STRUCTURAL AND GENETIC ANALYSIS OF THE STREPTOMYCES COELICOLOR DEVELOPMENTAL REGULATOR BLDB

By

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Abstract

Deletion of the *bldB* gene has demonstrated that this gene and its protein product are required for the development of aerial hyphae and the production of antibiotics in *Streptomyces coelicolor*. Addition of a single copy of the *bldB* gene restores the wild type phenotype, indicating that the phenotype is not due to polar effects of deleting the *bldB* gene. Using a bacterial two-hybrid system, analytical ultracentrifugation and gel filtration chromatography I have been able to determine that BldB forms a dimer *in vivo* and *in vitro*. Deletion mutagenesis has demonstrated that the central 58 amino acids are essential for this dimerization. The construction of *bldB* point mutants in the complementation plasmid pRA1 indicates that most of the highly conserved residues in the BldB sequence are indispensable for BldB function, with the exception of E44 and S58. The same mutations constructed in the bacterial two-hybrid assay suggest that while residues W30, R56 and W72 are part of a dimerization interface, the residues Y21 and F75 lie outside of this interface and are likely involved in interactions with BldB targets. Overexpression of the BldB protein results in a block in morphogenesis before sporulation, while overexpression of Y21A and F75A mutant proteins results in accelerated morphogenesis, causing hypersporulation. A comparison of gene expression between the wild type, *bldB* null mutant strain and the BldB overexpression strain revealed several genes that appear to be under the control of the BldB protein. Deletion of the *bldB* gene results in decreased expression of the antibiotic synthesis genes in *S. coelicolor*, while BldB overexpression results in the activation of various stress response proteins, and the downregulation of different secreted proteins.

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Introduction

Higher organisms are considered advanced because of their ability to coordinate the efforts of the varied cells in their body. Due to their dependence on multiple cells, their development requires a high degree of regulation, redundancy, and communication between cells. Multicellularity is not a trait exclusive to eukaryotes and higher organisms however, as many species of bacteria also have highly complex multicellular life cycles. Typically, bacterial morphogenesis is regulated by a large number of genes involved in a complex inter-dependant network that progresses through a life cycle that is unique to each species. One of the most interesting and complex cycles of morphogenesis occurs in the gram-positive soil-dwelling organism *Streptomyces coelicolor*. Not only does the genome of *S. coelicolor* encode many putative regulatory proteins, but its life cycle consists of three distinct cell types with separate fates, and the development of each cell type is tightly regulated by the expression of many specialized genes. Morphogenesis in *S. coelicolor* is made that much more complex by the fact that development appears to be coupled to secondary metabolism. Evidence has shown that these processes involve complicated signaling pathways between multiple cells of this organism, making it a model organism to study multicellular differentiation in prokaryotes.

Distinct cell types of *S. coelicolor*

The complexity of morphogenesis in *S. coelicolor* is most evident when examining the differences in form and function of its two primary cell types, and how their formation is regulated. At the onset of growth, *S. coelicolor* cells divide differently

than in other bacteria, whereby the cell grows via branching and elongation, with growth occurring exclusively at the cell tips (31, 36). This process begins with the germination of a spore under nutrient-rich conditions, resulting in the production two germ tubes, from opposite sides of the spore. The germ tubes develop into a dense mat of cells known as the substrate hyphae. Since septation in this cell type is rare, these long filamentous cells can contain dozens of copies of the chromosome per cell, and are therefore not ideal for propagation of the next generation of cells. Collectively, the substrate hyphae are referred to as the substrate mycelium, and the colony has a smooth appearance on solid media at this stage in the life cycle.

The substrate mycelium plays two main roles in the development of *S. coelicolor*. One role is to develop a protective environment in which to grow the second cell type, the aerial mycelium, in order to propagate the next generation of spores. The second is to lyse and provide nutrients to the developing aerial hyphae. The first role is accomplished by the production of secondary metabolites, some of which possess antimicrobial and/or antifungal properties (5). In *S. coelicolor*, there are at least four secondary metabolites that possess antimicrobial activity. These are undecylprodigiosin, actinorhodin, methylenomycin, and the calcium-dependant antibiotic (CDA). Actinorhodin has a blue pigmentation and diffuses into the surrounding medium, while undecylprodigiosin is pigmented red and is deposited on the surface of the cells (102). Evidence has shown that production of these secondary metabolites is coupled to the onset of the second stage of morphogenesis, growth of the aerial hyphae. Electron microscopy of cells at this stage of development shows degradation of the nucleoid and cytoplasmic compartments, while

the cell membrane remains intact, but hollow (70). This cellular degradation suggests that the substrate hyphae may be providing nutrients to the growing aerial hyphae (70). At this point, the role of the substrate mycelium is complete.

The growth of aerial hyphae requires the expression of a group of surface proteins known as the chaplins. There are two common features in all of the chaplins; they all contain a 40-residue hydrophobic domain known as "the Chaplin domain", and they all also possess a secretion signal. There are five short chaplins (ChpD,E,F,G,H) that contain only one chaplin domain. The other chaplins (ChpA,B,C) are longer, as each has two chaplin domains and a sorting signal on the C-terminus that is responsible for targeting it to the cell wall to be covalently attached by a sortase enzyme (18, 26). Deletion of the chaplin genes causes a delay in aerial hyphae formation, but this phenotype can be rescued by the addition of purified chaplin proteins (18). This suggests that the chaplins are important in aerial mycelium formation, and that the chaplins must coat the cell envelope in order for aerial hyphae to grow away from the aqueous environment into the air (26).

Once the foundation of a colony has been laid down by the substrate hyphae, and the chaplins have coated the surface, the aerial hyphae are free to grow, and they grow up into the air away from the colony, imparting a fuzzy white phenotype to the colony. This process is dependent on a group of genes collectively called the *bld* genes. Mutations in the *bld* genes block the formation of aerial hyphae, such that mutant colonies lack the wild type fuzzy appearance and look "bald". One of the *bld* genes, *bldB* will be the focus of this thesis. Mutations in *bldB* cause the most pleiotropic phenotype of any *S. coelicolor*

mutant as it is compromised in both aerial hyphae formation and antibiotic synthesis.

The sole purpose of the aerial hyphae is to generate spores to continue the propagation of the next generation. During this spore maturation process, the cells produce a polyketide pigment which imparts a grey color to the developing spores. Expression of a set of genes known as *whi* is required for spore maturation. Mutations in the *whi* genes block morphogenesis at various points following the erection of the aerial mycelium but before the production of the grey polyketide pigment, thus colonies then appear "white". Expression of the *whi* genes is crucial to the sporulation process because maturation of the aerial hyphae involves coiling of the cells to a right-handed helical conformation, followed by septation into uninucleoid compartments and ultimately sporulation (106). Mutations in the different *whi* genes have many different effects on the coiling of aerial hyphae, some prevent coiling and others cause either excessive or insufficient coiling for spore maturation. If all of the *whi* genes are expressed normally, the mature grey spores pinch off and begin the life cycle anew (16). Electron micrographs of the spores, the substrate hyphae and the aerial hyphae can be seen in figure 1.

Role of the *bld* genes in aerial hyphae formation

It has been demonstrated that the *bld* genes are required for the production of a small hydrophobic molecule, SapB, which reduces the surface tension of the air-water interface and coats the surface of the growing aerial hyphae, promoting aerial growth (37, 99, 107). Interestingly, most of the *bld* genes are involved in a hierarchical pathway where the function of one gene depends on the successful expression of the previous

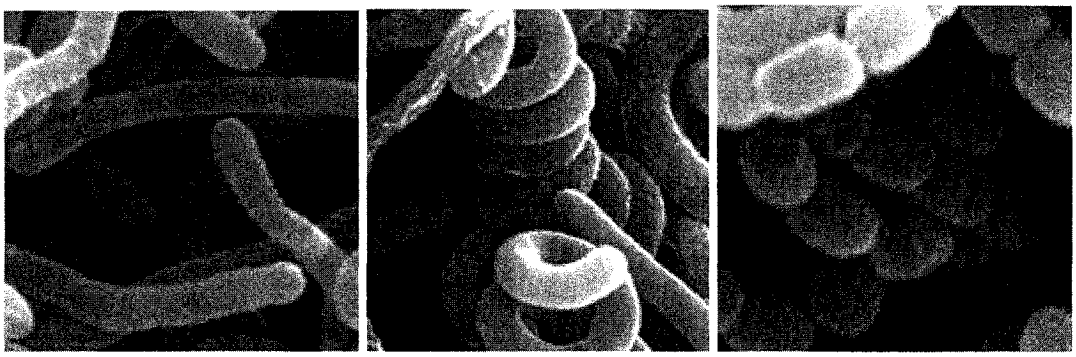
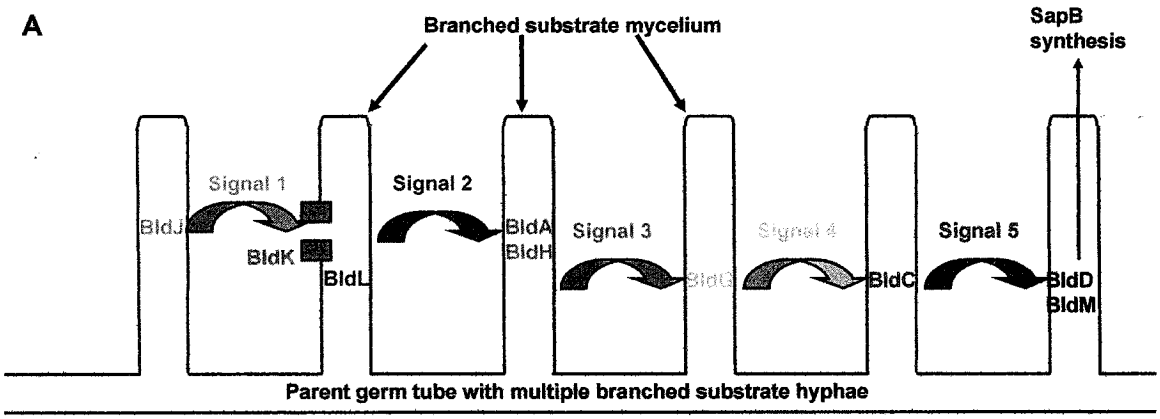


Figure 1. The three distinct cell types of the *S. coelicolor* life cycle. In the left panel, the substrate hyphae are long, smooth and straight cells. In the middle, the aerial hyphae form long, smooth, coiled structures. On the right, upon maturation, the coiled aerial hyphae septate to form long chains of spores which begin the cycle anew.

genes in the hierarchy. Many of the *bld* mutants can be complemented by growth in close proximity to other *bld* mutants, suggesting the exchange of an extracellular signal between mutants. In each case, one mutant serves as a donor of an extracellular molecule, while the other is the recipient, where only the recipient is complemented. This complementation is unidirectional in that certain mutants can only complement other mutants in a linear hierarchy (108). It is proposed that a series of small diffusible molecules, whose expression is either regulated by or responded to by the *bld* genes, govern the synthesis of SapB. In fact, there are as many as 5 extracellular signals involved in SapB production (75). A representation of the proposed model of extracellular complementation in *S. coelicolor* can be seen in figure 2 (71, 74, 108). Mutants on the left of the hierarchy synthesize only the first signal(s) and can be complemented by mutants higher up in the hierarchy (to the right), which produce the signals that the earlier mutants are unable to. Since early signals are required for, and induce production of later signals, mutants near the top of the hierarchy are capable of



B

Mutant	*bldJ*⁻	*bldK*⁻ *bldL*⁻	*bldA*⁻ *bldH*⁻	*bldG*⁻	*bldC*⁻	*bldD*⁻ *bldM*⁻
Signals produced		Signal 1	Signal 1 **Signal 2**	Signal 1 **Signal 2** Signal 3	Signal 1 **Signal 2** Signal 3 Signal 4	Signal 1 **Signal 2** Signal 3 Signal 4 **Signal 5**

Figure 2. The extracellular complementation hierarchy. (A) Representation of a branched substrate mycelium, where extracellular complementation occurs. The Bld proteins can be classified into 6 complementation groups, the first 5 are required for generating small molecule signals that are sensed and responded to by the other Bld proteins higher up in the hierarchy, ultimately leading to SapB synthesis. (B) Each *bld* mutant is blocked after the synthesis of the previous small molecule, with *bldJ* being unable to synthesize any of the signals as it is blocked before even the first signal is made.

complementing all of the *bld* mutants below them in the hierarchy, but can not be complemented by those mutants. The culmination of all of these extracellular signals ultimately results in the production of SapB, and disruption of this hierarchy at any point is sufficient to knock out its production and cause a *bld* phenotype in the cells.

Though this signaling cascade is absolutely required for SapB synthesis, an alternative role exists for this cascade in the developing cell. When SapB is added

exogenously to the *bld* mutants, aerial hyphae development is restored, however wild type morphogenesis and sporulation are not rescued, suggesting that the *bld* cascade plays another role in morphogenesis that is independent of SapB production (99). Also, several of the *bld* mutant phenotypes can be rescued by growth on alternative carbon sources, such as minimal media supplemented with mannitol (13). These strains were grown on minimal media and tested for production of SapB, and it was found that these strains, though able to erect aerial hyphae, did not produce SapB, and only *bldH* was rescued for antibiotic synthesis on alternative carbon sources (13, 107). These findings suggest that there are at least two distinct aerial mycelium formation pathways, which are either SapB-dependent or independent, however both require the extracellular signaling cascade. It has been proposed that *S. coelicolor* possesses this redundancy in order to ensure that sporulation is possible under multiple environmental conditions (107).

Current characterization of *bld* genes

A number of *bld* genes have been isolated and characterized. Many of these genes encode proteins with regulatory functions, including DNA binding proteins, sigma factors, and even a rare tRNA for the translation of a rare codon. Though many of these genes have been sequenced and their function investigated, the interaction between *bld* gene products and the method by which they regulate aerial hyphae formation remains unclear. Table 1 summarizes the characteristics of all of the currently isolated *bld* mutants. The existing *bld* mutants can be classified into groups based on their morphogenetic and metabolic phenotypes. The phenotypes are extremely variable

Table 1: Summary of *bld* gene products and antibiotic synthesis phenotypes

Mutant	Product	Antibiotic phenotype[a]	Reference
bldA	tRNA	Abs$^-$	Champness 1988
bldB	Putative regulator	Abs$^-$	Champness 1988
bldC	DNA-binding protein	Unable to make CDA	Pope 1996
bldD	Transcription factor (repressor)	Abs$^-$	Elliot 1998
bldG	Anti-anti-sigma factor	Abs$^-$	Champness 1988
bldH	Transcription factor	Carbon source dependent	Champness 1988
bldK	Oligopeptide transporter	Wild type	Nodwell 1996
bldM	Response Regulator	Wild type	Molle 2000
bldN	Sigma Factor	Wild type	Bibb 2000

[a]Abs$^-$: Antibiotic synthesis deficient

between mutants, and often conditional on the type of growth media. It has been suggested that the carbon source dependence of the *bld* mutants defines the role of the *bld* genes in sensing the nutritional state of the developing colony. More specifically, the involvement of the *bld* genes in the extracellular signaling cascade is thought to allow the bacteria to couple morphological differentiation with environmental cues such as nutritional state (13, 69, 83) and cell density (75). Many *bld* mutations also result in a defect in carbon catabolite repression in the cell by globally derepressing expression from the *gal*P1 promoter, which is usually repressed by glucose in wild type cells (83). This effect and that of the complementation of certain mutants on alternate carbon sources has not been adequately described to date, and requires further exploration.

Mutations in *bldB*, the subject of this thesis, cause the most severe developmental defect of any of the *bld* genes. These mutants are unable to grow aerial hyphae or efficiently produce any of the pigmented *S. coelicolor* antibiotics. Furthermore, and perhaps related to its biological role, *bldB* mutants are deficient in global carbon catabolite control (13). Clearly *bldB* is a critical gene in the development of *S. coelicolor*.

actinomycetes. Surprisingly, there are >20 homologous proteins encoded in the *S. coelicolor* genome, two of which, *abaA* and *whiJ*, have also been implicated in antibiotic production and sporulation respectively (13, 15, 34). An alignment of the sequences of these proteins with the sequence of *bldB* can be found in figure 3. Margaret Pope et al. demonstrated that expression from the *bldB* promoter is hyper-stimulated in a *bldB* mutant, suggesting that BldB somehow regulates its own expression, though there has been no success in demonstrating direct binding to its own promoter (82). It has been

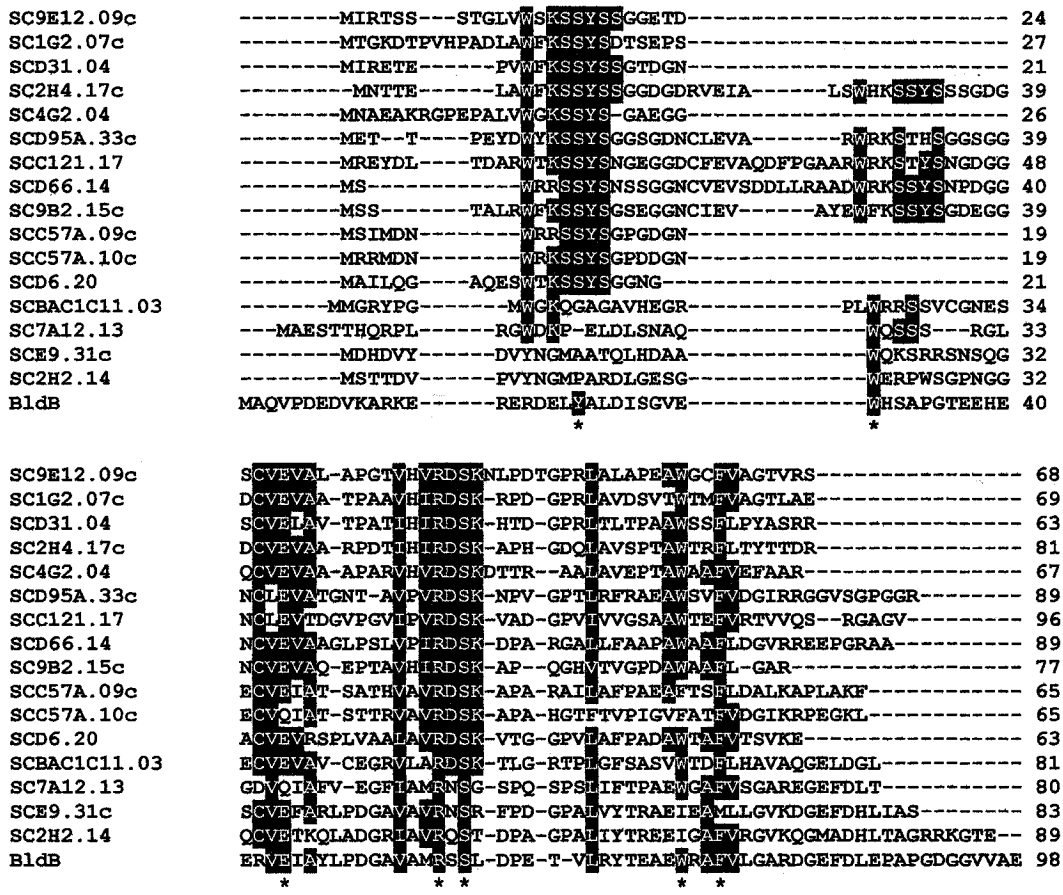


Figure 3. Sequence alignment of the closest homologues of BldB in *S. coelicolor* that were identified using BLAST searches against the BldB sequence. Highly conserved residues are red while the most universally conserved are marked with an asterisk.

suggested that BldB is a DNA-binding protein containing a helix-turn-helix motif typical of many transcription factors, but this has not been confirmed experimentally, and my evidence does not agree. Due to its novel nature, discovery of the function of this gene and its product is therefore very important.

Of the remaining *bld* genes, many perform similar functions, and the majority of the *bld* genes encode different types of regulatory proteins. Four of the *bld* genes (*bldC/D/H/M*) encode proteins that bind to DNA in order to regulate the expression of other genes. Two of the *bld* genes (*bldG/N*) encode proteins related to sigma factors, which direct RNA polymerase to target promoters, providing regulation by proper targetting. The *bldA* gene encodes a leucyl tRNA that is required for the translation of the rare UUA codon, which allows direct regulation of any coding sequence containing this codon. Finally, *bldK* encodes an oligopeptide permease that is required in the extracellular signaling cascade and likely imports one of the 5 signals represented in figure 2.

The *bld* genes that encode DNA binding proteins are *bldC*, *bldD*, *bldH* and *bldM*. The only one whose target promoter is well characterized is *bldD*. Mutations in this gene result in both a *bld* phenotype and a lack of antibiotic production implicating its function early in development. BldD is a DNA-binding transcription factor that acts as a repressor (27). The *bldD* open reading frame (ORF) contains a helix-turn-helix motif and binds its own promoter, as well as those of three σ-factor encoding genes:*bldN*, *whiG* and *sigH* (25). The first two of these are required for aerial mycelium formation and sporulation respectively and the third is involved in a stress response in *S. coelicolor*. *bldD* is only

one of the *bld* genes that encode a regulatory DNA-binding protein. *bldC* encodes a previously-uncharacterized family of DNA-binding proteins, with a DNA-binding domain similar to that of MerR transcriptional activators (49).

bldH encodes a protein that is homologous to the AdpA protein from *S. griseus*. AdpA is an activating transcription factor that is involved in the regulation of morphogenesis and antibiotic synthesis in *S. griseus* (98). In *S. coelicolor*, expression of the SCO0762 gene, which encodes a secreted protease inhibitor, was shown to be dependent on *bldH*, as it contains the established consensus binding sequence of BldH. *bldH* is one of the genes whose expression is known to be dependant on the rare UUA codon that requires the *bldA* tRNA for translation. Kim et al. have suggested that this protease inhibitor is required for the degradation of the substrate mycelia for nutrient supplementation of the developing aerial hyphae and that the *bldH* phenotype is due to a lack of expression of this protease (57). Interestingly, aerial mycelium formation in the *bldA* mutant can be restored by removing the UUA codon from the *bldH* gene, suggesting that *bldA*'s role in regulating this stage of morphogenesis may primarily be the regulation of BldH translation (73).

The *bldM* gene encodes a response regulator that is required for the expression or repression of genes that are currently unidentified (71). This protein is homologous to the FixJ family of response regulators. Surprisingly, though most response regulators are either activated or inhibited by phosphorylation, BldM is not phosphorylated on the conserved aspartate residue typical of this family of response regulators, and phosphorylation at this residue does not appear to be necessary for the function of this

protein (71). Expression of *bldM* however, is dependent on a sigma factor encoded by another member of the *bld* family, *bldN*.

bldG and *bldN* encode a different type of regulatory protein. Sigma (σ) factors are required for gene expression as they must direct the polymerase holoenzyme to the target promoter, but they themselves are often targets of post-translational regulation. Anti-σ factors regulate the activity of σ factors by binding to them directly and preventing their interaction with the core RNA polymerase. In some cases, anti-σ factors are themselves subject to post-translational regulation by proteins called anti-anti-σ factors. The *bldN* reading frame encodes a σ factor that is itself regulated by the transcriptional repressor *bldD* but whose target remains unknown (7, 25). Evidence suggests that *bldG* encodes an anti-anti-σ factor that interacts with an anti-σ factor encoded by the adjacent gene. The target σ factor is not known (9). Like other anti-anti-σ factors, it has been shown that BldG is phosphorylated by an unknown kinase, resulting in its inactivation (8).

One of the most studied of all of the *bld* genes is *bldA*, which encodes a tRNA that is required for the translation of the leucine UUA codon, which is rare in *S. coelicolor* (62). Disruption of this gene also results in a lack of antibiotic production, suggesting that *bldA* could act as a universal regulator of both morphogenesis and secondary metabolism. This is likely accomplished via the regulation of specific genes with the UUA codon at an early stage of development, before aerial hyphae formation and secondary metabolism occur (62, 69). Tsugimoto et al. identified that the insertion of a UUA codon into the enhanced green fluorescent protein (eGFP) caused a temporal delay in its expression and altered the spatial pattern of its expression (53). The *bldH*

gene was shown to contain the rare UUA codon, and is dependent on *bldA* for its expression (73). BldA has also been implicated in the regulation of expression of members of the *S. coelicolor* membrane and extracellular proteome (56).

