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Characterization of BldD and its targets in *Streptomyces coelicolor*

by

Marie Alaine Elliot 

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

Microbiology and Biotechnology

Department of Biological Sciences

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Abstract

Streptomyces are filamentous, spore-forming bacteria renowned for their morphological complexity and their ability to produce a vast array of commercially valuable secondary metabolites. A class of genes, termed the *bld* genes, that appears to regulate morphological differentiation, and in most cases antibiotic production, has been identified in *Streptomyces coelicolor*. The *bldD* mutant phenotype was shown to be due to a single point mutation within the *bldD* coding sequence, changing a tyrosine residue to a cysteine residue. This mutation adversely affects *bldD* transcription, as northern blot analysis and S1 nuclease protection assays showed constitutive *bldD* overexpression in the *bldD* mutant strain. This potential negative autoregulation by BldD was examined using BldD fusion proteins purified from *E. coli*. Gel mobility shift, DNaseI footprinting, and hydroxyl radical footprinting assays showed that BldD binds within its own promoter region, recognizing an inverted repeat overlapping the –10 promoter element.

Additional targets of BldD binding were identified through a variety of means: sequence scanning of regulatory genes involved in morphological and metabolic differentiation; gel mobility shift-based selection assays using chromosomal libraries; and personal communications from others working in this area. Among the targets identified were three different sigma factors: *bldN*, required for the erection of aerial hyphae; *whiG*, needed in the initiation of sporulation; and *sigI*, involved in the response to environmental stress. *ftsZ*, a cell-division gene needed for the subdivision of aerial hyphae into spores, was also found to be a possible target, as were two uncharacterized open reading frames, ORF 25.c and ORF 26.c, on the SCE68 cosmid. BldD binding to each of these genes was examined by gel mobility shift assays and DNaseI footprinting

assays (except for *ftsZ*), and permitted the determination of a consensus BldD binding sequence: an imperfect inverted repeat AGtgA (n) tcACC, separated by a variable spacer. The *in vivo* effect of BldD binding to each of these targets was also assessed using S1 nuclease protection or primer extension assays. It appears that BldD represses *bldD* and *bldN* expression, and acts as a dual function regulator of *whiG*, and ORF 26.c-25.c. Potential mechanisms of BldD regulation are discussed.

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List of Abbreviations

α	Alpha or anti (when referring to antibodies, like α-BldD or sigma factor antagonistic proteins, like α-sigma factor)
Δ	Deletion or delta (i.e. change in)
γ	Gamma
λ	Lambda bacteriophage
σ	Sigma factor
A	Adenine
Ab	Antibody
ABC	ATP-binding cassette
ATP	Adenosine triphosphate
bp	Base pair
BSA	Bovine serum albumin
C	Cytosine
C-	Carboxyl
cAMP	cyclic adenosine monophosphate
CDA	Calcium dependent antibiotic
cpm	Counts per minute
Da	Dalton
ddNTP	Dideoxynucleoside triphosphate
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
DNase	Dexoyribonuclease
dNTP	Deoxynucleoside triphosphate
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
emsa	Electrophoretic mobility shift assay
EtBr	Ethidium bromide
FPLC	Fast protein liquid chromatography
G	Guanine
GTP	Guanosine triphosphate
GST	Glutathione S-transferase
HPLC	High performance liquid chromatography
HTH	helix-turn-helix
IPTG	Isopropyl β-D-thiogalactopyranoside

kb	kilobase
K_d	Dissociation constant
kDa	kiloDalton
LB	Luria Bertani medium
LLP	LysR-like proteins
mRNA	Messenger RNA
N-	Amino
nt	Nucleotide
ORF	Open reading frame
P buffer	Protoplast buffer
PAG	Polyacrylamide gel
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
R2YE	Sucrose yeast-extract medium
RNA	Ribonucleic acid
RNase	Ribonuclease
rRNA	Ribosomal RNA
Sap	Spore associated protein
SDS	Sodium dodecyl sulfate
SELEX	Systematic evolution of ligands by exponential enrichment
SSC	Standard saline citrate
T	Thiamine
T_d	Denaturation temperature
T_m	Melting temperature
T buffer	Transformation buffer
TAE	Tris-acetate-EDTA buffer
TBE	Tris-borate-EDTA buffer
TCA	Trichloroacetic acid
TBP	TATA-binding protein
TE	Tris-EDTA buffer
tRNA	transfer RNA
TSB	Trypticase soy broth
tsp	Transcription start point
tsr	Thiostrepton resistance gene

U	Unit (measure of enzyme activity)
UV	Ultraviolet
v/v	volume/volume
w/v	weight/volume
w/w	weight/weight
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside
xylE	Catechol dioxygenase gene
YEME	Yeast-extract malt-extract medium
YT	Yeast-extract tryptone medium

Chapter 1

Introduction

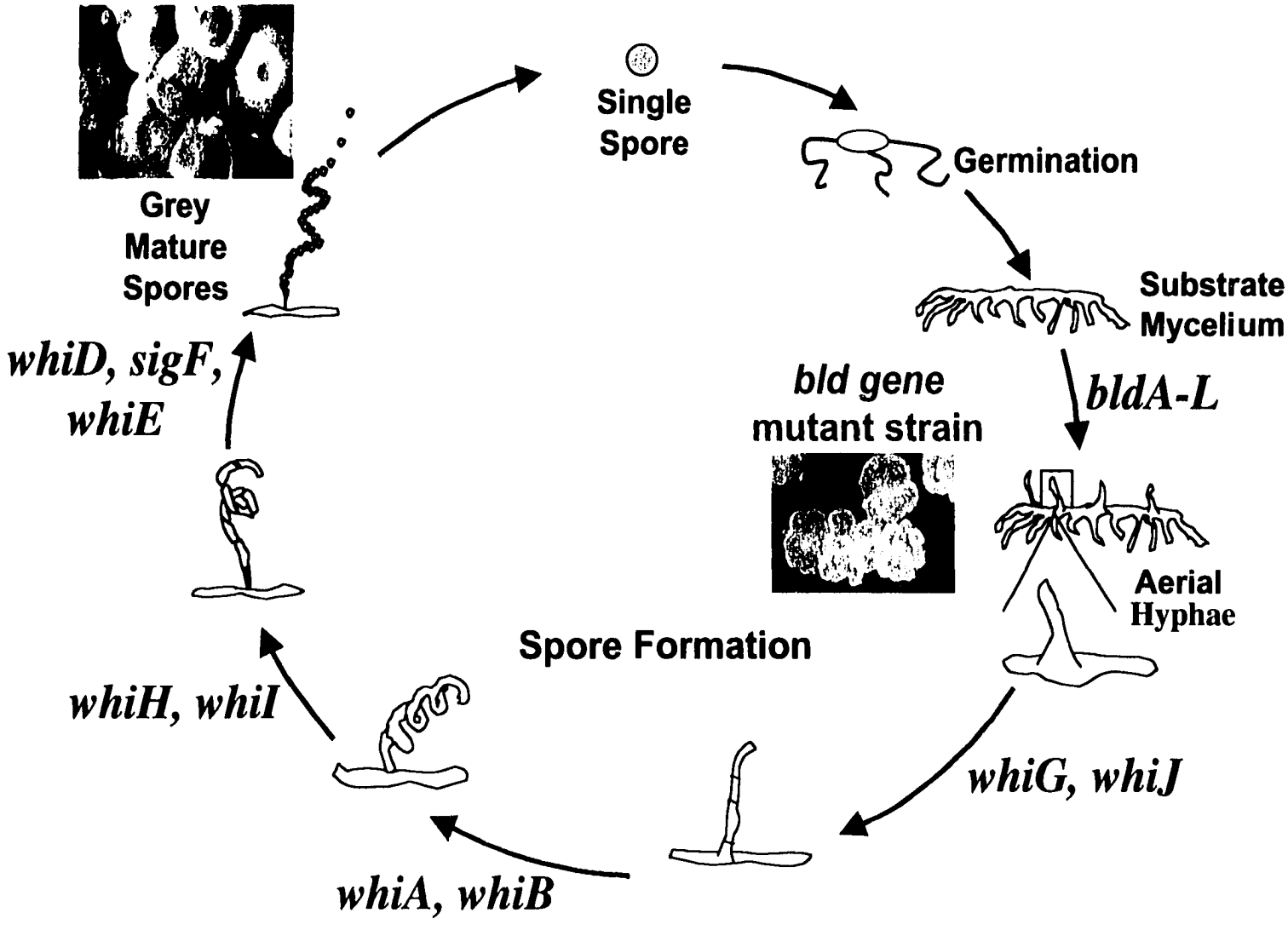
The actinomycetes are Gram positive organisms, defined by their ability to produce extensively branched substrate mycelium, as well as abundant secondary/aerial mycelium (Korn-Wendisch and Kutzner, 1992). Within the actinomycete family, are the streptomycetes, a soil-dwelling genus renowned for their morphological complexity and metabolic variability. They are well adapted for survival in the harsh soil environment: their mycelial growth habit is well suited for attachment to a variety of nutritional substrates; they possess a number of degradative capabilities, allowing them to successfully break down plant and animal material, such as polysaccharides, protein, and aromatic compounds, into useable forms; they are capable of surviving environmental extremes by forming spores that are resistant to frost, drought, pressure, and anaerobic conditions resulting from water saturation; and finally, they have acquired a diverse arsenal of weapons against other microbial competitors, in the form of antibiotics and a variety of other secondary metabolites (Korn-Wendisch and Kutzner, 1992).

The ability of the streptomycetes to produce secondary metabolites, and to form spores, has piqued the interest of scientists world-wide. The synthetic capabilities of these natural chemists are of great interest, and in many cases, have been appropriated by the pharmaceutical industry, to generate approximately $14 billion US annually in revenue, from the sale of the resulting antibiotics, immunosuppressants, herbicides, and anti-cancer agents. At the other end of the spectrum, studies into the formation of *Streptomyces* spores, and the differentiation process leading up to it, have revealed complex regulatory requirements, involving the integration of signals from a multitude of sources. An understanding of this differentiation process, and its connection to secondary metabolism, will allow for the manipulation of growth, thereby maximizing the

efficiency of secondary metabolite production. It will also help to further the understanding of regulation in the microbial world, possibly bridging the evolutionary distance between regulation in prokaryotes, and that in eukaryotes.

The *Streptomyces* growth cycle (Fig. 1.1) begins with a spore, which germinates under the appropriate nutritional conditions. The resulting mycelial outgrowth branches to form a mat of vegetative hyphae, with very few crosswalls. An unknown signal, believed to be an environmental stress of some sort, induces the streptomycete survival mode. The initial preparation for spore formation involves the erection of aerial hyphae, which coincides with the production of antibiotics, likely representing an attempt to eliminate potential competitors during this time of stress. At this point, *Streptomyces* are also seen to accumulate carbon and energy stores, in the form of glycogen and trehalose granules. It is believed that some of the glycogen stores are hydrolyzed to provide the turgor required to force the secondary hyphae into the air, and they may also provide some of the nutrients necessary for the growth of aerial hyphae, and/or production of secondary metabolites (Plaskitt and Chater, 1995). Additional nutrients required for these processes are likely provided by the substrate mycelium, which are lysed for this purpose. The erection of the aerial hyphae is followed by a coiling of the hyphae and a synchronous septation event, whereby the hyphae are uniformly divided into unigenomic compartments. The prespore compartmental walls then thicken and a second set of glycogen stores are hydrolyzed, both to permit the rounding out of the spores, and to provide the precursors required for the production of the grey spore pigment (in *S. coelicolor*). The final, mature spores are thick walled, to withstand environmental

Fig. 1.1 *Streptomyces coelicolor* life cycle. Genes controlling specific stages of development are indicated.



stresses, rounded, and dark grey in colour, as a result of the grey pigmented polyketide compound.

