THE BldB HOMOLOGUES OF *STREPTOMYCES COELICOLOR*

INVESTIGATION OF THE BLDB HOMOLOGUES OF *STREPTOMYCES COELICOLOR*: REGULATORS OF DEVELOPMENT AND ANTIBIOTIC PRODUCTION

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Abstract

The *Streptomyces* are invaluable as a natural source of antibiotics and other bioactive compounds used in medicine and agriculture. *S. coelicolor* is the model streptomycete, and is studied for its complex secondary metabolism and multicellular life cycle. The subject of this work is *bldB*, a gene essential for development and antibiotic production in *S. coelicolor*, and one of its many homologues, located in the *abaA* antibiotic regulatory locus. The aim was to study the transcriptional regulation of *bldB* using a luminescent reporter, and investigate the role of each of the genes in the *abaA* cluster in regulation of antibiotic production, in order to understand the function and mechanism of action of *bldB* and its homologues. Individual deletion of each of the four genes in the *abaA* cluster resulted in varying effects on production of the antibiotic CDA. The *bldB* homologue, *SCO0703*, was shown to be a positive regulator of CDA, as the null mutant was severely defective in CDA production. It was found that *bldB* is expressed in most other *bld* developmental mutants, with the exception of *bldD*. There was no direct interaction observed between BldD and the *bldB* promoter, and possible mechanisms of indirect regulation are proposed.

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1 Introduction

1.1 *Streptomyces* in the environment and medicine

The complex life cycle and growth habits of the streptomycetes defy many bacterial paradigms. They are not found as individual cells that reproduce by binary fission, but live in complex multicellular communities consisting of several differentiated cell types, and reproduce by an elaborate life cycle similar to filamentous fungi. Furthermore, they produce and excrete a vast array of secondary metabolites, making them a significant natural source for useful bioactive compounds such as antibiotics, chemotherapeutics, antifungals and herbicides (10).

Streptomycetes are ubiquitous in soil and are well adapted to growth in this competitive environment. Their spores are resistant to desiccation and germinate in response to favourable conditions (16). Early growth is characterized by the formation of a dense, filamentous network of cells known as the substrate mycelium. Interestingly, these cells grow with only infrequent septation to form multigenomic, highly branched hyphae. The growing colonies respond to depletion of nutrients and environmental stress by several concerted events culminating in sporulation, which ensures survival until favourable conditions arise. At the onset of differentiation, the substrate hyphae begin to produce numerous and varied secondary metabolites, including many clinically and agriculturally useful bioactive compounds. At the same time, a second cell type, the aerial hyphae emerge while the substrate mycelium undergoes programmed cell death. The aerial hyphae are coated by secreted hydrophobic surfactants, allowing their escape from the aqueous substrate into the air. Unlike the substrate hyphae, the aerial hyphae are

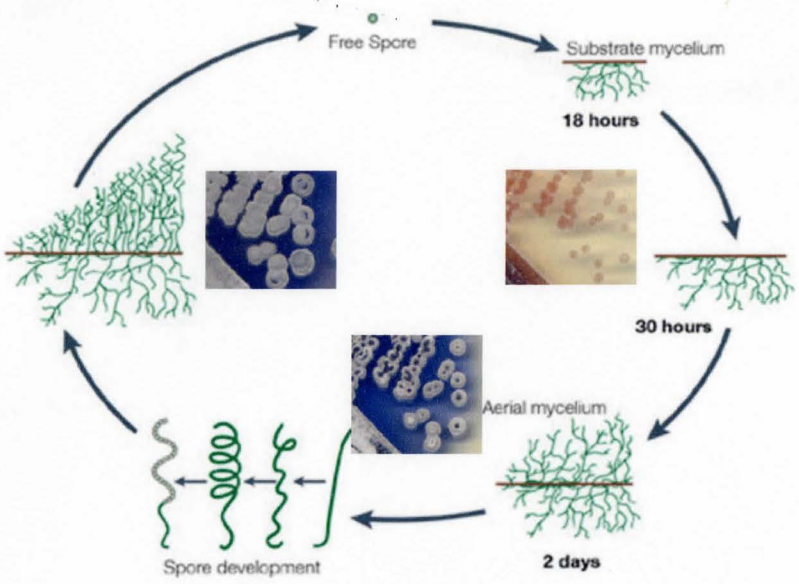

Figure 1 - Life cycle of *S. coelicolor*. Spore germination and growth by branching and tip elongation gives rise to the substrate mycelium. As the colony matures, aerial hyphae grow into the air, imparting a white fuzzy appearance to the colony. This stage in development is marked by the production of the blue and red pigmented secondary metabolites actinorhodin and undecylprodigiosin, respectively. Aerial hyphae coil, septate and mature into unigenomic spores, with accompanying production of a grey spore-associated pigment. Photographs depict *S. coelicolor* M145 grown on rich medium. Line drawings modified from (2).

less highly branched and undergo regular septation and cell division to generate unigenomic compartments which mature into individual spores that disperse into the environment (12).

S. coelicolor is an especially useful model organism for the study of development, as it provides many visual cues during its progression through the stages of its life cycle (Figure 1). Early substrate growth following germination yields smooth and shiny colonies whose surface becomes white and fuzzy as the aerial mycelium develops. The onset of secondary metabolism is visually marked by the production of the blue and red pigmented antibiotics actinorhodin and undecylprodigiosin, respectively. Finally, spore

maturation is accompanied by the deposition of a pigmented polyketide into the spore walls, turning the white aerial mycelium grey (12).

1.2 Discovery, identification and characterization of developmental genes in *S. coelicolor*

The concerted and appropriately timed initiation of morphogenesis requires, not surprisingly, an elaborate system of intercellular communication and the ability to sense and respond to extracellular signals. Loci involved in early stages of development were first discovered by analysis of developmental mutants generated by UV and chemical mutagenesis (36). Some of the resulting mutants were shiny and smooth in appearance, in contrast with the fuzzy wild type strains. These mutants were given the *bld* designation, reflecting their "bald" phenotypes (50). To date, many *bld* genes and loci have been identified, including *bldA*, *bldB*, *bldC*, *bldD*, *bldG*, *bldH*, *bldI*, *bldJ*, *bldK*, *bldL*, *bldM* and *bldN* (20, 27). These genes and their products are discussed in more detail below.

Early in the study of *bld* mutants it was observed that many are also unpigmented, indicating a defect in antibiotic production. This suggests that the onset of secondary metabolism and aerial development do not only coincide temporally, but are also regulated by at least some common pathways. It is believed that the production of antibiotics and other secondary metabolites confers an advantage for streptomycetes by limiting competition by other microorganisms at times of scarce nutrient availability, which also presumably signal the need for sporulation (15).

An interesting property of many *bld* mutants is carbon source-dependent phenotypic variation and defects in regulation of carbon utilization. Aerial development

and, in some cases, antibiotic production can be restored in all *bld* mutants, except *bldB*, by growth on poor carbon sources such as mannitol. Furthermore, it has been shown that *bld* mutants are defective in regulation of carbon source utilization (57). Therefore, it is proposed that the developmental defect of *bld* mutants is due to their inability to sense or respond to environmental signals that indicate starvation.

Additional evidence implicating the *bld* genes in environmental signal transduction is provided by the observation of extracellular complementation, which refers to the ability of certain *bld* mutants to restore normal morphogenesis to certain other *bld* mutants growing in close proximity (54, 67). Extracellular complementation is presumed to occur due to the diffusion of unidentified signaling molecules between cells, and is always unidirectional, meaning that mutants cannot be complemented by the strains that they themselves complement. Therefore, the extracellular complementation pattern takes the form of a hierarchy, and is commonly referred to as the "*bld* cascade." The hierarchy is: *bldJ* < *bldK* < *bldA/H* < *bldG* < *bldC* < *bldD/M* (54). Thus, *bldJ* can be complemented by *bldK* and all other mutants located downstream in the hierarchy, while *bldD* can complement all other mutants but itself cannot be complemented by any. Some *bld* mutations, such as *bldB* and *bldN*, do not fit clearly into the hierarchy. It is noteworthy that the *bld* cascade is applicable only to growth on rich media, and therefore the pathways to morphogenesis must be somewhat different on alternative carbon sources. This aspect of morphological differentiation is discussed in Section 1.3.

Although the *bld* genes are implicated in intercellular and environmental signaling, they encode a diverse range of gene products, most of which have regulatory

roles (20, 27). These include DNA-binding proteins (*bldC*, *bldD*, *bldH*), sigma factors and their regulators (*bldN* and *bldG*), an orphan two-component response regulator (*bldM*) and a tRNA (*bldA*). The sole structural element among the *bld* gene products is an oligopeptide permease encoded by the *bldK* locus. However, additional genes involved in aerial mycelium formation that encode structural components include the *ramCSAB* cluster and *chpA-H*. The *ram* genes are involved in the synthesis and export of a small lantibiotic-like peptide, SapB, which acts as a surfactant allowing emergence of aerial hyphae from the aqueous substrate (29, 68). The *chp* genes encode proteins known as the "chaplins," which similarly contribute to the hydrophobicity of aerial hyphae (29).

1.2.1 *bldA*: Developmental regulation by a tRNA

One of the best-characterized *bld* genes is *bldA*. The tRNA encoded by *bldA* specifies an unusual example of developmental regulation. *bldA* encodes the sole tRNA responsible for efficient translation of the TTA codon for leucine, which is extremely rare in streptomycetes due to the high-GC nature of their genomes. Presence of TTA codons in the biosynthetic regulators for the antibiotics actinorhodin, undecylprodigiosin and methylenomycin, *actII-orf4*, *redZ* and *mmyL/B*, respectively, accounts for the inability of *bldA* mutants to produce antibiotics (15).

The bald phenotype of *bldA* mutants is attributed to a TTA codon in *bldH* (64). BldH is not well characterized in *S. coelicolor*, but the *S. griseus* orthologue, AdpA, is a transcription factor which induces antibiotic production and aerial hyphae formation in response to A-factor, a γ-butyrolactone signaling molecule (10). Interestingly, AdpA also

regulates AdsA, the *S. griseus* orthologue of BldN, although no direct connection has been found between BldH and BldN in *S. coelicolor* (16).

1.2.2 *bldD* and *bldC*: *bld* genes encoding DNA-binding proteins

BldD is one of the better understood *bld* gene products. It is a dimeric DNA-binding transcription factor that negatively regulates the expression of the developmental and stress-induced sigma factor genes *bldN*, *whiG* and *sigH*, as well as itself (26, 41). The BldD orthologue of *S. griseus* also regulates the *amfTSBA* cluster, the equivalent of the *S. coelicolor ramCSAB* cluster involved in SapB production (68). Similarly to the AdsA/BldN and AdpA/BldH connection discussed above, this regulatory link is not direct in *S. coelicolor*.

The crystal structure of BldD was recently solved, and reveals a protein containing an XRE-type helix-turn-helix similar to the SinR sporulation repressor of *Bacillus subtilis* (43). Unexpectedly, both the dimerization and DNA-binding properties of BldD appear to be mediated by the N-terminal domain of the protein, while the C-terminal domain undergoes proteolytic cleavage at the onset of differentiation (47). It has been proposed that the C-terminal domain may also have uncharacterized DNA-binding properties, or may interact with an effector molecule or another protein, and cleavage acts as a switch between repressor and activator functions (26, 47). In comparison, repression of sporulation-activating genes by SinR is overcome by its interaction with SinI (26).

Another putative DNA-binding protein involved in early development is BldC, which is an interesting member of the MerR-like family of DNA-binding transcription factors. While most MerR-like proteins consist of a DNA-binding domain, an effector

recognition domain and a dimerization domain, BldC consists of only the putative DNA-binding domain. Its direct binding target is unknown, but it is indirectly required for expression of the pathway-specific regulators for actinorhodin and undecylprodigiosin synthesis (39). It is not known how this protein blocks morphological differentiation, and it may represent a novel mechanism of action for MerR-like proteins.

1.2.3 *bldB*: A widespread actinomycete-specific gene with unknown function

Mutations in *bldB* are the most pleiotropic among all *bld* mutations; these mutants are unable to produce antibiotics or aerial hyphae regardless of carbon source, and are globally deregulated for carbon utilization (57). Homologues of *bldB* are restricted to but widespread among other filamentous actinomycetes; 24 are found in the *S. coelicolor* genome alone (24). Multiple homologues are found in species of *Streptomyces*, *Saccharopolyspora*, *Salinispora*, *Frankia*, *Thermobifida* and *Nocardia*, totaling over 160. None of the *bldB* homologues have been studied or characterized. It has been speculated that BldB is also a DNA-binding protein, based on a predicted helix-turn-helix motif located in its C-terminus (56). Although it is frequently referred to as a DNA-binding protein in the literature, this is not supported by any experimental evidence.

1.2.4 *bldJ* and *bldK*: Evidence for extracellular signaling

Although it has become apparent that most *bld* genes do not encode direct components of a signaling cascade, as initially proposed, there is evidence for direct involvement of *bld* gene products in signal import. The *bldK* locus encodes an oligopeptide permease that imports a signaling molecule whose production depends on

bldJ (52). This signaling molecule has been purified and appears to be a modified peptide, but its precise structure has not been determined (52).

1.2.5 *bldG* and *bldN*: Developmental regulation by sigma factors

The *S. coelicolor* genome codes for 65 sigma factors, the most found in any organism to date (3). The presence of an unusually large number of sigma factors reflects the complex regulation of gene expression in response to developmental, stress and other environmental signals. Sigma factors involved in development include *bldN* and *whiG* (the latter is required for maturation of aerial hyphae into spores). In addition, a putative sigma factor regulator encoded by *bldG* is also implicated in developmental regulation.