Consistent with the signaling hypothesis, the *bldK* locus encodes an oligopeptide permease that is believed to import extracellular signals (75). BldK is a member of an ATP-binding cassette (ABC) family of oligopeptides, and *bldK* mutants are resistant to the toxic tripeptide bialaphos (75). The signaling molecule imported by BldK is proposed to be produced under the control of the wild type product of the *bldJ* gene, and was found to be a 655 Dalton (Da) peptide that is resistant to heat and proteases (74). In media conditioned by growth of the *bldK* mutant, the small peptide accumulates outside of the cell, and this media can be used to complement a *bldJ* mutant, as long as it encodes a functional *bldK* allele, confirming the dependence of BldK on this small peptide (74). Unfortunately, *bldJ* itself has not been characterized extensively to date and its role in controlling the synthesis of this signaling molecule is not known.

What remains to be discovered about the *bld* genes?

Though several of the *bld* genes have been well-studied, many questions about their function still remain. For instance, many *bld* mutants are unable to produce antibiotics, but their involvement in the regulation of antibiotic synthesis has not been defined. To complicate the analysis, some genes that have a well-defined role in the extracellular signaling cascade do not share metabolic phenotypes with the other mutants in the hierarchy. For example, the *bldK* mutant produces antibiotics at a comparable level

to wild type *S. coelicolor* while mutations on either side of *bldK* in the hierarchy are defective in antibiotic synthesis. This implies that *bldK*, among other genes in the signaling pathway may be dedicated to the formation of aerial hyphae and have little to no impact on antibiotic synthesis, placing the proteins in different morphogenetic and metabolic pathways. It is an important goal to understand how BldB works with the other *bld* gene products to ensure that aerial mycelium formation and antibiotic synthesis occur.

The extracellular signaling cascade model in figure 2, though comprehensive and based on extensive research, is still incomplete. It is believed that there are at least 5 extracellular signals (75), but only one of them has been isolated and characterized (74), and even in this case, the characterization was incomplete. All of the *bld* genes in the hierarchy have been classified, but none of them encode proteins that directly synthesize small molecules. Many of the *bld* genes are regulatory in nature, and the discovery of which genes or proteins are regulated by these factors could lead to identification of these small molecule signals and the proteins that produce them. It has also been shown that the SapB-independent morphogenetic pathway on minimal media requires an intact signaling cascade, yet this pathway remains unidentified.

The elucidation of the function of the *bld* genes is complicated further by the isolation of *bld* mutants that do not fit neatly into the extracellular complementation hierarchy. Some of these mutants have not been characterized (74), but *bldB, bldI,* and *bldN* have been and do not act as donors and recipients of the extracellular signals in any predictable way. Due to the existence of *bld* genes that do not fit into the known

hierarchy, they must act in more than one pathway in aerial hyphae formation (74). Basically, even with the extent of characterization to date, there simply aren't enough genes accounted for in the current collection to adequately explain aerial mycelium formation. For example, all of the *bld* genes are required for SapB synthesis, yet none of them are directly responsible for SapB production. It is known that SapB synthesis is not affected by the ribosomal inhibitor chloramphenicol (108) which suggests that SapB is produced by a non-ribosomal peptide synthetase (109). Evidence has demonstrated that the production of the SapB morphogen is reliant on the *ram* operon, containing the genes *ramA*, *ramB*, *ramC*, and *ramS*, under the control of the *ramR* response regulator, which is required for the expression of the entire operon (47, 59, 66, 77). The *ram* genes were originally of interest because their overexpression resulted in rapid aerial mycelium (ram) formation (66, 101). Mutations in any of the *ram* genes results in a *bld* phenotype, and thus the *ram* genes have been implicated in aerial hyphae formation.

The RamR protein is similar to most response regulator proteins in that it dimerizes and binds to promoter DNA in order to activate the expression of its target genes (*ramA/B/C/S*) (77). However, it has been shown that RamR is not phosphorylated as are most response regulators, despite sharing conserved residues typical of phosphorylated response regulators (78). The RamR protein binds cooperatively to three sites in the promoter upstream of the *ramCSAB* operon, activating the expression of these genes approximately 24 hours after germination of the spore (77). *ramA* and *ramB* encode two halves of an ABC transporter thought to be the SapB exporter. Our lab, in a collaboration with Joanne Willey's group have identified that the gene *ramS* encodes a

small lantibiotic-like pre-peptide that is post-translationally processed to become SapB (59, 109). The RamC protein is predicted to be responsible for the processing of RamS into SapB (59, 109). The *ramC* gene encodes a membrane-bound putative bifunctional enzyme, with three distinct domains (48). The amino-terminus of RamC contains amino acid residues and putative kinase motifs that are usually required to coordinate magnesium ions and phosphorylate target hydroxyl groups (48). The protein kinase-like sequence motifs in RamC are required for its function, as mutations in conserved residues in these motifs block RamC function *in vivo* (48). Despite the similarity to protein

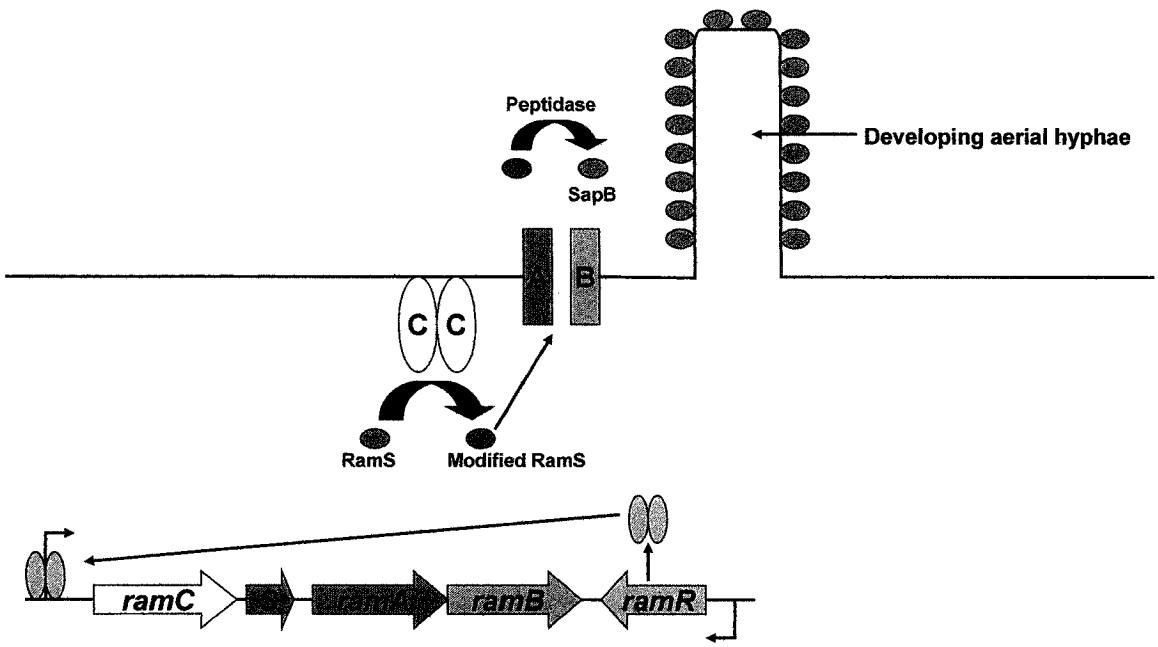


Figure 4. Model of the function of the ram operon. The RamR protein binds to the promoter upstream of the ramC gene, activating expression of ramC, ramS, ramA and ramB. ramA and ramB constitute two halves of an ABC transporter and are targeted to the membrane. RamC, a bifunctional modification enzyme is also membrane targeted. RamS is expressed and subsequently modified at the membrane by RamC. The modified peptide is exported via the ramA/B transporter, where it is cleaved with an unknown peptidase to form SapB. SapB then coats the developing aerial hyphae, permitting growth away from the substrate hyphae.

kinases, RamC is not able to autophosphorylate, and possesses several structural elements very different from typical serine/threonine kinases. The current model of Ram protein function, represented in figure 4, suggests that the kinase-like domain of RamC is responsible for the dehydration of serine residues in the RamS sequence (109). Just downstream of the kinase-like domain is a domain containing six consecutive repeats of the consensus amino acid sequence VDETTR. This repeat domain is required for the dimerization of the RamC protein (47). The C-terminus of the RamC protein is similar to some lantibiotic-processing enzymes and is believed to be required for further processing of RamS by forming covalent thioether lanthionine bridges between specific residues in the RamS sequence (59, 109).

Lantibiotics are small peptide molecules that possess antimicrobial activity and typically contain lanthionine bridges and the uncommon amino acid residues didehydroalanine and didehydrobutyrine derived from serine and threonine residues respectively. Recent evidence suggests that the RamS peptide is modified in a manner similar to the lantibiotics (59). Research in both the Willey and Nodwell lab suggest that SapB is processed from RamS by the dehydration of four serine residues to didehydroalanine residues and the formation of two lanthionine bridges in the peptide sequence. Though mature SapB contains didehydroalanine residues and two Lan bridges typical of lantibiotics, it possesses no antimicrobial activity and does not contain any didehydrobutyrine residues and is therefore not a true lantibiotic (109). Once RamC has processed the RamS peptide, it is exported through the RamA/B transporter, where an unidentified peptidase cleaves the leader sequence from RamS, producing the mature

SapB molecule which coats the developing aerial hyphae (109). It remains to be seen how *bldB* or any of the *bld* genes are involved in regulating or participating in this SapB synthesis pathway.

Regulation of morphogenesis in bacteria

Bacterial morphogenesis is highly variable among different species. The focus of this thesis is the *bldB* gene and how it is involved in the regulation of morphogenesis in *S. coelicolor*. Despite differences in morphogenesis between species, many of them share common developmental themes, so identification of how development occurs in *S. coelicolor* may provide insight regarding morphogenesis in other species.

Some bacteria undergo fairly simple morphogenetic programs. For example, *Escherichia coli* (*E. coli*) is a single cell gram negative bacterium that exists in a basic rod shape and reproduces by simple chromosome duplication and splitting into two daughter cells. However, as previously described, the life cycle of the gram positive *S. coelicolor* is much more complex and progresses through three distinct cell types whose growth is regulated by a complicated pattern of gene expression that is ultimately governed by the nutritional state of the surrounding environment. In fact, *S. coelicolor* morphogenesis is more akin to development in filamentous fungi than to the more simple prokaryotes. Though they belong to entirely different kingdoms that diverged early in evolution, *S. coelicolor* and filamentous fungi have both adopted similar lifestyles, in which a submerged feeding mycelium differentiates into aerial hyphae which then develop into resistant spores (110). Both of these processes require a small molecule to

decrease the surface tension of the air-water interface to permit the aerial hyphae to grow into the air, facilitating dispersion of spores. This suggests a common developmental theme between two distinct kingdoms, and that common mechanisms of morphogenesis exist between very different species of bacteria.

There are also similarities between some of the more complex prokaryotes, despite drastic morphological differences. For instance, several prokaryotes develop a cell type that is resistant to various environmental stresses. In the species *Myxococcus xanthus*, myxospore formation is facilitated by the production of a fruiting body, which requires an aggregation of multiple cells, demonstrating a reliance on multicellularity as in the *Streptomycetes*. In *Bacillus subtilis*, reproduction is highly dependent on the formation of spores as well, though this bacterium undergoes a much different cycle of differentiation to produce spores than *S. coelicolor*. Though the developmental cycle is different in *B. subtilis*, the *spo0* group of genes encode proteins that are required to assess the nutritional state of the cell and transduce this information to regulatory factors, or function as regulators themselves (65). This system ultimately results in activation of the sporulation mechanism, suggesting that *B. subtilis* and *S. coelicolor* sporulation share a dependence on the ability to assess the nutritional state of their environment. Because *bldB* is implicated in early development, and mutations cause a very pleiotropic phenotype, it is possible that it is involved in sensing the surrounding nutritional state, and mutations in *bldB* are pleiotropic because the response to depleted nutrients is compromised.

Despite significant evolutionary separation and morphogenetic differences, there

are striking similarities in the differentiation of *S. coelicolor*, *B. subtilis*, and *M. xanthus*, three of the better characterized complex prokaryotes. For example, it has been demonstrated that multiple extracellular signals are required to govern fruiting body formation in *M. xanthus* (39, 60). In fact, as in *S. coelicolor*, a cascade of intercellular signals regulates the series of chemotactic and morphogenetic events that are required for myxospore formation (50). Differentiation in *B. subtilis* is quite different from spore formation in *S. coelicolor*, but regulation of this process is accomplished with similar methods and even proteins. A series of pheromones are imported into differentiating cells which triggers the accumulation and activation of the transcription factors Spo0A and ComA (63). Import of small molecules in *B. subtilis* is highly dependent on a *bldK* homologue, called *spo0K*, which plays a crucial role in sensing the small molecule signal cascade (81, 88). Once imported, the small molecules in *B. subtilis* activate a three-protein phosphorelay and eventually activate a transcription factor, a role that could be filled in *S. coelicolor* by the unknown *bldL* gene product upon import of an unidentified small molecule through *bldK* (74, 79). It appears that these well-studied organisms employ similar small molecule signaling pathways to activate or repress transcription of genes vital to differentiation. As mentioned, it is still unclear how all of the *bld* gene products, including BldB, interact to carry out morphogenesis, so it is very important to determine this information in order to relate *S. coelicolor* morphogenesis to development in other bacteria.

Antibiotic synthesis in *S. coelicolor*

The *Streptomycetes* are particularly notable for their ability to produce a wide variety of pharmaceutically useful compounds as secondary metabolites. These compounds can be of use as antitumor agents, immunosuppressants, and almost 70% of naturally-isolated antibiotics (12). *S. coelicolor* produces 4 distinct antibiotics as secondary metabolites. Methylenomycin is encoded on one of the two chromosomal plasmids (SCP1) of *S. coelicolor* (58), while the other three are encoded on the chromosome. The calcium-dependent antibiotic (CDA) requires calcium in order to function effectively (42). The other two antibiotics are the red-pigmented undecylprodigiosin, which remains associated with the cytoplasm, and the blue-pigmented actinorhodin, which is deposited on the external surface of the cells (28, 40, 86, 102). The pigmentation of these antibiotics has greatly facilitated exploration of the synthesis of antibiotics and regulation of this process in *S. coelicolor*. Mutations in genes required for this process result in two distinct phenotypes. When alterations to biosynthetic or regulatory genes result in a defect in production of antibiotics (seen as a lack of pigmentation), the phenotype is known as Abs$^-$ (for Antibiotic synthesis minus). Mutations that have the opposite effect, an overproduction of antibiotics, are described has having a Pha (for Precocious hyperproduction of antibiotics) phenotype (3). *bldB* mutants exhibit an Abs$^-$ phenotype, and it is vital to determine how BldB affects antibiotic synthesis in *S. coelicolor*.

Due to their pigmented nature, actinorhodin and undecylprodigiosin are the most studied antibiotics produced by *S. coelicolor*. The tripyrrole undecylprodigiosin is

actually pigmented red due to a mixture of related prodigionines, of which undecylprodigiosin is the major component (100). There are at least 18 genes involved in production of undecylprodigiosin, the expression of which are tightly regulated and linked to the growth phase of the cells (19, 28, 87). The synthesis of actinorhodin also requires a large set of genes. In fact, the synthesis of actinorhodin provided the first evidence that biosynthetic genes for each actinomycete antibiotic are clustered together on the chromosome (68). It is well known that these biosynthetic pathways contain multiple peptides and enzymes encoded in large gene clusters, including several polyketide synthases, macrolides, and aminoglycosides (3). In fact, for most *Streptomycete* antibiotics, ten to thirty enzyme-catalyzed steps are required to convert primary metabolites to their final product (29). Each *Streptomyces* species contains multiple biosynthetic clusters and make their own distinct secondary metabolites. Interestingly, these species are sensitive to the lethal effects of their own secondary metabolites. It is therefore essential that the genome must also encode one or more self-protective resistance mechanisms, often encoded within the biosynthetic clusters themselves (21). In the *Streptomycetes*, the products of these complex biosynthetic pathways are usually expressed during stationary phase of growing liquid cultures and during differentiation on solid media (41). Expression of the genes in these biosynthetic clusters is subject to many different forms of regulation, including a general form of regulation provided by the control of the growth rate of the cells, and more specific regulation by many different encoded regulatory factors (97). The regulation of these processes by BldB is a primary focus of my research.

What genes are involved in regulation of antibiotic synthesis?

Antibiotic synthesis in *S. coelicolor* has been studied extensively, and the process has been found to be tightly regulated on many levels, by various pathways, and a multitude of genes. Despite this complexity of regulation, it has been found that expression of most of the genes in the biosynthetic pathways is directly under the control of pathway-specific regulation, in which a cluster-linked transcription factor (usually an activator) regulates expression of many genes in the cluster (3). The primary pathway-specific regulators for each *S. coelicolor* antibiotic have been identified. The activators required for the synthesis of actinorhodin, undecylprodigiosin, and CDA are *act*II-ORF4, *redD*, and *cdaR* respectively, while two regulators have been identified for methylenomycin biosynthesis, called *mmfL* and *mmyB* (3, 17, 29, 72). Interestingly, these factors not only perform the same function, but are structurally similar to one another, and form a family of proteins within the *Streptomycetes* known as SARP's, for *Streptomycete* antibiotic regulatory proteins (105).

One of the better studied and well characterized systems of regulation is that governing the synthesis of undecylprodigiosin. Mutations in the *redD* gene cause an inability of this strain to synthesize undecylprodigiosin, implying its essentiality to this process (87). Takano et al have demonstrated that transcription of the pathway-specific regulator *redD* increases dramatically during the transition from exponential to stationary growth phase in liquid culture (97). Increased translation of *redD* is immediately followed by an increase in transcription of *redX*, encoding a structural gene for an early step in the undecylprodigiosin biosynthetic pathway (38). This is followed immediately

by production of undecylprodigiosin (97). It has also been shown that extra copies of *redD* in the cell elicit a Pha phenotype, or overproduction of undecylprodigiosin (72). In this strain, excess *redD* causes an increase in transcripts of both *redD* itself, and its downstream target *redX*, suggesting *redD* is a positive regulator of these promoters. Consistent with this hypothesis is the fact that two other biosynthetic genes required for the O-methyltransferase activity of this pathway, *redE* and *redF* are not expressed in *redD* mutants (28). It appears as though the biosynthesis of undecylprodigiosin is therefore highly dependent on and positively regulated by the essential regulatory gene *redD*. It is possible that the inability of some of the *bld* mutants to synthesize undecylprodigiosin is due to misregulation of this gene.

Perhaps the best characterized system of antibiotic synthesis regulation is that of actinorhodin biosynthesis. The *act* biosynthetic cluster is separated into seven distinct regions, based on different classes of mutations. The regions are *act*I-IV, *act*Va, *act*Vb, *act*VI, and *act*VII, and each contain different sets of ORFs (29). The region most responsible for regulating actinorhodin biosynthesis is *act*II, as disruptions in this region knock out actinorhodin production completely, while extra copies of this region cause a Pha phenotype for actinorhodin synthesis (29, 67). The pathway-specific regulator *act*II-ORF4 is located within this region, and is responsible for the positive regulation of expression of genes within the *act* cluster (67). The other ORFs of the *act*II region encode proteins involved in export of the antibiotic. Regulation of this cluster appears to be very similar to regulation of the *red* genes, however, there is an additional level of regulation. Two of the ORFs in the *act*II region (ORF2 and ORF4) contain the rare UUA codon that

can only be translated by the BldA gene product. If this codon is altered in the *bldA* mutant to a UUG, which still encodes the amino acid leucine, but may be translated by higher abundance tRNA's, actinorhodin synthesis in this mutant is completely restored, implying that the precise role of *bldA* in actinorhodin production is regulation of these two ORFs (29). Since *bldA* is available late in the growth cycle of *S. coelicolor*, it provides a cellular "switch" to activate morphogenesis and antibiotic synthesis (62, 64). Due to the presence of a UUA in the primary regulator of actinorhodin synthesis and in a protein responsible for its export, the first and last steps of actinorhodin biosynthesis can be linked to temporal control by the *bldA* gene.

The actinorhodin pathway is not the only target of *bldA* regulation. In fact, out of 7825 genes in the *S. coelicolor* genome, 145 contain UUA codons (5). Proteomic analysis suggests that most of the genes that are substantially affected by mutations in *bldA* are involved in antibiotic synthesis. Specifically, UUA codons have been found not only in *act*IIORF2 and ORF4, but also in *redZ*, whose target is *redD*, and also in *mmfL* and *mmyB*, the two primary regulatory genes in methylenomycin biosynthesis (95). Since *bldA* mutants are unable to produce methylenomycin and altering the UUA codons in *mmfL* and *mmyB* restores the ability to produce this antibiotic, *bldA* regulation is implicated through the UUA codon in this pathway just as in the actinorhodin synthesis pathway. Because *mmfL* and *mmyB* are autoregulatory in nature, and they both contain UUA codons, large changes in expression pattern can be accomplished with only a small change in *bldA* tRNA levels (17). In 27 gene clusters for antibiotic synthesis in other *Streptomycetes*, the regulatory genes also contain a UUA codon, and are thus likely to be

regulated in a similar manner, providing a link between morphogenetic differentiation and antibiotic synthesis, as *bldA* is required for both, and its expression is detected at this phase of development (17).

Aside from pathway-specific regulation, and the *bldA* translational regulation, there are also several different kinds of global regulation of antibiotic synthesis in *S. coelicolor*. One locus in particular is responsible for facilitating the synthesis of all four antibiotics in *S. coelicolor*, and not surprisingly, this is the *abs* locus (for antibiotic synthesis). Point mutations in *abs* are deficient in the synthesis of all four antibiotics, giving the classic abs$^-$ phenotype (2). Inversely, when either of the two genes in this locus is knocked out completely, the result is early onset of antibiotic synthesis, and a Pha phenotype, where all 4 antibiotics are overproduced (3). Such phenotypes suggest that *abs* regulation of antibiotic synthesis is negative in nature. Interestingly, though this locus regulates production of all 4 antibiotics, the genes responsible are encoded on the chromosome within the biosynthetic cluster of only one of the antibiotics, CDA. This is highly unusual, as most regulators in the Streptomycetes appear to be pathway-associated in nature, or are generally unassociated in the case of global regulators.

The *abs* system consists of two genes that encode typical members of a two-component signal transduction system that regulates expression of multiple antibiotic synthesis gene clusters. *absA1* encodes a histidine kinase that is required for the phosphorylation of its cognate response regulator *absA2*. Typically, in response to a signal, the histidine kinase autophosphorylates, followed by the transphosphorylation of one subunit to another at a conserved histidine residue within the kinase. The phosphoryl

group is then transferred to a conserved aspartate residue on the response regulator, which usually contains a helix-turn-helix DNA binding motif, through which expression of target genes are activated or repressed. The *absA1/A2* system operates in such a manner, and when AbsA2 is in its phosphorylated form, it acts as a repressor of transcription of the genes responsible for general antibiotic synthesis (3, 93). Using chromatin immunoprecipitation and electrophoretic mobility shift assays, Nancy Sheeler has identified three putative targets of the AbsA2 protein. Not surprisingly, the three promoters identified by these experiments are the promoters for the pathway-specific regulatory genes *act*IIORF4, *redZ* and *cdaR* (unpublished data, personal communication). AbsA2 levels in the cell are controlled by the fact that AbsA2 is autoregulatory on its own promoter, ensuring that appropriate levels are available in the cell for AbsA1 phosphorylation, at the correct time. AbsA1 is also able to regulate this process as it is a bifunctional enzyme. It possesses the ability to act as both a kinase and a phosphatase, adding and removing the phosphoryl group from the AbsA2 response regulator as necessary (93). When AbsA1 is expressed to excessive levels in the cell, such that most of the protein is unable to respond to a signal, or there is not enough free phosphate in the cell to accommodate kinase activity, AbsA1 acts primarily as a phosphatase, stripping the phosphoryl group from phospho-AbsA2, causing derepression of biosynthetic gene expression, resulting in a Pha phenotype (3, 93). Consistent with previous evidence that links antibiotic production to growth phase, it appears that transcription of *absA1/A2* is also growth phase regulated such that they are expressed at the correct time to trigger antibiotic production.