The regulatory process that leads to these final mature spores is slowly being elucidated. While mutations in the genes involved in the sporulation process have been found to block spore development at various stages, these mutant strains share one major phenotypic commonality: lack of the final grey spore pigment, resulting in white-coloured hyphae. The genes responsible for this process have, as a result, been termed the "white", or "*whi*" genes. Thirteen different *whi* genes have been identified, and have been designated *whiA,B,D,E, G-O,* and these are grouped together with *sigF*, a *whi* gene named for its gene product rather than its mutant phenotypic characteristics (Potúcková *et al.*, 1995).

The different stages at which spore development was halted in each of the *whi* mutants was used to determine a hierarchy of *whi* gene expression: *whiG, whiJ* < *whiA, whiB* < *whiH* < *whiI* < *whiD, sigF, whiE* (Chater, 1998). The initiation of sporulation is apparently dependent upon *whiG* and *whiJ*, for mutations in these genes result in an inability to move beyond the erection of aerial hyphae: the hyphae appear straight and white, with neither coiling nor septation evident. Mutations in *whiA* and *whiB* halt spore development at the next stage, where hyphal coiling has initiated, but septum formation has not yet begun. Sporulation septae begin to appear in the *whiH* mutant strains, and are abundant in the *whiI* mutant strains. The final maturation of the spores appears to be controlled by *whiD, sigF* and *whiE. whiD* and *sigF* mutants are both capable of forming spores, albeit abnormal ones, with unusual spore wall characteristics, while *whiE* mutants produce normal spores that are deficient only in the production of the final spore

pigment. The most recently identified *whi* genes, *whiK, L, M, N,* and *O*, were found, when mutant, to exhibit significant phenotypic variation, making it difficult to assign them a defined position in the developmental progression (Ryding *et al.*, 1999).

Of the two earliest acting *whi* genes, *whiG* and *whiJ*, *whiJ* has yet to be cloned, and therefore the nature of its gene product remains to be elucidated. Phenotypically, *whiJ* mutants differ from *whiG* mutants in that they produce small amounts of the grey spore pigment, imparting a light grey colouring to the colonies (Chater, 1972), while *whiG* mutant colonies show no indication of grey pigment whatsoever. *whiG* has been well studied, and appears to encode a sigma factor, highly homologous to the motility sigma factor (σ^{28}) from *Bacillus subtilis*, rather than any of the *B. subtilis* sporulation sigma factors, as would be expected (Chater *et al.*, 1989). This may indicate that sporulation in *Streptomyces* represents not only an adaptation to survive unfavorable conditions, but also a means of moving to a more favorable environment, which is accomplished in other microbes by way of flagellar and cilial motility. It appears that the *whiG* sigma factor is not required for the germination of spores, growth of vegetative mycelium, or erection of aerial hyphae, as none of these processes are impaired in *whiG* mutant strains; however, it is believed that the amount of *whiG* product is the limiting factor in sporulation initiation. Not only does the *whiG* mutant phenotype reveal an early role for *whiG*, but when *whiG* is placed on a high copy number plasmid, hypersporulation and sporulation in the vegetative hyphae are observed. Surprisingly, high copy number *whiG* also affects secondary metabolite production, as it appears to suppress production of the antibiotic actinorhodin. This could be due to an increased number of hyphae that are dedicated to sporulation rather than secondary metabolite production, or could reflect

a decreased availability of precursors for metabolite production. Examination of *whiG* expression unexpectedly revealed the *whiG* transcript to be present at nearly constant levels throughout all stages of colony growth; it had been expected to be transcriptionally regulated, appearing when sporulation was required (Kelemen *et al.*, 1996). It is now thought that the *whiG* product is post-translationally regulated, likely by an anti-sigma factor, as is the case for σ^{FliA} (Ohnishi *et al.*, 1992), a sigma factor from *Salmonella typhimurium* bearing a high degree of homology to σ^{WhiG} (Tan and Chater, 1993). To better understand the role of *whiG* in sporulation, its relationship to other early *whi* genes was examined, through the generation of double *whi* mutants. It was found that the *whiG* mutation was epistatic to those of *whiA*, *whiB*, *whiH*, and *whiI*, suggesting that *whiG* may be involved in the expression of each of these genes (Chater, 1975).

Investigation of *whiH* began with the cloning and sequencing of the *whiH* gene. It was found to encode a regulatory protein, bearing strong similarity to a family of repressors that are responsive to carboxylate-containing intermediates in carbon metabolism (Ryding *et al.*, 1998). No direct targets of WhiH have yet been determined. The epistatic relationship between *whiG* and *whiH* was examined through transcriptional studies, where σ^{WhiG} was observed to direct the transcription of the *whiH* gene. A similar situation was observed for *whiI*, whose transcription was also directed by σ^{WhiG}. *whiI* specified another regulatory protein, an atypical member of the response regulator family involved in two-component signal transduction (Aínsa *et al.*, 1999). Typically, response regulators are paired in their chromosomal location with a sensor kinase, responsible for modulation of response regulator activity by phosphorylation (Hakenbeck and Stock, 1996); WhiI has not been found to associate with any known kinase. Interestingly, WhiI

and WhiH appear to cross-regulate the expression of each other, for relative to the wild type strain, *whiI* transcription is increased in the *whiH* mutant, and *whiH* transcription is increased in the *whiI* mutant. However, evidence that WhiH and WhiI directly mediate these effects is lacking. It is notable that while *whiH* and *whiI* are direct targets for σ^{WhiG}, *whiA* and *whiB* appear, based upon their mutant phenotypes, to provide the products necessary to move from the straight, white hyphae seen in the *whiG* mutant, to the loosely coiled, partitioned hyphae seen in the *whiH* and *whiI* mutants.

Based on the phenotypes of the *whiA* and *whiB* mutants, they were deemed to fall after *whiJ* and *whiG* in the *whi* gene cascade. Given the epistatic relationship between *whiG* and *whiA/B*, and the observed relationship between σ^{WhiG} and *whiH/whiI*, it is possible that *whiG* was required for the expression of *whiA* and *whiB* as well. *whiA* has been cloned but has not yet been characterized, and as such, nothing is known about it transcription or dependence on other *whi* genes (Ryding, 1995). *whiB*, on the other hand, has been examined in detail, and has been found to require none of the known *whi* genes for its expression, despite the fact that a *whiG* mutation is epistatic to a *whiB* mutation. *whiB* is transcribed from two different promoters, with both promoters having been found to be active in *bldA*, *bldB*, *whiA*, *whiB*, *whiG*, and *whiH* mutants (Soliveri *et al.*, 1992). Based on these observations, it has been predicted that the progression from aerial hyphae to mature spores is not a simple linear path, and that there may be more than one regulatory cascade involved. Despite this two-path prediction, it has been determined that the two paths must converge in order to permit septation in the aerial hyphae. *ftsZ*, required for septum formation, is dispensable for vegetative growth in *S. coelicolor*, but is absolutely required for spore formation (McCormick *et al.*, 1994). Transcription of

ftsZ proceeds from two different promoters in *S. coelicolor*, one termed the vegetative promoter, from which a constant level of transcription is seen, and a second deemed the sporulation promoter, from which transcription is up-regulated upon initiating sporulation. The upregulation from the sporulation promoter is absent in *whiG, whiH* and *whiB* mutants, and correspondingly, *ftsZ* rings are not seen to assemble in the aerial hyphae of these mutant strains (Schwedock *et al.*, 1997; Flärdh and Chater, 1998). It is not known whether the *ftsZ* defects in the *whiG* mutant strain are an indirect result of its effect on *whiH*, but as neither gene appears to influence *whiB* expression, the two paths must intersect at this point.

whiB, like *whiH*, appears to encode a transcription factor, however, it differs from WhiH in its small size (87 amino acid residues versus 295 residues for WhiH) and in its possession of an unusual combination of 4 central cysteine residues, all of which are localized to the same face of an alpha helix in the WhiB secondary structure (Davis and Chater, 1992). These cysteine residues may be sensitive to redox changes, and may be involved in metal ion coordination, perhaps leading to intramolecular interactions, such as disulfide bond formation. A general survey of actinomycetes has revealed that all examined species contain at least one *whiB*-like (*wbl)* gene, with at least five present in *S. coelicolor*, and six to seven in *Mycobacterium tuberculosis* (Soliveri *et al.*, 2000). This may indicate a novel mechanism of responding to environmental stresses, particular to the actinomycetes.

One of these *whiB*-like genes was found to play a role in the later stages of sporulation in *S. coelicolor*, in contrast to the early role played by *whiB* itself. Sequencing of *whiD* revealed it to be a homologue of *whiB* (Molle *et al.*, 2000), whose

product possesses the four conserved cysteine residues found in WhiB. A *whiD* null mutant was discovered to have reduced levels of sporulation, forming spores of irregular size and shape due to aberrant septum placement, resulting in some cases, in spore compartments that contained no chromosomal DNA. This may reflect a similarity between the function of *whiD* and that of the *min* locus of *E. coli* and *B. subtilis*, where mutations in the *min* genes result in division at the midcell and at the cell poles, to give some small, anucleoid compartments (Teather *et al.*, 1974). The *whiD* mutant spores were also observed to be defective in wall thickening, with most spore walls being abnormally thin (20-30 nm versus 60-80 nm), while unusually thick walls (up to 170 nm), were laid down between the spores. The spores were found to lyse extensively and were extremely sensitive to heat; these characteristics may have resulted from the spore wall defects.

In the *sigF* mutants, the spores were also malformed and had thinner walls than typically observed in the wild type strain; however, in contrast to the *whiD* mutant spores, the *sigF* mutant spores were not heat sensitive, although they were more sensitive to detergents than the wild type spores (Potúcková *et al.*, 1995). It was found that in the *sigF* mutant, the spore chains were difficult to fragment, indicating a potential defect in spore-spore separation; however, it was also observed that the spore chromosomes failed to condense, suggesting a pleiotropic role for the *sigF* gene product in sporulation. *sigF* was found to encode a sigma factor, showing similarity to σ^B from *B. subtilis*, which is involved in stationary phase gene expression (Potúcková *et al.*, 1995). Expression of *sigF* was found to require all of the "early" *whi* genes (*whiA, B, G, H, I,* and *J*) (Kelemen *et al.*, 1996), and given its dependence on *whiG*, was the first demonstration of a sigma

factor cascade necessary for sporulation in *Streptomyces* (such a cascade has been well documented in *B. subtilis* sporulation, although σ^B expression is not known to depend on σ^{28}). Further examination, however, revealed that the *sigF* dependence on *whiG* was indirect, with the *whiG* effects likely being mediated through *whiH* gene product activity. *sigF* is not expressed until the sporulation septum has formed, implying that σ^F is spatially restricted to the prespore compartment (Kelemen *et al.*, 1996). Septation may serve as a morphological check-point governing *sigF* expression, and this proposal is complemented by the observation that *ftsZ*, which is needed for septation, and *sigF*, appear to be coregulated by *whiH*. Various *sigF* targets have been suggested, including *whiE*, which encodes the enzymes specific for the synthesis of the grey spore pigment, as well as spore-specific penicillin binding proteins involved in spore wall thickening (Potúcková *et al.*, 1995).