BldG is similar to the well-characterized *B. subtilis* anti-anti-sigma factors RsbV and SpoIIAA (35). Anti-anti-sigma factors control cognate sigma factors by regulating the activity of corresponding anti-sigma factors, usually through phosphorylation. Despite high sequence similarity to other anti-anti-sigma factors, and experimental evidence for phosphorylation, the anti-anti-sigma factor function for BldG has not been experimentally verified (6). Genes for anti-anti-sigma factors are usually located adjacent to the sigma factors they regulate, but this is not the case for *bldG*. Its sigma factor target(s) have not been identified, and this is an important goal for future research.

bldN encodes an extracytoplasmic function (ECF) sigma factor that is synthesized as an inactive pro-protein, similar to σ^E and σ^K of *B. subtilis*, and undergoes cleavage to the active protein at the start of aerial mycelium development (5). As mentioned above, it is negatively regulated at the transcriptional level by BldD. It has been determined that a direct target for σ^{BldN} is one of the two *bldM* promoters, which in turn regulates chaplin

expression (5). There is very little known about *bldM*. It encodes a putative "orphan" two-component response regulator (16). No gene encoding a putative cognate sensor kinase is located nearby in the genome, and if a partner for BldM does exist, it is yet to be identified.

1.3 SapB, chaplins and rodlins: Morphogenetic proteins in *S. coelicolor*

There are two main classes of morphogenetic proteins in *S. coelicolor*: SapB and the chaplins. Their function is purely structural; they lower the surface tension at the air-substrate interface and confer hydrophobicity on the emerging aerial hyphae so that they are able to grow upwards into the air (29). A third class of cell surface proteins, the rodlins, are also partly responsible for the structural integrity and physical properties of aerial hyphae and spore surfaces, but they are not essential for aerial growth (29).

There are eight different chaplin proteins (an acronym for "*coelicolor* hydrophobic aerial proteins") – ChpA-C are the "long" chaplins, which are composed of two chaplin domains and a sorting signal for attachment to the cell wall, and ChpD-H are the "short" chaplins, composed of just one chaplin domain. It is believed that the chaplins polymerize on the cell surface and interact with the rodlins to form amyloid-like fibrils (29). The effect of *chp* gene deletions is additive – loss of one or two *chp* genes has little effect on the production of an aerial mycelium, while deletion of five or more results in a severe defect (30).

SapB is a lanthionine-containing peptide derived from the RamS protein, most likely through post-translational modification by RamC (44, 68). Upon export from the

cell, probably through the ABC transporter encoded by *ramAB*, SapB coats the aerial hyphae, promoting normal aerial mycelium formation.

Capstick et al (9) recently showed that aerial hyphae formation is SapB-independent and chaplin-dependent during growth on mannitol-containing media, while growth on rich medium involves both classes of proteins. This is consistent with the observation that many *bld* mutants that do not produce SapB are nevertheless able to sporulate on mannitol. The only *bld* mutants that do make SapB are *bldM* and *bldN*, and these genes have been shown to regulate chaplin expression. It has become evident that the formation of aerial hyphae can occur through multiple pathways, which depend on the perception of environmental signals such as carbon source availability.

1.4 Antibiotics and their regulation in *S. coelicolor*

Streptomyces are a significant source of natural antibiotics and other bioactive compounds, producing over half of these classes of drugs in clinical use (10). Along with other actinomycetes, they produce 70% of all known natural antibiotics. These include aminoglycosides (streptomycin, kanamycin), anthracyclines (doxorubicin), macrolides (erythromycin), glycopeptides (vancomycin), tetracyclines, antifungals (amphotericin), herbicides (bialaphos) and insecticides (10).

S. coelicolor produces four antibiotics: actinorhodin, undecylprodigiosin, calcium dependent antibiotic (CDA) and methylenomycin (Figure 2). Actinorhodin and undecylprodigiosin are both pigmented; actinorhodin is a blue-pigmented polyketide and undecylprodigiosin is a red-pigmented tripyrrole (10). CDA is a non-ribosomally synthesized cyclic lipopeptide that forms transmembrane channels and requires calcium

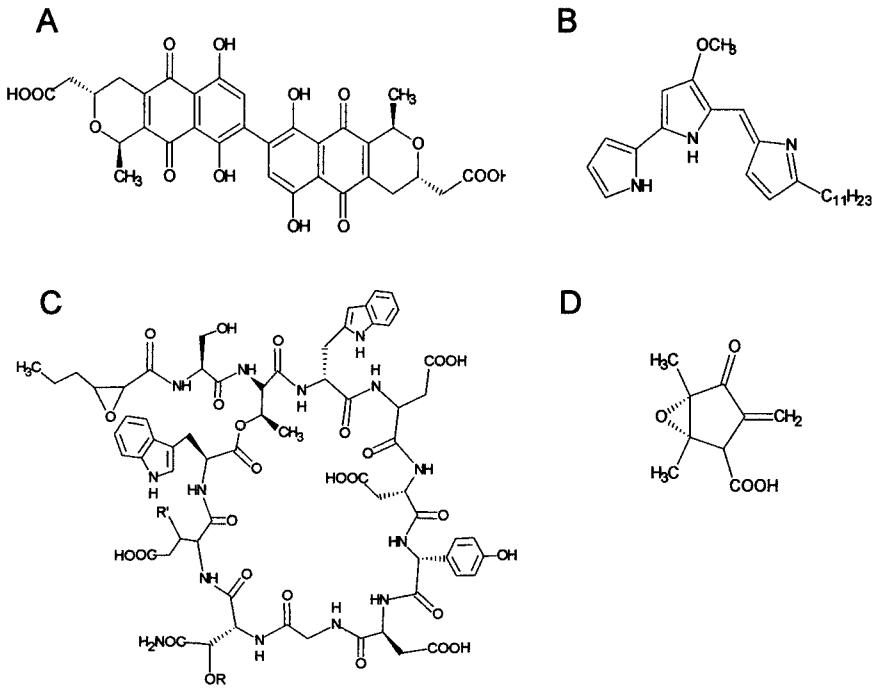

Figure 2 - Structures of the four antibiotics produced by *S. coelicolor*. A. actinorhodin, B. undecylprodigiosin, C. CDA, D. methylenomycin

ions for antimicrobial activity (19, 45). Methylenomycin, a cyclopentanone, is a rare example of a non-chromosomally encoded antibiotic, whose biosynthetic and resistance genes are located on the linear plasmid SCP1 (10).

Antibiotic production is regulated at multiple levels and is closely intertwined with life cycle progression. The highest level of control is exerted by *bld* genes which have pleiotropic effects on both antibiotic production and morphogenesis. More targeted regulation is achieved by global regulators of secondary metabolism, which affect synthesis of multiple antibiotics. Some examples include AfsR, a homologue of pathway-specific regulators that is phosphorylated by multiple kinases in response to various signals (37), and the two-components systems AbsA1-AbsA2, AfsQ1-AfsQ2 and CutR-CutS (10). The most specific regulators affect only one antibiotic; these are known as

pathway-specific regulators and are located in the respective biosynthetic gene cluster. ActII-ORF4 and RedD are the pathway-specific regulators for actinorhodin and undecylprodigiosin, respectively (4). They are members of a family of DNA-binding regulatory proteins known as SARPs (*Streptomyces* antibiotic regulatory proteins), which also includes AfsR. The CDA biosynthetic cluster encodes the pathway-specific regulator CdaR, as well as the aforementioned global regulators AbsA1-AbsA2 (62). Methylenomycin production is regulated by MmyR/MmyB, encoded by genes which are also embedded in the biosynthetic gene cluster (15).

1.5 The BldB homologues: Actinomycete-specific proteins in development and antibiotic production

Another putative pleiotropic regulator that affects all three chromosomally-encoded antibiotics is found in the *abaA* cluster, which consists of five open reading frames, originally named *orfCBADE*, and also referred to by the *S. coelicolor* genome annotation SCO numbers *SCO0700* through *SCO0704*, respectively. This cluster is believed to be a pleiotropic antibiotic regulator because *orfB* was shown to be required for antibiotic production in *S. coelicolor*; disruption of this gene abolished or significantly reduced production of all three chromosomally-encoded antibiotics (33). There is no other homologue of *orfB* in *S. coelicolor*, and only four were found among all bacteria sequenced to date – one in each of *S. avermitilis* (*SAV1046*), *S. griseus* (*SGR754*), *S. scabies* (*SCAB13571*) and *S. ambofaciens* (*SAMR0738*). *SAV1046* is found in an *abaA*-like cluster which is conspicuously missing an *orfA* homologue. There are no *abaA*-like genes located near *SGR754* and *SCAB13571*, and information is unavailable for *SAMR0738*.

This locus is of interest because one of the genes, *orfD (SCO0703)*, encodes a homologue of the highly pleiotropic developmental protein BldB. Interestingly, of the 23 chromosomal *S. coelicolor bldB* homologues, 18 are located in *abaA*-like clusters. The remaining five, including *bldB* itself, are not located near *abaA*-like genes. The only other gene in these clusters that has been studied is *whiJ*, a poorly understood sporulation regulatory protein located adjacent to the *bldB* homologue *SCO4542* (61). The regional organization of all *bldB* homologues is shown in Figure 3. The *orfC* gene, annotated as a probable ABC transporter, is not included in this figure or in future discussions because the original fragment cloned by Fernandez-Moreno et al (33) did not contain the entire

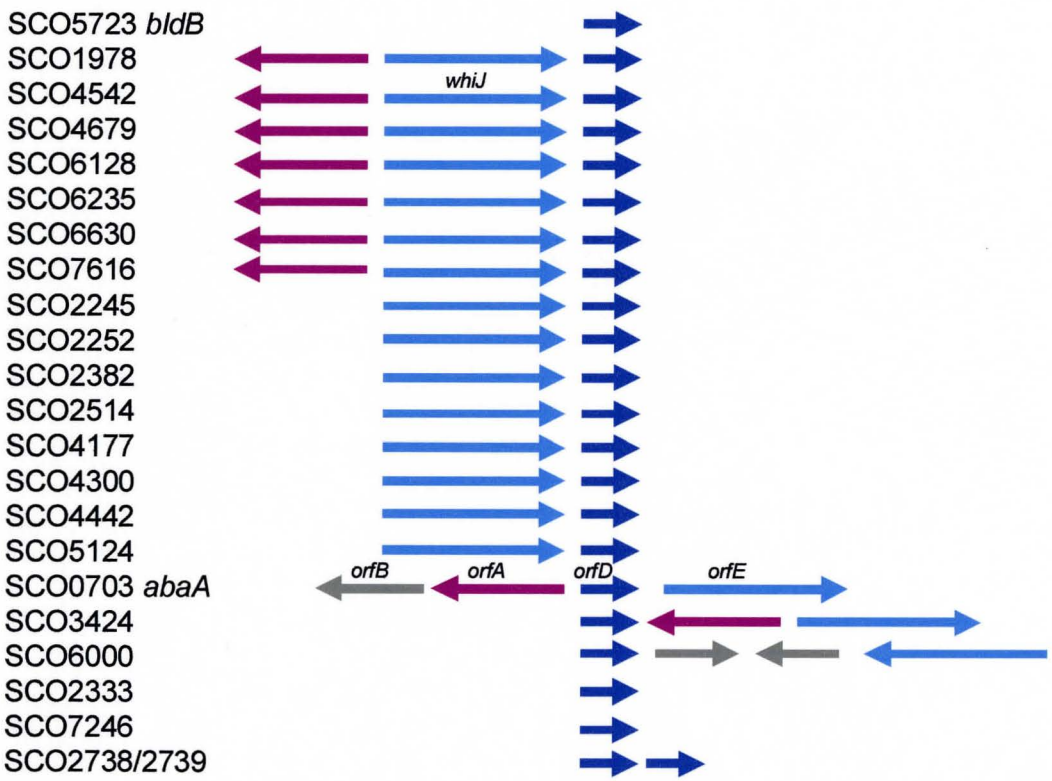

Figure 3 – Regional organization of *bldB* homologues on the *S. coelicolor* chromosome. Each arrow represents a gene, and homologous genes are colour coded. Each cluster is labeled with the *bldB* homologue present in that cluster, and alternative gene names are shown where applicable.

gene, and homologues of it do not appear in other *abaA*-like clusters.

Since the *abaA* cluster contains at least one antibiotic regulator and a *bldB*-like protein, it is possible that they function together in regulating antibiotic synthesis, and possibly some aspect of development. A systematic approach was taken to knock out each of these four genes (*orfBADE*, or *SCO0701-0704*) and investigate the phenotypes of the null mutants with respect to morphogenesis and antibiotic production. The goal of this work was to establish whether the *bldB* homologue is indeed a regulator, and gain insight into the regulatory role of the entire cluster. A related goal was to further clarify the role of *bldB* in development by studying its expression in other *bld* mutants using a luminescent reporter, and attempt to determine the nature of any interactions or interdependence. The long term goal beyond the scope of this work is to understand the mechanism of action of *bldB* and its homologues in the context of *abaA*-like gene clusters.

2 Methods

2.1 Strains, plasmids and culture conditions

The *E. coli* and *S. coelicolor* strains and plasmids used in this work are listed in Tables 1 and 2, respectively. *E. coli* was grown in LB medium at 37°C. *S. coelicolor* was grown on R2YE or MS solid media, or in YEME or R5 liquid media at 30°C (42). *S. coelicolor* and *E. coli* were manipulated following established protocols (8, 42).

Table 1 – *E. coli* and *S. coelicolor* strains used in this study.