In addition to *absA*, there is another global regulatory *abs* locus, designated *absB*, which encodes an RNase III homologue in *S. coelicolor*. When this gene is deleted, there is a global deficiency in antibiotic synthesis, similar to that in the *absA* null mutant (1, 84). There are also a fair number of other genes that are involved in the regulation of one or more antibiotics, but their precise role is much less well understood. Evidence has shown that mutations in the *rpsL* gene, which encodes the ribosomal protein S12, cause overproduction of actinorhodin in *S. coelicolor* (80). The gene *rpoB* encodes the RNA polymerase β-subunit, and mutations in this gene cause overproduction of actinorhodin, undecylprodigiosin, and CDA, but not methylenomycin (44). Another gene that regulates expression of multiple antibiotics is *afsR*. When this gene is knocked out, the synthesis of all the antibiotics except methylenomycin is reduced, but this phenotype was media-dependant (32). Surprisingly, though the absence of *afsR* has no effect on the transcription of the pathway-specific activators *act*II-ORF4 and *redD*, when extra copies of these activators are present, the *afsR* null mutant phenotype is suppressed. However, when *afsR* is overexpressed, transcription of *act*II-ORF4 and *redD* is increased, resulting in increased actinorhodin and undecylprodigiosin production (32). Since the same effect was not observed in *act*II-ORF4 and *redD* mutants, this suggests that the *afsR* induction of antibiotic synthesis is dependent on these activators. The specific regulatory role of these genes in regulation of antibiotic synthesis is poorly understood, and requires further research.

All of the types of regulation discussed so far specifically target antibiotic synthesis. Regulation of antibiotic synthesis can also be accomplished by a group of

genes that regulate both morphogenesis and antibiotic synthesis. Table 1 demonstrates that almost all of the *bld* mutants somehow play a role in regulating antibiotic synthesis, though some deficiencies may be rescued by growth on alternate carbon sources. The role played by BldB in this process is not known, but with the multiple levels of regulation, it is likely that BldB regulates one or more of these regulators of antibiotic synthesis.

Ongoing analysis of antibiotic production and the proteins involved

The list of genes involved in the synthesis of the four antibiotics produced by *S. coelicolor* continues to grow, and it is very likely that this list is not nearly exhaustive of all of the genes involved. As this list grows, many of the genes have been extensively characterized, but many of the newly-discovered genes play an as yet unidentified role. In most cases, it is only known that disruption or mutation of these genes has a phenotypic effect, either inhibiting or overproducing one or more antibiotics. To date, the number of proteins known to be involved in regulating antibiotic synthesis exceeds 15. The *absA1/A2*, *afsR/K* and *cutR/S* two component systems are known to be involved (2, 14, 43). As previously stated, the *absB* gene encoding an RNaseIII homologue also regulates antibiotic synthesis through a poorly understood mechanism (1). In the Streptomycetes, antibiotic synthesis also appears to be regulated by a group of molecules known as γ-butyrolactones. The *S. coelicolor* molecule is SCB1, whose synthesis and regulation is dependent on *scbA* and *scbR* respectively, but the exact target of this hormone is still unknown (96). Even within the biosynthetic clusters, expression of certain genes has been shown to be regulated by pathway-specific activators. Even the most characterized of

these activators do not directly regulate the expression of every gene accounted for in each cluster, so other regulators must be identified. Perhaps the most poorly understood mechanism of regulation of antibiotic synthesis is that provided by the *bld* genes. Though the role played by *bldA* has been well-determined, its role is based on the presence of a very specific UUA codon, and therefore this mechanism can not explain how the other *bld* genes are involved in regulation of antibiotic synthesis. Since mutations in *bldB* block antibiotic synthesis, and this phenotype can not be suppressed by growth on alternate carbon sources, it is one of the goals of this thesis to determine what role is played by the BldB protein in regulating antibiotic synthesis in *S. coelicolor*.

Chapter 2

Structural and genetic analysis of the BldB protein of *Streptomyces coelicolor*

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Abstract

I have shown that the previously uncharacterized *S. coelicolor* developmental regulator BldB is essential for both aerial hyphae formation and for antibiotic synthesis. I have also shown that in order to carry out its function, the BldB protein must form a higher order complex. Further research regarding this aspect of its function suggests that BldB forms a dimer *in vivo* and *in vitro*. Mutagenesis of different regions of the amino acid sequence of BldB identify that the central 58 amino acids are required for the dimerization of this protein. Comparison of the sequence of this region with the sequences of similar *S. coelicolor* proteins indicates that there is significant conservation in this family of proteins, particularly in this region. This data suggests that dimerization may be a common and essential feature in the BldB family of proteins.

Introduction

Based on previous studies, very little was known about the *bldB* gene, and it is my goal to elucidate the role this gene plays in the regulation of morphogenesis and antibiotic synthesis in *S. coelicolor*. It has been demonstrated that the synthesis of antibiotics and formation of aerial hyphae are developmentally linked, and that these events are coordinated at the molecular level (13). Mutations in the *bld* genes show that these genes, though non-essential for survival, are required for both developmental pathways (13, 69). Interestingly, mutations in the *bldB* gene have the most severe phenotypic consequences among all the *bld* mutations. Not only are *bldB* mutants unable to form aerial hyphae or synthesize antibiotics, they are also globally defective in catabolite control and do not fit into any of the well established groups within the *bld* mutant extracellular complementation hierarchy (13, 69, 76, 83, 107). Pope et al previously cloned the *bldB* gene, which encodes a 98-amino-acid protein with a molecular mass of 10,899 Da (82), illustrated in figure 1. There are at least 20 homologues of *bldB* in the *S. coelicolor* genome, of which *abaA* and *whiJ* are responsible for antibiotic synthesis and spore formation respectively (29, 34, 89). This family of proteins appears to be unique to the actinomycetes as there are no other homologues in the common databases.

The numerous defects caused by mutations in *bldB* suggest that BldB plays a role in controlling the expression of multiple genes. Expression of *bldB* is normally low during vegetative growth and increases during initial aerial hyphae formation. Pope et al have demonstrated that in *bldB* mutants, expression from the *bldB* promoter is constitutive, implying that BldB may regulate its own production in cells, via a currently-

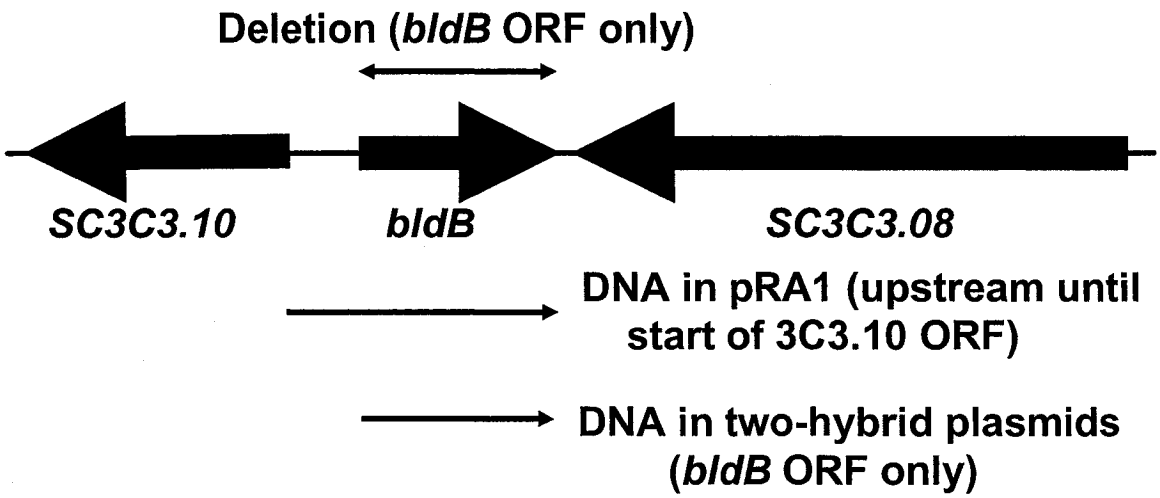


Figure 1. Chromosomal schematic of the *bldB* ORF and two flanking ORFs. *bldB* encodes a 98 amino acid protein in divergent orientation to flanking genes, thus it most likely has a distinct promoter. The 304 base pairs upstream of the *bldB* ORF was included in the plasmid used for complementing the *bldB* null mutation *in vivo* (pRA1). The exact ORF of BldB was cloned into a two-hybrid system to test protein interactions (pT18 and pT25).

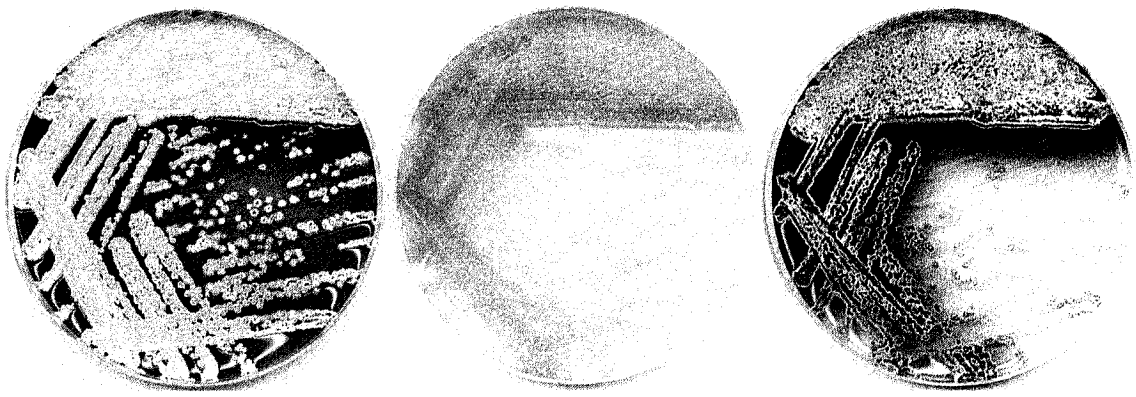
unknown mechanism (27, 82). An interaction between the BldB protein and the *bldB* promoter has not been demonstrated to date, suggesting that BldB regulates expression of its own promoter indirectly.

In order to determine the function of BldB in *S. coelicolor* development, I have constructed a chromosomal deletion of the *bldB* ORF. The phenotype of the null mutant is identical to that of the originally-isolated point mutants, implicating BldB in morphogenesis and antibiotic synthesis. Using two-hybrid analysis, gel filtration, and analytical ultracentrifugation, I have been able to discern that the BldB protein interacts with itself to form a homodimeric protein. I have also been able to determine that the amino acid residues within the conserved central region of BldB are required for this interaction. The conservation of this region among the proteins of the BldB family suggests that dimerization may be a characteristic shared by the proteins in this family.

Results

Construction of a genetic *bldB* knockout

Several distinct mutations in the *bldB* locus were made by Champness et al using UV irradiation and phenotypic selection (13). The mutations in the *bldB* locus varied in nature. Four of the mutations were amino acid substitutions or frameshift mutations within the coding sequence (Y21L and Y21C substitutions and W72-STOP and D50-frameshift respectively). Two mutations altered bases in the *bldB* promoter (-49:thymine-cytosine and -48:adenine-guanine substitutions), while the final mutation (-17:guanine-adenine substitution) created an alteration in the ribosome-binding Shine-Dalgarno sequence, which affects proper translation of the *bldB* transcript (69). Interestingly, the single amino acid substitution mutants within the sequence were point mutants at the same amino acid residue, a tyrosine that is the 21st residue in the sequence. Although the location of all of these mutants was different, all of them were defective in morphogenesis and antibiotic synthesis. However, none of these mutants is a null mutant. In order to be certain of the role of BldB in morphogenesis and antibiotic synthesis, I constructed a deletion of the *bldB* ORF from the *S. coelicolor* chromosome, replacing it with the neomycin resistance gene *aphI* (figure 1). The effects of this deletion can be seen in figure 2. The grey pigmentation, fuzzy appearance and pigmentation of wild type *Streptomyces* is evident in the wild type M145 strain containing the control plasmid pSET152. However, in the null mutant, aerial hyphae and the pigmentation associated with antibiotic production are absent. Since deletion of the entire coding sequence of *bldB* causes an identical phenotype to that of the point mutants, we can conclude that



M145 **N985 + pSET152** **N985 +pRA1**

Figure 2. Summary of the genetic analysis of *bldB* knockout in *S. coelicolor*. On the left is the wild type strain M145 containing the control vector pSET152, where aerial hyphae and antibiotic synthesis is normal. The middle plate contains the *bldB* null mutant strain (N985), lacking both of these functions. Addition of the *bldB* ORF and its natural promoter in Trans is sufficient to complement the null phenotype (N985 + pRA1)

these mutations prevent the normal function of BldB.

Complementation of the null mutant

Based on the orientation of the *bldB* gene and the SC3C3.08 ORF 29 nucleotides downstream of *bldB*, depicted in figure 1, it is unlikely that the drastic phenotypic difference between the null mutant and the wild type is the result of polar effects on downstream genes. To be certain that polar effects were not the cause of the *bldB* null phenotype, Reem Ali constructed the plasmid pRA1 containing the *bldB* ORF and the 304 base pairs upstream proposed to contain the *bldB* promoter region. Figure 1 illustrates that the entire upstream region between the *bldB* ORF and the next predicted ORF are incorporated to ensure that the *bldB* promoter was included. The pRA1 plasmid and the negative control vector pSET152 were introduced via transformation into both

the wild type strain (M145) and the *bldB* null strain (N985). Figure 2 demonstrates that the negative control plasmid alone had no effect on the *bld* phenotype. In contrast, the introduction of pRA1 into the *bldB* null strain restored morphogenesis and antibiotic synthesis was restored to near-wild-type levels.

Determination of oligomeric state of BldB

Since no structural information was available for the BldB protein, I chose to express the protein as an amino-terminal His_6 fusion protein with an adjusted molecular weight of 12,154 Da in order to test the proteins oligomeric behavior *in vitro* using gel filtration analysis. Upon purification using nickel affinity chromatography in common non-denaturing conditions (50mM Tris-HCl buffer pH 7.5, 0.2M NaCl), the purified protein was then applied to a Superdex H-75 gel filtration column. Using gel filtration, the Stokes radius of a protein can be calculated, which represents how compact a protein is, where higher Stokes radii values represent less compact proteins (61). The elution profile of the BldB protein when compared to proteins of known molecular weight suggested that the purified protein was behaving as though it were larger than predicted by its calculated molecular weight, as seen in figure 3. The trace in figure 3 illustrates the elution profile of BldB and of the molecular weight standard proteins albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and RNase A (13.7 kDa) as milliabsorbance units vs. time of elution, from which a Stokes radius can be calculated (61). The standard proteins have Stokes radii of 3.55, 3.05, 2.09, and 1.64 nanometers, respectively. According to the elution profile, BldB eluted at approximately 52.5 minutes,

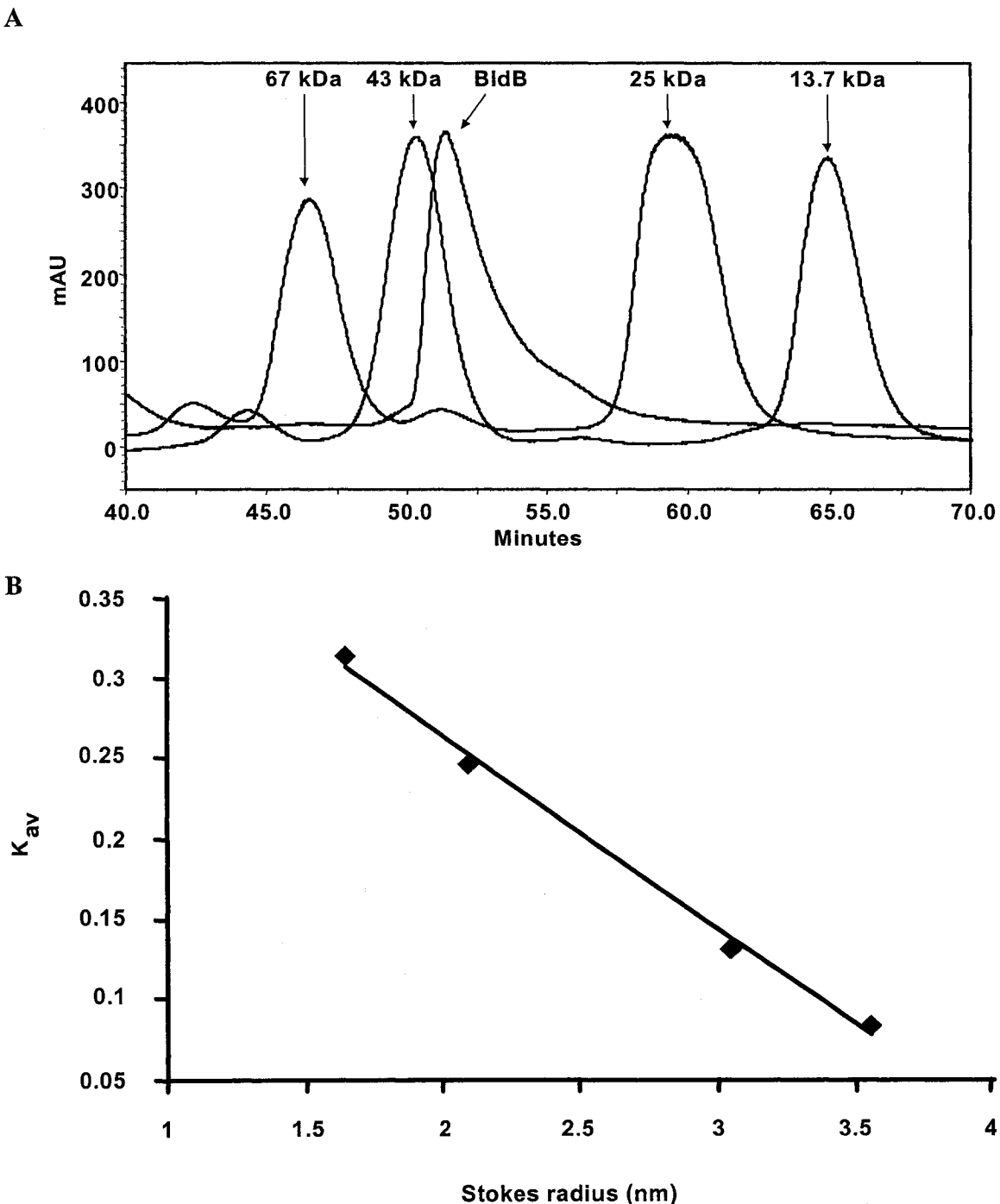


Figure 3. (A) Elution profile of molecular weight standards and BldB on Sepharose H-75 gel filtration column. BldB behaves as a protein between 43 and 25 kDa, though its predicted molecular weight is 12,154 Da. (B) A linear standard curve of Stokes radius of molecular weight standards in relation to elution volume expressed in terms of K_{av}. K_{av} is determined by $(V_e - V_o)/(V_e - V_t)$, where V_e is the elution volume, V_o is the column void volume, and V_t is the total column volume.

between ovalbumin and chymotrypsinogen A, resulting in a Stokes radius of approximately 3.00 for BldB, a rather large value for a protein with a predicted molecular weight of 12,154 Da. To explain this uncharacteristic result, we concluded that BldB was either entirely unfolded, and would thus elute more quickly than in a folded, globular form, or that BldB was forming a higher-order complex in solution.

A second common technique for determining the oligomeric state of proteins is analytical ultracentrifugation, which I used to determine the extent to which BldB interacted with itself. In an ideal solution, the hydrodynamic behavior of a protein in solution allows for the calculation of its molecular weight. Unfortunately, purified BldB did not behave as an ideal protein solution, so the results did not yield a precise degree of protein-protein interaction. This could be due to regions of the protein that possess a lot of random folding, or a less compact shape. Based on the fact that His_6-BldB is predicted to have a mass of 12,154 Da, the observed mass of 20,581 Da was 1.69 times higher than expected. However, if the BldB protein was monomeric in solution, this experiment would yield a mass much closer to the predicted molecular weight of 12,154 Da. Since the data implies that in solution, the BldB protein acts as though it has much more mass (20,581 Da), this strongly suggests the formation of a BldB-BldB dimer.

The advent of a bacterial two-hybrid system by Karimova and colleagues provided me with a system in which I could test whether BldB oligomerization occurs *in vivo* (51). This system also provided a novel *in vivo* assay for BldB, whereby I could test for loss of function mutations in the *bldB* sequence. The bacterial two-hybrid assay is based on the reconstitution of a signal transduction pathway, restoring adenylate cyclase

activity to a *cya*$^-$ mutant strain of *E. coli* called DHP1. The vectors pT18 and pT25 encode two halves (T18 and T25) of the catalytic domain of the *Bordetella pertussis* adenylate cyclase protein that are inactive when expressed on their own. However, if two interacting proteins are fused in frame to the T18 and T25 fragments, the interaction brings the two halves of the catalytic domain in close proximity to functionally reconstitute the adenylate cyclase activity in the DHP1 assay strain. This functional complementation results in the synthesis of cyclic AMP (cAMP), which in turn activates the expression of the specific sugar metabolizing maltose regulon. Maltose fermentation can be visualized by growth on MacConkey medium containing maltose as the sole carbon source, where maltose fermentation results in a red colony phenotype, and the lack of maltose fermentation confers a white colony phenotype to the *E. coli*.

The DNA encoding the ORF for BldB (NHB for non-His_6 tagged BldB) and His_6-BldB (BBH for BldB-His_6) was cloned in frame into both the bait (pT18) and target (pT25) vectors to yield plasmids pT18NHB, pT18BBH, pT25NHB, and pT25BBH respectively, which contain in frame fusions of the BldB protein to the two halves of the adenylate cyclase catalytic domain. These plasmids were introduced into the assay strain DHP1 and assayed for a BldB-BldB interaction (figure 4). As a negative control, colonies containing only a single BldB-fused protein (either T18-BldB or T25-BldB) remained white, and were unable to complement the *cya*$^-$ assay strain. In contrast, strains containing the two *bldB* fusions exhibited a strong red colony phenotype, indicative of a positive BldB-BldB interaction, as illustrated by the *in vitro* experiments (figure 4). Figure 4 also shows that this interaction is not affected in any way by the presence or

absence of the amino-terminal His_6 tag, suggesting that this tag does not interfere with the function of the BldB protein.

The two-hybrid system interaction provided a novel assay of BldB binding. In order to identify the amino acids in the BldB sequence required for this interaction, I carried out random mutagenesis, in which residues in the pT25BBH plasmid were altered randomly and screened against pT18BBH to look for loss of protein-protein interaction. Mutagenesis was carried out by growing the pT25BBH plasmid in the mutagenic *E. coli* strain XL-1 Red, which is defective in the DNA repair genes *mutD*, *mutS*, and *mutT*, which causes this strain to have an increased tendency to create point mutations

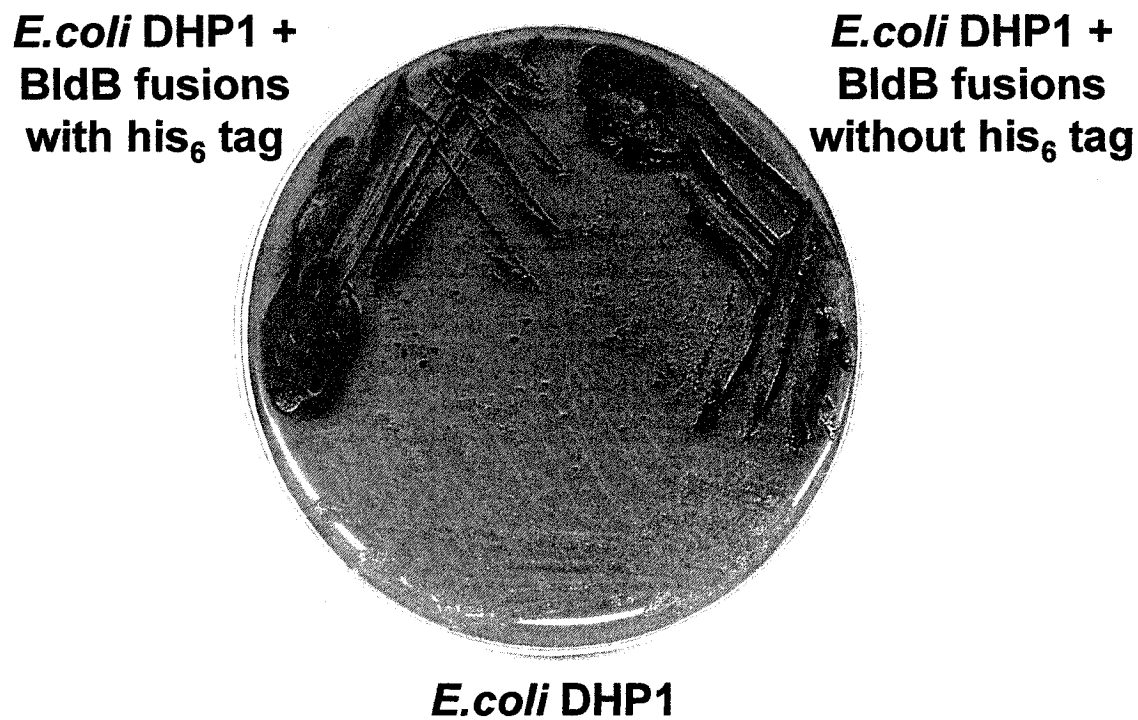


Figure 4. The bacterial two-hybrid system assay. The BldB ORF was cloned into the bait and target vectors, and both were introduced into the *E. coli* DHP1 assay strain. On the bottom of the plate is the assay strain alone. The colonies appear white as they are unable to ferment the maltose in the medium. Both the His_6- and non-His-tagged versions of BldB are able to interact and can thus ferment maltose, making the colonies red.

without repairing them during DNA replication (20, 35, 92). After screening a random pool of approximately 8000 colonies against the wild type pT18BBH plasmid in DHP1 for a loss of the interaction, I identified two *bldB* mutations. In both cases, a single base pair had been deleted, the first at position 49 (FS1) and the other at position 73 (FS2) of the *bldB* coding sequence, indicated in figure 5. The frameshift caused by both of these mutations resulted in a nonsense coding sequence for the last 54 and 30 amino acids of BldB in FS1 and FS2 respectively.