Investigations of *whiE*, the complex locus that encodes the enzymes responsible for synthesizing the grey spore pigment, have found its expression to be developmentally regulated, and like *sigF*, its product may be confined to the spore compartments (Kelemen *et al.*, 1998). Eight different genes define the *whiE* locus, and these are divided into two divergent operons controlled by two promoters, *whiEP1* [Open reading frames (ORFs) I–VII] and *whiEP2* (ORFVIII). Mutations in *whiA*, *whiB*, *whiG*, and *whiI* completely blocked expression from both promoters, while mutations in *whiH* and *whiJ* resulted in lower levels of transcription from both promoters. This is consistent with the light grey mutant colony appearance of *whiH* and *whiJ* mutants. When the *whiE* dependence on σ^F was examined, it was found that expression from *whiEP1* appeared to

be unaffected by *sigF* disruption, but transcription from *whiEP2* was undetectable in the *sigF* mutant.

The final five identified *whi* genes, *whiK-O,* were recently isolated (Ryding *et al.*, 1999), and have significant phenotypic variation associated with mutations in each. In the case of *whiL*, one of the isolated mutants was found to have pale grey colonies with long spores, both curved and straight, while a second mutant was found to completely lack grey pigmentation, although the spore morphologies were otherwise identical to the first. The *whiM* mutant phenotype was characterized by white, undifferentiated aerial hyphae, indicating that it was likely an early acting *whi* gene. The hyphae were found to lyse rapidly, and this mutant strain was poorly viable. Mutations in the *whiO* locus resulted in colonies that were medium grey in colour, having some undifferentiated aerial hyphae, together with spore chains of normal appearance. Both *whiK* and *whiN* have now been cloned and characterization of them is underway. The *whiK* locus was originally identified by virtue of two different mutant strains, one of which had long, tightly coiled aerial hyphae with frequent septation, while the second had a more severe phenotype, with straight, undifferentiated aerial hyphae, indicating that the *whiK* gene product was likely required early in the sporulation process. *whiN* mutants were found to be of two different varieties, either appearing pale to medium grey with frequent spores that were longer than normal, or alternatively, like *whiK*, appearing white with long, straight, undifferentiated hyphae. Cloning and sequencing of *whiK* and *whiN* revealed that they encode a response regulator, and a sigma factor, respectively. Surprisingly, however, when null mutations were generated for each of these genes (Molle and Buttner, 2000; Bibb *et al.*, 2000), the resulting colonies were completely lacking not only

in spores, but also in aerial hyphae. Consequently, their gene designations have been changed from *whiK* and *whiN* to *bldM* and *bldN*, respectively (see below).

Deficiencies in aerial hyphae formation are typically seen to result from mutations in a class of genes termed the *bld* genes, as the loss of aerial hyphae confers a smooth, shiny, "bald" appearance to the otherwise furry-appearing streptomycete colonies (Merrick, 1976). Thus far, *bldA-L* have been identified in *S. coelicolor* (Merrick, 1976; Champness, 1988; Nodwell *et al.*, 1996; Nodwell *et al.*, 1999), and with the apparent bald phenotypes of *whiK* and *whiN* null mutants, they were renamed *bldM* and *bldN*, respectively. For several of the *bld* mutant strains, including *bldA*, *bldD*, and *bldG*, secondary hyphae do appear to be produced, however, they appear to be incapable of extending into the air, and instead, lie prostrate on the colony surface. Interestingly, the bald phenotype is not an absolute one, as it has been found that growth of a number of *bld* mutants on minimal medium supplemented with a poorly utilized carbon source such as mannitol, restored their ability to erect aerial hyphae, and proceed on to sporulation (Champness, 1988; Merrick, 1976). The exceptions to this observation were *bldB* (although with extended incubation times, sparse hyphae were observed regardless of the media used), *bldC*, *bldF*, *bldI*, *bldM*, and *bldN*. This suggests that there may be at least two different paths by which aerial hyphae are erected/formed, one being absolutely dependent on all of the *bld* genes, and the other being dependent on select *bld* genes. It therefore appears that while the *bld* genes were identified and grouped on the basis of a common morphological characteristic, they, like the *whi* genes, exhibit significant phenotypic variation as well. The pleiotropic nature of the *bld* genes is emphasized by the observation that defects in the *bld* genes not only impact the erection of aerial hyphae,

but in many cases, also influence the production of secondary metabolites. The timing of aerial hyphae is coincident with the timing of antibiotic production, suggesting a co-regulation of these processes, at least in part, by a number of the *bld* genes. *S. coelicolor* is known to produce four different antibiotics, two of which are pigmented, and are therefore obvious markers for antibiotic production. Actinorhodin is a blue pigmented antibiotic (under alkaline conditions) that diffuses into the medium surrounding the colonies, while undecylprodigiosin is a red, cell-associated antibiotic. The two other antibiotics produced by *S. coelicolor* are the plasmid encoded methylenomycin A and a calcium dependent antibiotic (CDA). All of the identified *bld* genes influence the expression of at least one of these antibiotics, except for *bldM* and *bldN*, which do not appear to be involved in antibiotic production. Mutations in each of *bldA, B, D, G, H,* and *I* result in defects in the ability of the colony to produce all four antibiotics, and unlike the conditional aerial hyphae defect, only the *bldH* antibiotic defects are completely reversed when the mutant is grown on minimal medium with mannitol; while the *bldA* defect in undecylprodigiosin production is restored when grown on low phosphate-containing medium. Three of the four antibiotics were absent in *bldE* and *bldF* mutant strains, with only undecylprodigiosin being produced. *bldK* mutants were defective in the production of actinorhodin, although this defect was medium-dependent, as growth on minimal glucose medium restored blue pigment production (Nodwell *et al.*, 1996), while the *bldC* mutant was defective in the production only the calcium dependent antibiotic (Hopwood and Wright, 1983).

The *bld* genes, like the *whi* genes, are at an early stage of characterization, with only *bldA, B, D, G,* and *K* having been cloned and sequenced, together with the newly

discovered *bldM* and *bldN*. The best characterized of the *bld* genes is *bldA*, which has been found to encode a leucyl tRNA, specific for the TTA codon, which is rare in the G+C-rich *Streptomyces* chromosome (Lawlor *et al.*, 1987). Interestingly, this TTA codon has been found to be largely absent from genes required for vegetative growth, and is only present in genes required later in development; TTA codons have been identified in genes needed for antibiotic production, antibiotic resistance, differentiation, degradative enzyme production, and plasmid integration and transfer (Trepanier, 1999). The *bldA* tRNA is developmentally regulated; while it is transcribed during vegetative growth, it does not accumulate in its processed, mature form until later on (Leskiw *et al.*, 1993), thereby delaying the expression of TTA-codon-containing genes, at least in some cases, until after vegetative growth (Leskiw *et al.*, 1991). In *S. coelicolor*, the pathway-specific regulators of actinorhodin and undecylprodigiosin production, *act*II-ORF4 and *redZ*, have been found to contain TTA codons, suggesting a direct regulatory role for *bldA* in the production of each of these antibiotics (Fernández-Moreno *et al.*, 1991; White and Bibb, 1997; Guthrie *et al.*, 1998). *bldX*, the *S. coelicolor* homologue of *nrsA* from *S. griseus*, discussed in greater detail below, has also been found to contain a TTA codon, linking *bldA* to morphological differentiation as well (Babcock and Kendrick, 1990; McCue *et al.*, 1996). A number of the *bld* genes have been examined for their dependence on *bldA*, and it appears that only *bldI* expression requires a functional *bldA* gene (Leskiw and Mah, 1995). With the *S. coelicolor* genome sequencing project expected to be completed this year (2000), all targets of *bldA* in the *S. coelicolor* chromosome will be known, and the study of these genes should help to further the understanding of this unusual mode of regulation by a tRNA.

The regulation of aerial hyphae formation appears to be exerted on many levels: translational control by *bldA*, post-translational control by phosphorylation and ADP-ribosylation (Okamoto *et al,* 1998; Shima *et al.*, 1996), and transcriptional control by the newly characterized *bldB, bldD, bldM,* and *bldN*. It is interesting that of the 5 *bld* genes that have been cloned and sequenced, three of them, *bldB, bldD,* and *bldM* all appear to specify DNA-binding transcription factors. *bldB* and *bldD* encode small proteins, of 98 and 167 amino acid residues, respectively, with apparent helix-turn-helix DNA-binding motifs localized in the C-terminal domains of each protein (Harasym *et al.*, 1990; Pope *et al.*, 1998; Elliot *et al.*, 1998). BldB appears to negatively regulate its own expression; however, to date, no additional targets of BldB regulation have been identified (Pope *et al.*, 1998). This thesis has focussed on the characterization of BldD, and its targets in the chromosome, and these are discussed at length in the Results (3) and Discussion (4) sections. The BldM regulator is larger than either of BldB or BldD, at 203 amino acids, and is a member of the family of response regulators (Molle and Buttner, 2000). Like WhiI, BldM is not paired with a sensor kinase. Questions have been raised as to whether BldM activity is controlled by phosphorylation, for while its phosphorylation pocket has been conserved, and point mutations in this conserved region have resulted in the white phenotype observed originally, mutation of its conserved Asp residue, the residue typically phosphorylated in other 2-component signal transduction systems, did not impact its ability to erect aerial hyphae or produce wild type spores. While the post-translational state of BldM is not completely understood, a much clearer picture exists for its transcriptional regulation; *bldM* expression is controlled by the sigma factor specified by *bldN* (Bibb *et al.*, 2000). *bldN*, like *bldM*, was originally identified as a *whi* mutant,

with point mutations adversely affecting the ability to sporulate, while a null mutation eliminated the ability to erect aerial hyphae. The observation that different alleles of these two genes arrested development at different stages, suggested either these genes have dual functions in the differentiation process, or, point mutations in these genes constitute weak alleles that allow the formation of aerial hyphae, but prevent the maturation of the hyphae into spores. Interestingly, defects in either of these genes had no effect on the ability of the colony to produce antibiotics, making them the first identified *bld* genes to be solely dedicated to aerial mycelium formation.

Examination of *bldN* expression has revealed it to be absolutely dependent on the *bldG* and *bldH* gene products. *bldH* has not been characterized, however, *bldG* has been found to encode a protein with similarity to anti-anti sigma factors (Bignell *et al.*, 2000). Directly downstream from *bldG* was found a putative anti-sigma factor, but surprisingly, no sigma factor was found to be encoded within this *bldG* locus. BldG, and its putative anti-sigma factor binding partner, are not expected to regulate the activity of σ^{BldN}, as its transcription, not its activity, depend on a functional *bldG* gene; however, it is attractive to propose that a σ-factor regulated by the *bldG* regulatory pair is required for the expression of *bldN*, either directly, or indirectly. If true, this suggests that a minimum of four σ-factors are required for the erection of aerial hyphae and the development of spores: $\sigma^{BldG\text{-}dependent} \longrightarrow \sigma^{BldN} \longrightarrow \sigma^{WhiG} \longrightarrow \sigma^{SigF}$. The sigma factor(s) controlled by *bldG*, must however, be involved in more than just transcription of *bldN*, as *bldN* does not impact antibiotic formation, and yet *bldG* mutants are defective in the production of all four antibiotics produced by *S. coelicolor*.

The final *bld* locus to be characterized thus far is *bldK*, which comprises a locus encoding a member of the ATP-binding cassette (ABC) family of membrane spanning transporters (Nodwell *et al.*, 1996). The BldK transporter appears to be an oligopeptide importer, and is proposed to import an extracellular signaling molecule, whose production is believed to be dependent on *bldJ* (formerly *bld261).* Potential import targets were isolated from the medium on which a *bldK* mutant was grown, using high performance liquid chromatography (HPLC) to separate the components in the *bldK* "conditioned" medium (Nodwell and Losick, 1998). The resulting fractions were applied to the surface of a *bldJ* mutant, as this mutant was expected to be unable to produce the BldK target. Two separate fractions/factors were found to restore hyphae formation to the *bldJ* mutant: the first factor was not abundant enough to characterize, however, the second factor was found to be a 655 Da molecule, containing serine and glycine residues. Determination of whether this molecule is encoded by *bldJ*, awaits the cloning and sequencing of the *bldJ* gene.