Strain	Genotype/ description	Reference/ source
E. coli		
XL-1 Blue	General cloning host; *Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac*	Stratagene
ET12567	Host for production of non-methylated DNA; *dam dcm hsdM hsdS hsdR cat tet*	(48)
BW25113	Δ(*araD-araB*)*567* Δ*lacZ4787*(::*rrnB-4*) *lacIp-4000*($lacI^Q$) λ^- *rpoS369*(Am) *rph-1* Δ(*rhaD-rhaB*)568 *hsdR514* *OR* Δ*araBAD* Δ*rhaBAD*	(22)
BT340	DH5α/pCP20; FLP recombinase strain	(17)
JM109	Host for His_6-BldD expression; *recA1 supE44, endA1 hsdR17 gyrA96 relA1 thi-1* Δ*(lac-proAB)*	(70)
S. coelicolor		
M145	Wild type Prototroph $SCP1^-$ $SCP2^-$	(3)
M600	Wild type Prototroph $SCP1^-$ $SCP2^-$	(3)
J1501	Wild type *hisA1 uraA1 strA1* Pgl^- $SCP1^-$ $SCP2^-$	(13)
N985 (*bldB*)	M145 *bldB::aphI*	(23)
J1700 (*bldA*)	*bldA39 hisA1 uraA1 strA1* Pgl^- $SCP1^-$ $SCP2^-$	(46)
J660 (*bldC*)	*bldC18 mthB2 cysD18* NF SCP2*	(50)
HU66 (*bldD*)	*bldD53 hisA1 strA1* Pgl^- NF SCP2*	(67)
C103 (*bldG*)	*bldG103 hisA1 uraA1 strA1* Pgl^- $SCP1^-$ $SCP2^-$	(11)
HU261 (*bldJ*)	*bldJ261 hisA1 uraA1 strA1* NF SCP2*	(67)
NS17 (*bldK*)	M145 *bldK::aadA*	(53)
J2151 (*bldM*)	M145 *bldM::hyg* Δ*glkA119*	(51)
Δ*bldD1*	M600 Δ*bldD*	(28)
Δ*bldG* 1DB	M145 Δ*bldG*	(7)
SCO0701::apra	M145 *SCO0701*::(FRT-*oriT-aac(3)IV*-FRT)	This work
SCO0702::apra	M145 *SCO0702*::(FRT-*oriT-aac(3)IV*-FRT)	This work
SCO0703::apra	M145 *SCO0703*::(FRT-*oriT-aac(3)IV*-FRT)	This work
SCO0704::apra	M145 *SCO0704*::(FRT-*oriT-aac(3)IV*-FRT)	This work
Δ*SCO0701*	M145 Δ*SCO0701*	This work
Δ*SCO0702*	M145 Δ*SCO0702*	This work
Δ*SCO0703*	M145 Δ*SCO0703*	This work
Δ*SCO0704*	M145 Δ*SCO0704*	This work

Table 2 – Plasmids used in this study.

Plasmid	Description	Reference/ source
E. coli		
pBlueScript II SK+	General cloning vector; *bla*	Stratagene
pIJ773	pBlueScript KS(+) P1-FRT-*oriT*(RK2)-*aac(3)IV*-FRT-P2	(34)
pIJ778	P1-FRT-*oriT-aadA*-FRT-P2	(34)
pIJ790	λ-RED (*gam bet exo*) *cat araC rep101*^{ts}	(34)
pQE9-bldD	Expression of his_6-BldD	(31)
pUZ8002	Non-transmissible mobilizing plasmid for conjugation; *tra neo* RP4	(55)
S. coelicolor		
pMU1	*luxCDABE aac(3)IV oriT*(RK2) *int attP*(ϕBT1)	(21)
phrdBlux	pMU1 containing *hrdB* promoter	(21)
pEM101	pMU1 containing *bldB* promoter	This work
pWHM3	High copy number plasmid; *tsr bla lacZ rep*(pIJ101) *ori*(pIJ101) *ori*(pUC19)	(66)
pEM0701	pWHM3 containing *SCO0701* and its promoter	This work
pEM0703	pWHM3 containing *SCO0703* and its promoter	This work
2StF42	Supercos-1 containing *abaA* locus	(59)
St9C5	Supercos-1 containing *bldD*	(59)

For *E. coli*, antibiotics were used at the following concentrations: 100 μg/mL ampicillin/ carbenicillin, 50 μg/mL apramycin, 25 μg/mL chloramphenicol, 50 μg/mL kanamycin, 25 μg/mL nalidixic acid, 50 μg/mL spectinomycin, 50 μg/mL streptomycin. For *S. coelicolor*, antibiotics were used at the following concentrations: 50 μg/mL apramycin, 50 μg/mL kanamycin (in MS medium; 200 μg/mL in R2YE), 25 μg/mL nalidixic acid, 400 μg/mL spectinomycin (200 μg/mL for overlay on MS), 10 μg/mL streptomycin, 50 μg/mL thiostrepton. All antibiotics were from Sigma. Primers (Table 3) were from Sigma Genosys or Invitrogen, except M13for and M13rev, which were from the Mobix Lab. Sequencing was done at the Mobix Lab at McMaster University.

Table 3 – Primers used in this study.

Primer name	Sequence (5' to 3')
reda-up	ACTCGGTCCGCTCCCCCGAACAGCAAGGAGACCGCGGTGATTCCGGGGATCCGTCGACC
reda-dn	TACAGGGACAGGTGAGTTGAGGCCCGGAGGAAACACGTGATTCCGGGGATCCGTCGACC
red0701-up	CCCCGTACCACCACCCTGCTCGCGGGCCTGGACGGCGTGATTCCGGGGATCCGTCGACC
red0701-dn	TGGAGGGCGCGGGTCCCGGTCCAGGCGGTGCCGGTCTCATGTAGGCTGGAGCTGCTTC
red0702-up	CGACGCCCCGTTCTGACGTCGCATCAGGGAGATGCTGTGATTCCGGGGATCCGTCGACC
red0702-dn	CTGCGCATGGTGTGGGGCCCTTCCGCGGCAGTCGGGTCATGTAGGCTGGAGCTGCTTC
red0704-up	CTTTCCTTCAAGTCTGCCGGTCTGCTGGGAGTCAGGATGATTCCGGGGATCCGTCGACC
red0704-dn	GCGCCGCGCGTGGCCCCTCCTCCGTCGTCCGTGCCGCTATGTAGGCTGGAGCTGCTTC
redas1	GGCTATTATCCACGCTTAGAGCGCTC
redas2	GTGACTCCCAGCAGACCGGCAGAC
red0701sf	ACACCATGCGCAGCGCTCCGA
red0701sr	GCTTTCCTACCCTGTCGGCCGGGAG
red0702sf	TTCGCGCAATTTCTCGTGCAATTGC
red0702sr	GGGCGGCGTCTGACGGTCGGT
red0704sf	GGAAGCGGAGCCCAACTACCCGATTTC
red0704sr	GCGGACTGGGGCGTCTGCGCT
intrev	AACCCATTCAAAGGCCGGCATTTTCAG
intfor	CGACGCTGCATCTTGCCGAGTTGAT
0703w3	GTGAATTCACAGCATCTCCCTGATGC
0703w4	CGCCGCTCTAGACCTACACCAGGTG
0701w1	CTCAGAATTCCCGACTGCCGCGGAA
0701w2	CCAGGCTCTAGAGGTCTCAGTCCCCGAG
luxbldB1	CTCTTGCGGATCCTCTGAAGCACTGGTG
luxbldB2	CTCGTCCGGTACCTGGGCCATGGC
bldBE1	AGTTCGGGGGCAGGGGGCCG
bldBE2	CATGGCGGAGGTGTCCTTTCAGGC
bldBE3	CCCTGCCTCGGTGGCGGAAG
bldBE4	AGGCAGGGGCAGGGCCAGTAAGGT
bldB-ef	TGCTGCGGTACACCGAGGCGGA
BldB-EcoR	CGAGCAGAATTCTCACTCGGCGACG
bldDE1-b	TTCGGCTTGACGCAGCAGAGTAA
bldDE2-b	AAGGTGTCGACGCGGCAGGCTG
1489red1	CAGCCTGCCGCGTCGACACCTTGTCCGGGGAGCCATATGATTCCGGGGATCCGTCGACC
1489red2	ACCCCGGCGGCACGTTTCTGCTGAGGTGGTGGGGGCTCATGTAGGCTGGAGCTGCTTC
1489sf	CATTCGGCTTGACGCAGCAGAGTAAC
1489sr	GCCGAGCCTACGACGGGTAAAAGC
M13for	GTAAAACGACGGCCAGT
M13rev	CAGGAAACAGCTATGAC

2.2 Construction and analysis of *abaA* mutants

2.2.1 REDIRECT: PCR-targeted gene replacement

Each of the genes in the *abaA* cluster, *SCO0701-0704*, was individually knocked out in *S. coelicolor* M145 using the Redirect method described by Gust et al. (34). For each gene, PCR was used to generate a disruption cassette containing the apramycin resistance gene *aac3(IV)* and an origin of transfer, *oriT*, flanked by FRT recombination sites and having a 39 nucleotide extension complementary to sequences flanking the gene of interest. The following primer pairs were used: red0701-up and red0701-dn for *SCO0701*; red0702-up and red0702-dn for *SCO0702*; reda-up and reda-dn for *SCO0703*; red0704-up and red0704-dn for *SCO0704*. The disruption cassettes were introduced by electroporation into *E. coli* BW25113 containing the λRed recombination plasmid pIJ790 and the cosmid 2StF42, which contains the region of the chromosome including the *abaA* cluster. The resulting transformants were screened for replacement of the gene of interest on the cosmid by the respective resistance cassette, generating disruption cosmids. The primer pairs used for screening by PCR were red0701sf and red0702sr for *SCO0701*; red0702sf and red0702sr for *SCO0702*; redas1 and redas2 for *SCO0703*; red0704sf and red0704sr for *SCO0704*, generating ~1.5 kb products.

The disruption cosmids were transformed into *E. coli* ET12567 containing the non-transmissible mobilizing plasmid pUZ8002, and subsequently introduced into *S. coelicolor* M145 by conjugation. For conjugation, *E. coli* strains were grown overnight in 25 mL LB containing the appropriate antibiotics, harvested and washed twice with 5 mL LB and resuspended in 2 to 4 mL LB. *S. coelicolor* M145 spores (10-20 μL of concentrated suspension) were heat shocked for 10 min at 50°C in 500 μL of 2x YT

medium, and then mixed with 500 µL of the resuspended *E. coli* cells. The mixtures were plated on MS medium, incubated at 30°C for 16-20 h, and then overlaid with nalidixic acid and apramycin. Resulting exconjugants were screened for apramycin resistance and kanamycin sensitivity ($Apra^R$ Km^S), indicating replacement of the gene of interest with the resistance cassette in the *S. coelicolor* chromosome. Chromosomal DNA from $Apra^R$ Km^S candidates was screened by PCR using the appropriate screening primer pairs for each gene. Successful gene replacements were indicated by the presence of ~1.5 kb products. In some cases, the disruption was also confirmed by PCR using either the upstream or downstream screening primer (priming outside the recombination region for each gene), with a primer specific to the apramycin resistance cassette (intfor or intrev). PCR using these primers generated ~500 bp products.

Scar cosmids were constructed in which *aac(3)IV* and *oriT* were removed, leaving an 81 bp scar sequence in place of the disruption cassette. To generate the scar cosmids, the disruption cosmids were transformed into *E. coli* BT340 containing the FLP recombinase system, and recombination was induced by incubation at 42°C. Resulting clones were re-streaked on LB at 37°C and screened for apramycin sensitivity and kanamycin resistance, and were further confirmed by PCR and digestion with BamHI. Scar cosmids were passaged through *E. coli* ET12567 and were transformed into protoplasts of the disruption mutants. Protoplasts were generated from *S. coelicolor* disruption strains grown in 50 mL R5 medium for 2-3 days at 30°C. Cells were washed with 10.3% sucrose and treated with 2 mg/mL lysozyme in P-buffer at 30°C for 45 min to 1.5 hours to generate protoplasts, which were simultaneously mixed with ~10 µg of non-

methylated scar cosmid DNA and 100 µL 25% PEG-1000. The mixture was grown on R2YE medium overnight, then overlaid with kanamycin. Resulting transformants were grown non-selectively on MS medium, then screened for sensitivity to both apramycin and kanamycin. Candidates were confirmed positive by the presence of ~300 bp products following PCR using the same set of screening primers.

2.2.2 Overexpression of *SCO0701* and *SCO0703*

SCO0701 and *SCO0703* were overexpressed on the high-copy plasmid pWHM3 under the control of their own promoters. Each gene with its promoter was amplified from the *S. coelicolor* M145 chromosome using *Pfu* (Fermentas) or Vent (New England Biolabs) DNA polymerase and primer sets 0701w1/0701w2 and 0703w3/0703w4, respectively, introducing EcoRI and XbaI restriction sites. The PCR products were purified using a MinElute kit (Qiagen) or by gel extraction using a QiaexII kit (Qiagen), and then cloned into the EcoRV site of pBlueScript II SK+. The cloned inserts were sequenced (primers M13for and M13rev) and subcloned into pWHM3 using EcoRI and XbaI, generating plasmids pEM0701 and pEM0703. Non-methylated plasmid DNA, isolated from *E. coli* ET12567 using the Qiaprep kit (Qiagen), was introduced into *S. coelicolor* M145 by protoplast transformation.

2.2.3 Phenotypic analysis

Sporulation and production of the pigmented antibiotics was evaluated on various media: R2YE, R2YE without calcium, ONA, ONA with 12 mM $Ca(NO_3)_2$, Bennett's, SMMS, MM + glucose and MM + mannitol, (42). In addition, low-calcium versions of Bennett's and SMMS were prepared by substituting peptone for casaminoacids. Spores

of each strain were streaked to yield a dense lawn. Plates were incubated at 30°C and observed daily for 4 days.