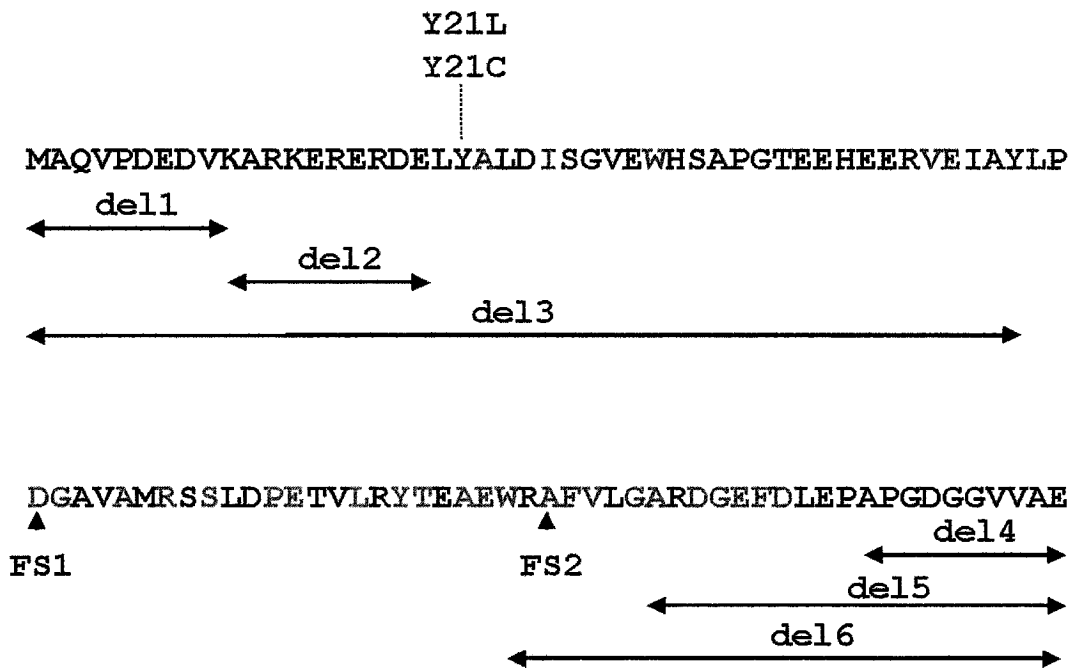


Figure 5. A representation of the BldB amino acid sequence and all of the mutations that were assayed in the bacterial two-hybrid system. All of the alterations except del1, del2, del4 and del5 block the BldB-BldB interaction *in vivo*. The central 58 amino acids are essential for this interaction. Residues in red represent the highly conserved residues in the BldB family of proteins.

Since random mutagenesis only yielded proteins with biologically irrelevant sequences due to the shift in the reading frame, I chose to identify smaller fragments of the BldB protein that retained the capacity to dimerize. In order to do this, I created sequential deletions in the BldB ORF in pT25BBH plasmid. These plasmids were tested for the ability to interact with wild type pT18BBH and restore a red colony phenotype to the *E. coli* strain DHP1. The BldB-BldB protein interaction was not affected by the deletion of residues 1 to 10, 11 to 20, 78 to 98, or 88 to 98 in the BldB sequence. However, deletion of a larger fragment of the amino terminus, from residues 1 to 47 blocked interaction, implicating the importance of residues 21 to 47. Interestingly, the deletion of residues 71 to 98 also abolished dimerization, even though only 7 additional residues are deleted than in the 78 to 98 residue deletion, suggesting a crucial role for one or more of these 7 residues in the folding and stability of BldB or in the BldB-BldB interaction. The data suggests that the 58 amino acids between residues 20 and 78 are likely sufficient to bring about dimer formation *in vivo*.

As mentioned previously, two of the originally-isolated *bldB* point mutants are located within the coding sequence at the same residue at position 21, replacing the tyrosine with a leucine and a cysteine residue (82). This residue is therefore implicated in the function of BldB, and to test whether it is required for dimerization, these substitutions were made in the plasmids pT18BBH and pT25BBH. Upon expression of these plasmids in DHP1, it was evident that the tyrosine residue was not essential for BldB dimerization, as the red colony phenotype was not altered, even when both plasmids carried mutations in BldB at tyrosine 21. This result implicates that this residue

is required for something other than BldB dimerization, likely interaction with another target, or perhaps this residue is the target of a crucial phosphorylation event.

The BldB family of regulatory proteins

Two of the strongest homologues of BldB in *S. coelicolor* are proteins of known function. AbaA is a protein involved in the regulation of antibiotic synthesis, while WhiJ is one of the proteins required for the maturation of aerial hyphae into spores. Since BldB is required for both morphogenesis and antibiotic production, similarity to these two

```
WhiJ     1   ----------------------MTVRPRWRKS   10
7A12.13  1   ---MAESTTHQRPLRGWDKP-ELDLSNAQWQSS   29
E9.31c   1   --------MDHDVYDVYNGMAATQLHDAAWQKS   25
AbaA     1   ------------MPPVRNGVQAS-LLDACWKKS   20
BldB     1   MAQVPDEDVKARKERERDELYALDISGVEWHSA   33
                              **    *****    ****

WhiJ     11  SYSEGGDGNTCVEIAAL-HTRIAVRDSKAPSQGT  43
7A12.13  30  S---RGLG--DVQIAFV-EGFIAMRNSGSPQSPS  57
E9.31c   26  RRSNSQGS--CVEFARLPDGAVAVRNSRFPDGPA  57
AbaA     21  RHSNAEGN--CVEVA-LVDGGIAMRNSRDPDGPA  51
BldB     34  PGTEEHEE--RVEIAYLPDGAVAMRSSLDPET-V  64
               **        **** **** **** * ***

WhiJ     44  -VTVPIGSFAALIQSLKRSTV-------------  63
7A12.13  58  LIFTPAEWGAFVSGAREGEFDLT-----------  80
E9.31c   58  LVYTRAEIEAMLLGVKDGEFDHLIASESAAERSG  91
AbaA     52  LVYTPAEVAAFLAGAKDGEFDHLVAPGDGGVVA-  84
BldB     65  LRYTEAEWRAFVLGARDGEFDLEPAPGDGGVVAE  98
             * ** **  **************  *********
```

Figure 6. An alignment of BldB with its four closest homologues. This alignment highlights the conserved residues in BldB, AbaA, WhiJ, and two homologues of unknown function, SCE9.31c and SCE7A12.13. Conserved residues are indicated by asterisks under the amino acid sequence.

proteins is not surprising. The amino acid sequences of two other strong *S. coelicolor* BldB homologues SCE9.31c and SCA7A12.13, which are of unknown function were aligned with the BldB, AbaA, and WhiJ sequences, as seen in figure 6. This alignment revealed a high degree of conservation among these polypeptides, including the residues in the core dimerization region between residues 20 and 78. The conservation in this region suggests that these BldB-like proteins may also function as dimers, or that BldB may even form heterodimers with other family members.

Discussion

Research into the function of the *bldB* gene was limited before this work. Seven point mutants had been isolated, all of which shared a similar phenotype. All of the mutations blocked the formation of aerial hyphae and antibiotic production, but since the *bldB* gene was being expressed, whether at inappropriate levels or in a mutant form, this phenotype could not be attributed to the absence of the *bldB* protein. It was therefore necessary to observe the phenotypic effects of removing the *bldB* gene entirely from the *S. coelicolor* chromosome. Upon deletion of the *bldB* ORF, it was confirmed that this gene is absolutely essential for aerial hyphae formation, and that it is involved either directly or indirectly with the synthesis of the pigmented antibiotics of *S. coelicolor*. A single copy of the *bldB* gene was supplied in Trans to the null mutant to ensure that the observed phenotype was solely due to the replacement of *bldB* with *aphI* and not any other anomaly or polar effects on expression of other downstream genes. The restoration of the wild type phenotype to the null mutant by pRA1 and not the control vector

pSET152 confirms that the *bldB* gene is essential for the aforementioned functions, and that polar effects are not the cause of the aberrant phenotype.

It can be noted that the phenotype of the *bldB* null mutant was not completely restored upon transformation with pRA1. The slight difference in phenotype is most likely due to the fact that the complementation vector inserts itself into the *S. coelicolor* chromosome at a very specific location, the integration site for the phage ϕC31. Since this is not the natural *bldB* location on the chromosome, the level of expression of this gene could be affected, and is probably the reason for the slight delay in the onset of morphogenesis and antibiotic production.

The expression of BldB as a His_6-tagged fusion protein permitted me to perform *in vitro* analysis of how this protein behaved in solution. The results of the hydrodynamic experiments suggest that BldB might form an asymmetric dimer. Often when proteins have an elongated or asymmetric shape, they migrate more quickly during gel filtration than they would if they were globular, resulting in an exaggerated estimate of their molecular weight (33). Meanwhile, increased friction with buffer molecules during high-speed centrifugation causes asymmetrical proteins to move more slowly, ultimately resulting in an underestimation of the molecular mass of such proteins (33). If a protein is non-spherical, the degree of deviation from spherical shapes can be estimated using the frictional ratio (f/f_0) of a protein, which compares its frictional behavior with that of a theoretically spherical protein of similar size, and a Perrin factor that is required to account for the hydration of such a protein. Gel filtration of BldB suggested the protein

had a Stoke's radius (r^s) of 3.00. Using this value, I could calculate the frictional ratio of BldB using the equation

$$f/f_0 = r^s/(3\nu M/4\pi N_A)^{1/3}$$

where ν is the partial specific volume (0.725 ml/g for BldB), M is the molecular mass predicted by the protein sequence (12,154, 24,308, 36,462, and 48,616 for monomer, dimer, trimer and tetramer respectively), and N_A is Avogadro's number (94). The partial specific volume of most proteins falls within the range of 0.7 and 0.75 ml/g and when a specific value is not known, a partial specific volume of 0.725 is assumed (91). The resulting frictional ratios for a monomeric, dimeric, trimeric and tetrameric protein were calculated to be 1.97, 1.57, 1.40 and 1.24 respectively as any one of these oligomeric states were possible given a Stoke's radius of 3.00. Using this data, another factor that is useful in determining the oligomeric state of a protein is the Perrin factor (F), which can be calculated using the equation

$$F = f/f_0\,(1 + \delta/\rho\nu)^{-1/3}$$

where δ represents the protein hydration (0.35 ml/g is a standard protein value), and ρ is 1 (the solvent density, estimated to be unity). The Perrin factor of each species was calculated to be 1.73, 1.37, 1.2 and 1.09 for the monomer, dimer, trimer, and tetramer species respectively (11). Typically, spherically folded proteins yield frictional ratio values between 1.2 and 1.4, and Perrin factors close to 1.0. The values calculated for BldB therefore suggest that it is a monomer of extremely irregular shape, a dimer or trimer of moderately irregular shape or a spherical tetramer. Based on the specificity of the ultracentrifugation data, I suggest that BldB forms an asymmetric dimer.

Once it was evident that BldB possesses the capacity to form homodimers, I decided to determine what elements of the BldB structure were required for this interaction. The bacterial two-hybrid system provided a novel method to create mutations in the *bldB* sequence to test for disruption of this newly-determined behavior of BldB. Fortunately, the presence of a His_6-tag on the amino terminus of BldB did not disrupt the dimerization activity, which provided confidence that the tagged protein was behaving in a similar manner to the untagged protein *in vivo* and that *in vitro* results would suggest how the protein behaves in living cells. By making sequential deletions along the length of the BldB sequence, I was able to determine the core region of the amino acid sequence that was essential for BldB dimerization and localize it to the amino acids 20 through 78. Interestingly, though the deletion of residues 78 to 98 did not disrupt dimerization, the deletion of 7 more residues, from 71 to 98 caused a disruption in this activity. Since this deletion occurs at the same position as one of the isolated frameshift mutations, this suggests that one or more of the residues in this region are crucial to the dimerization behavior or folding and stability of BldB.

Interestingly, even though the tyrosine residue at position 21 in the BldB sequence is essential for differentiation and antibiotic synthesis, it appears as though it is dispensable in the dimerization function of BldB. This suggests that dimerization is only one step in the natural function of the BldB protein, and that this particular residue is likely involved in another critical aspect of BldB function such as protein-protein interactions, or it is a site of post-translational modifications, including phosphorylation. The fact that protein dimerization remains intact *in vivo* suggests that the protein is likely

correctly folded, but due to the phenotype of tyrosine 21 mutants, it is evident that correct folding and dimerization is not sufficient for wild type activity of BldB and that this residue is central to whatever events follow BldB folding in the cell. The exact role of BldB has not yet been determined in the developing *Streptomyces* cell so it is difficult to predict precisely what happens after folding or why the tyrosine residue is crucial, but discovering the role of this residue in BldB function would greatly facilitate the understanding of BldB function as a whole.

As previously stated, there are many homologues of BldB in the *S. coelicolor* genome, but figure 6 shows the 4 homologues that share the highest degree of homology to BldB. In this alignment 56 of the compared amino acids (57%) are shared by at least two of the five proteins BldB, AbaA, WhiJ, SCE9.31c and SCE7A12.13. The similarity between BldB and the proteins AbaA and WhiJ is significant because these proteins are involved in regulating antibiotic production and morphogenesis respectively, both processes that are also dependent on BldB expression (30, 89). Thus, by elucidating the mechanism by which BldB functions in the cell, this function may be extrapolated to other members of this protein family.

Of particular importance in the alignment in figure 6 is the location of the most highly conserved residues, or residues shared by 3 or more of these proteins. Starting at position 43 of the BldB sequence and ending at position 85, many of the residues are either identical in all 5 of these proteins, or they possess similar biochemical characteristics due to the similar polar and/or hydrophobic character of the amino acid side chains. Based on the determination that amino acids in this central region are

necessary and sufficient for dimerization of BldB, this suggests that the other proteins may behave in a similar manner by forming dimers, and that this region is equally important to this process in other members of this protein family.

This alignment also illustrates a major difference between BldB and its closest homologues at position 21 in the sequence. It has been demonstrated that the tyrosine at this position is essential for BldB function in regulating antibiotic synthesis and morphogenesis, but is dispensable for dimerization (69). The absence of this residue in the closest homologues of BldB implicates its role in the specific function of BldB. I have suggested that this tyrosine may be a site of interaction with a target protein, or may itself be a target for phosphorylation, but whatever its purpose, it appears to be unique to BldB among this family of proteins. The absence of BldB homologues outside of the actinomycetes suggests this is a new family of developmental regulators, and determining how BldB functions would greatly facilitate understanding the regulation of morphogenesis in *S.* coelicolor.

Materials and Methods

Bacterial strains and culture conditions. The strains used in this work are listed in Table 1. *Escherichia coli* were grown on Luria-Bertani medium at 37°C for routine purposes. *S. coelicolor* was grown at 30°C in yeast extract with malt extract medium or on R2YE solid medium (55). For two-hybrid analysis, *E. coli* strain DHP-1(51) was grown on MacConkey agar supplemented with 1% maltose at 30°C. *S. coelicolor* protoplasts were transformed (55) with unmethylated plasmid DNA isolated from Er^2-1

cells. Ampicillin, apramycin, chloramphenicol, and neomycin were used at 100, 50, 25, and 10 μg/ml, respectively.

Plasmids, primers, and sequencing. The plasmids used in this study are listed in Table 2. The Mobix Laboratory at McMaster University performed primer syntheses and DNA sequencing. PCR was performed using Vent DNA polymerase from New England Biolabs and *Pfu*, *Pfu turbo*, and Herculase polymerases (Stratagene).

Table 1: Strains employed in this study.

Strain	Genotype	Reference
Streptomyces coelicolor		
N985	*bldB*::*aphI* SCP1$^-$ SCP2$^-$	This work
M145	Prototroph SCP1$^-$ SCP2$^-$	13
Escherichia coli		
BL-21(DE3)	F$^-$ *dcm ompT hsdS*(r_B^- m_B^-) *gal met* λ(DE3)	Novagene
Er^2-1	F′ *lacI*^{Q} *leuB6 thi*-1 *fhuA31 lacY1 tsx-78 galK2 galT22 supE44 hisG4 rpsL136* (Str^r) *xyl-5 mtl-1 dam13*::*Tn9* (Cam^r) *dcm-6 mcrB1 mcrA hsdR2* ($r_K^-m_K^+$)	J. McCormick
DHP-1	F$^-$ *cya*$^-$ *glnv44(A5) recA endA1 gyrA96 Nal*^{r} *thia1 hsdR17 spoT1 rfbD1*	11
XL-1 Blue (Stratagene)	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac*[F′ *proAB lacI*^{Q}ZΔM15*Tn10* (Tet^r)]	Stratagene
XL-10 Gold (Stratagene)	Tet^r Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* The [F′ *proAB lacI*^{Q}ZΔM15*Tn10* (Tet^r) Amy Cam^R]^a	Stratagene
XL-1 Red (Stratagene)	*EndA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac mutD5 mutS mutT* Tn*10* (Tet^r)^a	Stratagene (6, 10, 20)

Table 2: Plasmids employed in this study.

Plasmid	Description	Phenotype[a]	Reference
Streptomyces coelicolor			
pRA1	0.62-kb EcoRI/XbaI amplicon from *S. coelicolor* containing *bldB* and its promoter inserted into pSET152	Apr^r	This work
pSET152	*lacZ*α MCS[b] *rep*^{pUC} *ori*T ϕC31 *int attP aac(IV)3*	Apr^r	2
pOJ260	*rep*^{pUC} Am^r *ori*T *lacZ*α MCS	Apr^r	2
pBKO	2-kb HindIII/NdeI amplicon from *S. coelicolor* chromosome of DNA upstream of *bldB*; *aphI* inserted at NdeI site; 1.2-kb NdeI/EcoRI amplicon of DNA downstream of *bldB* inserted into pOJ260	Apr^r	This work
Escherichia coli			
pPCR-Script Amp	Stratagene plasmid based on pBluescript IISK$^+$ Unique SrfI site for insertion of blunt-end amplicons	Amp^r	Stratagene
pBluescriptNeo	Derived from pIISK$^+$Bluescript; 0.9-kb AseI amplicon containing *aphI* inserted into pBluescript	Amp^r	This work
pT18	*bla ori colE1 f1 origin T18* MCS	Amp^r	13
pT25	*cat ori p15A T25* MCS		
pBB801	*lac*I *bla ori* pBR322; MRGSH6-BldB under T7 promoter/terminator expression		R. Seyler
pT18BBH	*bla ori colE1 f1 origin T18* MCS 0.32-kb KpnI amplicon from pBB801 containing *his$_6$-bldB* inserted into pT18	Amp^r	This work
pT25BBH	*cat ori p15A T25* MCS *his$_6$-bldB*; 0.32-kb *Kpn*I amplicon from pBB801 containing *his$_6$-bldB* inserted into pT25	Chl^r	This work
pT18NHB	*Bla ori colE1 f1 origin T18* MCS 0.32-kb KpnI amplicon from pBB801 containing *bldB* inserted into pT18	Amp^r	This work
pT25NHB	*cat ori p15A T25* MCS *bldB*; 0.32-kb *Kpn*I amplicon from pBB801 containing *bldB* inserted into pT25	Chl^r	This work

[a] Antibiotic resistance markers are apramycin (Apr^r), ampicillin (Amp^r), and chloramphenicol (Chl^r).
[b] MCS, multiple cloning site.

Construction of a *bldB* null mutant.

2-kb and 1.2-kb DNA fragments upstream (Bup) and downstream (Bdown) of *bldB* were amplified by PCR and introduced into pOJ260 so that they were separated by an *Nde*I site introduced during amplification. The resulting plasmid was cut with *Nde*I and ligated to an *aphI* gene with *Ase*I ends to produce pBKO. Unmethylated pBKO was introduced into protoplasts of *S. coelicolor* strain M145, and transformants were selected

with neomycin. These were screened for sensitivity to apramycin to identify strains in which *bldB* was replaced with *aphI*; 2% of the screened transformants exhibited the Neo^r Apr^s phenotype. Chromosomal DNA from these candidates was digested with *Pst*I and subjected to Southern analysis with the Bdown DNA fragment as a probe (90).

Complementation of the *bldB* null mutant.

A 0.62-kb DNA fragment containing *bldB* and its promoter region was amplified by PCR with primers Bcomp A and B. This fragment was inserted into pPCR-Script Amp (Stratagene), cut out with *Eco*RI, and ligated into plasmid pSET152 digested with the same enzyme to generate pRA1. The *bldB* null mutant N985 and its wild-type parent M145 were transformed with pRA1 and pSET152 and selected for resistance to apramycin. Phenotypic analysis was carried out by growth on solid R2YE medium for 2 days at 30°C.

Purification of BldB.

Plasmid pBB801 is based on the commercial vector pET15b. pET15b was digested with *Nco*I and *Bam*HI to remove the His tag-encoding sequence. The open reading frame encoding BldB was inserted at these sites, and a His_6 tag (MRGSHHHHHH-) was cloned into the *Nco*I site preceding the N terminus of the BldB coding region. This plasmid with His_6-BldB under the control of a T7 promoter was introduced into *E. coli* BL21(DE3). Two liters of these cells was grown in liquid Luria-

Bertani medium at 37°C until the optical density at 600 nm reached 0.6, at which point the cells were induced with 1 mM isopropyl-ß-D-thiogalactopyranoside for 3 h. The induced cells were harvested by centrifugation and lysed by passage through a French pressure cell. The lysate was treated with DNase I and RNase A for 30 min, and insoluble material was pelleted by centrifugation. This lysate was applied to a 1-ml nickel sulfate column (Amersham Pharmacia). The column was washed with 50 mM imidazole and 500 mM NaCl in 50 mM Tris-HCl buffer (pH 7.5), and bound proteins were eluted with 0.5M imidazole and 0.5 M NaCl in 50 mM Tris-HCl buffer (pH 7.5). Protein samples were concentrated, and the buffer was exchanged with 0.2 M NaCl and 1 mM dithiothreitol in 50 mM HEPES buffer (pH 7.5). The protein concentration was determined as described by Bradford (10). BldB was further purified using a 1- by 30-cm Superdex H-75 column (Amersham Pharmacia). The mobile phase was 0.2 M NaCl and 1 mM dithiothreitol in 50 mM HEPES buffer (pH 7.5), and the flow rate was 0.1 ml/min. Blue dextran was used to determine the void volume. A single peak of BldB was detected, and its identity was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with both Coomassie staining and Western analysis with anti-His_6 antibody.

Analytical ultracentrifugation of native BldB.

Velocity sedimentation analysis was performed at the Ontario Cancer Institute. Sedimentation was performed at 20°C in a Beckman XLI analytical ultracentrifuge using an AN50-Ti rotor and sapphire windows. The sedimentation equilibrium experiments

using six-channel charcoal-Epon cells were performed at 23,000, 28,000, and 33,000 rpm. Samples were left for 24 h at each centrifugation speed before absorbance measurements were taken. Global analysis of the data was performed using XL-A and XL-I data analysis software (version 4.0) from Beckman Instruments.