Extracellular communication, through the production, excretion and uptake of signaling factors, has been proposed to play an important role in differentiation, with a cascade of signals mediated by the *bld* genes, beginning with the *bldJ*-dependent signal, and culminating in the erection of aerial hyphae (Willey *et al.*, 1993). This proposal is based, in part, on observations that wild type colonies are able to "complement" aerial mycelium formation in *bld* mutant colonies when plated in close proximity (Willey *et al.*, 1991), with hyphae appearing on the *bld* mutant strain at the juncture between the two colonies. The proposal was furthered by the discovery that different *bld* mutant strains are capable of "complementing" other *bld* strains when plated side-by-side (Willey *et al.*,

1993; Nodwell *et al.*, 1999; Bibb *et al.*, 2000). It has been suggested that this occurrence is a result of one strain, termed the donor strain, providing an extracellular factor that is missing in the recipient strain. The provision of this factor is believed to alleviate the developmental block in the recipient strain, permitting the erection of aerial hyphae and formation of spores. A hierarchy of *bld* genes has been determined, based upon the extracellular complementation profile of each: *bldJ* < *bldK* < *bldA, bldH* < *bldG* < *bldC* < *bldD, bldM,* with each strain complementing those to the left, and being complemented by those to the right. A screen by Nodwell *et al.* (1999) has resulted in the isolation of 50 new *bld* mutant strains, most of which map to the *bldK, bldC,* or *bldD* complementation groups. A number of the new *bldK* group strains were found to be *bldK* alleles, however, a subset of the new *bldK* group strains were mapped to a different position on the chromosome, and thus were designated *bldL*. Conversely, most of the new *bld* strains belonging to the *bldC* and *bldD* complementation groups were not alleles of *bldC* or *bldD*, potentially representing targets of the *bldC* and *bldD* gene products. Some of the *bld* strains assigned to the *bldD* complementation group presumably correspond to the *bldM* gene, which was not examined during the screening process.

A number of genes, however, do not conform to this simplistically linear cascade hierarchy. These include *bldB, bldI, bldN*, and 6 new *bld* strains isolated by Nodwell *et al.* (1999). Assessment of the *bldB* mutant strain revealed it to belong to the *bldC* complementation group, except for the fact that it failed to complement, or be complemented by, *bldK, bldA* or *bldH* mutants. *bldI*, on the other hand, complemented only the *bldJ* mutant strain, and neither complemented, nor was complemented by, any of the *bldK, bldA, bldH, bldG, bldC,* or *bldD* mutant strains; while *bldN* was found to be

capable of complementing *bldJ*, *bldK*, and to some extent, *bldH*, but had no effect on, and was not affected by, *bldA*, *bldG*, *bldC*, or *bldD* mutant strains (Bibb *et al.*, 2000). Given that *bldM* was shown to be a direct target of the *bldN* sigma factor, and that *bldM* was observed to fit into the *bldD* complementation group in the complementation cascade, while *bldN* did not, suggests that the effects of *bldN* are wide ranging, and are not limited to *bldM* regulation. The six new mutant strains identified by Nodwell *et al.* (1999) had diverse complementation profiles, differing from each other, and from those observed for *bldB*, *bldI* and *bldN* mutants (see Table 1.1).

The extracellular complementation cascade is believed to culminate in the production of SapB, a spore associated protein found to coat the aerial hyphae of *Streptomyces* colonies (Willey *et al.*, 1993). In the studies by Willey *et al.* (1993), SapB was not produced by any of the *bld* mutants, however, SapB production was found to be restored in the recipient (hyphal producing) strain, when one mutant strain successfully complemented another. It was also discovered that the application of purified SapB to the surfaces of the *bld* mutant strains transiently restored the appearance of aerial hyphae; however, these hyphae failed to mature into spore chains. Interestingly, a fungal hydrophobin, SC3 from *Schizophyllum commune*, was also able to restore aerial mycelium formation to the *bld* mutants (Tillotson *et al.*, 1998). Recent studies have revealed that SapB, like SC3, is a surfactant, aggregating at the air-water interface, and reducing the surface tension of water at the colony surface, consequently allowing the hyphae to escape from the colony surface and extend into the air. Involvement of hydrophobin-like proteins, such as SapB, in the emergence of aerial hyphae is a feature common to both prokaryotic and eukaryotic filamentous organisms. Surprisingly, SapB

Table 1.1 Extracellular complementation phenotype of nonconforming mutant strains

Complementation Group	*bldB*	*bldI*	*bldN*	MY292	MY304	MY343	MY512	MY537	MY590
bldJ	Donor	Donor	Donor	Donor	Donor	Donor	Donor	Donor	Donor
bldK/L	Neither	Neither	Donor	Donor	Donor	Donor	Neither	Neither	Donor
bldA/H	Neither	Neither	Neither/Donor	Neither	Neither	Donor	Neither	Donor	Donor
bldG	Donor	Neither	Neither	Donor	Donor	Recipient	Donor	Donor	Donor
bldC	Neither	Neither	Neither	Recipient	Neither	Recipient	Recipient	Neither	Neither
bldD/M	Recipient	Neither	Neither	Recipient	Neither	Neither	Recipient	Neither	Neither

is not required for erection of aerial hyphae on minimal medium supplemented with mannitol, supporting the notion that two different paths may be followed on the way to aerial hyphae formation.

Four other spore associated proteins have also been identified, and have been designated SapA, C, D, and E. Saps C, D, and E have all been found to be encoded by the large, linear SCP1 plasmid, but since SCP1 is dispensable for aerial hyphae formation, none of these proteins are believed to play a major role in the developmental process (Guijarro *et al.*, 1988). Transcription of SapA has been found to be decreased in *bldC, bldD,* and *whiH* mutants, indicating that it may be involved in differentiation (Guijarro *et al.*, 1988).

While the extracellular complementation theory has significant merit, especially in light of BldK and its target factors, it falls short of explaining the diverse phenotypes of each of the *bld* mutants. Its simplistic, linear arrangement fails to take into account the increasing number of *bld* genes that do not fit neatly into any given complementation category, and it does not address the antibiotic deficiencies that accompany most *bld* mutations.

An alternative, or possibly complementary, theory to that proposed by the extracellular complementation model, involves sensing of the environment, and an ability to manipulate environmental conditions. It is known that during vegetative growth, the pH of the medium drops significantly, from a pH of ~7, to a pH of ~4.7, due to the excretion of organic acids, such as α-ketoglutarate and pyruvate. In studies by Süsstrunk *et al.* (1998), wild type *S. coelicolor* strains were able to neutralize the medium, possibly through the excretion of cAMP, or through the uptake of the organic acids; however,

bldA, B, C, D, G, and *H* mutants were not, and as a result, the medium remained acidic. One gene believed to influence this neutralization process is the cAMP synthesis gene, *cya*. Mutation of this gene was found to result in a conditional bald phenotype, with an absence of aerial hyphae when grown on unbuffered media, as well as a conditional antibiotic defect, with no pigmented antibiotic observed when grown on unbuffered media (Susstrunk *et al.*, 1998). This developmental block has been correlated with a pH dependent growth arrest, where an absence of cAMP, apparently required for medium neutralization, does not allow for the resumption of growth, resulting in a block to aerial mycelium formation and antibiotic production. The aerial hyphae and antibiotic deficiencies seen in the *cya* mutant strain could be rescued either by growth on buffered media, or alternatively, by treatment with cAMP. It was also found that the *cya* mutant phenotype could be rescued by growing a *cya* mutant strain next to a wild type strain. It is not known whether this "extracellular complementation" was due to the provision of an extracellular signal, by the wild type strain that was taken up by the mutant strain, or was due to medium neutralization by the wild type strain, through the uptake of the acids or excretion of cAMP. The relationship between the *bld* mutants and the *cya* mutant, in terms of an ability to restore aerial hyphae formation to each other, was not assessed, so where *cya* would fit into the previously determined *bld* gene cascade is not known. It has, however, been discovered that the *bld* mutants were all capable of making cAMP, and were not rescued by growth on buffered media, implying that the *bld* mutant phenotypes were not a result of abnormal *cya* expression.

In *Escherichia coli*, cAMP has been associated with the ability to relieve glucose catabolite repression; however, such a role has not been demonstrated in Gram positive

bacteria. Despite the lack of a clear connection between cAMP and catabolite repression in the *Streptomyces*, it is obvious that the sensing of environmental and metabolic signals is imperative for proper development, as defects in both cAMP production and carbon catabolite control have been associated with an inability to erect aerial hyphae, and produce antibiotics. Studies by Pope *et al.* (1997) revealed that mutations in the *bld* genes result in defects in the regulation of carbon utilization. Mutant strains of *bldA, B, D, G,* and *H* were found to be incapable of regulating *galP1*, the glucose-sensitive, galactose-dependent promoter of the galactose utilization operon. In each of these *bld* mutant strains, *galP1* expression was found to be expressed at a high level in the presence of glucose or galactose. The *bldA* mutant strain was subjected to further examination, and it was determined that growth on a poorly utilized carbon source, such as mannitol, rescued not only the ability to form aerial hyphae, but also rescued the defect in *galP1* regulation. Conversely, the *bldB* mutant phenotype was not rescued when grown on mannitol, and correspondingly, the *bldB* defects in carbon source utilization were also not restored (Pope *et al.*, 1997). It has been suggested that the *bldB* mutation may confer a global defect in carbon catabolite control, as additional studies have revealed the *bldB* mutant strain to be unregulated not only for galactose utilization, but also for glycerol and agarase metabolism. One role of the *bld* genes may therefore be to evaluate the nutritional environment, and sense and/or signal starvation. The evaluation of the nutritional cycle is likely mediated, in part, by the central metabolic cycle, which would be capable of responding rapidly to nutritional changes. The ability to initiate aerial hyphae formation, and ultimately sporulation, would depend on nutrient depletion, or some otherwise unfavorable environmental condition, together with sufficient metabolic

and energy reserves to complete sporulation. The importance of the metabolic enzymes is emphasized by work conducted by Schwartz *et al.* (1999), who observed that defects in the Krebs cycle aconitase gene (*acnA*) in *S. viridochromogenes* Tii494, result in a bald and antibiotic deficient phenotype.

In addition to carbon source sensing, nitrogenous compounds also appear to play a role in the ability of *S. coelicolor* to undergo differentiation. Asparagine limitations have been found to promote spore formation in *S. coelicolor* (Karandikar *et al.*, 1996), and nitrate limitation/exhaustion has been associated with glycogen accumulation and an increase in surface hydrophobicity (Karandikar *et al.*, 1997), characteristics which are symptomatic of a move into aerial hyphae formation.