2.2.4 CDA assay

Concentrated spore suspensions of *abaA* mutant and overexpression strains were diluted 1:10 in 0.85% saline, and 5 µL of each strain was spotted on ONA and ONA + 12 mM $Ca(NO_3)_2$. After 24h incubation at 30°C, the plates were overlaid with 4 mL of soft agar (1:1 ONA: ON broth) containing a 1:100 dilution of a *Staphylococcus aureus* (ATCC 29213) overnight culture. Plates were incubated at room temperature for one hour, followed by 37°C overnight, and the inhibition of *S. aureus* growth was assessed.

2.3 Investigation of *bldB* promoter activity in *bld* mutants

2.3.1 Construction of luminescent reporter

The *bldB* promoter, spanning the region from –290 to +43 relative to the transcriptional start site [as reported by Pope et al. (56)], was amplified from the *S. coelicolor* M145 chromosome using Vent DNA polymerase (New England Biolabs) and primers luxbldB1 and luxbldB2, introducing BamHI and KpnI restriction sites. The resulting fragment was cloned into the EcoRV site of pBlueScript II SK+ and sequenced, then subcloned into the BamHI and KpnI sites of pMU1 [encoding the *luxCDABE* operon, (21)], yielding plasmid pEM101. The positive control construct, phrdBlux, containing the promoter for the major vegetative sigma factor gene *hrdB* upstream of the *lux* operon, is described in reference (21). The plasmids were introduced by conjugation into the *S. coelicolor bld* mutant strains listed in Table 1. Conjugation into sporulating strains was performed as described above. Non-sporulating strains were grown on R2YE

for 2-3 days and mycelia were scraped and recovered in 3-5 mL 0.85% saline. An aliquot of the mycelia (500 µL) was mixed with 500 µL of washed and resuspended *E. coli* cells (ET12567/pUZ8002 containing the plasmid to be transferred), and the mixture was grown on MS at 30°C overnight before overlaying with nalidixic acid and apramycin. Eight exconjugants of each strain were streak-purified twice on $R2YE_{apra}$, and assayed for bioluminescence.

2.3.2 Luminescence assay

Solid media - Strains containing pMU1, phrdBlux or pEM101 were grown on $R2YE_{apra}$ at 30°C for 2-3 days. Two to four colonies of each strain were crushed in 50 µL 0.85% saline. Additional saline (200 µL) was added and the suspension was mixed and allowed to settle. Each strain (10 µL of the cell suspension) was inoculated into six replicate wells of white 96-well plates (#353296, BD) containing 200 µL solid R2YE medium per well. Plates were incubated at 30°C and luminescence was read at ~8 to 16 hour intervals using an EnVision multilabel plate reader (Perkin Elmer) at the McMaster High Throughput Screening Lab.

Liquid media – Strains of M145/pMU1 and M145/pEM101 were grown on $R2YE_{apra}$ at 30°C for 2-3 days. Three colonies of each strain were inoculated into 50 mL YEME and grown at 30°C with shaking. Aliquots of 300 µL were removed at 8 to 16 hour intervals, and luminescence was read using a Lumat luminometer (Berthold).

2.4 Interaction between BldD and the *bldB* promoter

2.4.1 Purification of BldD

His-tagged BldD was expressed from plasmid pQE9-bldD and purified as described by Elliot (25). *E. coli* JM109 containing pQE9-bldD was grown in LB_{amp} medium at 37°C to OD_{600} ~ 0.8, induced with 0.5 mM IPTG and grown for an additional 5 hours. The cells from 500 mL culture were resuspended in 15 mL of 50 mM NaH_2PO_4 pH 7.8, 300 mM NaCl, 1 mg/mL lysozyme, incubated on ice for 30 min and lysed by sonication. The lysate was incubated with 5 mL Ni-NTA (Qiagen) slurry for one hour at 4°C, and the mixture was poured into a BioRad Econocolumn (1 x 30 cm). The column was washed three times with 10 mL of 50 mM NaH_2PO_4 pH 7.8/ 300 mM NaCl buffer containing 10, 30 or 40 mM imidazole. Purified protein was eluted with 350 mM imidazole-containing buffer. The eluted fractions (12 x 0.5 mL) were analyzed for BldD quantity and purity by SDS-PAGE and staining with Coomassie Brilliant Blue R-250. The fractions of interest were pooled, the buffer was exchanged to 20 mM Tris-Cl pH 8, 150 mM NaCl using an Amicon Ultra-4 centrifugal filter (10,000 MWCO) and glycerol was added to 10% final concentration. Protein concentration was determined by the Bradford assay and single-use aliquots were frozen at -80°C.

For the Bradford assay, 10 µL protein sample was mixed with 200 µL of Bradford reagent (1:4 BioRad Protein Assay Reagent Concentrate: water) in triplicate in 96-well plates, and the absorbance was read at 595 nm. Protein concentration was determined based on a standard curve constructed using 0-500 µg/mL BSA.

2.4.2 Electrophoretic mobility shift assays

Three DNA fragments from the *bldB* promoter region were amplified from plasmid pEM101 (containing the *bldB* promoter) using the primers specified in Figure 4, to generate probes 1, 2 and 3 (123, 61 and 70 bp, respectively). The *bldD* promoter was amplified from M145 chromosomal DNA using primers bldDE1-b and bldDE2-b to generate probe D (91 bp). The PCR products were extracted from 8 or 12% polyacrylamide gels, end-labeled with γ^{32}P-ATP using T4 polynucleotide kinase (New England Biolabs), and purified using a MinElute kit (Qiagen). Non-specific control DNA was a 119 bp fragment from the end of the *bldB* coding region, amplified using primers bldB-ef and bldB-ecoR.

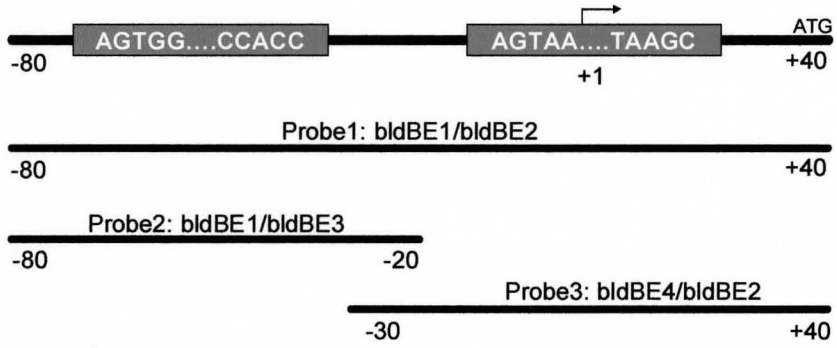

Figure 4 – Schematic representation of *bldB* promoter probes used to test BldD binding by EMSA. Numbering is with respect to the transcription start site (+1), and possible BldD recognition sites are represented by the grey boxes. The primer pairs used to generate each probe are specified.

EMSA reactions contained 1 ng labeled probe, 0 to 1 μM His-BldD and 90 ng poly(dI-dC) in a buffer consisting of 10 mM Tris-Cl pH 7.8, 150 mM NaCl, 2 mM DTT and 10% glycerol, in a total volume of 20 μL. For competition assays, 500x excess (500 ng) unlabeled probe DNA or unlabeled non-specific DNA (from the coding region of *bldB*) was also added. The reactions were incubated at 30°C for 20 min and resolved on

8% polyacrylamide gels at 70V in a buffer consisting of 1X TBE with 1.5% glycerol. Gels were exposed to a phosphor screen and analyzed using a Typhoon Phosphorimager (Amersham/ GE Healthcare).

3 Results

3.1 Construction and analysis of *abaA* mutants

3.1.1 Construction of *abaA* null mutants

Disruption cosmids based on the 2StF42 cosmid were individually constructed for *SCO0701-SCO0704* by λ-Red recombination using disruption cassettes generated by PCR. The presence of a ~1500 kb product following PCR with the appropriate screening primers or the presence of a ~750 kb product following digestion with SacI confirmed successful recombination. Following introduction of the disruption cosmids into M145, multiple exconjugants were screened for apramycin resistance and kanamycin sensitivity, indicating replacement of each gene by the disruption cassette. Typically, ~20-30% of the exconjugants were $Apra^R$ Km^S.

Chromosomal DNA from three to six clones per strain were analyzed by PCR using the screening primers; results are shown in Figure 5. For some clones, no specific PCR products were obtained; this is likely due to low quality chromosomal DNA preparations. For the *SCO0701::apra*, *SCO0702::apra* and *SCO0703::apra* mutants, products confirming successful gene replacement were obtained with primers specific to sequences ~100 bp outside each recombination region. Specific products were not obtained for the *SCO0704::apra* candidates using a primer pair priming ~100 bp outside the *SCO0704* gene. Therefore, the *SCO0704::apra* candidates were also screened using

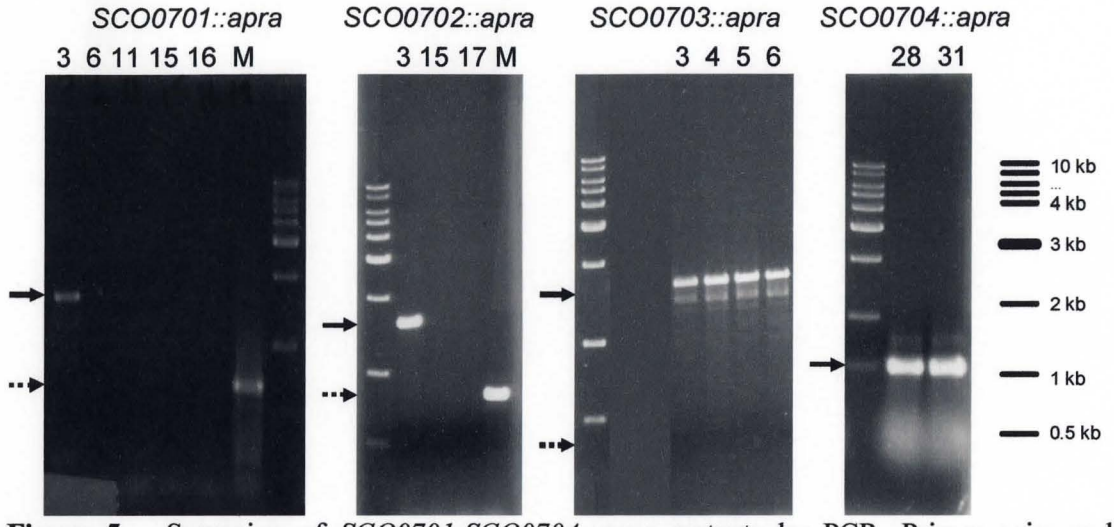

Figure 5 – Screening of *SCO0701-SCO0704::apra* mutants by PCR. Primer pairs and expected products were *SCO0701*: red0701sf/red0701sr (~1.5 kb), *SCO0702*: red0702sf/red0702sr (~1.5 kb), *SCO0703*: redas1/redas2 (~1.5 kb), *SCO0704*: red0704sr/intfor (~0.5 kb). Lanes are labeled with the clone number or M (for M145). A schematic representation of the molecular weight ladder is shown to the right. Solid arrows indicate expected products for positive $Apra^R$ Km^S double crossover recombinants. Dashed arrows indicate expected products for M145 controls (~650 bp for *SCO0701*, ~750 bp for *SCO0702*, ~400 bp for *SCO0703*).

one primer ~100 bp upstream or downstream of the gene, and one primer internal to the apramycin cassette (two sets of primers: red0704sr/intfor and red0704sf/intrev, each producing ~0.5 kb products). Two clones were confirmed positive using the latter two sets of primers.

Markerless in-frame deletion mutants (Δ*SCO0701*–Δ*SCO0704*) were constructed by transformation of the disruption mutants with the respective scar cosmids and screening for $Apra^sKm^s$ transformants. $Apra^sKm^s$ transformants were obtained with a frequency of 15-40% after re-streaking twice on non-selective MS medium. $Apra^sKm^s$ candidates were screened by PCR using the same screening primers that were used to confirm the initial gene replacement. Successful replacement of the disruption cassettes by the scar sequence was indicated by ~300 bp PCR products (Figure 6).

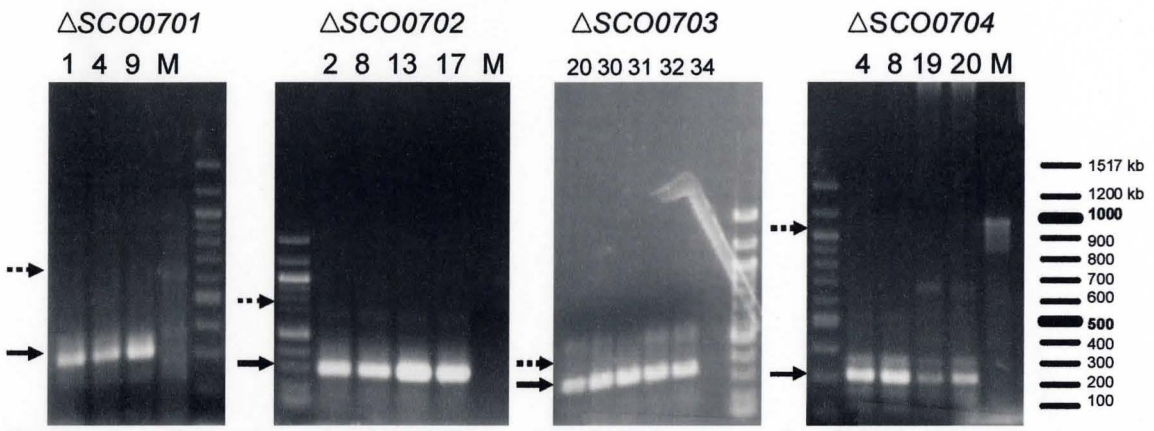

Figure 6 – Screening of Δ*SCO0701* – Δ*SCO0704* mutants by PCR. Primer pairs were Δ*SCO0701*: red0701sf/red0701sr, Δ*SCO0702*: red0702sf/red0702sr, Δ*SCO0703*: redas1/redas2, Δ*SCO0704*: red0704sf/red0704sr. Expected products were ~300 bp for all strains. Lanes are labeled with the clone number or M (for M145). A schematic representation of the molecular weight ladder is shown to the right. Solid arrows indicate expected ~300 bp products. Dashed arrows indicate expected products for M145 controls (~650 bp for *SCO0701*, ~750 bp for *SCO0702*, ~400 bp for *SCO0703*, ~1100 bp for *SCO0704*).