Two-hybrid analysis.

The *bldB* gene, with and without a His_6 tag, was amplified from pBB801 with primers HisB A and B and NHB, respectively, to introduce *Kpn*I sites at the ends. These primers replaced the stop codon at the end of *bldB* with a GGG to permit expression of the carboxy-terminal T18 adenylate cyclase fusion. The amplified products were digested with *Kpn*I and ligated into plasmids pT18 and pT25 digested with *Kpn*I to yield plasmids pT18BBH and -NHB and pT25BBH and -NHB. All four plasmids were introduced into DHP-1, and the resulting cells were tested for a red-colony phenotype.

Mutational analysis of BldB-BldB interaction.

E. coli XL-1 Red (Stratagene) was transformed with pT25BBH. The mutagenized plasmid was isolated and digested with *Pst*I and *Nco*I to release the mutated *bldB* gene. This was purified and ligated to fresh pT25BBH. The resulting clones were pooled, and the plasmid was recovered. DHP-1 containing the pT18BBH plasmid was transformed with the pooled, mutagenized pT25BBH, and the transformants were screened for white-

colony producers. pT25BBH plasmids from these were then retested, and DNA that produced white colonies was isolated and sequenced through the *bldB* region.

Amino-terminal deletions (Figure 5) were generated in *bldB* by PCR amplification with 5'-phosphorylated oligonucleotides. After the amplified products were digested with *Dpn*I, the DNA ends were ligated together using T4 DNA ligase from New England Biolabs. Carboxy-terminal deletions in *bldB* were generated by the introduction of stop codons at relevant sites in *bldB* in pT25 using QuikChange mutagenesis (Stratagene).

Table 3: Oligonucleotides employed in this study

Primer name	Primer function[a]	Sequence of oligonucleotide primer partners[b]
Bup A/B	Amplification of BldB upstream region from the *S. coelicolor* chromosome	5' –CAGGCGG**CATATG**AACGATCCCCGACAACCTTAC and 5' –GTCGAG**AAGCTT**TCGGGTGAGGCGCTG
Bdown A/B	Amplification of BldB downstream region from the *S. coelicolor* chromosome	5' –CTCGACGAGCTCTGGCAGGGCACGCGC and 5' –GCGACGGGG**CATATG**TCGCCGAGTGAC
Bcomp A/B	Amplification of BldB and its promoter from the *S. coelicolor* chromosome	5' –GCGACGC**GAATTC**GCTACGTCGCACCG and 5' –GTGCTCATCTCGGGTACGGGAGTGGTCCTGC
HisB A/B	Amplification of His-tagged *bldB* from pBB801	5' –GGAG**GGTACC**AATGAGAGGATCGCAT and 5' –TCGA**GGTACC**CCCTCGGCGACGACGCC
NHB	Amplification of non-his-tagged *bldB* from pBB801	5' –ATCA**GGTACC**AATGGCCCAGGTGCCG
pT18seq	Sequencing plasmid pT18	5' –GACCATGATTACGCCAAGCG
pT25seq	Sequencing plasmid pT25	5' –GATTCGGTGACCGATTACCTG
NT10 A/B	BldB a.a. 1-10 deletion in pT25BBH	5' –GTGATGGTGATGGTGATGCGATC and 5' –GCCCGCAAGGAGCGCGAGC
NT20 A/B	BldB a.a. 11-20 deletion in pT25BBH	5' –TTTGACGTCCTCGTCCGGCAC and 5' –TACGCGCTCGACATCTCGGGTG
NTVar	BldB a.a. 1-47 deletion in pT25BBH	5' –CTGCCCGACGGAGCCGTGGCCATG
CT10 A/B	BldB a.a. 89-98 deletion in pT25BBH	5' –ACCTGGAGCCGTAGCCGGGCGAC and 5' –GTCGCCCGGCTACGGCTCCAGGT
CT20 A/B	BldB a.a. 79-98 deletion in pT25BBH	5' –TCGTCCTGGGTTAGCGGGACG and 5' –CGTCCCGCTAACCCAGGACGA
Framedel A/B	BldB a.a. 72-98 deletion in pT25BBH	5' –GAGGCGGAGTGACGGGCTTTC and 5' –GAAAGCCCGTCACTCCGCCTC
Y21L ½	Y21L mutagenesis in pT18BBH	5' –CGAGCTG**CT**CGCGCTCGAC and 5' –GTCGAGCGCGA**G**CAGCTCG
Y21C ½	Y21C mutagenesis in pT18BBH	5' –CGAGCTGT**G**CGCGCTCGAC and 5' –GTCGAGCGCG**C**ACAGCTCG

[a]a.a., amino acids.
[b]Non-homologous regions and introduced restriction sites are in boldface type

Point mutations altering Y21 were also generated by QuikChange PCR. All mutations were confirmed by sequencing with pT18seq and pT25seq. The mutated plasmids were introduced into DHP-1 cells and tested for BldB-BldB interaction as described above. The primers used for this work are listed in Table 3.

Personal contributions (multiple author publication)

The majority of the work in this chapter was carried out by myself, the primary author of this publication. Reem Ahmed Ali constructed the single-copy complementation vector pRA1 and successfully complemented the *bldB* null mutant. I would like to thank Richard Seyler and Janet Westpheling for providing the BldB expression vector that was used to generate purified BldB protein.

Chapter 3

Dominant effects of *bldB* overexpression on sporulation-associated septation in *Streptomyces coelicolor*

A version of this chapter was submitted to the Journal of Bacteriology in July, 2006

Abstract

The *bldB* gene of *Streptomyces* encodes the best characterized member of an actinomycetes-specific family of small proteins (the BldB family) that have very low iso-electric points. The BldB protein forms dimers and is required for efficient production of antibiotics and spore-forming cells known as aerial hyphae. The biochemical roles of BldB and the rest of the proteins of this family are unknown. In this chapter I identify seven amino acids in BldB that are highly conserved among this family and demonstrate that five of these residues are important for BldB function at normal expression levels. Three of the five are required for dimerization and the other two are not. I demonstrate that the overexpression of *bldB* in *S. coelicolor* causes a morphogenetic phenotype similar to that caused by the *whi* mutations, which block sporulation but not the formation of aerial hyphae. Surprisingly, overexpression of the dimerization-competent but functionally defective alleles caused a phenotype opposite to that of the *bldB* overexpressor where colonies were instead hypersporulating. These results suggest that the role of BldB may be linked to sporulation-associated septation and that in carrying out its intracellular role, the protein has at least one important interaction with another protein.

Introduction

It has been well established that the *bldB* gene is required for morphogenesis and antibiotic synthesis in *S. coelicolor* (23, 69), however, little is known regarding its method of action. Null mutations in this gene, or deletion of the entire ORF result in a similar phenotype, where aerial hyphae formation and antibiotic synthesis are both compromised. These two processes have previously been linked to growth in *S. coelicolor* (54), but the details of this linkage are poorly understood. The *bldB* mutations are not the only ones that cause defects in both morphogenesis and secondary metabolism. In fact, mutations in many of the *bld* genes result in deficiencies in both pathways, further supporting a link between the two (13, 69). Mutations in the *bldB* gene appear to be more severe than in any of the other *bld* mutants however, as morphogenesis can not be rescued by growth on different media, and carbon catabolite control is also globally compromised (83). The pleiotropic nature of these mutations suggests that the *bldB* gene is involved in an early step in morphogenesis or that it regulates multiple processes in development. It was my goal to determine how mutations in the *bldB* gene have such widespread effects when compared to the other *bld* mutants. In order to establish the specific role of *bldB*, I must understand what functions the BldB protein is required for specifically, and how this protein facilitates this function.

Due to a lack of similar genes in common databases, the best lead in establishing a specific role for the BldB protein is the fact that there are a large number of similar proteins encoded in the *S. coelicolor* genome (5, 34). For the most part, the functions of these proteins are unknown, however, two of the more similar proteins, AbaA and WhiJ,

are known to be involved in antibiotic synthesis and sporulation respectively, and they may function in a manner similar to BldB (30, 89).

The pleiotropic nature of *bldB* mutants suggests that BldB regulates multiple genes, and that the BldB family of proteins may constitute a new class of regulatory proteins in *S. coelicolor*. The similarity between these proteins in the core dimerization region is extensive, but these proteins most likely have distinct protein or DNA targets, and none of these targets have been identified. The difference in specificity of targets and function is likely localized to the regions that are not homologous in this family. A good example is the tyrosine residue at position 21 in the BldB sequence that is essential for BldB function, but not dimerization (23). This residue is not conserved at all in the four closest homologues of BldB discussed in chapter 2, and many of the other members of the BldB family do not have a tyrosine residue at this location, suggesting this residue may be required for the specific function of BldB and not the other proteins.

Based on the high degree of conservation in the central region of the sequences in the BldB family of proteins, I chose to construct mutations altering the six most conserved residues. Residues selected for mutagenesis were hydrophilic or aromatic suggesting that their side chains might be surface associated. I therefore constructed *bldB* mutations that changed each residue to an alanine in an effort to determine their role in the function of proteins in this family. I also changed the essential tyrosine residue at position 21 in the BldB sequence to an alanine to further explore its role in the specific function of BldB. These mutations were then assayed in both the two-hybrid system and in the *bldB* null mutant strain to test their effects on dimerization and BldB function.

The effects of removing the BldB protein from developing *S. coelicolor* have now been well established, and BldB is required for normal development and secondary metabolism. The next logical assay is to assess what effects there are, if any, of overexpressing the BldB protein. By cloning the *bldB* ORF and its promoter into a plasmid with a high copy number in *Streptomyces* (pIJ486), I effectively elevated the number of copies of the *bldB* gene in the cell to up to 150, relative to the *S. coelicolor* chromosome (97, 103). Upon construction of the plasmid pIJ486BldB, it was then possible to create the same point mutations in the conserved residues as in the *in vivo* complementation and two-hybrid assays such that overexpression of mutant BldB proteins could also be assayed. When BldB is overexpressed in wild type M145 cells, it appears to hinder morphogenesis, blocking development after the growth of aerial hyphae. Interestingly, overexpression of two mutant forms of the BldB protein, Y21A and F75A results in a phenotype opposite to that of wild type protein overexpression. Instead of the whi phenotype seen when wild type BldB is overexpressed, cells overexpressing the mutant proteins Y21A and F75A demonstrate accelerated development, causing hypersporulation. The phenotypes of these strains were examined by electron microscopy. Despite the differences in morphogenesis mentioned, the cells appeared to be in tact and displayed no defects in surface structure or membrane integrity, suggesting that the effects of BldB overexpression are internal to the cells.

The effects of BldB overexpression are not easily explained. Because the downstream targets of BldB are not known, and the function of BldB is poorly understood, it is unclear how the overexpression phenotypes arise. Since it is known that

BldB plays a regulatory role in morphogenesis, it is important to isolate what developmental pathways may be affected by BldB overexpression. I have therefore introduced the wild type and point-mutant pIJ486BldB plasmids into several different developmental mutant strains of *S. coelicolor* to see if their developmental phenotypes are bypassed or exaggerated.

Results

Mutagenesis of highly conserved residues in BldB

While the biochemical role of BldB proteins is not currently known, the BldB protein itself has been shown to form dimers and that this protein is required for both antibiotic production and the formation of aerial hyphae in *S. coelicolor*. To explore the role of the highly conserved amino acids in the BldB family of proteins I introduced point mutations into the *bldB* gene that changed six highly conserved residues that possess hydrophilic or structurally complex side chains (W30, E44, R56, S58, W72 and F75) and the tyrosine residue previously identified to be important for function (Y21) to alanines. These residues, which are conserved among the majority of proteins in this family are marked with an asterisk in figure 1. The mutagenesis of these residues was carried out in both the pRA1 plasmid to test for effects on protein function, and in the two-hybrid plasmids pT18NHB and pT25NHB to assay effects of these mutations on BldB dimerization *in vivo*.

Each mutant was introduced into the *bldB* complementation vector pRA1, and the resulting vectors were introduced into the *bldB* null mutant strain (N985) to assess the

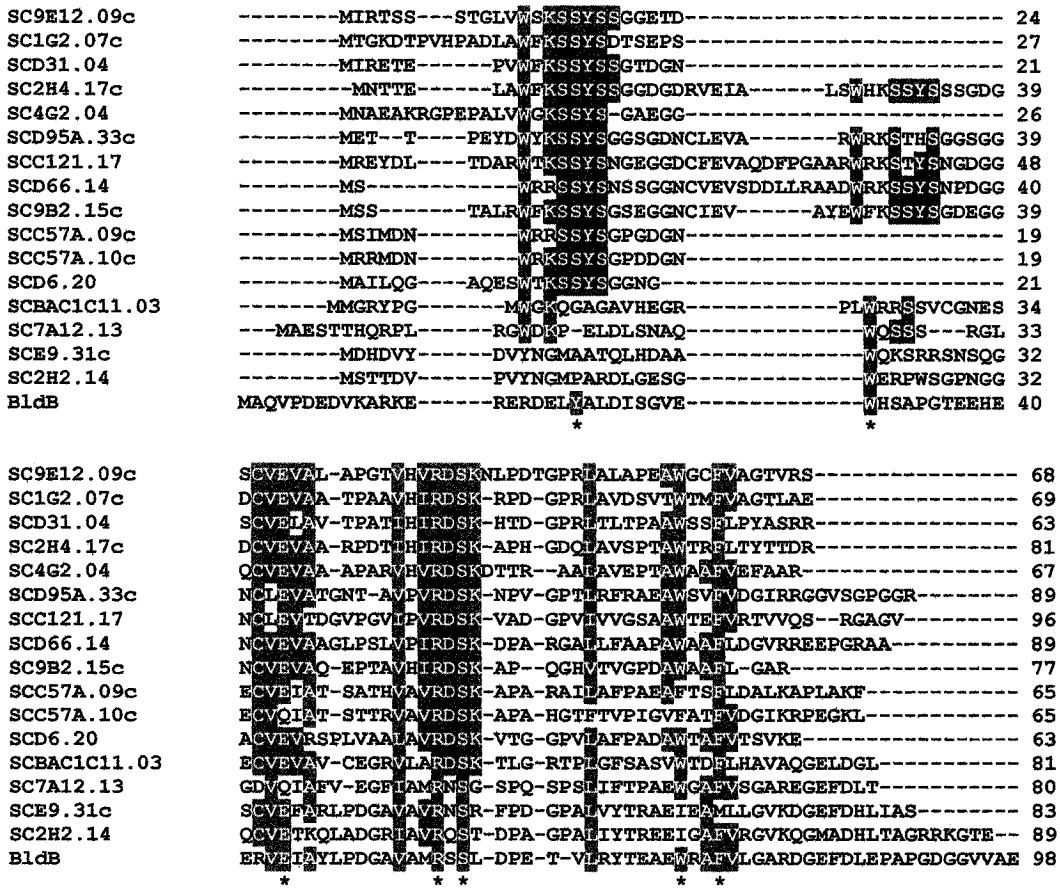


Figure 1. Mutagenesis of highly conserved residues in the BldB family of proteins. Residues that are marked with an asterisk which are generally conserved in the majority of these proteins, were substituted with alanine residues in both the *in vivo* functional complementation and two-hybrid assays.

ability of each allele to drive aerial mycelium formation relative to the wild type *bldB* gene. Examples of the resulting data are shown in figure 2. Mutations changing amino acids E44 (plate 2) and S58 (not shown) had little or no effect on gene function suggesting that though highly conserved, these residues are not crucial to the proper function of BldB. In contrast, mutations changing Y21, W30, R56, W72 and F75 (plate 3, shown in figure 2) dramatically reduced the capacity of *bldB* to function *in vivo* suggesting that these residues are important for BldB function. A summary of the effects

Figure 2. Single copy complementation assay. Point mutations in the *bldB* gene in the pRA1 plasmid were introduced into the *bldB* null strain. When wild type BldB (pRA1) is introduced, the wild type phenotype is restored. The E44A mutation has little to no effect on the ability of the pRAE44A plasmid to complement *bldB*. When pRAF75A is introduced, there is no complementation, suggesting F75A is essential for BldB function.

TABLE 1. Effects of *bldB* mutations on function *in vivo*

Allele	Dimerization (Two-hybrid system)	Single copy Complementation	Overexpression Phenotype
Wild Type (wt)	+	+	Whi[a]
(Y21A)	+	-	Hyp[b]
(W30A)	-	-	WT
(E44A)	+	+	Not Tested
(R56A)	-	-	WT
(S58A)	+	+	Not Tested
(W72A)	-	-	WT
(F75A)	+	-	Hyp

[a] Whi phenotype: develops aerial hyphae but does not sporulate
[b] Hyp phenotype: hypersporulating, accelerated morphogenesis

of introducing these BldB mutations into the *bldB*⁻ null strain can be seen in table 1.

Bacterial two-hybrid analysis: dimerization assay

I have previously used two-hybrid analysis to demonstrate that the BldB protein (23) and one of its homologues, AbaA (Eccleston and Nodwell, unpublished observations) form dimers. To determine whether mutations in conserved residues had an effect on dimer formation, I introduced each sequence change into the bait and target plasmids pT18NHB and pT25NHB and assessed the products of each allele to interact in *E. coli*. The mutant alleles were assayed against wild type BldB and against the same mutations in each plasmid (example: Y21A bait and Y21A target). Consistent with the fact that both alleles functioned normally in morphogenesis, BldB(E44A) and BldB(S58A) were both able to interact *in vivo*. In contrast, three of the defective alleles, W30, R56 and W72 exhibited defects in dimer formation using this assay, suggesting that these residues may form part of the dimer interface. Importantly, two of the alleles that were unable to restore aerial hyphae formation and antibiotic synthesis to the *bldB* null mutant, Y21A and F75A encoded proteins that maintained the ability to interact *in vivo* in the two-hybrid system. Table 1 summarizes the effects that each mutation had in the two-hybrid dimerization assay.

BldB overexpression assay

In parallel with the two functional assays, I assessed the effects of placing *bldB* (under the control of its own promoter) on a high copy number vector (pIJ486) that

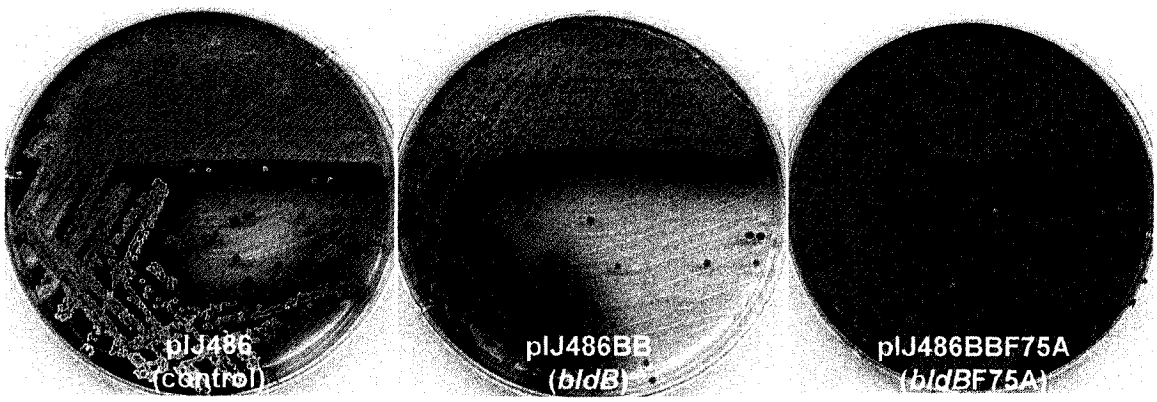


Figure 3. Overexpression of BldB. The *S. coelicolor* high copy vector pIJ486 was introduced into wild type M145. The plasmid alone had no effect on M145. However, when the high-copy pIJ486BB plasmid containing the wild type *bldB* gene and its natural promoter were introduced, a Whi phenotype resulted and sporulation was blocked. Introduction of the high copy vector pIJ486F75A containing an F75A mutation in the *bldB* gene results in accelerated morphogenesis and a hypersporulation phenotype.

results in overexpression in *S. coelicolor*. Surprisingly, I found that when the wild type allele was overexpressed in wild type *S. coelicolor*, colonies exhibited a delay in aerial hyphae formation reminiscent of a Whi phenotype, suggesting a defect in sporulation. Figure 3 shows a comparison between the wild type strain M145 containing the high copy plasmid alone (Plate 1) to the same strain overexpressing the wild type BldB protein (Plate 2).

To determine the effects of *bldB* mutations on this phenotype, I constructed overexpression vectors for each of the five defective *bldB* alleles and introduced them into the wild type *S. coelicolor* strain M145 (Adam Beveridge, a senior thesis student in our lab, constructed the W30A, R56A, and W72A vectors). The *bldB*(W30A), *bldB*(R56A) and *bldB*(W72A) alleles had no discernible effect on morphogenesis when overexpressed, consistent with the fact that their products were either devoid of

biochemical activity, the mutant proteins weren't folded correctly or they were unstable and were degraded in the cell. This data demonstrates that the phenotypic effects of overexpression are not due to the high copy number of the *bldB* promoter. In marked contrast, the *bldB*(Y21A) and *bldB*(F75A) alleles both caused a rapid acceleration of morphogenesis such that, within 48 hours of growth on R2YE medium the colony was completely covered in a mature deep gray aerial mycelium. The third plate in figure 3 illustrates the excessive degree of gray spore pigment formation when compared to plates 1 and 2.

Microscopy: phenotypic effects of BldB overexpression

To observe the effects of BldB overexpression on the phenotype of *S. coelicolor*, I elected to view the cells using both scanning and transmission electron microscopy. Wild type M145 cells containing the control vector pIJ486, *bldB* null strain cells containing pIJ486, and M145 cells containing the BldB overexpression vector pIJ486BB were grown for 48 hours on R2YE medium. At this stage, the phenotypic difference is most evident. The cells were fixed using glutaraldehyde and prepared for microscopy. Scanning electron microscopy of these colonies revealed that excess *bldB* blocked morphogenesis prior to the coiling of aerial hyphae and sporulation septation that is evident in the wild type strain (figure 4a): all hyphae were smooth and lacked the regularly spaced perforations of septating hyphae (figure 4c). Using transmission electron microscopy, I was unable to detect cross wall formation in cells where BldB was overexpressed, while these could be readily identified in cells bearing the control vector

(data not shown). This data further supports the suggestion that excess BldB blocks septation events in developing spores.

The above treatment was repeated using the pIJ486 plasmid containing the F75A mutation in the *bldB* sequence. The plasmid was introduced into M145 and the resulting colonies were grown on R2YE medium for 48 hours, then fixed with glutaraldehyde, and prepared for microscopy. Upon viewing under the scanning electron microscope, the surface of this strain was indeed covered with mature spores. Sporulation in this strain at this time point was much more advanced than that observed in the wild type strain M145 (compare figure 4a to figure 4d). Figure 4 shows scanning electron micrographs that highlight the phenotypic differences observed upon BldB overexpression. This phenotype remains consistent over time, each spore harvested from these colonies germinates to form equally rapidly sporulating colonies indistinguishable from their progenitors.