Coupled to the apparent metabolic requirements for differentiation, are the proteolytic events that also appear to be necessary for development. A trypsin-like protease is believed to be required for the digestion of substrate mycelium under conditions of nutrient limitation (Kim and Lee, 1996), providing some of the substrates needed for the assembly of aerial hyphae. The contribution of proteolytic activity to the differentiation process does not, however, appear to be limited to the digestion of the substrate hyphae, as the Clp ATP-dependent protease appears to exert intracellular control over differentiation as well (de Crécy-Lagard *et al.*, 1999). The Clp protease is composed of two regulatory subunits, ClpX and ClpC, and a catalytic subunit, ClpP. Two *clpP* genes have been identified in *S coelicolor*, *clpP1* and *clpP2*, and overexpression of either of these two genes serves to accelerate the formation of aerial mycelium, relative to the wild type strain. Conversely, the disruption of *clpP1* completely abolishes aerial hyphae formation, giving a bald phenotype that is

unconditional in terms of carbon source. The mutant strain can, however, be complemented extracellularly by growth adjacent to a wild type strain, suggesting the provision of a missing stimulatory, extracellular factor, or the removal/neutralization of an inhibitory factor from the medium. How, or whether, *clpP1* fits into the overall *bld* gene complementation cascade has not yet been examined. Interestingly, overexpression of the *clpX* regulatory subunit accelerates actinorhodin production, implying a role for the Clp protease in both morphological differentiation and secondary metabolism.

One possible target for the Clp protease may be the gene product of the *bldX* gene in *S. coelicolor*, or that of its better studied homologue, *nrsA* in *S. griseus*. The *nrsA* gene encodes two different products, P56, an apparent DNA-binding regulatory protein, and P49.5, a shorter protein version of P56, generated by translation initiation at a site within the P56 coding sequence, downstream from the helix-turn-helix DNA-binding motif (McCue *et al.*, 1996). Translation of both of these proteins was found to be dependent on *bldA*, due to the presence of a TTA codon within the coding sequence of each protein. This gene was originally identified based on its ability to suppress the defects in aerial hyphae formation and antibiotic production of a number of *S. griseus bld* mutant strains (Babcock and Kendrick, 1990). Attempts to disrupt *nrsA* were unsuccessful except in the presence of a second *bld* mutation, implying that *nrsA* was required for viability (McCue *et al.*, 1996). It has been postulated that the larger of the two proteins, P56, is responsible for the suppression of the *bld* phenotype, and that the smaller protein, P49.5, somehow modulates the activity of P56, for an overall effect of preventing premature sporulation. It is plausible that degradation, perhaps of the smaller protein, by the Clp protease allows sporulation to proceed.

A number of other genes have been identified that appear to influence the erection of aerial hyphae, but are not required for the process. The *ram* gene cluster from *S. coelicolor,* was isolated because it confers *r*apid *a*erial *m*ycelium formation to *S. lividans* when introduced at low copy number (Ma and Kendall, 1994). The *ram* locus comprises three genes, *ramA, ramB*, and *ramR*, with *ramA* and *ramB* encoding proteins similar to ATP-dependent membrane-translocating proteins, and RamR resembling a family of two component response regulators. Disruption of *ramB* resulted in severe defects in aerial mycelium formation, although hyphae formation was not entirely lost, as well as overproduction of a red pigmented antibiotic, presumed to be undecylprodigiosin. It is possible that together, the *ramA* and *ramB* gene products are responsible for the translocation of an important morphogenic factor, perhaps functioning like the BldK membrane-spanning transporter which has been shown to be essential for the formation of aerial hyphae. The apparent stimulatory effect of *ramB* disruption on undecylprodigiosin production may simply be the result of a metabolic imbalance, or may indicate that RamB has a role in activating the formation of aerial hyphae, while suppressing the production of undecylprodigiosin. A similar three-gene complex, whose genes were termed *amfR, amfA,* and *amfB (*for *a*erial *m*ycelium *f*ormation), was discovered in *S. griseus,* and again appeared to encode a response regulator protein, and membrane translocation proteins, respectively (Ueda *et al.*, 1998). Disruption of *amfR* completely abolished the formation of aerial mycelium, giving a bald colony phenotype, and it is conceivable that disruption of *ramR* would have the same effect. One distinct difference between *amfR* and *ramR* is in their dependence on *bldA*: *amfR* appears to be

regulated by *bldA*, as it contains a TTA codon; however, *ramR* does not possess this codon, and as such would not be under the direct control of *bldA*.

An additional *amf* gene, *amfC*, was discovered in *S. griseus*, where it was isolated as a result of its ability to restore the formation of aerial hyphae to an *S. griseus* strain with a bald mutant phenotype; an *amfC* homologue has been identified in *S. coelicolor*, however, its role in differentiation has not yet been examined (Kudo *et al.*, 1995). Inactivation of *amfC* in *S. griseus*, resulted in a severe reduction in sporulation, with no effect on antibiotic production. Interestingly, it was discovered that the *nrsA* gene, at high copy number, could restore the *amfC*-mutant defects; however, none of *amfR*, *amfA*, or *amfB* were able to suppress the sporulation deficiencies.

Given the apparent similarities between the regulation of aerial hyphae formation, and sporulation, in two distantly related streptomycetes, *S. coelicolor* and *S. griseus*, it is conceivable that the sporulation process is regulated in a similar fashion in all *Streptomyces*. One of the best-studied systems for regulation of differentiation is the A-factor-dependent cascade, defined by studies in *S. griseus*; although, it is becoming increasingly apparent that such a system exists in *S. coelicolor* as well. A-factor, or autoregulating factor, is a γ-butyrolactone microbial hormone required for sporulation, antibiotic production and resistance, as well as yellow pigment production in *S. griseus* (Hara and Beppu, 1982). A-factor was originally identified by virtue of the fact that one group of streptomycin non-producing strains had their ability to produce streptomycin restored in the presence of a factor excreted by mutants of the other group (it is conceivable that A-factor-like compounds are involved in the extracellular complementation of the *bld* mutants in *S. coelicolor).* It was discovered, however, that

A-factor could not directly regulate streptomycin biosynthesis (Hara and Beppu, 1982). To elucidate its mechanism of regulation, A-factor was radiolabeled, and was found to associate with a protein termed the A-factor receptor protein, or ArpA (Miyake *et al.*, 1989). The ArpA/A-factor interaction was deemed to be a highly specific one, as ArpA was incapable of recognizing other γ-butyrolactone compounds similar to A-factor, such as VB-C, from *S. virginiae* (Miyake *et al.*, 1989). Studies of the mode of ArpA activity revealed that ArpA repressed streptomycin production, as well as sporulation, in the absence of A-factor. It was proposed that ArpA repressed its target gene(s) until bound by A-factor, at which time ArpA was released from the DNA, allowing transcription of its previously repressed target(s) to proceed (Miyake *et al.*, 1990; Onaka *et al.*, 1995). An ArpA target, *adpA* (*A*-factor *d*ependent *p*rotein), has been identified, which influences streptomycin production, streptomycin resistance and aerial hyphae formation in *S. griseus* (Ohnishi *et al.*, 1999). Sequencing of the *adpA* gene revealed that it shows similarity to positive transcriptional activators (Ohnishi *et al.*, 1999), and AdpA was found to bind upstream from the *strR* promoter, and activate the transcription of both *strR* and *aphD* (the regulators of streptomycin production and resistance) (Vujaklija *et al.*, 1993). A recent search for additional targets of AdpA has revealed a potential one in an extracytoplasmic function sigma factor, designated *adsA*, for *A*-factor *d*ependent extracytoplasmic function *s*igma factor (Yamazaki *et al.*, 2000). The phenotypic effects of *adsA* were revealed by disruption of the gene, which resulted in complete abrogation of aerial hyphae formation. *adsA* has since been shown to be the orthologue of *bldN* in *S. coelicolor*, again, emphasizing the regulatory similarities between these two distantly related strains. The *S. coelicolor* AdpA equivalent has just recently been identified by the

S. coelicolor genome sequencing project, and an intriguing observation has placed the *S. coelicolor adpA* gene under the control of *bldA*, as it contains a TTA codon; however, *bldN* expression is not dependent on *bldA*, so whether AdpA plays a role in *bldN* expression in *S. coelicolor* awaits further investigation.

At least three different ArpA-like proteins have also been found in *S. coelicolor*, and of these, two have been analyzed for their contributions to differentiation. Like *arpA*, *crpA* and *crpB* were both proposed to encode DNA-binding proteins, and due to extensive similarities in their DNA-binding motifs, were predicted to bind to the same sequence (Onaka *et al.*, 1998). Surprisingly, disruption of these genes resulted in opposing/contrasting mutant phenotypes. The *crpA* mutant produced less actinorhodin and undecylprodigiosin relative to the wild type, and sporulation was delayed by a day, while the *crpB* mutant produced copious amounts of actinorhodin, wild type levels of undecylprodigiosin, and accelerated sporulation relative to the wild type. This suggested that CrpA functions as a positive regulator of differentiation and antibiotic production, and that CrpB acts as a negative regulator of the same processes. Given their similarities to ArpA, these proteins are likely regulated by a γ-butyrolactone signaling molecule(s), of which at least four have been identified in *S. coelicolor* (Bibb *et al.*, 1996).

Extracellular signaling and environmental sensing are recurring themes in the initiation of differentiation in *Streptomyces*, from nutritional sensing and pH detection, to microbial hormones, and other small signaling factors. Each of these extracellular stimuli, must however, be associated with an appropriate intracellular signal and response. The Krebs cycle is known to serve as one such intracellular checkpoint, however, many other factors must contribute to the overall cellular response, and

presumably include the stringent response, and the assessment of energy stores/levels. The Obg protein in *S. coelicolor* is a membrane bound protein, believed to be involved in sensing of the intracellular GTP pool (Okamoto *et al.*, 1998). Obg is essential for viability in *S. coelicolor*, and is present in the membrane until the end of vegetative growth and the onset/beginning of aerial hyphae formation, after which it is no longer detectable, perhaps being targeted for degradation by the Clp protease. When Obg binds GTP, aerial hyphae formation is prevented, but when GDP is bound, or Obg is in a nucleotide-free form, hyphae formation is permitted. The nucleotide-bound state of Obg is believed to act as measure of energy levels in the cell: if energy levels are high, it likely indicates that nutritional sources are abundant, environmental conditions are favorable and sporulation is not necessary, but if they are low, and GTP is not available to be bound, then the cell is likely in the throes of nutritional angst, and sporulation is necessary. When multiple copies of *obg* were introduced into *S. coelicolor*, aerial mycelium formation was suppressed, and actinorhodin production was decreased; a similar phenotype was observed with an *obg* point mutant, believed to be defective in its GTPase activity. Mutations that were proposed to increase the affinity of Obg for GDP, or increase its affinity for a guanine nucleotide dissociation stimulator, resulted in the acceleration of aerial hyphae development coupled with increased actinorhodin production. Taken together, all of these finding suggest that Obg monitors the intracellular GTP pool, thereby sensing changes in the nutritional environment, which leads to the promotion, or repression, of morphological differentiation, and antibiotic production (at least actinorhodin production) (Okamoto *et al.*, 1997).