3.1.2 Effect of *abaA* null mutations on development and antibiotic production

Phenotypes of the *abaA* disruption mutants (*SCO0701::apra – SCO0704::apra*) and markerless mutants (Δ*SCO0701* – Δ*SCO0704*) were assessed visually throughout development on the solid media listed in Section 2.2.3. On most media there was no effect on pigmentation or development in comparison with M145. The *SCO0701::apra*, *SCO0702::apra* and *SCO0703::apra* strains produced less pigment on R2YE, R2YE-Ca and ONA, while aerial hyphae and sporulation were unaffected. The delay in antibiotic production was also apparent in liquid R5 media (not shown). The phenotypes of all disruption and markerless mutants on R2YE and ONA are shown in Figure 7.

CDA production by the Δ*SCO0701*-Δ*SCO0704* mutants was assessed on ONA+Ca. Two clones of each strain were tested, and representative results are shown in Figure 8. Compared to M145, CDA production was increased in Δ*SCO0702*, slightly decreased in Δ*SCO0701* and Δ*SCO0704*, and greatly decreased in Δ*SCO0703*. There was

no CDA production in Δ*SCO0703* at 24 h, but this strain did produce some CDA at later time points (30 and 48 h; results not shown). *S. aureus* growth was not affected by any of the strains in the absence of calcium (on ONA medium), indicating that antibiotic activity is due to CDA. The Δ*SCO0701* and Δ*SCO0702* mutants were completely defective in CDA production (not shown), therefore the apramycin insertion mutants were not studied further.

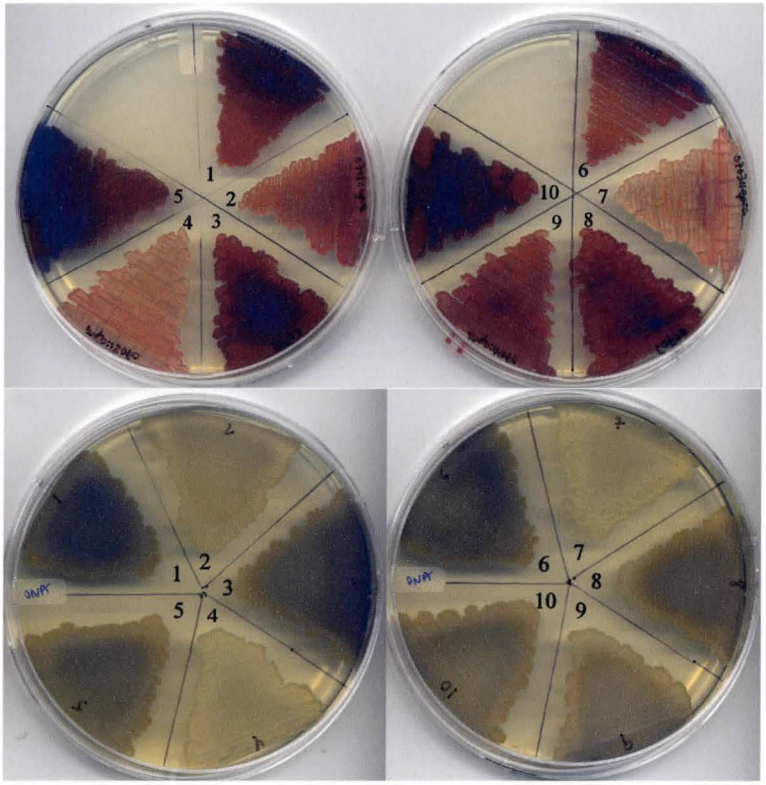

Figure 7 – Phenotypes of *SCO0701::apra-SCO0704::apra* and Δ*SCO0701*-Δ*SCO0704* strains on R2YE (2 days, top) and ONA (4 days, bottom). The *SCO0701::apra*, *SCO0702::apra* and *SCO0703::apra* strains were less pigmented on both media. Pictures are from the bottoms of the plates. 1,6. M145, 2. *SCO0701::apra*, 3. Δ*SCO0701*, 4. *SCO0702::apra*, 5. Δ*SCO0702*, 7. *SCO0703::apra*, 8. Δ*SCO0703*, 9. *SCO0704::apra*, 10. Δ*SCO0704*

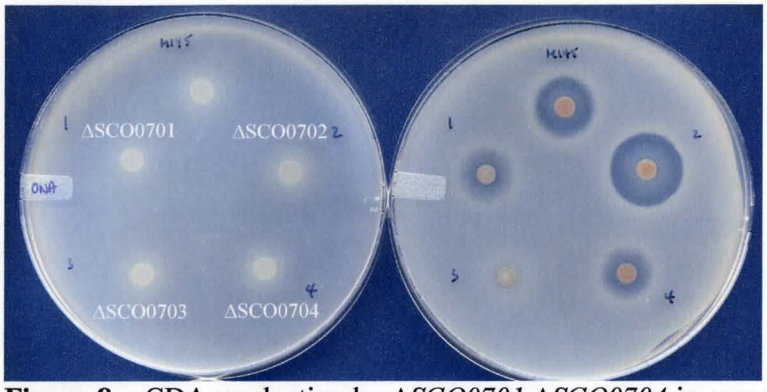

Figure 8 – CDA production by Δ*SCO0701*-Δ*SCO0704* in comparison with M145 after 24 hours growth on ONA (left, negative control) and ONA+Ca (right).

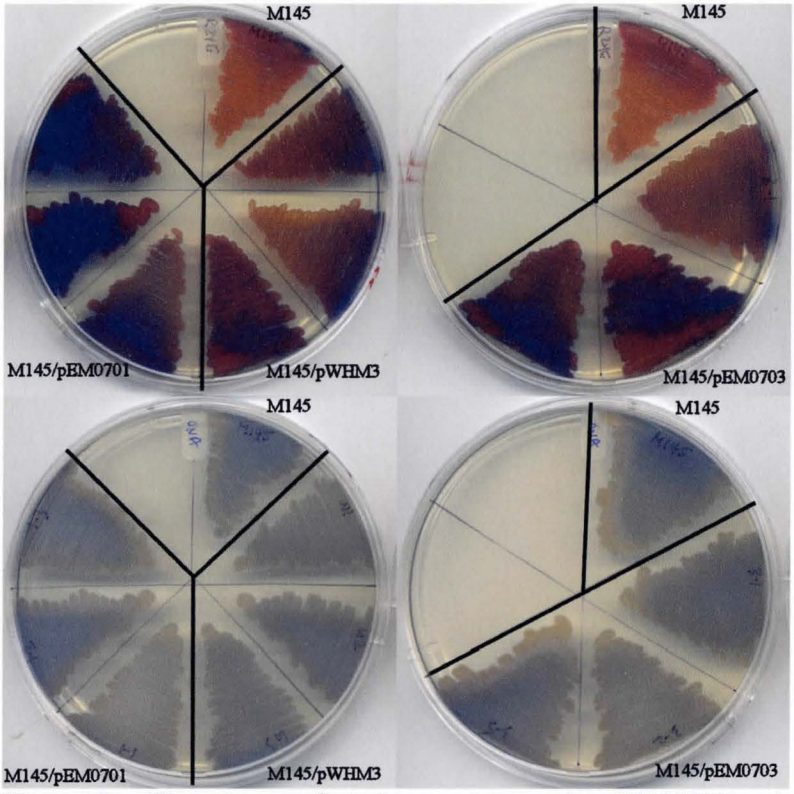

Figure 9 – Phenotypes of strains overexpressing *SCO0701* and *SCO0703*. Three thiostrepton resistant transformants of each strain (M145/pWHM3, M145/pEM0701, M145/pEM0703) are shown. There was no observable effect of overexpression on R2YE (2 days, top) and ONA (4 days, bottom). Pictures represent the bottom of the plates.

3.1.3 Effect of *SCO0701* and *SCO0703* overexpression on phenotype and CDA production

Phenotypes of the M145/pEM101 and M145/pEM0703 strains were assessed on R2YE and ONA, in comparison with M145/pWHM3 and M145 (Figure 9). Introduction of pWHM3 into M145 caused slightly elevated pigmentation. Overexpression of *SCO0701* and *SCO0703* did not affect pigmentation or sporulation relative to M145/pWHM3. The effect of overexpression on CDA production was inconclusive; eight thiostrepton resistant transformants of each strain were tested, and zone sizes were variable.

3.2 Investigation of *bldB* promoter activity in *bld* mutants

3.2.1 Activity of *bldB* promoter in wild type strains M145 and J1501

Two wild type strains of *S. coelicolor* (M145 and J1501) were tested to ensure that the *bldB* promoter-*lux* construct in pEM101 is functional in reporting expression from the *bldB* promoter. Consistent with the dependence of antibiotic production and aerial hyphae formation on BldB, it was expected that the *bldB* promoter would be active prior to or coincident with production of the pigmented antibiotics and aerial hyphae. Eight clones of each strain were assayed for luminescence on solid R2YE in 96-well plates over the course of development, and representative results are shown in Figure 10. In M145, maximal signal from the *bldB* promoter occurred at the time of red pigment appearance, at approximately 24 h post-inoculation; aerial hyphae and blue pigment were visible by 48 h, after *bldB* promoter activity had ceased. As expected, there was no significant luminescence from pMU1, and the *hrdB* promoter in phrdBlux was active throughout the life cycle. In J1501, which develops more slowly than M145, red pigment

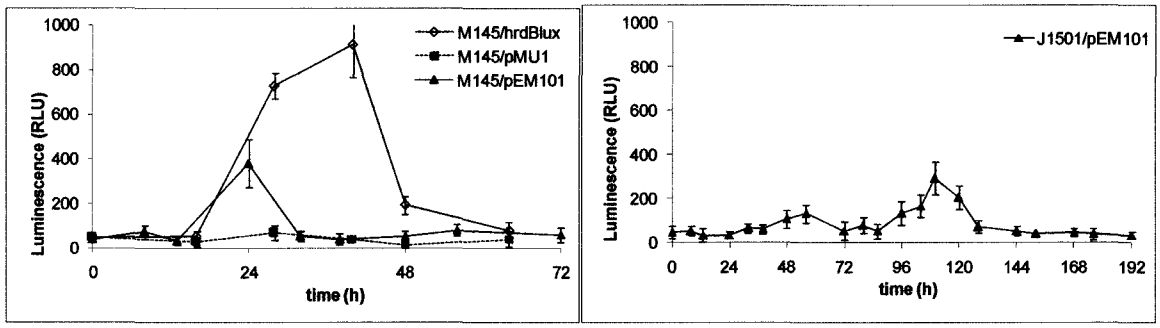

Figure 10 – Luminescence from pMU1, pEM101, and phrdBlux in *S. coelicolor* M145 (A) and pEM101 in J1501 (B) on solid R2YE medium in 96-well plates. Error bars represent the standard deviation of six replicate wells.

was produced by 80h, and aerial hyphae and blue pigment appeared around 144 h, while the *bldB* promoter was active between 100-120 h.

3.2.2 Activity of *bldB* promoter in liquid medium

In liquid YEME medium, maximum activity from the *bldB* promoter was observed at approximately 16 h in M145. There was no significant luminescence from pEM101 in the *bldB* null mutant N985. Three clones of each strain were assayed, and representative results are shown in Figure 11.

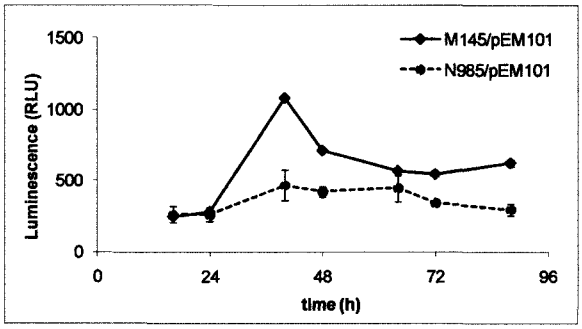

Figure 11 – Luminescence from pEM101 in *S. coelicolor* M145 and N985 in liquid YEME medium. Error bars represent the standard deviation of three replicate aliquots.

3.2.3 Activity of *bldB* promoter in *bld* mutants

An initial screen was conducted to determine which *bld* mutants express *bldB* based on transcription of the *lux* genes from the *bldB* promoter in pEM101. The *bld* mutants tested are in a variety of genetic backgrounds, therefore side-by-side comparison with a matched wild type strain is not possible in many cases. Therefore, deviations in the time or amplitude of maximal luminescence cannot be considered significant. It was necessary to screen eight clones per strain because in some cases multiple clones did not exhibit identical luminescence profiles. For example, six *bldJ*/pEM101 clones peaked at 152 h, with luminescence maxima ranging from 280 to 600 RLU (results not shown for multiple clones). However, out of the remaining two clones, one exhibited two peaks (at 120 and 192 h, with a minimum at 152 h), and one peaked later than the rest (168 h). For all strains, clones representative of the majority are presented here.