Overexpression of BldB in developmental mutants

The overexpression of *bldB* has distinct inhibitory effects on the sporulation program of *S. coelicolor*. Since *bldB* mutants are pleiotropic in their phenotype, this suggests that *bldB* is responsible for regulating multiple genes involved in development. In order to test whether overexpression of wild type or mutant forms of BldB are able to bypass various developmental mutants, the pIJ486BldB and pIJ486BldBF75A plasmids were introduced into several developmental mutants, including *bldK*, *whiA*, *whiB*, *whiG*, *whiH*, *sigF*, *ramC*, and *ssgB*. Another class of genes has also been implicated in differentiation in *S. coelicolor*, the chaplin genes (*chp*) which are required for the

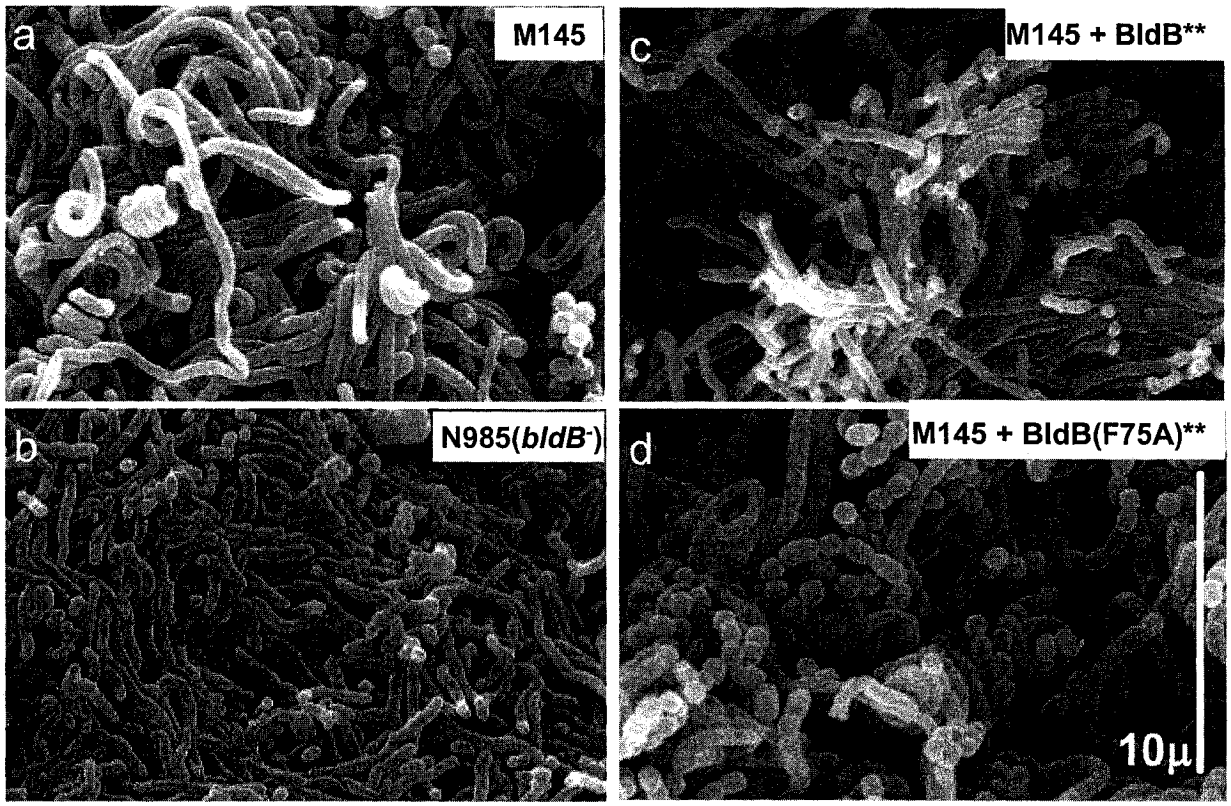


Figure 4. Scanning electron microscopy of *bldB* null mutant, M145, and M145 overexpressing wild type BldB and BldBF75A after 48 hours growth. a) Aerial hyphae have begun to coil and septate, representing early stages of sporulation. b) N985, the *bldB* null strain not only lacks the coiled aerial hyphae, but the substrate hyphae are misshapen. c) M145 strain containing the high copy pIJ486BB plasmid, resulting in overexpression of BldB (**=overexpression). Aerial hyphae are formed, but do not begin to coil after only 48 hours of growth, and no spores are formed. d) M145 cells containing pIJ486BBF75A, which overexpresses the F75A mutant form of BldB. Almost all of the cells have formed mature spores after 48 hours.

secretion of small molecules that are necessary for aerial hyphae formation (26). Since the chaplin genes make up a distinct pathway, not all of the null strains were assayed. The three strains utilized in this assay were: a *chpH* null mutant; a generic null of most of the chaplin genes with the exception of the essential *chpE*; and a strain containing the minimal chaplin genes required for aerial hyphae formation, *chpE*, *chpC*, and *chpH*. Both

the mutant and wild type versions of the overexpression plasmid were introduced into all of these developmental mutants and the resulting colony phenotypes were compared to the same strains containing the control vector pIJ486. Upon analysis in each of these strains, it appeared that no significant phenotypic difference was observed when either wild type or mutant *bldB* was overexpressed suggesting that the excess BldB is not sufficient to bypass any of these null mutations and that the hypersporulation phenotype may be dependent on any or all of these genes.

Discussion

The complementation of the *bldB* null with a single copy of the gene not only confirmed the role of the *bldB* gene in aerial hyphae formation and antibiotic synthesis, but it also provided a novel assay for the function of BldB *in vivo*. Using this assay, I chose to alter the amino acid residues that are most conserved among the members of the BldB family of proteins, and one residue that has previously been implicated in BldB function, Y21 (69), changing them to alanines on the pRA1 plasmid. By introducing these mutant versions of BldB into N985, I was able to screen for mutations that blocked the ability of the pRA1 plasmid to complement the N985 strain. As shown in table 1, all but two of the altered residues (E44A and S58A) severely compromised the ability of pRA1 to drive aerial mycelium formation, suggesting that the other conserved residues, and Y21 are either all essential for BldB function, or these mutations caused the protein to fold incorrectly, affecting its stability and hence its ability to drive aerial mycelium formation. These results are the first implication of the importance of the residues W30,

R56, W72, and F75 in the function of BldB. In order to determine the functional importance of these residues and the Y21A residue, it will be important to determine where these residues are situated in the overall folding pattern of BldB. It is unknown whether these residues are located at a dimerization interface or if they are exposed on the surface of the homodimeric protein, and available for interaction with other proteins.

The two-hybrid system created by Karimova et al provided a second novel BldB functional assay in which I was able to test the effects of point mutations in the *bldB* sequence on dimerization of BldB *in vivo* (51). Only three of the mutations (W30A, R56A, and W72A) blocked the dimerization of BldB, even though the majority of these mutations are within the previously established minimal dimerization domain (23). Interestingly, though they are required for function in the complementation assay, the Y21A and F75A alleles maintained that ability to interact in this assay. These data suggest that Y21 and F75 in the BldB sequence are important for its function, but dispensable for dimer formation. Because these alleles are unable to restore aerial hyphae formation, this suggests that they may be crucial for the undetermined function of BldB, such as protein interaction, or DNA binding. The combination of the results from both of these assays provides an early framework for the putative structure of the BldB protein. Figure 5 summarizes that the W30, R56 and W72 residues likely are located along the BldB-BldB dimerization interface, as their deletion blocks dimerization. The Y21 and F75 residues are dispensable for dimerization, but are required for protein function and are therefore probably exposed on the surface of the protein, and are distant from the dimerization interface in the 3-dimensional structure of the protein. The simplicity of this

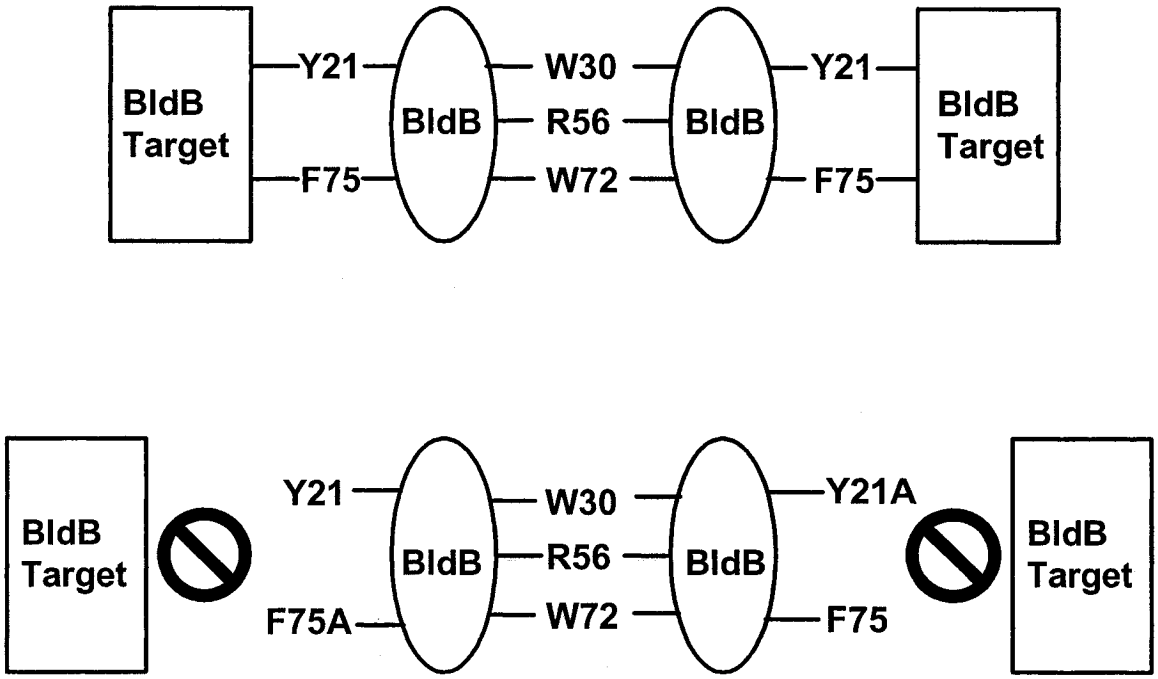


Figure 5. Basic model of BldB structure with key residues highlighted. Based on the two-hybrid assay, the W30, R56, and W72 residues are implicated to be required for dimerization and are likely located along the dimerization interface. The Y21 and F75 residues are not required for dimerization and are likely excluded from this interface. They are likely to be exposed on the protein surface to allow interaction with a target DNA or protein.

model emphasizes the need to determine the actual 3-dimensional structure of this protein, as a structure would illustrate exactly where these residues lie and how they may be involved in dimerization or protein interaction.

My earlier work has demonstrated that deletion of the *bldB* gene blocks morphogenesis and antibiotic synthesis early in development, suggesting that the *bldB* gene positively regulates both functions. However, when the *bldB* gene was overexpressed, the phenotype was surprising in that the resulting Whi phenotype suggests that the role of *bldB* in morphogenesis is contrary to this supposed function, and *bldB* is

required for negatively regulating morphogenesis. When the dimerization-competent but functionally defective alleles of BldB (Y21A and F75A) were overexpressed in the wild type M145 strain, the Whi phenotype was reversed. In fact, the overexpression of these mutant forms of BldB appeared to accelerate sporulation when compared to the wild type strain containing the pIJ486 vector alone. This new data further supports the thought that the *bldB* gene and gene product are likely required to negatively regulate a critical step in morphogenesis at the sporulation stage. It is still unclear precisely what pathways or genes are targets of BldB regulation, but determining these targets would greatly facilitate the exploration of how the BldB protein functions in the cell.

The observed phenotypes of the Y21A and F75A overexpression strains provide some insight into their importance in BldB function. Because both of these mutations cause hypersporulation, it is likely that these residues are close together on the surface of the folded protein, and that they may be required for interaction with the same BldB target, be it DNA or protein. Both Y21 and F75 are aromatic residues, which may be important in determining how they interact, and why both mutations cause a similar phenotype. For this reason, it is crucial to establish a three-dimensional structure to determine where these residues are located in the protein. It is also very important to determine what the BldB targets are in the cell, and how the Y21 and F75 residues are involved in this interaction.

The search for a BldB target may be facilitated by the discovery that many of the BldB homologues are commonly located on the chromosome adjacent to two specific putative proteins. Amy Gehring discovered that the BldB homologues in general are

encoded in small three-ORF clusters containing a BldB-like protein, a histidine kinase-like ATPase, and a DNA-binding domain-containing protein (unpublished results). These genes are not likely transcribed from one promoter as the orientation of the three genes varies for each BldB homologue. This clustering is not true for all of the homologues, and indeed BldB is not encoded in one of these clusters. However, there are other orphaned BldB homologues, DNA-binding proteins and ATPases dispersed throughout the chromosome that are similar in sequence to the BldB-cluster-related proteins. I suggest that any of these orphaned proteins might be primary candidates as targets of the BldB protein.

To gain further insights into the effects observed by overexpressing both wild type and mutant BldB in the cell, I introduced the overexpression plasmids into strains bearing null mutations in the genes *bldK*, *whiA*, *whiB*, *whiG*, *whiH*, *sigF*, *ramC*, *ssgB*, and into various chaplin mutant strains including *chpH*$^-$, *chp*$^-$, and the minimal chaplin strain containing only the *chpC* and *chpH* genes. Plate assays did not reveal any effect of overproduction of the wild type allele on any of these mutants, all appeared identical to strains containing the control vector alone. This data implies that BldB functions in a manner that is either dependent on these genes for hypersporulation, or there is no relation between the function of BldB and the absence of these genes from the cell. In order to determine what genes are dependent on BldB and vice versa, further research in this area is suggested.

Materials and Methods

Site-directed mutagenesis in the *in vivo* complementation assay

Mutagenesis was carried out on the plasmid pRA1 at the amino acid residues marked by asterisks in figure 1. The coding sequence at these seven locations was altered to encode for an alanine residue at each position. The polymerase chain reaction (PCR) was used to amplify the pRA1 plasmid, introducing each of the individual mutations in the *bldB* coding sequence using the respective oligonucleotides listed in table 3. The non-mutagenized template plasmid was removed from the PCR reaction by digestion with the *Dpn*I restriction endonuclease, which cuts only methylated DNA, and therefore will not digest DNA amplified *in vitro* in the absence of methylation enzymes.

Site-directed mutagenesis in the bacterial two-hybrid system

All of the mutations created in the plasmid pRA1 were duplicated in both of the two-hybrid plasmids pT18NHB and pT25NHB, using the same site-directed mutagenesis technique described above, including the oligonucleotides listed in table 3.

Cloning of the BldB overexpression vector pIJ486BB

Since the pIJ486 vector alone does not contain an *E. coli* origin of replication, the plasmid was originally isolated from *Streptomyces* according to methods developed by Kieser et al. (55). Due to the ease of cloning and selection in *E. coli*, I chose to utilize the origin of replication from the pRA1 vector to aid in the generation of a pIJ486 plasmid that can replicate in *E. coli*. Therefore, I digested a large fragment from the pRA1

plasmid with the restriction endonucleases *Bam*HI and *Nhe*I, which encompasses not only the *bldB* gene and its promoter, but also an apramycin resistance cassette to allow for selection of positive clones, and the pUC18 *E. coli* origin of replication to permit screening for candidate plasmids in *E. coli*. The cohesive ends generated by digestion with *Nhe*I are compatible for ligation with the cohesive ends of *Hind*III-digested DNA. The presence of unique *Bam*HI and *Hind*III recognition sequences in the target vector pIJ486 permitted direct cloning of the *Bam*HI/*Nhe*I fragment from the pRA1 vector into the pIJ486 vector digested with *Bam*HI and *Hind*III. The same cloning procedure was repeated for all of the point mutants in pRA1 by myself and Adam Beveridge, generating overexpression plasmids for wild type *bldB* and for all of the mutant forms of the protein from pRA1.

Scanning and transmission electron microscopy

Four strains were selected to be observed using electron microscopy. The wild type M146 containing the pIJ486 plasmid alone was used to illustrate normal development. I also elected to view the *bldB* null strain, the wild type M145 strain containing the pIJ486BB *bldB* overexpression vector, and the wild type M145 strain containing the pIJ486BBF75A *bldBF75A* overexpression vector with both scanning and transmission electron microscopy. Each strain was streaked on R2YE solid media and allowed to grow for precisely 48 hours.

Electron microscopy sample preparation

After 48 hours, the cells were fixed by addition of a layer of 2% glutaraldehyde in 0.1M sodium cacodylate buffer pH 7.4 for 30 minutes at room temperature. The samples were then rinsed in 0.2M sodium cacodylate buffer pH 7.4. Once rinsed, they were post-fixed in 1% osmium tetraoxide in 0.1M sodium cacodylate buffer, pH 7.4 for 30 minutes are room temperature. Upon completion of the fixation procedure, the samples were isolated and dehydrated in an ethanol series of 50, 70, 95 and 100% Ethanol. All samples were prepared by Michael Moore, a technician at the electron microscopy facility in the McMaster campus hospital research centre.

Preparation for transmission electron microscopy

Once dried with ethanol, the samples were further dehydrated with propylene oxide, infiltrated with Spurr's resin and polymerized in 100% Spurr's resin at 60°C overnight. The samples were then cut into thin (70nm) sections, and stained with uranyl acetate and lead citrate. After staining, the samples were ready to be imaged on the JEOL 1200EX Toyoko transmission electron microscope.

Preparation for scanning electron microscopy

After drying with ethanol, the samples were critical point dried and gold coated to 20-40nm. Once coated with gold, samples were ready to be viewed on the JEOL 840 Toyoko scanning electron microscope.

Overexpression of BldB in developmental mutants

The cloning of the overexpression vector pIJ486 was completed as mentioned previously. The pIJ486 vector alone, the vector containing the *bldB* coding sequence, and the vector containing the *bldBF75A* coding sequence were all introduced into the various developmental mutants via the transformation of protoplasts, as described by Kieser et al. (55). Primary transformants were restreaked on R2YE media containing apramycin to maintain selection for the plasmid. Once it was confirmed that successful transformation had taken place, the strains were streaked on R2YE solid media without selection and observed for phenotypic differences over 96 hours, and they were photographed periodically.

TABLE 2. *Plasmids used in this study*

Plasmid	Description	Phenotype[a]	Reference
S. coelicolor			
pIJ486	*ter neo tsr ori pIJ101 rep pIJ101* MCS[b]	Apr^r/Thio^r	(55)
pRA1	*lacZα aac(3)IV ori pUC18 oriT* (RK2) *intϕC31 ϕC31attP* MCS *bldB* + 304 b.p. promoter region	Apr^r	(23)
E. coli			
pT18NHB	*bla ori colE1 f1 origin T18* MCS 0.32-kb *Kpn*I amplicon from pBB801 containing *bldB* inserted into pT18	Amp^r	(23)
pT25NHB	*cat ori p15A T25* MCS 0.32-kb KpnI amplicon from pBB801 containing *bldB* inserted into pT25	Chl^r	(23)

[a] Antibiotic resistance markers are apramycin (Apr^r), ampicillin (Amp^r), thiostrepton (Thio^r) and chloramphenicol (Chl^r).
[b] MCS: Multiple cloning site.

TABLE 3. Oligonucleotides used for mutagenesis in this study

Oligonucleotide	Sequence (5'-3')	End use
Y21P1	CGAGCTGGCCGCGCTCGAC	Y21A mutagenesis
Y21P2	GTCGAGCGCGGCCAGCTCG	Y21A mutagenesis
W30P1	CTCGACATCTCGGGTGTGGAGGCGCACAGCGCG	W30A mutagenesis
W30P2	CGCGCTGTGCGCCTCCACACCCGAGATGTCGAG	W30A mutagenesis
E44P1	GGAACACGAGGAGCGGGTGGCGATCGCCTATC	E44A mutagenesis
E44P2	GATAGGCGATCGCCACCCGCTCCTCGTGTTCC	E44A mutagenesis
R56P1	CCGACGGAGCCGTGGCCATGGCGTCGTCGCTG	R56A mutagenesis
R56P2	CAGCGACGACGCCATGGCCACGGCTCCGTCGG	R56A mutagenesis
S58P1	GAGCCGTGGCCATGCGGTCGGCGCTGGATCC	S58A mutagenesis
S58P2	GGATCCAGCGCCGACCGCATGGCCACGGCTC	S58A mutagenesis
W72P1	CTGCGGTACACCGAGGCGGAGGCGCGGGCTTTC	W72A mutagenesis
W72P2	GAAAGCCCGCGCCTCCGCCTCGGTGTACCGCAG	W72A mutagenesis
F75P1	CCGAGGCGGAGTGGCGGGCTGCCGTCCTGGGTG	F75A mutagenesis
F75P2	CACCCAGGACGGCAGCCCGCCACTCCGCCTCGG	F75A mutagenesis

Personal contribution (multiple author publication)

The majority of the work in this chapter was carried out by myself, the primary author of this submitted article. I would like to thank Adam Beveridge, a third year thesis student for his contributions in constructing the W30A, R56A, and W72A overexpression vectors.

Chapter 4

Effects of *bldB* deletion and overexpression on gene expression in *S. coelicolor*.

Abstract

The *bldB* gene is essential for the regulation of both morphogenesis and antibiotic synthesis, but the mode of regulation is still unknown. The pleiotropic nature of *bldB* mutants suggests that the gene affects these processes early in development, or that the BldB protein directly or indirectly governs the expression of multiple genes required for the natural development of *S. coelicolor*. In order to determine what effects the removal of *bldB* would have on gene expression patterns, microarray gene chips containing approximately 97% of the genes of *S. coelicolor* were used to compare gene expression in the *bldB* null strain and the wild type M145 strain grown liquid media. I also compared the gene expression profiles of the wild type M145 strain with that of a strain that contains multiple copies of the *bldB* gene (M145 plus the high-copy plasmid pIJ486BldB). When the *bldB* gene is removed from the chromosome, many of the genes in the wild type strain that are required for antibiotic synthesis appear to either be repressed or simply lack the activation that was observed in the wild type control strain. There also appeared to be a number of uncharacterized genes whose expression was severely altered in the absence of the *bldB* gene. Surprisingly, the overexpression of the *bldB* gene had numerous effects on large groups of genes that have not been previously associated with the function of *bldB*, including cold shock proteins and ribosomal proteins, suggesting a link between BldB overexpression and stress response in *S. coelicolor*. The data provide important leads for future work in determining what genes are under the control of BldB.

Introduction

Despite recent discoveries of essential and non-essential aspects of BldB protein structure and function, the specific function of the protein remains elusive, due among other reasons, to the fact that the downstream targets of BldB are unknown. Since there are currently no known targets of BldB, it is paramount to establish what unique effects BldB has on development of *S. coelicolor*. As one approach to this question, I have chosen to analyze the effects that the *bldB* gene exerts on gene expression in *S. coelicolor*. The sequencing of the *S. coelicolor* genome by Bentley et al. provided the entire 8.7 Mb *S. coelicolor* chromosomal DNA sequence that is available at the Sanger Centre (http://www.sanger.ac.uk/Projects/S_coelicolor/) (5). The generation of this sequence and the development of efficient methods for genomewide analysis of expression profiles using DNA microarrays enabled our global analysis of factors that affect the transcription of *Streptomyces* genes (22). In collaboration with David Weaver and Camilla Kao at Stanford university, I used DNA microarrays to identify alterations in gene expression that occur during *S. coelicolor* growth both in the overexpression and knockout constructs of *bldB*.

Due to the phenotypic effects of *bldB* deletion and overexpression (see chapter 3), the expression levels of various genes required for morphogenesis and antibiotic synthesis are likely to be altered when BldB is either absent or in excess in the cell when compared to wild type levels. The genes required for antibiotic synthesis in *S. coelicolor* have been extensively characterized and grouped into distinct biosynthetic gene clusters.

The work of Arias et al states that clustered genes on the chromosome that encode enzymes of individual secondary metabolic pathways are commonly subject to multiple levels of regulation (4). There are two common types of regulation of secondary metabolism in *Streptomyces*. Generally, each cluster contains at least one gene that encodes a regulator protein that controls the expression of all of the genes within the cluster known as "pathway- or cluster-specific regulators". The regulatory genes *redZ*, *redD*, *cdaR*, and *act*II-ORF4 are typical members of this family of regulators (3, 29, 72). So called "higher level" or pleiotropic regulation is usually carried out by genes that are situated outside of the biosynthetic clusters which exert pleiotropic effects on the production of multiple secondary metabolites or on both secondary metabolism and morphological development. An important question in *Streptomyces* biology is how the multiple levels of control of antibiotic synthesis pathways are co-ordinated (6). The co-ordination of this control is made extremely complex due to the presence of multiple biosynthetic clusters in the genome and the multiple regulators present in many of these clusters. Interestingly, the *absA* locus is encoded in the CDA biosynthetic cluster but it is known to regulate the synthesis of antibiotics globally in *S. coelicolor*. The *absA1/absA2* two-component system is a typical "higher level" regulator, and initial inspection would suggest that the *bldB* gene should also be classified in this manner due to the fact that mutations in *bldB* disrupt the synthesis of multiple antibiotics. DNA microarray analysis was employed to determine what role the *bldB* gene plays in the regulation of secondary metabolism.