The intracellular response to changes in the nutritional environment is the basis of the stringent response as well, which is typically induced by a depletion of amino acids, although it can also be artificially induced by the addition of serine hydroxamate to culture media (Strauch *et al.*, 1991). During the stringent response, there is a global reduction in RNA synthesis, mediated by ppGpp. ppGpp is synthesized by a ribosome-associated enzyme, the [p]ppGpp synthetase I, encoded by *relA*, and its synthesis is stimulated when an uncharged codon-specific tRNA is bound to the ribosomal A site, indicating a paucity of amino acids. There is a separate *relA*-independent pathway associated with ppGpp formation, and it is believed to respond to carbon and energy source depletion. The *relC* gene, encoding ribosomal protein L11, has also been implicated in ppGpp production, as *relC* mutants make reduced levels of ppGpp. There has been significant debate about the effects of ppGpp and *relA*/*relC*, and whether they are associated with antibiotic production or morphological differentiation. Strauch *et al.* (1991) observed that increased ppGpp levels were not sufficient to initiate antibiotic biosynthesis in *S. coelicolor*, and this was supported by Chakraburtty *et al.* (1996), who found that a *relA* mutant had little effect on actinorhodin production (however, ppGpp was still observed to be produced, so this conclusion may be misleading). It was later discovered that disruption of *relA* had no phenotypic effects, but deletion of *relA* served to abolish actinorhodin production, without affecting the synthesis of undecylprodigiosin or CDA (Ochi, 1990a; Chakraburtty and Bibb, 1997; Martinez-Costa *et al.*, 1998). Interestingly, the *relA* null mutant phenotype in *S. coelicolor*, was conditional, in that decreased phosphate concentrations in the media were able to restore actinorhodin production. *relC* mutations were also found to have contradictory phenotypic effects, for

in *S. lavendulae* (Ochi, 1987; Ochi, 1990b) and *S. antibioticus* (Ochi, 1987; Kelly *et al.*, 1991), antibiotic production was adversely affected in a *relC* mutant; however in *S. clavuligerus,* the absence of *relC* had no effect on antibiotic production (Bascarán *et al.*, 1991). Isolation of a *relC* mutant from *S. coelicolor* revealed the strain to be deficient in actinorhodin and undecylprodigiosin, as well as in its ability to make aerial hyphae (Ochi, 1990a).

The intracellular levels of ppGpp have also been found to be influenced by *rpsL,* which encodes the ribosomal protein S12 (Shima *et al.*, 1996). Mutations in this protein decrease ppGpp accumulation, and confer both streptomycin resistance and precocious actinorhodin production to *S. coelicolor* and *S. lividans.* The correlation of decreased ppGpp levels and increased actinorhodin production in the *rpsL* mutant, contrasts with the observations made for *relA* and *relC* mutants, where decreased ppGpp was associated with reductions in antibiotic production. This only serves to underscore the complexity in the regulation of antibiotic production. Surprisingly, it was found that mutations in *rpsL* were able to circumvent the effects of *relA* mutations in *S. coelicolor*, as well as the *relC* defects in *S. griseus.* This ability was apparently exerted at the translational level; however, the effects on secondary metabolism, and in some cases morphological differentiation, resulting from mutations in ribosomal proteins, are difficult to assess, as they may be due to a ppGpp-mediated effect, or alternatively, may be the result of modified growth, stemming from altered protein synthesis capabilities.

As outlined above, *S. coelicolor* produces four different antibiotics, actinorhodin (Wright and Hopwood, 1976a), undecylprodigiosin (Rudd and Hopwood, 1980), methylenomycin A (Wright and Hopwood, 1976b) and a calcium dependent antibiotic

(Hopwood and Wright, 1983). While these antibiotics are not used therapeutically, the well-defined genetics of *S. coelicolor*, and the pigmented nature of actinorhodin and undecylprodigiosin, constitute valuable tools in advancing the understanding of antibiotic regulation in the streptomycetes as a whole.

Actinorhodin is a blue pigmented, diffusible antibiotic. The polyketide class of antibiotics, to which actinorhodin belongs, is synthesized through a succession of condensation reactions between small acyl units (O'Hagan, 1991), similar to that seen for fatty acid synthesis. By varying the condensation units, the number of condensation reactions, and the modifications (reduction, dehydration, and cyclization) to the carbon skeleton, it is possible to generate a wide variety of polyketide antibiotics. The study of these polyketide antibiotics was initiated with the cloning of the actinorhodin biosynthetic gene cluster on a 25 kb fragment. The ability to clone the antibiotic biosynthetic genes, regulatory genes, and resistance genes together on a single DNA fragment, provided the first evidence for the clustering of antibiotic biosynthetic genes (Malpartida and Hopwood, 1984). This gene clustering is now known to be a common organizational theme (Seno and Baltz, 1989; Martín and Liras, 1989), and has allowed for the isolation of entire gene sets dedicated to antibiotic biosynthesis. The *act* mutants have been divided into seven classes, with the second class representing the regulatory locus. The *act*II region comprises four different genes, designated ORFs 1-4, with *act*II-ORF2 and *act*II-ORF3 encoding the actinorhodin export complex, *act*II-ORF1 specifying a transcriptional repressor responsible for the control of *act*II-ORF2 and *act*II-ORF3 expression, and *act*II-ORF4 coding for the major transcriptional activator for the *act* gene cluster. ActII-ORF4 has been classified as a member of the SARP (for *Streptomyces*

*a*ntibiotic *r*egulatory *p*rotein) family of proteins, together with the activator of undecylprodigiosin production, *redD*, and a number of proteins from other *Streptomyces* species (Wietzorrek and Bibb, 1997). These proteins share a high degree of homology, and have been shown to possess a DNA-binding domain similar to that of OmpR. Recent studies have demonstrated that ActII-ORF4 is capable of binding DNA at two different sites within the *act* cluster: the operator regions of *act*III-*act*I, and *act*VI-ORF1-ORFA (Arías *et al.*, 1999) These two gene groups have divergently arranged promoters, and are specific for either the early (*act*III-*act*I) or late (*act*VI-ORF1-ORFA) acting biosynthetic steps, with ActII-ORF4 having greater affinity for the operator of the late-acting genes, than that of the early-acting genes (Arías *et al.*, 1999).

The other well-studied antibiotic produced by *S. coelicolor* is commonly referred to as undecylprodigiosin, although technically, this red-pigmented antibiotic is a mixture of three different prodigionines: undecylprodigiosin, butylcycloheptylprodigionine, and dipyrrolyldipyrromethane (Tsao *et al.*, 1985), of which undecylprodigiosin is the major component. The biosynthetic genes for this tripyrrole antibiotic are clustered on a 35.7 kb segment of the chromosome (Malpartida *et al.*, 1990), and while its genes have been cloned, an understanding of undecylprodigiosin synthesis is not as advanced as it is for actinorhodin. There is evidence to suggest that proline may be a biosynthetic precursor and that the production of the secondary metabolite, undecylprodigiosin, may therefore be a response to excess proline produced by the primary metabolic system (i.e. is a sink for proline) (Hopwood, 1994). The first identified regulator of this gene cluster was *redD* (Narva and Feitelson, 1990), which like *act*II-ORF4, was assigned to the SARP family (Wietzorrek and Bibb, 1997; Takano *et al.*, 1992). A second regulator was recently

discovered, termed *redZ*, and is believed to be a transcriptional activator of *redD*, as it is transcribed prior to *redD*, and *redD* expression depends upon a functional *redZ* gene (White and Bibb, 1997). In contrast to RedD and ActII-ORF4, RedZ is not a member of the SARP family, instead resembling a response regulator from the two component response regulator family, although lacking the conserved residues that make up the phosphorylation pocket of typical response regulators (Guthrie *et al.*, 1998).

The final two antibiotics produced by *S. coelicolor*, methylenomycin A and a calcium dependent antibiotic (CDA), are not as well characterized as the pigmented antibiotics. The methylenomycin A biosynthetic genes have been cloned, and have revealed it to be the only known plasmid-encoded antibiotic in the actinomycetes, with its biosynthetic genes clustered on the linear, self transmissible SCP1 plasmid (Wright and Hopwood, 1976b). Methylenomycin is believed to be produced in response to stress, as its accumulation has been found to coincide with maximal glucose consumption, and acidification of the medium (Hobbs *et al.*, 1992). Methylenomycin A is unusual in that the antibiotic itself appears to be responsible for stimulating expression of the resistance genes (Hobbs *et al.*, 1992). Little is known about CDA, although it is thought to be a lipopeptide, that functions as a calcium ionophore (Lakey *et al.*, 1983). It is a chromosomally encoded antibiotic, and is not produced by any of *bldA, B, C,* or *D* mutant strains. The recent cloning of the CDA biosynthetic gene cluster (Chong *et al.*, 1998) (sequence is available in the *S. coelicolor* database: http://www.sanger.ac.uk/Projects/S.coelicolor) should help to further the understanding of its regulation and biosynthesis.

While the regulation of actinorhodin and undecylprodigiosin production are reasonably well characterized in terms of their pathway-specific activators, ActII-ORF4 and RedD/RedZ, respectively, an understanding of the physiological signals responsible for controlling the expression of these pathway-specific activators, has not progressed as rapidly. Antibiotic production appears to be a growth phase-dependent process; however, the timing of expression for the individual antibiotics is not coincident, indicating a response to different signals in each case. At least in liquid culture, methylenomycin A is the first antibiotic to be synthesized, appearing before the onset of the stationary phase of growth (Hobbs *et al.*, 1992). The entry into stationary phase is followed immediately by the appearance of undecylprodigiosin, while actinorhodin production is typically delayed by several hours (Strauch *et al.*, 1991). The cessation of growth that defines the stationary phase is thought to contribute to the initiation of antibiotic production. The finding that ppGpp may impact antibiotic production seemed to support this proposal, although actinorhodin was the only antibiotic consistently affected by the alteration of ppGpp levels through ribosomal proteins and associated enzymes. Medium acidification may also influence antibiotic production, as the accumulation of methylenomycin has been found to coincide with maximal glucose consumption, and acidification of the medium. Antibiotic production has been found to be restored in the *cya* mutant, by buffering of the medium, suggesting that pH may mediate the initiation of antibiotic production, however, buffering of the media upon which the *bld* mutants were grown did not alleviate their antibiotic deficiencies. Carbon and nitrogen source depletion have also been associated with the onset of antibiotic production. Any influence on antibiotic production that is mediated by these above factors suggests that environmental stresses

may be a major contributing factor in the decision to initiate antibiotic production. The response by *S. coelicolor* to these environmental stresses may be achieved through a number of pleiotropic regulators controlling antibiotic production, and in some cases, also morphological differentiation.

A number of antibiotic global regulatory proteins in *S. coelicolor* were originally identified as a result of their ability to "complement" *S. griseus* strains deficient in their ability to produce A-factor, in a way similar to that described for the *bld* gene extracellular complementation cascade. These genes have thus been designated *A-f*actor *s*ynthesis (*afs*) genes. Mutation of the *S. coelicolor afsB* gene was found to abolish actinorhodin and undecylprodigiosin production, while not influencing methylenomycin or CDA production (Hara *et al.*, 1983). Attempts to clone the *afsB* gene have lead to the isolation of a number of different genes capable of complementing the *afsB* phenotype, including *afsR/afsK*, *afsS*, and *afsQ1/afsQ2*, although the *afsB* gene was never identified in the complementation screen. Curiously, the introduction of extra copies of the major vegetative sigma factor of *S. coelicolor*, *hrdB*, was also able to restore the antibiotic deficiencies of the *afsB* mutant strain (Shiina *et al.*, 1991).