The *bldB* promoter was active in all *bld* mutants tested, with the exception of *bldB* (N985), *bldD* and *bldG* (Figure 12). The timing of peak activity varied from 16-24 h in the M145-based strains (*bldK* and *bldM*), to as late as ~150 h in *bldJ* and *bldA*. *bldC* showed an unusually long period of *bldB* promoter activity that may indicate constitutive expression, but this cannot be considered significant without a matched wild type control.

bldB promoter activity in *bldD* and *bldG* mutants was investigated further to confirm that these strains do not express this promoter. A *bldG* null mutant, Δ*bldG* 1DB, was employed because its parent is M145, allowing direct comparison. Unlike in the first *bldG* strain tested, the *bldB* promoter was active in Δ*bldG* 1DB, although deregulated compared to M145 (Figures 10 and 13). Instead of a single peak of *bldB* promoter activity at ~24 h, this strain showed two distinct maxima, at ~40 h and ~90 h.

Unexpectedly, the *hrdB* promoter was dramatically upregulated compared to its activity in M145; in Δ*bldG* 1DB, the maximum was approximately ~25,000 RLU.

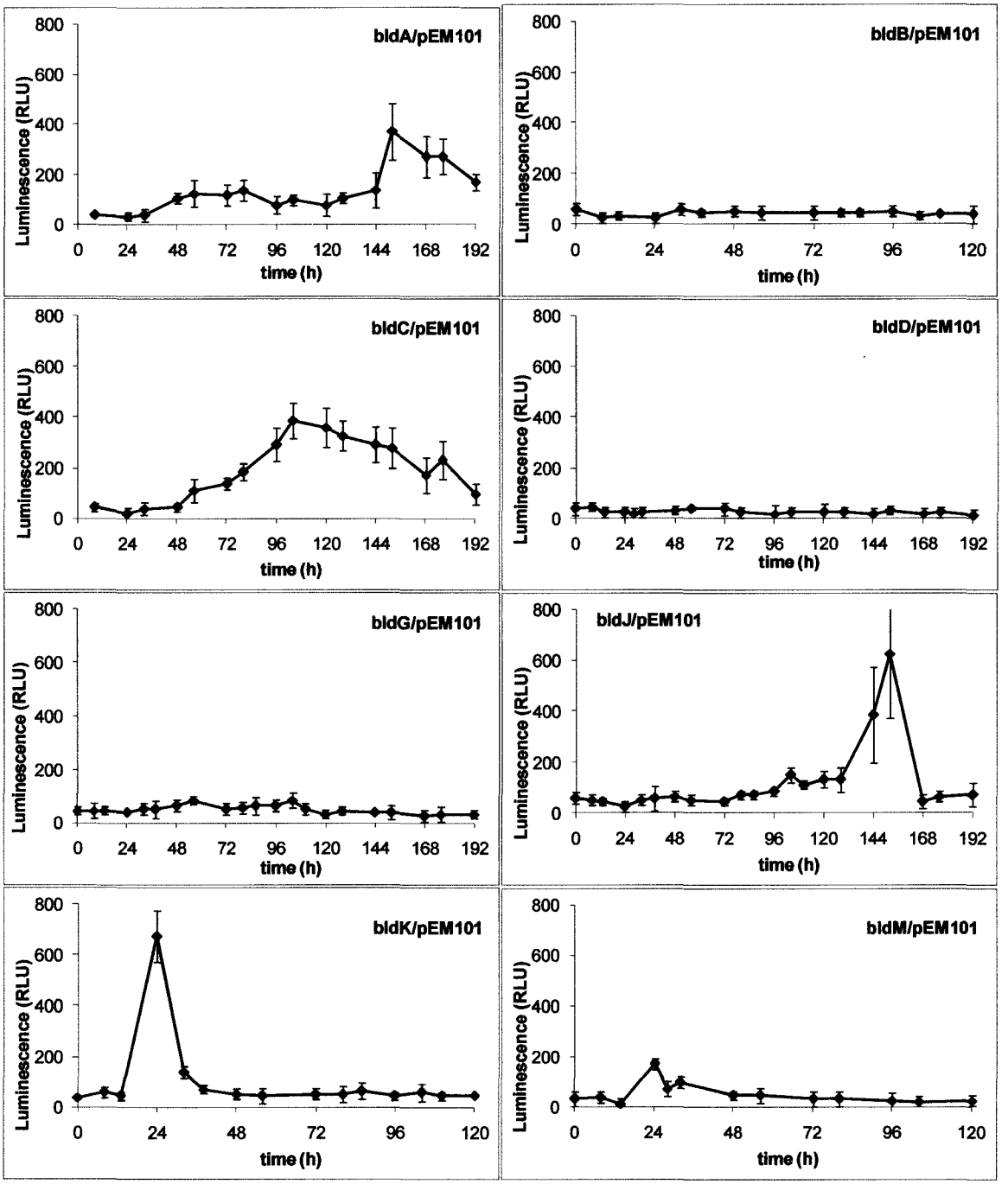

Figure 12 – Activity of the *bldB* promoter-*lux* fusion from pEM101 in *bld* mutants. Error bars represent the standard deviation of six replicate wells.

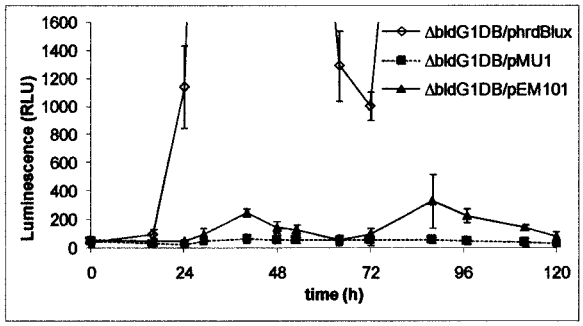

Figure 13 – Luminescence from pMU1, pEM101, and phrdBlux in Δ*bldG* 1DB on solid R2YE medium in 96-well plates. Error bars represent the standard deviation of six replicate wells.

To further investigate *bldB* promoter activity in a *bldD* mutant, a markerless *bldD* null strain, Δ*bldD1*, was used, which is in the M600 background. pMU1, pEM101 and phrdBlux were introduced into both Δ*bldD1* and M600 and assayed for luminescence (results are shown in Figure 14). As in the first *bldD* mutant tested, the *bldB* promoter was inactive in Δ*bldD1*. The *hrdB* promoter was active in both strains. However, there was no detectable luminescence from pEM101 in M600 at any time point in any of the clones tested, in contrast with the other wild type strains M145 and J1501. A second attempt at conjugation of pEM101 into M600 also did not yield any luminescent clones. The assays were repeated with addition of the luciferase substrate decanal to increase the signal (by inverting the 96-well plates over a container containing 1% decanal solution for 10 seconds); however, this did not increase the signal above baseline. To rule out the possibility of luminescence from pEM101 being very brief and possibly occurring between the ~8 to 16h timepoints, another assay was conducted with 2 h timepoints. There was no luminescence detected from pEM101 in M600, N985 or Δ*bldD1*, while M145 and Δ*bldG* 1DB exhibited luminescence at the expected timepoints (data not shown).

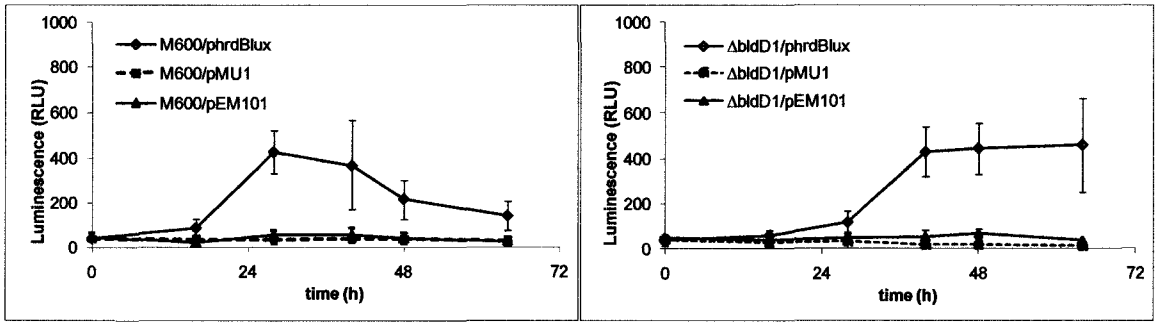

Figure 14 – Luminescence from pMU1, pEM101, and phrdBlux in M600 and Δ*bldD1* on solid R2YE medium in 96-well plates. Error bars represent the standard deviation of six replicate wells.

An attempt was made at constructing a *bldD* deletion mutant in the M145 background using Redirect, to allow direct comparison between a *bldD* mutant and its congenic parent. The gene was successfully replaced with the spectinomycin/ streptomycin resistance cassette on the St9C5 cosmid, and the cassette was subsequently removed by FLP-mediated recombination. However, conjugation of the disruption cosmid into M145 was unsuccessful (no $Spec^RSm^R$ exconjugants were obtained following conjugation of the cosmid into M145) , and the construction of this mutant was not completed.

3.3 Interaction between BldD and the *bldB* promoter

3.3.1 Electrophoretic mobility shift assays for BldD binding to the *bldB* promoter

Direct interaction between BldD and the *bldB* promoter was tested by EMSA because the *bldB* promoter contains two sites bearing similarity to the BldD binding consensus sequence (26). Purification of His-tagged BldD yielded a product of ~20 kDa, which was judged to be sufficiently pure (Figure 15).

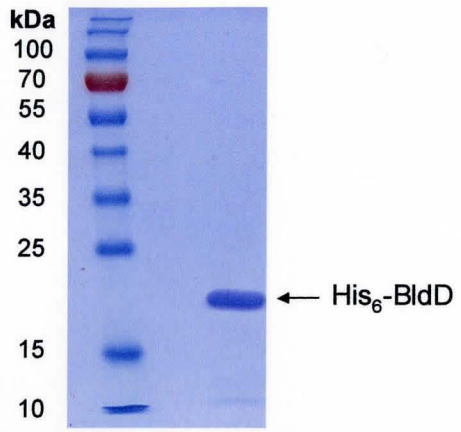

Figure 15 – His-tagged BldD (4.5 μg) purified from pQE9-bldD expressed in *E. coli* JM109, resolved on a 12% SDS-PAGE gel and stained with Coomassie Brilliant Blue R-250.

The EMSA conditions were as described by Elliot et al (26) and were validated by demonstration of specific binding of BldD to the *bldD* promoter, one of its known targets (26, 28). Figure 16 shows that the presence of BldD (0.25 to 1.0 μM) resulted in shift of the radiolabeled *bldD* promoter probe, indicating protein-DNA interaction. Two distinct shifted bands were detected, which could indicate sequential binding to the two half-sites. The interaction is specific, as indicated by the absence of a shift in the presence of 500x unlabelled *bldD* promoter DNA, and the inability of 500x excess non-specific DNA (from the *bldB* coding region) to abolish binding.

Interaction between BldD and the *bldB* promoter was tested under the conditions shown to permit specific binding. The EMSA of BldD binding to each of the three probes is shown in Figure 17. At sufficiently high concentrations (2 to 3 μM), BldD bound each of the three probes. The presence of excess non-specific DNA abolished binding and excess unlabeled probe partly abolished binding, to a lesser extent than excess non-specific DNA. Therefore, binding of BldD to the *bldB* promoter was found to be non-specific.

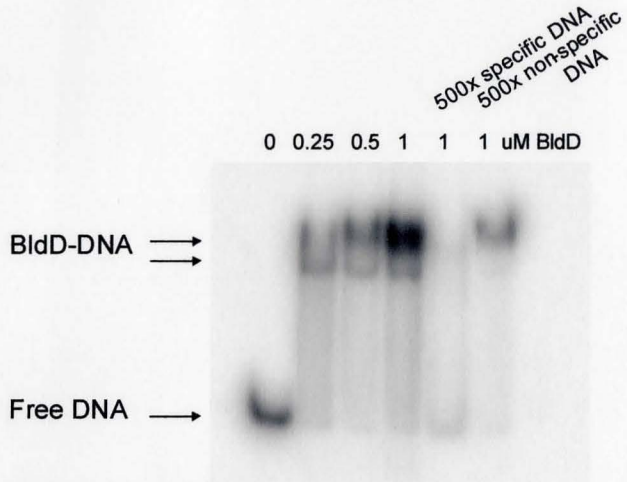

Figure 16 – EMSA of BldD binding to its own promoter. His_6-BldD (0 to 1 μM) was incubated with 1 ng ^{32}P-labeled probe in a buffer consisting of 10 mM Tris-Cl pH 7.8, 150 mM NaCl, 2 mM DTT and 10% glycerol. For competition assays, 500x excess (500 ng) unlabeled probe DNA or unlabeled non-specific DNA also added. The reactions were incubated at 30°C for 20 min and resolved on 8% polyacrylamide gels at 70V in a buffer consisting of 1X TBE with 1.5% glycerol.