In order to establish what genes are under the control of the BldB protein, I

compared the relative abundance of all transcripts between the wild type strain M145 and the *bldB* null strain. To obtain this information, I compared the hybridization of genomic RNA samples from both strains isolated from liquid media using DNA microarrays. The first experiments were performed on samples isolated from liquid media due to the difficulty of isolating stable intact RNA from cells grown on solid media. Since *S. coelicolor* does not undergo differentiation in liquid media, this analysis likely does not encompass all of the genes affected by the absence of *bldB*, namely the genes involved in differentiation. This information can only be obtained by DNA microarray analysis on genomic RNA isolated from the same strains grown on solid media that permits differentiation. However, this analysis provided important information regarding the antibiotic synthesis genes and all of the other genes not associated with differentiation that may be affected by the absence of BldB. Not surprisingly, the activation of antibiotic synthetic genes in wild type cells is not present in the *bldB* null strain. Interestingly, some genes appear to be up-regulated in the absence of *bldB* suggesting the BldB exerts negative regulation on some genes in the chromosome. One of these genes encodes a putative regulatory protein which may describe how BldB is able to regulate multiple differentiation pathways.

Results

Time course RNA isolation from liquid media

In order to establish gene expression profiles of the wild type and *bldB* null strains, it was essential to analyze mRNA transcript abundance from these strains at various time points during the growth cycle of *S. coelicolor* to determine what genes are affected by the absence of *bldB*. This was most easily accomplished by inoculating liquid cultures of the wild type and null strains in parallel, and subsequently removing samples for RNA isolation at different time intervals. Because of the changes in gene expression during the logarithmic phase of growth, samples were taken at shorter intervals during early development, and the final sample was taken when the cultures had reached stationary phase. To compare the gene expression of the *bldB* null strain and M145, equal volumes (25 ml) of cells from the cultures were removed at 18, 36, 48, 72, and 108 hours after inoculation, and genomic RNA was isolated from these cells and used in DNA microarray experiments in order to assess gene expression patterns that varied between these strains.

To obtain more information about the effects of *bldB*, I chose to examine what effects the overexpression of the *bldB* gene would have on gene expression in *S. coelicolor*. In order to examine these effects, I isolated RNA from both the wild type M145 strain and the same strain containing the high copy plasmid pIJ486BB, which contains the *bldB* gene and its natural promoter, effectively increasing the number of copies of the *bldB* gene. As in the comparison between the wild type and *bldB* null strain,

it was crucial to examine the gene expression profile of the wild type and *bldB* overexpression strains at various time points during growth. To this end, the two strains were grown in parallel liquid cultures, and equivalent volumes of cells were taken from each at 24, 48, 72, and 96 hours, and genomic RNA was isolated.

Due to its sensitivity to degradation by RNase enzymes, RNA is a highly unstable molecule and the isolation process often results in severe damage to the sample. However, successful protocols have been developed by Kieser et al to ensure the isolation of pure and intact RNA from *Streptomyces* (55). RNA stability was confirmed by running samples on 1.2% agarose gels containing formaldehyde. RNA samples purified from the *bldB* null strain in liquid media are shown in figure 1. Once pure RNA had been isolated, the intact genomic RNA samples were sent to David Weaver at Stanford University. The RNA harvested at various time points was used to prepare fluorescent-labeled cDNA for microarray analyses. In both of the experiments comparing either wild type M145 to the *bldB* null or M145 to the *bldB* overexpression strain, the earliest available wild type M145 sample was chosen to represent a time=0 starting point for comparison of expression to all other time points and samples. This cDNA was labeled with the green fluorescent dye Cy3, while cDNA samples of all the other time points in all of the strains were labeled with the red fluorescent dye Cy5. The Cy3-dCTP green fluorescence-labeled cDNA from M145 at the earliest time point was hybridized on *S. coelicolor* DNA microarrays with Cy5-dCTP red fluorescence labeled cDNA from the *bldB* null and overexpression strains at varying time points and from the wild type M145 at the same time points. Based on this labeling scheme, any gene that is up-regulated when compared

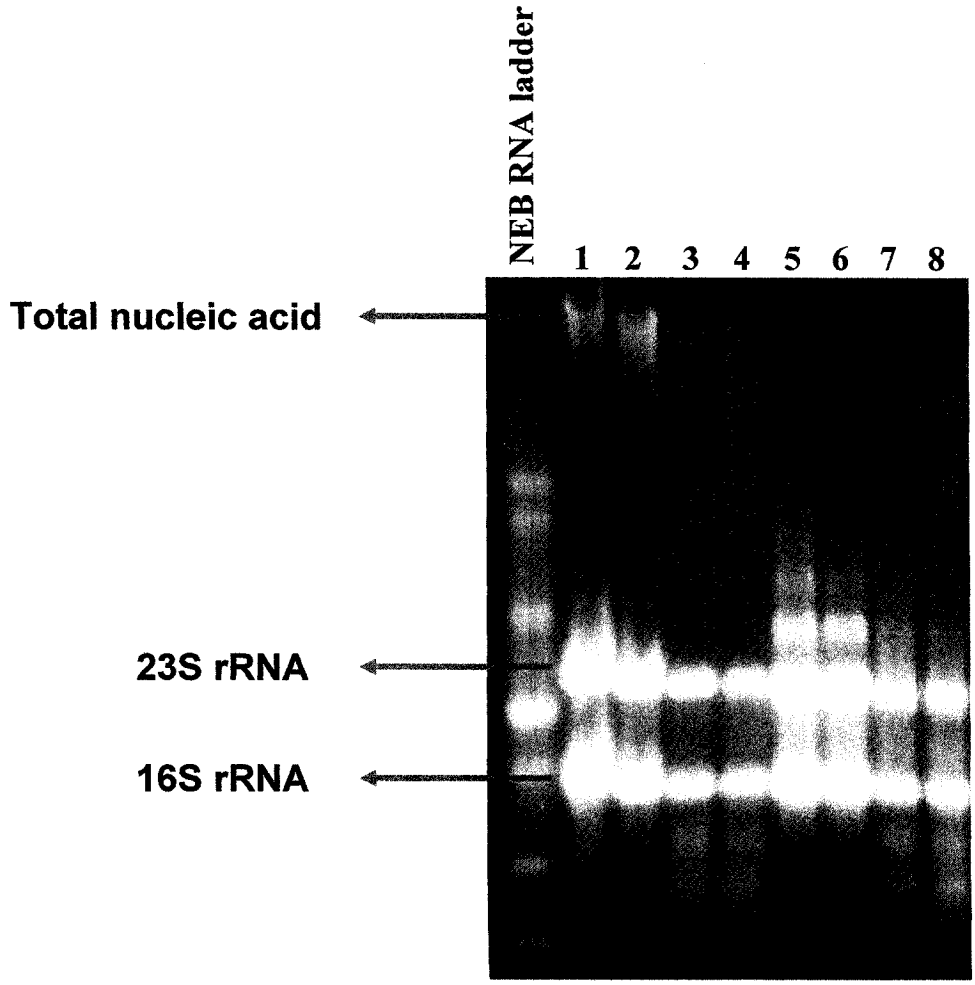


Figure 1. Total nucleic acid and RNA samples isolated from the *bldB* null strain and run on 1.2% agarose gel with formaldehyde. Lanes 1-4 represent the total nucleic acid isolated from *bldB*⁻ at 18, 24, 36 and 48 hours. Lanes 5-8 represent the same samples after treatment with DNase and purification using the QIAGEN RNeasy kit.

repressed would appear green, and the level of fluorescence quantitatively represents the amount of hybridized cDNA.

Comparison of wild type *S. coelicolor* vs. *bldB*⁻ null

Labeled mRNA transcripts in the *bldB* null strain were compared to the copy

numbers in the wild type M145. It appeared that many genes in the null mutant demonstrate different expression than in the wild type strain, however several genes and gene clusters in particular appear to be affected by the absence of the *bldB* gene. Most of the known genes in the antibiotic synthesis pathways for actinorhodin, undecylprodigiosin, and CDA were up-regulated after 72 hours of growth in the wild type M145 strain, as shown in figure 2. In the *bldB* null strain however, this up-regulation was largely eliminated. Figure 2 contains the DNA microarray fluorescence readout that demonstrates that even at 108 hours, long after the wild type strain has activated the biosynthetic genes, the *bldB* null strain still lacks this activation. Overall, the genes that

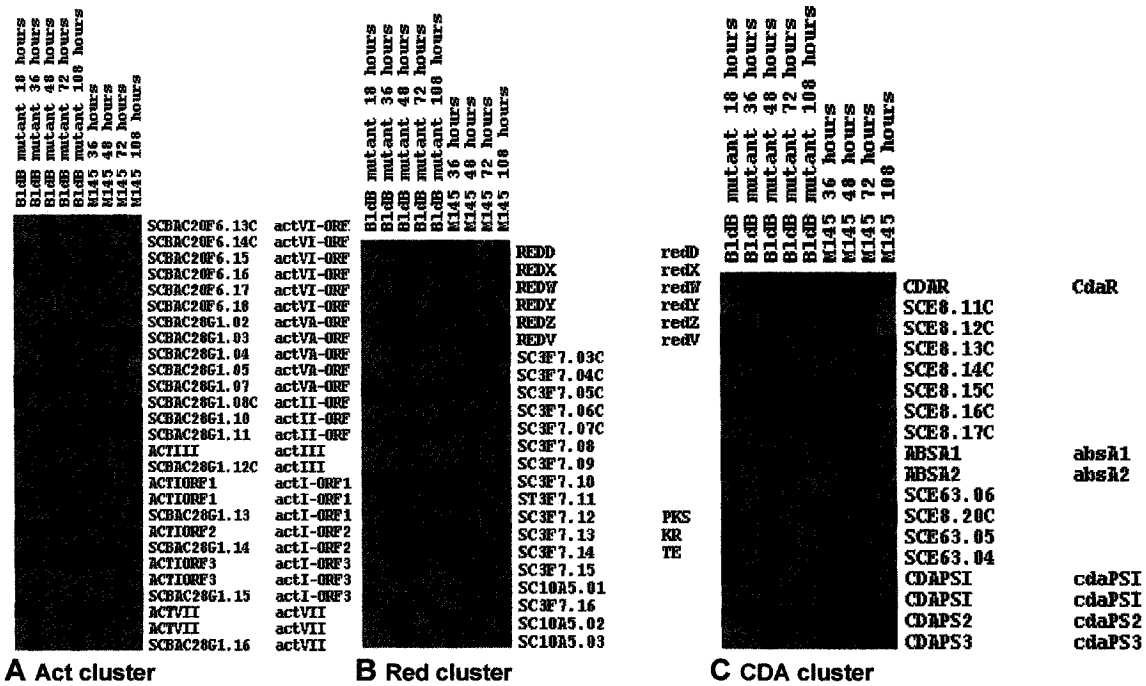


Figure 2. M145 vs. *bldB* null microarray comparison of antibiotic cluster activation. Red tiles indicate transcript levels higher than that of M145 at 18 hours, while green tiles represent transcript levels that are lower than M145 at 18 hours. In each of **A**, **B** and **C**, antibiotic synthesis genes are up-regulated (red) starting at 72 hours in the M145 strain, but not in the in the *bldB* mutant strain

are responsible for synthesis of all of the genome-encoded antibiotics were found to be repressed or simply not activated in the *bldB* null strain when compared to the wild type.

Interestingly, the antibiotic synthesis clusters were not the only gene clusters affected by the absence of *bldB*. Indeed, figure 3A illustrates that another putative cluster containing polyketide synthases (typical of biosynthetic pathways) and neighbouring

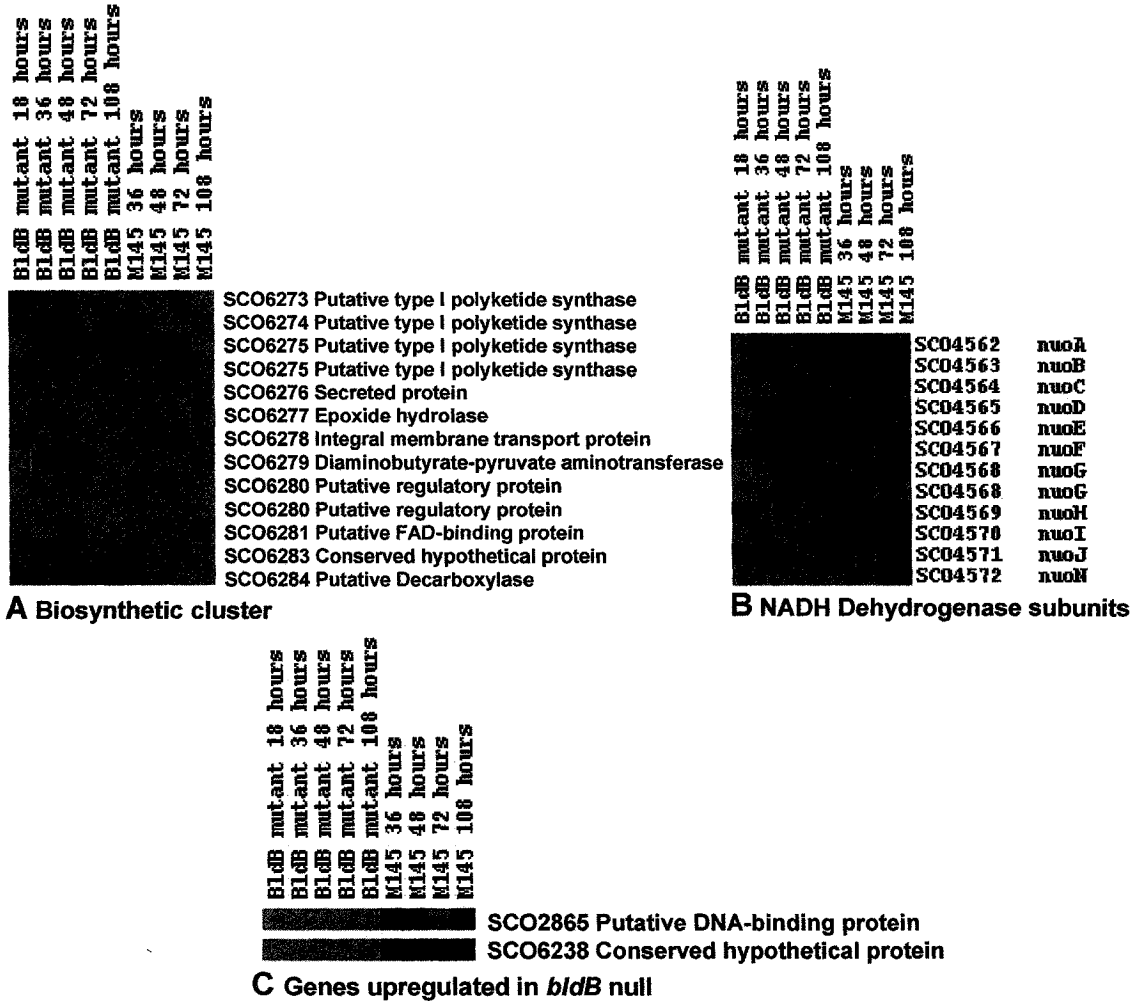


Figure 3. Down-regulation of gene clusters not associated with antibiotic synthesis in the *bldB* null strain. A) The genes that are activated (red) in the M145 strain as early as 36 hours encode proteins typical in biosynthetic clusters. This activation does not occur in the *bldB* null strain. B) The *nuo* genes which encode the various subunits of NADH dehydrogenase also appear to be activated in M145 but not in the *bldB* null strain. C) Two genes are actually up-regulated in the *bldB* null strain, one is a putative regulator.

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synthetic genes appears to be either repressed, or at least lacks activation in the *bldB* null strain. The *S. coelicolor* sequencing project carried out by Bentley et al. has demonstrated that *S. coelicolor* may encode more than 20 separate secondary metabolites but not all of them have been characterized (5). This polyketide synthase cluster may well be required for synthesis of an as-yet unidentified secondary metabolite which is also under the control of the BldB protein.

Further analysis of the microarray data indicated the *nuo* genes, which encode a set of NADH dehydrogenase subunits, appear to be activated at the later time points in the wild type M145 strain, predominantly at 108 hours. However, as seen in figure 3B, these genes are not activated in the *bldB* null strain. All of the data so far suggest that the *bldB* gene exerts positive regulation of several clusters of genes, and in its absence, these genes are not activated as they are in wild type cells. The nature of this positive regulation remains unknown at this time.

Surprisingly, despite the positive regulation exhibited on the antibiotic and other putative synthetic pathways, the microarray comparison strongly implicated the *bldB* gene in the negative regulation of some specific targets. In particular there are two genes that appeared to be strongly activated in the absence of the *bldB* gene, while the expression levels of these genes in the wild type strain did not appear to change over time. The two genes that were overexpressed in the *bldB* null strain encode proteins that are currently not identified, but the intensity of the difference in the microarray data

suggests that these may be targets of BldB regulation, as the majority of genes in the analysis are not activated to this extent. The fluorescence microarray data in figure 3C indicates the high level of activation of these genes in the absence of the *bldB* gene. The Sanger database-labeled SCO2865 gene encodes a putative DNA-binding protein, with a well-characterized DNA-binding motif from the xenobiotic response element (XRE) family of transcriptional regulators. The second gene, SCO6238, has no motifs common to any known protein and its function can not be inferred by comparison to proteins in the known databases. This gene is however similar to various hypothetical proteins, in particular to various proteins in *Mycobacterium tuberculosis*. This data is the first genetic evidence that suggests that *bldB* may act as a negative regulator of gene expression.

Comparison of wild type *S. coelicolor* vs. BldB overexpression

The microarray data comparing gene expression in the *bldB* null strain and M145 indicated that the *bldB* gene may exert both positive and negative regulation of genes. In order to gain more information regarding direct effects that the BldB protein has on gene expression, I elected to assay the gene expression profile of a strain that contains multiple copies of the *bldB* gene in comparison with the same strain (M145) that contains only a single copy of the gene. To this end, parallel YEME cultures were inoculated with each strain and 25ml samples were removed at 24, 36, 48, 72 and 96 hours. Unfortunately, during the fluorescence labeling experiment, the 96 hour time point proved to be unusable in the *bldB* overexpression strain, but all other time points were examined.

The data obtained from these experiments yielded surprising information

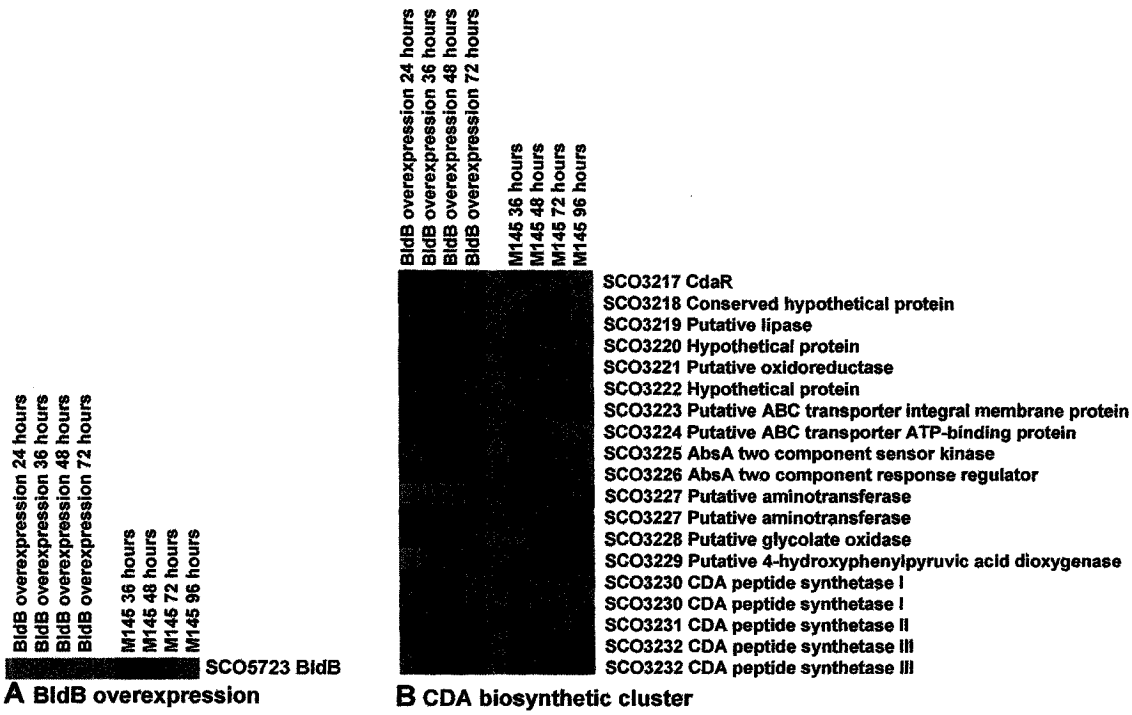

Figure 4. BldB overexpression microarray analysis. A) The red colour indicates that expression of the *bldB* gene is highly activated in relation to the same gene in M145 alone. B) Surprisingly, despite BldB overexpression, there appears to be little to no effect on the activation of antibiotic synthesis, represent by the genes in the CDA cluster here.

overexpression strain when compared to M145. Surprisingly, despite the fact that deletion of the *bldB* gene resulted in repression of the antibiotic synthesis genes, when *bldB* was overexpressed, no significant effects were observed on the antibiotic synthesis clusters. It is evident in figure 4B that genes in the CDA biosynthetic cluster were neither up-regulated nor down-regulated in comparison to wild type, suggesting that the BldB regulation of antibiotic synthesis may be indirect, and that the transcript levels of the *bldB* gene are not significant in this regulation.

The microarray data from this experiment also highlighted many genes that have not been previously associated with *bldB* function. For instance, the genes encoding

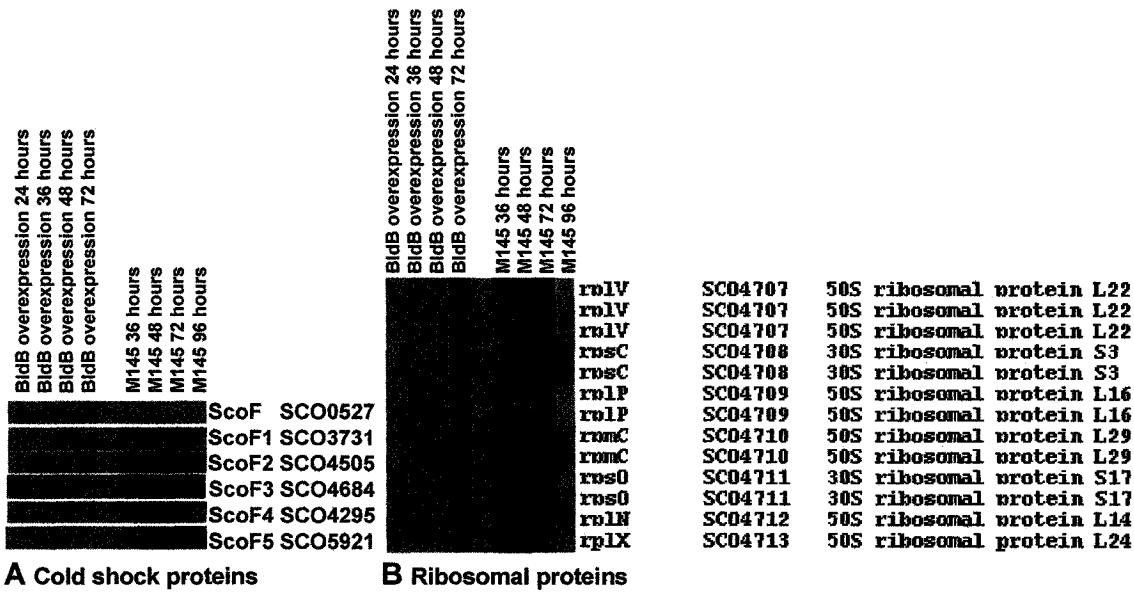

Figure 5. Genes that are up-regulated when BldB is overexpressed. A) Many of the genes that are known to be involved in the *S. coelicolor* cold shock response demonstrate activation in the presence of BldB overexpression relative to the wild type M145. B) A small subset of the genes encoding ribosomal proteins that are strongly activated when BldB is overexpressed. There are at least 36 additional genes involved in protein translation and folding that are up-regulated in a similar manner due to BldB overexpression.

known cold shock proteins (listed in figure 5A) in *S. coelicolor* were all up-regulated when the *bldB* gene was overexpressed, suggesting that BldB exerts positive regulation on these particular genes. The data in figure 5B demonstrates that in addition to the cold shock protein genes, *bldB* overexpression also up-regulated the transcription of a large number of ribosomal proteins that are adjacent to each other on the chromosome. The group represented in figure 5B is only a small subset of the more than 40 genes that were significantly up-regulated in the *bldB* overexpression strain. These up-regulated clusters also contain other genes involved in gene expression, including *rpoA*, and translation factors such as the initiation factor 1 (*infA*), and the elongation factors G and TU-1 (*fusA*

and *tuf1* respectively). The significance of these findings is yet to be determined, as further analysis of the effects of these target genes is required to understand their function in the development of *S. coelicolor*.

