets and the connecting loop between them. A hydrophobic core stabilizes the structure of the MtaN DNA-binding domain (20), and Y41 forms part of this core. The fact that a Y49C change in BldC is highly disruptive but a Y49F change is tolerated suggests that Y49 may serve the equivalent hydrophobic core function in BldC.

Apart from their effects on sporulation, *bldC* mutations also affect production of actinorhodin and undecylprodigiosin. Thus, *bldC* is required not only for the normal differentiation of aerial hyphae, but also for antibiotic production in the substrate mycelium. Our results show that transcription of *actII-orf4* depends on *bldC*, explaining the lack of actinorhodin production in a *bldC* null mutant. In contrast, *bldC* does not appear to be necessary for normal transcription of the undecylprodigiosin pathway-specific activators *redD* and *redZ* during vegetative growth. However, levels of *redD* transcripts were high in the wild type during aerial mycelium formation and sporulation but were severely reduced at the equivalent time points in the *bldC* mutant, while *redZ* transcription continued unaffected. This suggests that *bldC* may be required for maintenance of *redD* transcription during differentiation. RedZ is required for transcription of *redD*, and RedD in turn activates the *red* biosynthetic genes (49), yet *bldC* mutations appear to affect transcription of *redD* and not *redZ*. These results show that regulatory inputs relating to differentiation can be integrated at the level of *redD* transcription, at least in the case of *bldC*.

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