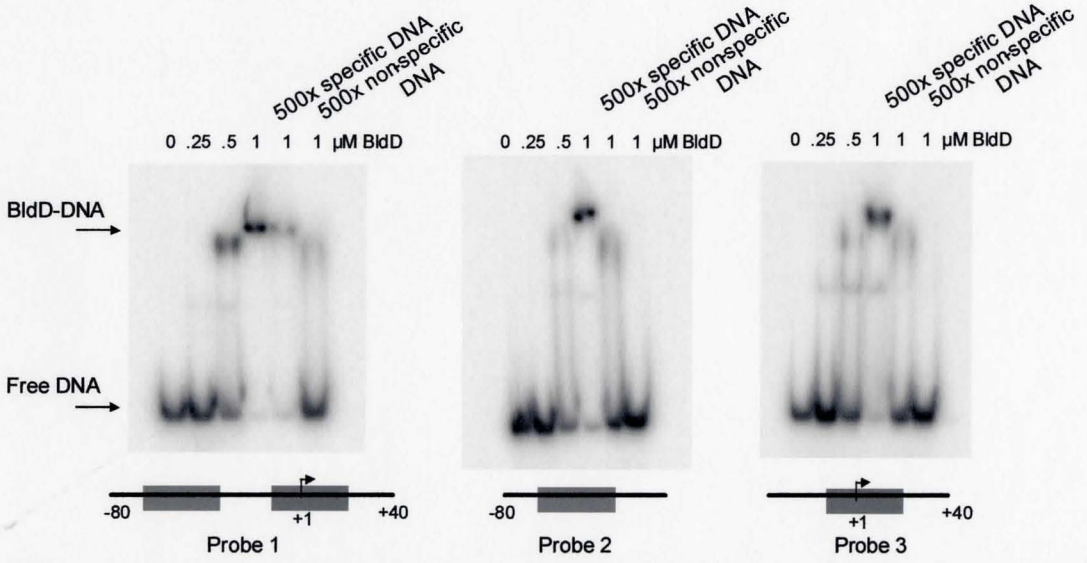

Figure 17 – EMSA of BldD binding to the *bldB* promoter Probes 1, 2 and 3. His_6-BldD (0 to 1 μM) was incubated with 1 ng ^{32}P-labeled probe in a buffer consisting of 10 mM Tris-Cl pH 7.8, 150 mM NaCl, 2 mM DTT and 10% glycerol. For competition assays, 500x excess (500 ng) unlabeled probe DNA or unlabeled non-specific DNA also added. The reactions were incubated at 30°C for 20 min and resolved on 8% polyacrylamide gels at 70V in a buffer consisting of 1X TBE with 1.5% glycerol. A schematic representation of the region corresponding to each probe is shown. Grey boxes represent putative BldD binding sites, and right-angle arrows represent the *bldB* transcription start site. Numbering is with respect to the transcription start site. The putative binding sites are shown in more detail in Figure 4.

4 Discussion

4.1 Role of the *abaA* locus in antibiotic production

It has been reported that *SCO0701* (*abaA-orfB*) pleiotropically regulates antibiotic production in *S. coelicolor*. Fernandez-Moreno et al (33) found that disruption of *SCO0701* by a ϕC31 prophage insertion resulted in decreased production of actinorhodin, undecylprodigiosin and CDA. Therefore, we were interested in the function of each of the genes in this cluster, particularly the *bldB* homologue *SCO0703* (*abaA-orfD*).

Initial observations, based on the phenotypes of the apramycin cassette insertion mutants (*SCO0701::apra-SCO0703::apra*), were that disruption of these genes negatively affects production of the blue-pigmented actinorhodin. Mutants were delayed, but not completely defective in actinorhodin production compared to the wild type, on R2YE and ONA solid media. However, upon removal of the apramycin cassette from these mutants, their phenotypes reverted to wild type pigmentation on all media tested. These results are not completely in agreement with previous observations that the disruption of *SCO0701* significantly reduced blue pigmentation (33). The differences observed may be due to the different strain background (M145 vs. J1501), culture conditions or the variability of media prepared in different labs. The current results indicate that individual deletion of these four genes does not negatively affect production of the pigmented antibiotics in *S. coelicolor* M145.

The reason for the reduction in pigmentation and CDA production in some of the disruption mutants is unknown, but illustrates the need to be cautious when interpreting the phenotypes of mutants generated by insertion of antibiotic resistance cassettes. It is

recommended that complete in-frame deletions be constructed whenever possible. A method has recently been developed for FLP-mediated recombination in *Streptomyces*, and it may significantly facilitate marker removal in the future (32).

While there was no visible effect on actinorhodin or undecylprodigiosin production in the Δ*SCO0701*-Δ*SCO0704* deletion mutants, CDA production was significantly affected in all four mutants. CDA production was reduced in Δ*SCO0701*, consistent with the observations of Fernandez-Moreno et al (33), and also in Δ*SCO0703* and Δ*SCO0704*. The most dramatic effect was seen in Δ*SCO0703*, which produced virtually no CDA after 24 hours growth on ONA+Ca. Deletion of *SCO0702* had the opposite effect – CDA production was elevated in comparison with M145. It will be necessary to complement these mutants by re-introducing the respective genes, in order to show that the effect on CDA production is specific to the deleted genes.

The effect of overexpression of *SCO0701* and *SCO0703* on CDA production was inconclusive because variable zone sizes were observed in the CDA assay, and a trend was not apparent. The variability may arise from instability of pWHM3 in the absence of selection (the CDA assay is performed on ONA media without added antibiotics). In the future it will be worthwhile to attempt overexpression on other high-copy plasmids, or place the genes under the control of a constitutive promoter such as *ermE* on an integrating vector. Overexpression of *SCO0703* in related streptomycetes may be significant for increasing industrial production of antibiotics or the discovery of novel antibiotics.

Observations of the production of the pigmented antibiotics and CDA in the four *abaA* deletion mutants indicate that, under the conditions tested, this cluster regulates CDA production in *S. coelicolor*. *SCO0702* appears to be a negative regulator, while *SCO0701*, *SCO0703* and *SCO0704* are likely positive regulators. Future research will focus on understanding the mechanism by which these genes and their products act to regulate CDA production. Since the loss of *SCO0703* has the most severe effect on CDA production, this gene product may be a direct activator of *cdaR*, the pathway-specific regulator for CDA biosynthesis. Alternatively, the negative regulator encoded by *SCO0702* may be a repressor of *cdaR*. Further, the activity of a *cdaR* transcription factor may be regulated by one or more genes in the *abaA* cluster. It is also possible that some of the *abaA* gene products regulate the expression or activity of other genes in the cluster. It will be necessary to identify any protein-protein and protein-DNA interactions between the *abaA* gene products, their promoters, and other CDA regulators.

4.2 Expression of *bldB* in *S. coelicolor* wild type and *bld* mutants

Consistent with the dependence of antibiotic production and aerial hyphae formation on BldB, it was found that *bldB* promoter activity was coincident with the initiation of development in both wild type strains M145 and J1501. The *bldB* promoter is turned on for a very short period of time at the start of morphological differentiation, and likely functions as a switch to activate other pathways leading to secondary metabolite production and morphogenesis. At this time the mechanism of BldB function is unknown.

The *bldB* promoter was not active in the *bldB* null mutant N985. This result is not in agreement with previously published data, which indicated that *bldB* promoter expression is upregulated in a *bldB* mutant (56). This observation was based on the activity of a *bldB* promoter/*xylE* reporter fusion in the *bldB15* mutant, which contains a single amino acid substitution at a highly conserved tyrosine residue (Y21L). It is known that a Y21A mutation in BldB permits dimerization, but alters protein function; a Y21A mutant allele cannot restore sporulation to a *bldB* null mutant, but causes hypersporulation when overexpressed (24). Upregulation of the *bldB* promoter in a Y21 mutant may occur if this protein retains the ability to regulate expression from its promoter, either through interaction with another protein, or direct DNA binding. According to this model, the promoter in the N985 null mutant would not be active due to complete lack of BldB protein. This hypothesis should be tested experimentally before we draw any conclusions on auto-regulation of *bldB*.

The *bldB* promoter was active in most *bld* mutants tested, including *bldA*, *bldC*, *bldG*, *bldJ*, *bldK* and *bldM*, suggesting that the developmental block in these mutants is downstream of the action of *bldB*. This observation, together with the observation that the *bld* phenotype cannot be rescued under any growth conditions, shows that BldB functions very early in the sequence of checkpoints controlling the initiation of morphogenesis and secondary metabolism.

In the *bldC* mutant, the *bldB* promoter was on for most of the time course, in contrast with the sharp peaks observed in the wild type and other mutants. This may indicate that *bldB* expression is not turned off in a *bldC* mutant, an event that may

normally be mediated, directly or indirectly, by *bldC*. The phenotype of *bldC* is also bald and unpigmented, similar to *bldB*. Overexpression of *bldB* has been shown to block sporulation, but allow aerial hyphae formation (24), therefore the phenotype of *bldC* is likely not due to upregulation of *bldB* caused by loss of *bldC*. It is likely that the *bldC* mutation has wide-ranging effects on gene expression, including an indirect effect on *bldB* expression.

The *bldB* promoter appeared to be inactive in the *bldG* mutant C103, but was subsequently shown to be expressed in the *bldG* null mutant strain Δ*bldG* 1DB. The absence of detectable luminescence in *bldG* C103 may have been due to low signal rather than complete loss of *bldB* expression. However, in Δ*bldG* 1DB the *bldB* promoter was deregulated in comparison with its congenic parent M145, as indicated by late and prolonged expression (40-96 h in Δ*bldG* 1DB). BldG is a putative anti-anti-sigma factor, and it is proposed that its cognate sigma factor may be involved in *bldB* expression, or expression of other proteins regulating *bldB*. The *bldG* mutation, similar to *bldC*, likely has an indirect effect on *bldB* expression, and this observation also illustrates that there is a widespread complex deregulation of *bld* genes in *bld* mutants.

The only *bld* mutant that failed to show luminescence directed by the *bldB* promoter was *bldD*. This was true for both *bldD* mutants tested (strain HU66 containing the *bldD53* allele, and the null mutant Δ*bldD1*). It is a concern that M600, the parent strain of Δ*bldD1*, also failed to show *bldB* promoter activity. Therefore, it cannot be concluded with certainty that *bldB* is not expressed in *bldD* mutants. The high-GC *lux* operon used as the reporter may not be sensitive enough to low levels of transcription.

Nevertheless, the possibility of *bldB* regulation by BldD was investigated further by testing the ability of BldD to directly bind the *bldB* promoter.

4.3 Interaction between BldD and the *bldB* promoter

Preliminary results using the *bldB* luminescent reporter indicated that the *bldB* promoter is not expressed in a *bldD* mutant, and therefore BldD may be required for *bldB* expression. Analysis of the *bldB* promoter region revealed two possible BldD recognition sites (Figure 18), suggesting that BldD may activate expression by direct binding to this region. Activation of the erythromycin biosynthetic genes by the BldD orthologue of *Saccharopolyspora erythrea* has been reported to be due to binding at a site overlapping the transcription start site (18). It is also interesting to note that two of the original *bldB* mutations (*bldB28* and *bldB17*) altered nucleotides in the AGTAA half site that we propose to bind BldD (56). Therefore, this hypothesis was tested by EMSA to determine whether purified BldD can bind the *bldB* promoter.

EMSA results indicated that while BldD can bind the *bldB* promoter at high protein concentrations (>1 μM), this interaction is not specific. Therefore, if BldD does regulate *bldB* expression, it must do so indirectly. It is also possible that specific binding requires a ligand, which was not present in the in vitro assay.

-80
AGTTCGGGGGCAGGGGGCCGGGCGTCAGTGGCCGTCGGCTGCTTCCGCCACCGAGGCAGGG
+1 +40
GCAGGGCCAGTAAGGTTGTCGGGGATCGTTGGTAAGCCGCCTGAAAGGACACCTCCGCCATG

Figure 18 – Sequence of the *bldB* promoter, showing the transcription start point +1 (56) and possible BldD binding sites (highlighted). The consensus sequence for BldD recognition is AGTgA $(n)_m$ TCACc (26).

The most likely mode of indirect regulation of *bldB* is through a sigma factor. It is known that BldD negatively regulates several sigma factors, including σ^{BldN}, σ^{WhiG} and σ^{H} by directly binding their promoters and repressing transcription (26). σ^{BldN} is implicated in the control of chaplin expression on some media (30), σ^{WhiG} in regulation of the sporulation genes *whiH* and *whiI* (14), and σ^{H} in sporulation and stress response (49). None of these sigma factors are likely candidates for regulation of *bldB* because they all function later in development.

The absence of *bldB* expression in *bldD* mutants suggests that BldD is an activator of *bldB*. If there is a BldD-controlled sigma factor that in turn activates *bldB* transcription, then BldD would have to be an activator of this sigma factor (this model is shown schematically in Figure 19A). While BldD acts as a repressor of all targets published to date, there is evidence that it can activate at least one target, *sigQ* (Marie Elliot, personal communication). There is evidence that BldD undergoes developmentally regulated proteolysis (47), which may be responsible for activating or inactivating it, or switching between repressor and activator functions. Therefore, it is possible that under some conditions BldD activates a sigma factor which in turn activates BldB, either directly or indirectly. Another possible model for regulation of *bldB* by BldD that

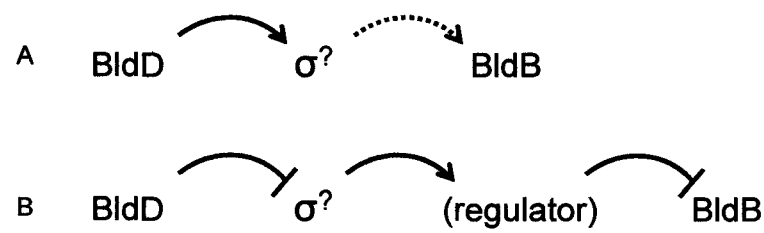

Figure 19 – Two possible models for activation of BldB by BldD. BldD may activate a sigma factor that is directly or indirectly required for *bldB* transcription (A), or it may repress a sigma factor that activates a negative regulator of BldB (B).

involves the more common role of BldD as a sigma factor repressor is shown in Figure 19B. In this model, the sigma factor may be required for expression of a *bldB* repressor protein or antagonist.

Identification of the sigma factor regulating *bldB* is not a trivial task in an organism as complex as *S. coelicolor*, which encodes 65 sigma factors, the greatest number found in any sequenced genome (3). Most sigma factors have not been characterized, and the *bldB* promoter does not contain known sigma factor binding consensus sequences (in comparison with consensus sequences reported in references (40, 49, 65, 69). Elucidation of the mechanism of *bldB* regulation by BldD, identification of the sigma factor regulating *bldB*, and identification of BldB-binding proteins are all targets for future research.