The first microarray experiment indicated that BldB may act as a negative regulator of gene expression in some cases. The overexpression microarray experiment also revealed possible negative regulation when BldB was in excess. The mRNA transcript levels of several genes encoding secreted proteins, listed in figure 6, were significantly down-regulated in the *bldB* overexpression strain in comparison to those in M145. Two of the genes in particular (*chpE* and *chpH*) encode two of the chaplin proteins, which have been identified previously to be crucial for aerial hyphae formation

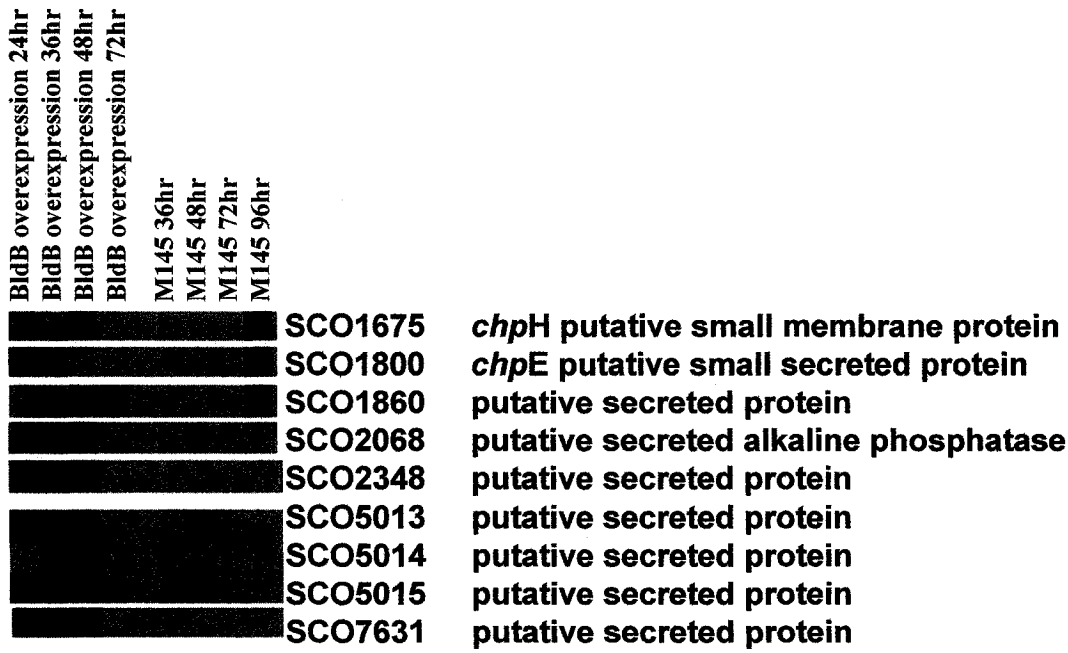

Figure 6. Several genes encoding small secreted proteins are down-regulated when BldB is overexpressed. The *chp* genes are essential for aerial hyphae formation (26). BldB overexpression represses all of these genes, suggesting that it acts as a negative regulator of their expression.

overexpression represses all of these genes, suggesting that it acts as a negative regulator of their expression.

(18, 26). Despite the fact that genomic RNA was isolated from non-differentiating *S. coelicolor*, the down-regulation of these genes relative to their expression in M145 may have been due to the vast excess of BldB in this strain, suggesting BldB may repress the expression of these genes. Many of the putative secreted proteins that are down-regulated when BldB is overexpressed have not been characterized, so it is not currently possible to determine how the control of these genes contributes to the function of *bldB*.

Discussion

A complex regulation programme governs gene expression during the development of *Streptomyces*. The goal of the microarray experiments is to determine what role the *bldB* gene plays in this complex programme by observing the effects of *bldB* gene deletion and overexpression on gene regulation in *S. coelicolor*. Because the genomic RNA was isolated from strains grown in non-differentiating media, many of the genes required specifically for differentiation were not expressed, but all of the genes required for primary and secondary metabolism and *S. coelicolor* development were expressed normally. Previous work has implicated the *bldB* gene in secondary metabolism, so any genes involved in secondary metabolism that are under the control of BldB regulation would have been identified in these experiments.

Antibiotic production in *Streptomyces* species generally involves the expression of physically clustered biosynthetic genes on the chromosome, often containing the genes

necessary for regulation of the cluster embedded within it (52). It has previously been suggested that "higher level" pleiotropic regulators activate the "pathway specific" regulators located within chromosomal gene clusters that encode biosynthetic machinery for antibiotic production (46). Some examples of such proposed "higher level" regulators include the *absA1/2* locus, the *afsR2/afsS* locus, and the *abaA* gene, all of which regulate the expression of multiple antibiotics. Based on the fact that multiple antibiotic synthesis clusters are not activated in the *bldB* null strain, it would appear as though BldB could be classified as a "higher level" or pleiotropic regulator of antibiotic synthesis. It remains to be determined whether the *bldB* gene regulates the expression of the pathway-specific activators for each biosynthetic cluster. Since expression of the *redD* and *redZ* genes in figure 2 are down-regulated in the *bldB* null strain, this may suggest that BldB regulates undecylprodigiosin synthesis in *S. coelicolor* by activating these pathway-specific regulators. It is possible that BldB binds to the promoter of these genes, but due to the pleiotropic regulation of multiple antibiotics, it is more likely that BldB regulates expression of these genes via interaction with one or more regulatory proteins.

Completion of the genome sequence of *S. coelicolor* led to the prediction of at least twenty different biosynthetic pathways for secondary metabolites. These include not only antibiotics but also different pigments, siderophores, signaling molecules and complex lipids (5). Interestingly, as depicted in figure 3, it appears that at least one other secondary metabolite biosynthetic pathway is also potentially under the control of the *bldB* gene, as transcript levels from this cluster containing several polyketide synthases and other typical biosynthetic genes are much lower in the *bldB* null strain than in M145.

Unfortunately, because this cluster has not been extensively characterized, further study is required in order to determine exactly what this pathway is dedicated to synthesizing, and how these genes are related to the function of *bldB*. Regulation of this cluster is most likely similar to the regulation of the antibiotic synthesis clusters, probably involving the interaction between BldB and a protein that specifically regulates this cluster.

The down-regulation in the *bldB* null strain of the *nuo* genes, which encode various NADH dehydrogenase subunits, raises similar questions about *bldB* regulation. The NADH dehydrogenase enzyme is the first complex in the mitochondrial electron transport chain, and is therefore vital to primary metabolism in the cell. NADH dehydrogenase reduces the coenzyme Q (CoQ) in the following reaction:

$$2NADH + CoQ + 2\ H^+ \rightarrow 2\ NAD^+ + CoQH_2$$

This reaction results in the translocation of protons across the inner mitochondrial membrane, building the electrochemical potential across the membrane that is required for the synthesis of ATP. In the wild type M145, these genes are activated later in development, coinciding with the induction of the antibiotic synthesis genes. The down-regulation of the *nuo* genes in the *bldB* null strain may therefore play a role in the dysfunction of the mutant. It has been demonstrated that morphogenesis is linked to the nutritional state of developing cells (54). When *bldB* is deleted, my data suggest that the *nuo* genes are down-regulated, and primary metabolism is compromised. If metabolism provides a cue to initiate aerial hyphae formation and antibiotic synthesis, disruption of metabolism could result in termination of morphogenesis before these processes commence. Further insight into the regulation of the *nuo* genes could yield significant

information regarding the role of *bldB*.

Based on the phenotype of the *bldB* null strain, it was not surprising to note a marked decrease in activation of the antibiotic synthesis genes, but some of the genes that show differential expression in the *bldB* null strain were previously not associated with BldB function. The two genes in figure 3C that appear to be up-regulated in the absence of the *bldB* gene imply that it must act as a negative regulator, either directly or indirectly. The dramatic overexpression of SCO2865 and SCO6238 in the *bldB* null strain relative to M145 suggests that their expression in M145 is actively repressed when *bldB* is present, but once deleted, these genes are highly and constitutively activated at all time points during logarithmic growth and stationary phase. Mutants in the *bldB* gene are known to be extremely pleiotropic, implying that the *bldB* gene either regulates multiple genes or it regulates a step early in morphogenesis that if blocked, causes multiple downstream effects, resulting in the pleiotropic phenotype. Due to the fact that both the SCO2865 and SCO6238 genes encode putative regulatory proteins, this suggests the possibility that BldB may exert regulation over several pathways involved in morphogenetic differentiation and secondary metabolism by controlling the expression of various different regulatory proteins within the cell that are required for distinct but specific functions during development. In this way, BldB could act as a primary regulator of multiple developmental pathways, and mutations in this protein would have numerous consequences, such as the ones observed in all of the *bldB* mutants.

It was not surprising that the deletion of the *bldB* gene resulted in down-regulation of the antibiotic synthesis genes because of the lack of antibiotic production in

bldB mutants. However, it was surprising to note that the overexpression of the *bldB* gene had little to no effect on the expression of the biosynthetic genes in the microarray comparison to M145, as demonstrated in figure 4B. Since there is already a copy of the *bldB* gene in M145, it is likely that excess copies of *bldB* do not exert an extra degree of regulation, suggesting that the regulation of antibiotic synthesis by BldB is not stoichiometric, and that transcript levels of the *bldB* gene are inconsequential in their activation.

There were many genes in the *bldB* overexpression microarray analysis whose up-regulation was surprising however. In figure 5A, it is evident that genes involved in the cold shock response of *S. coelicolor* are up-regulated in the presence of excess *bldB*. In fact, the genes in this figure encompass all of the characterized *S. coelicolor* genes known to be involved in this process. Though some of the genes appear to be more highly activated than others, there is a noticeable trend that suggests activation of the cold shock response. Based on all that is currently known about BldB, there is no information that implicates this developmental regulator in stress response so the significance of these findings is not immediately apparent. Equally surprising is the large number of genes encoding ribosomal and translational proteins that are also up-regulated in the the *bldB* overexpression strain. More specifically, at least 43 genes involved in protein translation appear to be strongly activated according to this microarray analysis. The *rps* and *rpl* genes make up the majority of the genes affected by *bldB* overexpression, and these encode the large and small subunit ribosomal proteins required for efficient global protein translation in *S. coelicolor*. As with the cold shock proteins, this response to excess BldB

is a novel discovery, and its significance is yet to be determined. However, if *bldB* is indeed involved in the regulation of global protein translation machinery, this would provide a logical explanation for how mutations in a single gene, *bldB*, cause the pleiotropic phenotype observed.

Previous studies in *S. coelicolor* have shown that differentiation is highly dependent on the production and export of a series of extracellular signals (75, 108). More recent studies have implicated a class of genes known as the chaplin genes (*chp*) that all contain secretion signals, and share a common hydrophobic "chaplin" domain (18, 26). These genes encode proteins that are exported and targeted to the cell surface, where they are required for aerial hyphae formation. Interestingly, several genes encoding putative secreted proteins, and two of the chaplin genes (*chpE* and *chpH*) appear to be repressed when the *bldB* gene is overexpressed, as seen in figure 6. When *bldB* is overexpressed in M145, it causes a delay in aerial hyphae formation, and blocks the developmental process before sporulation via an unknown mechanism. The negative regulation of the genes encoding putative secreted proteins and chaplin genes may suggest a mechanism by which this phenotype arises. If these secreted proteins are required for differentiation, and they are normally repressed by a controlled level of BldB in the cell, the overexpression of BldB could exert excessive negative regulation on these crucial signaling molecules, resulting in expression levels too low to carry out morphogenetic differentiation. Further analysis of the specific function of these signaling molecules will help to discern how regulation of their expression is involved in *bldB* function and differentiation.

The method of regulation exerted by the BldB protein on all of these genes is currently unknown. It is possible that BldB is a transcriptional regulator that directly binds to and regulates the target promoters. However, there is very little evidence to support this possibility, and experiments in our lab have not been able to demonstrate BldB DNA-binding. Typically, transcription factors that regulate multiple genes bind to consensus target sequences to carry out regulation. Analysis of the sequences upstream of various genes whose expression is strongly affected by the deletion of *bldB* in the microarray data does not identify a common consensus sequence for BldB binding. This suggests that the regulation of gene expression in *S. coelicolor* by BldB is more likely indirect. The BldB protein is more likely to act as a check point in development, where the cellular activity blockage of the null mutant is manifested at the level of gene expression. Further examination of BldB function is needed to discern whether BldB acts directly as a transcription factor or indirectly by interacting with one or more protein targets.

Materials and methods

RNA isolation

In order to perform DNA microarray hybridization experiments that compare gene expression between two strains, it is important to maintain similar growth conditions for both strains being examined. Therefore, the strains whose gene expression patterns were to be compared were grown in parallel liquid cultures, and samples were isolated at precise time points. All strains were grown in 500ml of YEME media and

25ml samples were harvested for each time point. RNA was isolated from *S. coelicolor* using the modified Kirby mix protocol, with several modifications (55). 70% ethanol, prepared from RNase-free (DEPC-treated) water and 95% ethanol, was used throughout the protocol. The mycelium were immediately vortexed with 5g of glass beads and 5ml of modified Kirby mix, which contained N-lauroylsarcosine instead of sodium-triisopropylnaphthalene sulphonate (TPNS) (D. Weaver, personal communication). The organic extractions and initial precipitations were performed as described previously (55). After the second RNA precipitation step, the pellet was resuspended in 200–300 µl of RNase-free water. To digest the DNA, I used the commercial RNA purification kit available from QIAGEN, which combines a DNase enzyme with RNA binding columns for high level purification. The RNA was then precipitated as described and resuspended in 100 µl of RNase-free water (55). The RNA concentration was measured using spectrophotometry, and the sample was stored as an isopropanol precipitate. For microarray analysis, the appropriate volume was taken from the isopropanol stock and centrifuged for 15 min. The pellet was washed twice with 70% ethanol, air dried and resuspended in water to the appropriate concentration (104). RNA stability was assessed by 0.67% formaldehyde and 1.2% agarose gel electrophoresis.

Microarray hybridizations

All of this work was done by David Weaver at Stanford university, and the methods used have been previously described (104). DNA microarrays were fabricated as described previously (45). Some 97% of the predicted genes in *S. coelicolor* were

represented on the microarrays. For each microarray experiment, two different nucleic acid samples were labeled with Cy3-dCTP or Cy5-dCTP, respectively, and hybridized to a single microarray.

15 μg of total RNA and 5 μg of random hexamers (72% GC composition) were mixed with water to obtain a volume of 7 μl. The mixture was heated at 75°C for 15 min and cooled immediately on ice. The following components were added to a final volume of 15 μl, at the given final concentrations: Cy3- or Cy5-dCTP (1.7 mM; Amersham Pharmacia Biotech); dATP, dTTP (400 μM each); dGTP (1 mM); dCTP (20 μM); SuperScript II buffer (1×); dithiothreitol (10 mM); and SuperScript II (400 U; Gibco BRL). The reaction was incubated at room temperature for 10 min, and then 42°C for 2.5 h. A sample of 500 μl of TE (10 mM Tris, pH 7.4, 1 mM EDTA, pH 8.0) was added to the reaction, and the mixture was concentrated in a microcon-10 filter (Amicon) to a final volume of ≈6 μl. (This wash step was repeated for RNA labelings.) For each microarray experiment, the Cy3 and Cy5 samples were mixed together. SSC (final concentrations 0.6 M NaCl, 0.06 M trisodium citrate dehydrate), SDS (0.2% final concentration) and polyA (10 μg) were added. The hybridization mixture was heated at 100°C for 2 min and applied to a microarray. The microarray was incubated at 65°C for 10–12 h and then scanned with a GenePix 4000B (Axon Instruments). Microarray data were visualized using the program treeview (24).

Chapter 5

Determining the function of BldB—future research into putative interactions and protein structure.

Putative BldB interactions and isolation of targets

When very little information is available regarding how a protein functions, the determination of that function can be facilitated by establishing whether or not that protein interacts with other proteins in the cell. If it is determined that other proteins are involved, it is crucial to establish what functions are attributed to the target proteins. The data collected so far suggests that *bldB* likely functions by regulating a large number of genes involved in different processes. Due to the small size of BldB (98 amino acids), it is unlikely that BldB possesses enough distinct domains to regulate all of these processes directly without recruiting the aid of other proteins. The work in chapters 2 and 3 argue that BldB interacts with another protein because though the protein forms a stable dimer *in vivo*, this activity is insufficient to complement the phenotype of the null mutant. Because the F75A and Y21A mutations maintain the ability to fold and dimerize, but are unable to complement the *bldB* null strain, this suggests that these residues could be surface associated and likely constitute part of an ineraction interface. I have also demonstrated that the deletion of *bldB* results in the activation of a putative regulatory protein using DNA microarray analysis. It is likely that BldB exerts its regulation of multiple differentiation pathways by regulating the expression various proteins that act as regulators of more specific pathways, or via interactions with such proteins. In this chapter I will discuss some of the methods that can be employed to try to discern what interactions facilitate the function of BldB.

Continuation of microarray analyses

To gain more information about the function of BldB in morphogenesis, it is important to determine not only what proteins interact directly with BldB, but also what genes may be regulated by BldB either directly or through interaction with a protein target. DNA microarrays have proven to be a valuable tool in observing global gene expression, and can be used to determine changes in gene expression based on changes in *bldB* levels in the cell. The microarray experiments carried out thus far have compared the gene expression profiles of the wild type M145 strain to both a *bldB* null strain, and the M145 strain overexpressing the *bldB* gene. In order to both confirm genes that were affected in the previous experiments, and to isolate other putative gene targets for BldB, the next microarray experiments should examine the effects of artificial induction of *bldB* expression on transcript levels in M145. The construction of a plasmid containing the *bldB* gene under the control of the *tipA* promoter (pIJ6902BldB—data not shown) has made this analysis possible. By comparing gene expression both before and after a brief induction, it may be easier to isolate genes that are specifically and rapidly altered by the upregulation of the BldB protein, identifying targets that respond to changing BldB levels in the cell.

It is important to note that all of the microarray experiments conducted to this point have been carried out using samples isolated from non-differentiating *S. coelicolor* for ease of RNA isolation. Due to this fact, the microarray results reported so far are focused on antibiotic synthesis and metabolism. In order to learn more about the role of BldB in regulating morphogenesis, it is important to repeat the microarray experiments

using RNA isolated from *S. coelicolor* grown on solid (R2YE) media, as it differentiates fully under these conditions. Until these experiments are performed, it will be difficult to determine what morphogenetic genes may be regulated by BldB. Though these experiments individually often yield a large number of potential targets, the combination of the data from the various experiments can help to narrow the list of potential targets and provide a basis for further research. Once potential targets have been identified, further biochemical and genetic experiments can be planned to determine precisely how they interact with BldB to help it carry out its function.

BldB homologues AbaA and WhiJ

The two BldB homologues AbaA and WhiJ are fairly well characterized, and are known to be involved in antibiotic synthesis and sporulation respectively. At this time, their method of action is also unknown. Many of the BldB homologues are located on the chromosome adjacent to two very specific types of genes, in various orientations. These pseudo-clusters most often consist of a small acidic BldB-like protein, a DNA-binding domain-containing protein, and a protein containing a histidine kinase-like ATPase. The fact that a majority of the isolated homologues exist in these clusters suggests that their proximity is more than a coincidence and may have some bearing on the function of these proteins. The *abaA* and *whiJ* genes are located in such clusters, but BldB is not. Despite the fact that *bldB* is not adjacent to either of these genes, there are numerous unpaired genes of this nature throughout the chromosome that may be coupled to BldB or other homologues that are also distant. By knocking out the genes in the *abaA* and *whiJ*

clusters, we hope to determine the relationship between the BldB-like proteins and these associated genes. Further experiments can be applied to determine the purpose of these genes once it has been established that there is interplay between the BldB-like genes and their associated (adjacent) cluster genes.

Since very little is known about the BldB family of proteins, one way to learn more about AbaA and WhiJ is to repeat the microarray experiments using these two homologues. Since both homologues are involved in similar processes to BldB, there may be some overlap in the changes in gene expression when they are deleted, but there will also be different genes affected that may be more specific to the function of each of the homologues. The results from these experiments may simply demonstrate that these genes regulate entirely different sets of genes, but they may also identify similarities that could lead to significant insight into how this family of proteins affect gene expression.

Future characterization of interactions: TAP tag

As previously stated, BldB likely exerts regulation of multiple pathways during development so it is unlikely that it does so by interacting with a single protein. To learn more about what proteins or complexes that BldB interacts with, the Tandem Affinity Purification (TAP) tag method created by Rigaut et al is an ideal procedure to identify such targets (85). The TAP tag system allows the purification of proteins expressed at their natural level under native conditions. Therefore, any proteins isolated that copurify with BldB are binding to the same amount of BldB that is usually present in developing *S. coelicolor*, and they will bind in natural stoichiometric levels. The target protein (BldB) is fused to the high affinity TAP tag, which contains two distinct purification

domains that isolate proteins in very mild, non-denaturing conditions that allow bound proteins to remain bound during purification. The TAP tag is fused to the target protein under the control of the target protein's natural promoter so as not to cause false interactions due to overexpression from a synthetic promoter. The TAP tag contains a calmodulin binding peptide (CBP), a TEV protease cleavage site, and a protein A component that allow for high-affinity purification (85). Cell extracts are first bound to IgG beads, to bind the protein A component of the tag, and all non-binding proteins are washed away. The target protein and remaining bound proteins are then eluted by cleavage with TEV protease, leaving the target protein fused to the CBP domain. Passage over a calmodulin affinity column further washes away any remaining non-target-specific proteins. After elution from the calmodulin beads with EGTA, the resulting proteins can be analyzed via SDS-PAGE, and proteins that co-purify with the target protein can be isolated from the gel and analyzed by mass spectrometry to determine their identity. This method can identify any proteins that interact specifically with BldB and may yield significant information about how BldB is able to regulate multiple processes during development. Because there are no proteins of similar function in the common databases, the isolation of a protein of known function from *S. coelicolor* extracts that binds to BldB would greatly aid in the understanding of how BldB functions to regulate development.

Continuing analysis of BldB function—future directions

The work in this thesis has revealed information about the structure of BldB and uncovered relationships between the structure and function of this novel protein. It is now

known that BldB forms a dimer, that the central region of the coding sequence is essential to this dimerization, and that deletion of certain amino acids abolishes dimerization, and therefore function of BldB. The Y21 and F75 residues have been implicated in the function of BldB, but not in its dimerization. I have observed that overexpression of BldB causes a block in morphogenesis prior to sporulation, suggesting negative regulation of morphogenesis. However, there is still much to be discovered regarding the function of BldB. In particular, the role of the amino acids that have been identified as crucial to BldB function is not known. The substitution of alanine for either the Y21 or F75 residues could result in any number of structural changes in the BldB protein, or they may specifically alter a protein-protein interaction interface on the surface of the protein. To discern the importance of these amino acids, and to learn more in general about the structure of BldB, the determination of the three-dimensional crystal structure of BldB would prove vital. By analyzing the structure of BldB, putative interaction surfaces and key functional residues can be located and identified, and the dimerization surface can be properly mapped. Pope et al suggested that BldB may be a DNA-binding transcription factor, as a structural prediction program they used identified a typical helix-turn-helix DNA binding motif in BldB (82). My data does not support this, but the determination of a crystal structure will facilitate comparison of that structure with other proteins in common databases and may allow the extrapolation of a protein function based on structural homology with other proteins of known function. Because it is still not known whether BldB is a DNA-binding protein or is involved in various protein-protein interactions, a structure would facilitate the determination of what kind of protein BldB is

likely to be.

In addition to structural information, it remains crucial to identify targets of the BldB protein, whether they are other proteins or DNA sequences. If the BldB protein does not interact with DNA elements, then it must function by binding to at least one other protein. The most ideal method for isolating target proteins is the TAP-tag method, which facilitates the purification of BldB from cellular extracts bound to any of its target proteins or complexes.

In summary, though I have uncovered significant information about the structure and function of BldB, there is still much that remains unclear. The information gathered to date has helped to establish what should be undertaken in future research into this novel regulator, and the other members of the BldB family of proteins in *S. coelicolor*.

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