The genes capable of complementing the *afsB* mutant phenotype were then individually assessed for their contributions to antibiotic production. *afsS*, is a small gene downstream from *afsR* (discussed below), that encodes a product with some similarity to region 3 of sigma factors from a variety of prokaryotes, although it is significantly smaller than all known sigma factors, and lacks the most highly conserved region 2, needed for promoter recognition and DNA binding (Helmann and Chamberlin, 1988). Introduction of *afsS* into *S. lividans* on a high copy plasmid was found to

stimulate actinorhodin and undecylprodigiosin production (Vogtli *et al.*, 1994). *S. lividans* is often used for the study of genes believed to influence antibiotic production, for despite sharing ~99% DNA similarity with *S. coelicolor*, it does not make visibly appreciable levels of either pigmented antibiotic, and as such, subtle effects on antibiotic production can be assessed. The AfsS stimulatory effect is significant, however, as an increased copy number in *S. coelicolor* also resulted in a discernable increase in actinorhodin production.

afsR, upstream from *afsS*, was found to encode another member of the SARP family, sharing homology with RedD and ActII-ORF4 in its amino terminus (Fernández-Moreno *et al.*, 1991; Wietzorrek and Bibb, 1997), although, it cannot substitute for the activity of either. Disruption of the *afsR* gene showed that it plays an important role in antibiotic production, as a complete loss of pigment production was observed (Hong *et al.*, 1991). AfsR appears to be regulated by phosphorylation, however, unlike the typical two component signal transduction system commonly used in bacteria, where a phosphate group is transferred from a sensor histidine kinase, to a conserved aspartate residue of a response regulator, AfsR is phosphorylated on serine and threonine residues, seen more commonly in eukaryotic systems. AfsK is believed to encode the serine/threonine protein kinase necessary for phosphorylation of AfsR; however, its function appears to be redundant, as disruption of *afsK* did not abolish AfsR phosphorylation (Matsumoto *et al.*, 1994). Correspondingly, the *afsK* disruption phenotype was not as severe as that of *afsR*, as undecylprodigiosin production was unaffected, and actinorhodin production was significantly reduced, rather than abolished completely (Matsumoto *et al.*, 1994). It seems likely that the membrane associated AfsK

responds to some environmental stress signal, and this signal is transmitted to AfsR via a phosphorylation cascade, to initiate antibiotic production.

It also appears that three typical prokaryotic signal transduction systems also play a role in the production of antibiotics in *S. coelicolor*. *afsQ1/Q2*, *cutR/S*, and *absA1/A2* (discussed later), all typify classical prokaryotic two component signal transduction systems. In this system, a membrane associated sensory histidine protein kinase responds to environmental signals by autophosphorylating a conserved histidine residue. The phosphate is then transferred from the histidine residue of the sensor kinase to an aspartate residue in the response regulator protein, and this is believed to activate the response regulator. The *afsQ1/Q2* system is thought to stimulate the production of actinorhodin, and undecylprodigiosin; however, it is likely part of a redundant activation cascade, as disruption of either *afsQ1* or *afsQ2* has no effect on antibiotic production or morphological differentiation (Ishizuka *et al.*, 1992). The *cutR/S* system, however, appears to repress actinorhodin production, as mutations in either of these genes was found to stimulate actinorhodin production in *S. lividans* (Chang *et al.*, 1996). It is not known whether phosphorylated CutR represses actinorhodin production directly, through control of *act*II-ORF4, or whether its effects are mediated through another regulator.

The introduction of DNA from *S. coelicolor* into *S. lividans*, for the purpose of identifying regulators of antibiotic production, has been a useful and well-utilized tool. It was used to assess effects on antibiotic biosynthesis of *afsR2/afsS* and *afsQ1/Q2*, and was also used in the isolation of a DNA fragment containing the *abaA* locus (for *a*ntibiotic *b*iosynthesis *a*ctivator). Introduction of *abaA* on a multicopy plasmid, into *S. lividans*, was found to stimulate actinorhodin production (Fernández-Moreno *et al.*, 1992).

Disruption of *abaA* in *S. coelicolor* resulted in the elimination of actinorhodin, and a severe reduction in both undecylprodigiosin and CDA, although there was no effect on methylenomycin production.

The most global of the antibiotic regulatory genes, *absA* and *absB*, were identified through a mutagenic screening for *S. coelicolor* colonies deficient in pigmented antibiotic production. Mutations in these genes were found to not only eliminate the production of the pigmented antibiotics, but also to abolish the expression of methylenomycin and CDA (Adamidis *et al.*, 1990; Adamidis and Champness, 1992). Cloning and sequencing of these loci have revealed *absA* to encode a eubacterial two-component signal transduction system (Brian *et al.*, 1996), and *absB* to specify an RNaseIII homologue (Price *et al.*, 1999).

The *absA* locus was subdivided into *absA1* and *absA2*, corresponding to the sensor histidine kinase and the response regulatory protein, respectively. This locus was found to map to the CDA cluster, and may have originally been a regulator of *cdaR*, the pathway specific activator, before evolving to have a more global effect on the other antibiotic pathways as well (W. Champness, personal communication). Strangely, it was found that disruption of *absA1* did not result in the elimination of antibiotic production that had been seen in the previously identified point mutant strains, but rather, appeared to cause premature and precocious expression of actinorhodin and undecylprodigiosin (the other two antibiotics were not examined) (Brian *et al.*, 1996). It was therefore predicted that the original *absA* mutant strains were locked into a negative mode of regulation. Recently it has been shown that strains with point mutations in *absA1* readily acquire suppressive mutations, also found to be within the *absA1* coding sequence (Anderson *et al.*, 1999). Conversely, point mutations within *absA2* gave the same antibiotic overexpression phenotype seen with the *absA* disruptant, suggesting that

mutations within *absA2* are epistatic to those in *absA1* (Anderson *et al.*, 1999). An interesting correlation to note is that the *absA* antibiotic defective phenotype can be partially suppressed by multiple copies of the *afsQ1/Q2* locus, or the *afsR-K-S* gene cluster, restoring actinorhodin and undecylprodigiosin production. These two gene clusters may represent redundant means of activating antibiotic production, and whose effects may parallel, at least in part, those of the *absA* products.

Examination of *absB* mutants revealed that, unlike the *absA* situation, they had a consistent antibiotic deficient phenotype. It was also found that an *absB* disruption resulted in an accumulation of 30S rRNA precursors, although given the function of RNaseIII in regulating rRNA processing as well as controlling the half life and activity of many mRNAs, this was not surprising (Price *et al.*, 1999). Due to the timing of its expression, a second RNase, RNaseES, has recently been proposed to play a role in antibiotic production as well, however, such a role has yet to be demonstrated (Hagège and Cohen, 1997). Interestingly, *absB* missense mutations were discovered to decrease the transcription of *act*II-ORF4 and *redD*, which is contrary to what would be expected from mutation of an RNase.

The simplest hypothesis that could account for all of these diverse observations is that the pleiotropic regulators exert their effects through the pathway specific activators. Mutations in *afsB, afsS,* and *absB* have all been found to adversely affect the expression of *act*II-ORF4 and *redD*, while mutations in *bldA* prevent the translation of ActII-ORF4 and RedZ. Signal transduction cascades appear to be a prevalent theme in antibiotic regulation, with three different two-component signal transduction systems, and one serine/threonine eukaryotic-like system. Interestingly, the regulation of morphological development involves an abundance of response regulators without associated sensor kinases: WhiI, BldM, and AmfR/RamR. This may reflect a greater flexibility in

phosphorylation control, or alternatively, may indicate a move away from regulation by phosphorylation, perhaps to prevent the cross-regulation of morphological and physiological differentiation, although the *bld* genes appear to exert global regulation over both differentiation processes. The connections between the *whi* genes, the *bld* genes, the antibiotic regulatory genes, and the various extracellular and intracellular stimuli, are only now beginning to be investigated.

The primary goals of this thesis were to characterize the *bldD* gene, and elucidate its role in the regulation of differentiation in *S. coelicolor*. Characterization of BldD involved determining the nature of the only known *bldD* mutation, found to be a single point mutation, changing a tyrosine residue to a cysteine residue. It also involved assessing *bldD* transcription, both in the wild type and mutant strains. *bldD* was determined to be expressed as a monocistronic transcript, in a temporally regulated manner. It appears to be subject to negative autoregulation, as the *bldD* transcript was far more abundant in the mutant strain than the wild type strain. Finally, analysis of the BldD protein revealed it to be a cytoplasmically-localized protein, whose predicted secondary structure suggested the presence of a DNA-binding helix-turn-helix motif. In determining the role played by BldD in differentiation, it was necessary to identify targets of BldD regulation, and thereby begin to understand the interactions of the various regulatory components. BldD has been found to govern the expression of itself, *whiG*, *bldN*, a stress response sigma factor, *sigI*, and two previously uncharacterized open reading frames.

Chapter 2

Materials and Methods

2.1 Bacterial strains, plasmids and growth conditions

2.1.1 *Streptomyces* and *Escherichia coli* strains and plasmids

Streptomyces strains used are listed in Table 2.1. *E. coli* strains used are listed in Table 2.2.

2.1.2 Plasmids used in *E. coli* and in *Streptomyces* are listed in Table 2.3.

2.1.3 Propagation of *E. coli* strains

E. coli strains were all maintained as frozen glycerol stocks. Five hundred microlitres of sterile 40% glycerol was added to 500 µL of overnight LB (1% w/v tryptone, 0.5% w/v yeast extract, 1% w/v NaCl) culture, to give a final glycerol concentration of 20%. Plasmid-containing strains were grown in LB, supplemented with ampicillin (100 µg/mL; Sigma), apralan (100 µg/mL; Provel) or kanamycin (50 µg/mL; Sigma). Glycerol stocks were flash frozen in ethanol-dry ice, and were stored at –80°.

2.1.4 Propagation of *Streptomyces* strains

Streptomyces strains were maintained as frozen spore or mycelial stocks. They were grown on cellophane discs overlaying R2YE agar (Hopwood *et al.*, 1985), supplemented with thiostrepton (5-50 µg/mL; gift from S. Lucania at Squibb and Sons, Inc., Institute for Medical Research) or apralan (10 µg/mL) for strains containing plasmids, at 30°C for 4-7 days. Harvesting of these plate cultures was accomplished by scraping the spores or the mycelial growth from the cellophane with a sterile spatula, and resuspending them in sterile milli-Q H_2O in a glass universal bottle. For spore stocks, the

Table 2.1. ***Streptomyces*** **strains used in this study**

Streptomyces **strain**	**Genotype**	**Reference or Source**
Streptomyces coelicolor **A3(2)**		
J1501	*hisA1, uraA1, strA1, pgl,* SCP$^-$, SCP2$^-$	Chater *et al.*, 1982; John Innes Institute
HU66	*hisA1, uraA1, strA1, pgl, bldD53,* NF, SCP2*	Willey *et al.*, 1993; J. Willey
J916	*hisA1, mthB2, pheA1, strA1,* SCP1^{NF-} (SCP2*?)	Wright, 1976; John Innes Institute
1169	*hisA1 mth32, pheA1, strA1, bldD53,* SCP1^{NF-} (SCP2*?)	Wright, 1976; John Innes Institute
MY262	putative bald mutant derived from *S. coelicolor* 1147	Nodwell *et al.*, 1999
MY405	putative bald mutant derived from *S. coelicolor* 1147	Nodwell *et al.*, 1999
MY486	putative bald mutant derived from *S. coelicolor* 1147	Nodwell *et al.*, 1999
Streptomyces lividans **66**		
1326	SLP2, SLP3	Lomovskaya *et al.*, 1980; John Innes Institute

Table 2.2. *Escherichia coli* strains used in this study

***E. coli* strain**	**Genotype**	**Reference or Source**
DH5α	F⁻, *supE44, ΔlacU169, (ϕ80lacZΔM15), hsdR17, recA1, endA1, gyrA96, thi-1, relA1*	Hanahan, 1983; Gibco BRL
ET12567	F-, *dam-13::Tn9, dcm-6, hsdM, hsdR, recF143, zjj-202::Tn10, galK2, galT22, ara14, lacY1, xyl-5, leuB6, thi-1, tonA31, rspL136, hisG4, tsx78, mtl-1, glnV44*	MacNeil *et al.*, 1992; gift from D. MacNeil, Merck Sharp and Dohme Research Laboratories
JM109	*recA1, supE44, endA1, hsdR17, gyrA96, relA1 thiΔ(lac-proAB)*	Yanisch-Perron *et al.*, 1985; gift from Karen Anthony
One Shot™ TOP10		Invitrogen; gift from Anne Sharpe