5 Conclusions

The purpose of this study was to clarify the role of the *abaA* cluster, particularly the *bldB* homologue, in antibiotic production in *S. coelicolor*, and to discover regulatory relationships between *bldB* and other *bld* genes by probing *bldB* promoter activity in *bld* mutants.

Analysis of null mutants in each of the four genes in the *abaA* cluster revealed that, under the conditions tested, this locus regulates production of CDA, but not actinorhodin and undecylprodigiosin. *SCO0701*, *SCO0703* and *SCO0704* were found to positively regulate CDA production. Loss of *SCO0702* increased CDA production, and it is proposed that this gene product is a negative regulator of CDA. A consequent goal is

to study interactions between the *abaA* genes and their products and elucidate the mechanism of CDA regulation by the *abaA* cluster. The possibility of increasing antibiotic production in other streptomycetes by overexpression of *SCO0703* may also be explored.

The study of *bldB* expression in *bld* mutants showed that *bldB* is expressed in most *bld* mutants, with the exception of *bldD*, and likely deregulated in *bldC* and *bldG* mutants. There is also evidence suggesting auto-regulation of *bldB* expression. Since *bldB* is active in almost all *bld* mutants studied, it is likely that it functions very early in the initiation of development. The dependence of *bldB* expression on BldD was tested by EMSA to detect direct interaction between BldD and possible recognition sequences in the *bldB* promoter. Specific interaction between BldD and the *bldB* promoter was not detected, and we conclude that BldD may regulate *bldB* indirectly. The mechanism of regulation is likely through an unidentified sigma factor.

Future directions arising from this study include clarifying the mechanism of *bldB* regulation by BldD, and elucidating the mechanism of CDA regulation by the *abaA* cluster. This study contributes to our understanding of the regulation of development and antibiotic production in the model streptomycete *S. coelicolor*.

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Appendix I – Construction and expression of a Tandem Affinity Purification (TAP) tag in *S. coelicolor*

Tandem Affinity Purification

Tandem affinity purification (TAP) is a technique for studying protein-protein interactions by purifying and analyzing complexes of a target protein associated with unknown interacting proteins (60). The bait protein is purified by exploiting the high affinity and high specificity properties of a tag fused to it. Under gentle purification conditions, native proteins interacting with the bait are co-purified and can be subsequently identified. The TAP tag of interest in this work consists of streptavidin and calmodulin binding peptide (SBP and CBP) domains separated by a cleavage site for tobacco etch virus (TEV) protease [Figure 20A; (1)]. A hemagglutinin (HA) epitope is also present to permit detection of the tag by Western blot. Purification of target complexes from complex mixtures is achieved by first binding to streptavidin resin, eluting specifically bound material using TEV protease, then purifying further on calmodulin resin in the presence of calcium, and finally eluting the purified proteins by chelation of the calcium using EGTA (Figure 20B). Advantages of this method include its widespread applicability, the high degree of purification and specificity achieved by the two-step affinity purification, and preservation of native protein structure and interactions under the mild conditions of binding and elution. The high degree of purification afforded by this method allows for purification of proteins present in very low abundance in the cell, and therefore provides the option of expressing the protein at or near physiological levels in order to preserve natural protein interactions (63).

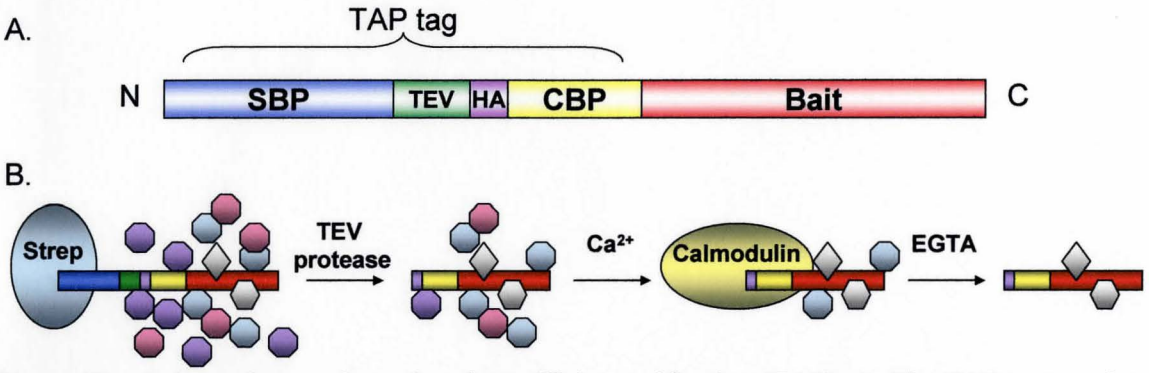

Figure 20 – Schematic overview of tandem affinity purification (TAP). A. The TAP tag consists of SBP and CBP domains separated by a TEV protease cleavage site and HA epitope. B. The bait protein (red) is expressed as a fusion with the TAP tag. The first step in purification involves binding to streptavidin. Elution from streptavidin is achieved by cleavage with TEV protease. Eluted material is passed over calmodulin beads, and specific binding is promoted by the presence of calcium. EGTA is added to release bound proteins. The final eluted fractions contain the bait protein and proteins that directly or indirectly interact with it, which are then fractionated by SDS-PAGE and identified by mass spectrometry. The grey diamond and hexagon represent specifically bound proteins, and coloured octagons are components of the crude cell lysate. Adapted from (1).

Construction of TAP vector

The TAP tag was amplified from the vector pGLUE (provided by R.T. Moon) using Vent DNA polymerase (New England Biolabs) and primers TAPtag1 (5'TTAAGCCCATATGGACGAGAAGACCACC) and TAPtag2 (5'ACGCGGCTCTAG ATATCTGTACAGG), which introduced NdeI and XbaI restriction sites, respectively. The blunt-ended PCR product was cloned into pBlueScript II SK+ digested with EcoRV to generate plasmid pEM1, and the construct was sequenced to ensure fidelity. The TAP tag (311 bp) was subcloned into the NdeI and XbaI sites of pIJ6902 [an integrating *S. coelicolor* vector with a thiostrepton inducible promoter upstream of the MCS (38)], to generate plasmid pEM5 (Figure 21).

A TAP-BldB vector (pEM6) was constructed by cloning *bldB* into the KpnI and EcoRI sites of pEM5. The *bldB* gene was amplified from the chromosome of *S.*

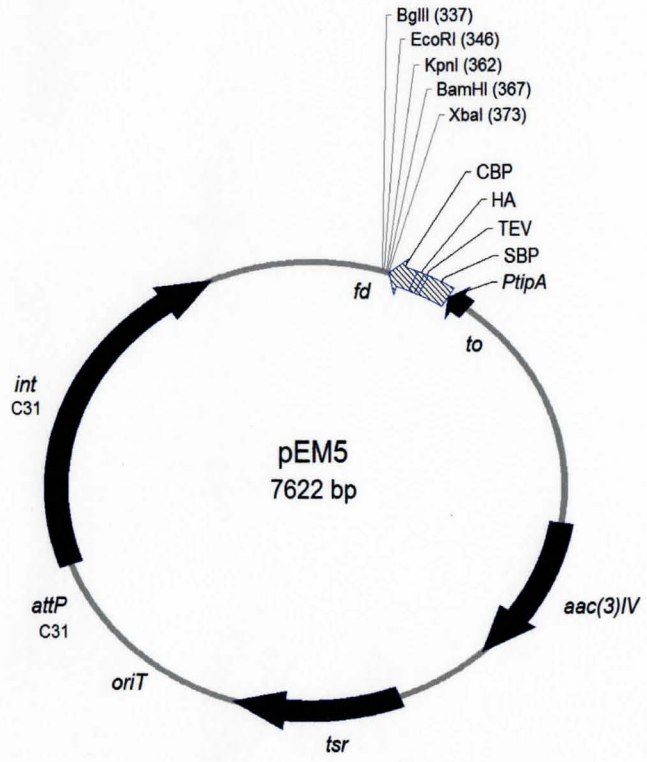

Figure 21 – Plasmid pEM5 for expression of TAP-tagged proteins in *S. coelicolor*. fd – transcription terminator, CBP – calmodulin binding peptide, HA – hemagglutinin epitope, TEV – Tobacco Etch Virus protease cleavage site, SBP – streptavidin binding peptide, P*tipA* – thiostrepton inducible promoter, *aac(3)IV* – aminoglycoside acetyltransferase gene for apramycin resistance, *tsr* – thiostrepton resistance, *oriT* – origin of transfer RK2, *attP* and *int* ϕC31 – attachment site and integrase gene of ϕC31.

coelicolor M145 using primers bldB-KpnF (ACACCGGTACCATGGCCCAGGTG) and bldB-EcoR (5'CGAGCAGAATTCTCACTCGGCGACG), cloned into the EcoRV site of pBlueScript II SK+, sequenced, and subcloned into pEM5 to generate pEM6.

Expression of TAP-tagged BldB in *S. coelicolor*

pEM5 and pEM6 were introduced into *S. coelicolor* strains M145 (wild type) and N985 (*bldB::aphI*) by conjugation from *E. coli* ET12567/pUZ8002, and exconjugants

were selected with apramycin and thiostrepton. Phenotypes of all eight strains on R2YE and R2YE+thiostrepton are shown in Figure 22.

Introduction of pIJ6902 negatively affected development in both M145 and N985. M145/pIJ6902 developed a dense aerial mycelium, but did not sporulate as profusely as M145, indicated by significantly less grey pigmentation. N985 developed some pigmentation upon extended growth on R2YE, but introduction of pIJ6902 completely eliminated all pigmentation in this strain. M145/pEM5 and M145/pEM6 did not differ significantly from M145/pIJ6902 either in the absence or presence of thiostrepton

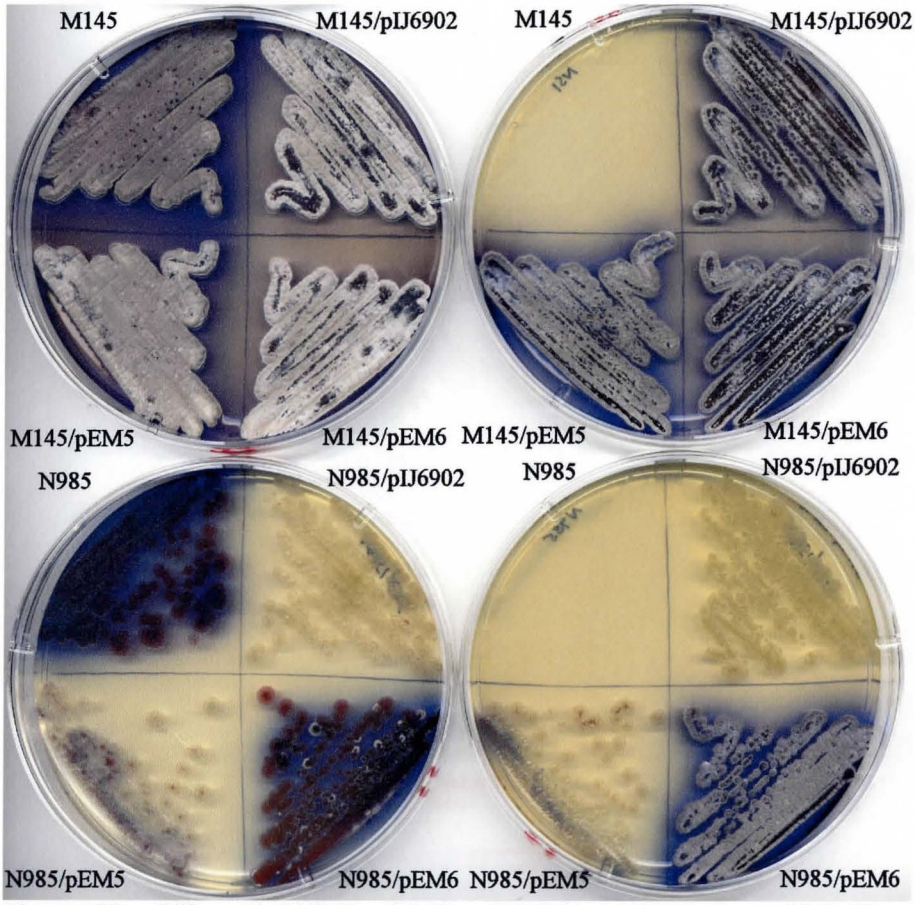

Figure 22 – Effect of pIJ6902, pEM5 and pEM6 on phenotypes of M145 and N985 after 4 days. Top and bottom left: R2YE. Top and bottom right: R2YE+thiostrepton. All images are of the tops of the plates.

induction. However, in N985, thiostrepton induction of the TAP-BldB construct in pEM6 resulted in sporulation at a level above what was observed in M145/pEM6. There was some complementation of the *bldB* mutation in N985 even in the absence of thiostrepton, likely due to leaky transcription from the *tipA* promoter.

The TAP-BldB construct is functional in restoring development in *S. coelicolor*. The increased level of sporulation in N985/pEM6 is reminiscent of the hypersporulating phenotype caused by overexpression of some *bldB* mutant alleles [Y21A, F75A; (23)]. It is possible that the tagged BldB protein, while capable of restoring morphogenesis, has a slightly altered physiological function.

Attempts to purify TAP-BldB using published methods (1, 58, 63) from cells grown on R2YE overlaid with cellophane were unsuccessful. However, since it has been shown that TAP-tagged proteins can be functional in *S. coelicolor* when expressed from pEM5, it would be worthwhile to attempt purification of other TAP-tagged protein complexes.