Table 2.3. ***Escherichia coli*** **and** ***Streptomyces*** **plasmids used in this study**

Plasmid	Relevant characteristic	Reference and Source
Streptomyces **plasmids**		
pIJ486	high copy number cloning vector (*tsr*)	Ward *et al.*, 1986; John Innes Institute
pIJ4083	high copy number promoter probe vector (*tsr*, promoterless *xylE*)	Clayton and Bibb, 1990; John Innes Institute
E. coli **plasmids**		
pUC118/119	high copy number cloning vector (*amp*)	Vieira and Messing, 1987; J. Vieira, Waksman Institute of Microbiology, Piscataway, N.J.
pIJ2925	pUC18 derivative with *Bgl*II sites flanking the polylinker *(amp*)	Janssen and Bibb, 1993
pAU5	pIJ2925 with *tsr* marker (*amp*)	Geibelhaus *et al.*, 1996
pAU171	pIJ2925 containing *bldD* on a 4.5 kb insert	Elliot *et al.*, 1998
pAU184	pUC119 containing *bldD* on an *Sph*I-*Xmn*I fragment	Elliot *et al.*, 1998
pAU193	pAU184 containing the apramycin resistance gene cassette	this study
pAU194	pAU184 containing the thiostrepton resistance gene cassette	this study
pK184	high copy number cloning vector (*kan*)	Jobling and Holmes, 1990
pQE9	high copy number 6xHis tag fusion protein overexpression vector (*amp*)	Qiagen; gift from Dr. Stemke
pQE9BldD+	pQE9 vector containing the *bldD* coding sequence (*amp*)	this study
pQE9BldD-	pQE9 vector containing the mutant *bldD* coding sequence (*amp*)	this study
pGEX-2T	high copy number GST fusion protein overexpression vector (*amp*)	Pharmacia; gift from J. Manchuk
pCR®-TOPO	cloning vector for PCR products with 3'-T overhang (*amp*)	Invitrogen; gift from A. Sharpe
pBSApr	pBluescript derivative containing the apramycin resistance gene cassette	gift from A. Paradkar

E. coli-Streptomyces **shuttle vector**		
pSET152	high copy vector in *E.coli*; integrates into the *att* site in *Streptomyces* (*apr*)	Bierman *et al.*, 1992; Northern Regional Research Center, Peoria, Ill.
pAU181	pSET152 containing *bldD* on an *Sph*I-*Xmn*I fragment	Elliot *et al.*, 1998

resulting spore suspension was placed in a water bath sonicator for 5-10 minutes, to separate the spores from the vegetative biomass. The mixture was allowed to settle, and the liquid portion was filtered into a sterile universal bottle through sterile non-absorbent cotton wool packed into a conical tube with a hole drilled in the bottom. The filtered suspension was then centrifuged at 2900 rpm, at 4°C, for 10 minutes in a PRJ International Centrifuge (swinging bucket rotor), the supernatant was poured off, and the spore pellet was resuspended in glycerol, to give a final concentration of 20%. The spore stock was then dispensed into sterile Bijou bottles as 1-2 mL aliquots, and stored at –20°C. Mycelial stocks were homogenized (Kontes, ground glass homogenizer), centrifuged at 2900 rpm, resuspended to give a 20% glycerol final concentration, aliquoted and stored at –20°C.

2.2 Transformation and DNA isolation

2.2.1 Preparation of *E. coli* competent cells

Calcium chloride competent cells were prepared for use in plasmid transformation according to the procedure of G. Rizzel (personal communication). A sterile toothpick was used to pick an isolated *E. coli* colony into 2 mL of LB broth, which was allowed to sit at 37°C overnight. Twenty microlitres of this overnight culture were then added to 20 mL LB, and the culture was grown with shaking at 37°C, for 3-4 hours. The cells were poured into a sterile Universal bottle, and were centrifuged at 2000 rpm for 5 minutes in the swinging bucket centrifuge described above. The cell pellet was then subjected to two separate washes; the first wash involving resuspension of the cell pellet in 5 mL of 100 mM $MgCl_2$, followed by a 5 minute centrifugation step, as described above. The

second wash utilized 5 mL of 100 mM $CaCl_2$ for resuspension of the cell pellet, and was followed again by a 5 minute centrifugation. Calcium chloride and glycerol were added to this resulting cell pellet, to a final concentration of 100 mM and 20%, respectively, in a final volume of 1 mL. One hundred microlitre aliquots of the final cell suspension were dispensed into 1.5 mL Eppendorf tubes. The cells were flash frozen (as described previously) and were stored at –80°C.

2.2.2 Transformation of *E. coli*.

E. coli DH5α competent cells were obtained from Gibco BRL, and were stored as 50 μL aliquots at –80°C. All other competent cells were prepared as outlined in 2.2.1. The competent cells were thawed on ice, after which 1-6 μL of plasmid DNA were added. The cells and DNA were mixed gently, and left on ice for 30 minutes. The cells were then heat shocked at 37°C for 20 seconds for the commercial cells, and at 42°C for 60-90 seconds, for prepared cells, and placed on ice for 2 minutes before LB broth was added to 1 mL. The cells were incubated at 37°C for 1 hour on a rotating shaker (Bellco Biotechnology), before 1-100 μL aliquots of the cells were spread on LB agar plates supplemented with the appropriate antibiotic [ampicillin (100 μg/ml); apralan (100 μg/mL); or kanamycin (50 μg/mL)]. If blue/white selection was to be used in the identification of insert-containing clones, IPTG (100 mM) and X-gal (40 μg/mL) were added to the plates in addition to the antibiotic. The plates were incubated at 37°C overnight. Subculturing was performed in LB broth with the appropriate antibiotic.

2.2.3 Isolation of *E. coli* plasmid DNA

Plasmid DNA from *E. coli* was typically isolated on a small scale, using a standard alkaline lysis procedure outlined in Sambrook *et al.* (1989). When high purity DNA was required, Qiagen tip 100 columns were used for small scale plasmid preparations, while Qiagen tip 500 columns were used for large scale preparations (both were used according to the manufacturers recommendations).

2.2.4 Preparation of *Streptomyces* Protoplasts

To generate protoplasts, *S. coelicolor* and *S. lividans* strains were grown and harvested as outlined in Hopwood *et al.* (1985). Briefly, glycerol spore or mycelial stocks were used to inoculate a 5 mL TSB (3% trypticase soy broth) seed culture in Universal bottles with springs, and these were grown for 36-72 hours at 30°C and 250 rpm on a platform shaker. This starter culture was then added to a 25 mL mixture containing 15 mL of YEME [0.3% Yeast Extract (Difco), 0.5% peptone (Difco), 0.3% Malt Extract (Difco), 1% glucose, 34% sucrose] supplemented with 5 mM $MgCl_2$-$6H_2O$, 0.5% glycine, and Tiger's milk (called "Super" YEME), and 10 mL TSB, in 250 mL spring-containing flasks, and grown at 30°C and 250 rpm. After 36-48 hours, the culture was poured into a Universal bottle, and was centrifuged at 2900 rpm for 10 minutes (in the swinging bucket centrifuge described previously). The resulting mycelial pellet was washed with a 10.3% sucrose solution, and centrifuged again. The mycelia were then resuspended in 4 mL of lysozyme solution (1 mg/mL of lysozyme in sterile P-buffer) and incubated at 30°C for 60 minutes. After 45 minutes, the mycelium/protoplast suspension was mixed by thrice passing the suspension up and down in a 5 mL pipette, before

incubating for the remaining 15 minutes. At the conclusion of this incubation, 5 mL of P-buffer were added, and the suspension was filtered through non-absorbent cotton wool, as described in 2.1.3. The protoplast suspension was then centrifuged in a bench top clinical centrifuge for 7 minutes at 3150 rpm. The supernatant was discarded, the protoplasts were resuspended in 5-10 mL P-buffer, and dispensed as 1 mL aliquots into 1.5 mL Eppendorf tubes. The aliquots were frozen slowly, being placed in a bucket of ice at –80°C, where they were then stored.

2.2.5 Transformation of *Streptomyces*

An aliquot of protoplasts was thawed quickly at 37°C, and transferred to a 15 ml round bottomed plastic tube (Corning). The 1 mL aliquot was washed using 5 mL of P-buffer, and was centrifuged at 3150 rpm in the bench top centrifuge. The supernatant was poured off, and the protoplasts were resuspended in the remaining buffer. DNA, in up to 20 μL of TE or water, was added to the protoplast suspension, followed immediately by 500 μL of T-Buffer (25% PEG-1000 in P-buffer) to aid in the DNA uptake. This solution was mixed once, using a micropipet (with a 1 mL tip), before the addition of 5 mL of P-buffer and a subsequent 7 minute spin in the bench top centrifuge. The protoplast pellet was finally resuspended in 500 μL P-buffer, and 100 μL were spread onto R2YE plates, which were incubated at 30°C. After 16-20 hours of incubation, the transformants were overlaid with 1 mL of milli-Q H_2O containing either thiostrepton or apralan, to give a final concentration of 50 μg/mL or 100 μg/mL, respectively. The plates were then incubated at 30°C until the antibiotic resistant colonies

were large enough to streak onto R2YE plates containing thiostrepton (50 μg/mL), or apralan (100 μg/mL).

2.2.6 Isolation of *Streptomyces* chromosomal DNA

Streptomyces chromosomal DNA was isolated exactly as described in Procedure 3 of Hopwood *et al.* (1985).

2.3 DNA analysis and purification

2.3.1 Cloning and digestion of DNA

Restriction enzyme digestion for both plasmid and chromosomal DNA was carried out as recommended by the enzyme supplier (Roche, New England Biolabs, or Promega), and by Sambrook *et al.* (1989). Ligations were performed using an insert to vector ratio of ~3:1 for sticky-end ligations, and of ~5:1 for blunt end ligations. All ligation reactions were performed using 1 mM ATP/reaction, and 0.5 U of T4 DNA ligase (Roche) in a buffer of 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 5% PEG 8000, and 1 mM DTT. Cohesive end ligations were incubated at 15°C overnight (usually for 12-24 hours), while blunt end ligation reactions were performed at room temperature for 4-7 hours. Reactions requiring both blunt end and cohesive end ligations were incubated at room temperature for 2-4 hours before being placed in a bucket containing 2-4 litres of room temperature water, which was allowed to cool to 4°C overnight.

2.3.2 Polymerase chain reaction

Polymerase chain reaction (PCR) was used to amplify DNA fragments required for cloning, as well as DNA probes needed for S1 nuclease mapping, Northern and Southern blot analysis, electrophoretic mobility shift assays, and footprinting assays. Amplification reactions were carried out in 100 μL volumes, in 0.6 mL Eppendorf tubes using the Techne PHC-2 thermocycler, or in 0.2 mL tubes (Rose) using the MJ Research Minicycler™. Typical amplification reactions comprised 0.2 mM dNTP's (Roche), 4% DMSO (Sigma), 3.5 mM $MgCl_2$, 40 pmol of each oligonucleotide primer (Table 2.4), 50 mM Tris-HCl (pH 9.2), 16 mM $(NH_4)_2SO_4$, +/- detergents (as in the manufacturer supplied buffer), either 1 μg of chromosomal DNA or 10 ng of plasmid DNA as template, and 0.7 μL (2.5 units) of EXPAND High-Fidelity Polymerase™ (Roche). Reactions carried out using the Techne thermocycler were overlaid with 3 drops of mineral oil (the reactions performed in the Minicycler™ did not require oil overlay, as a heated lid was utilized) before being subjected to 5 minutes of denaturation at 95°C. Thirty cycles of denaturing for 30 seconds, primer annealing at 48-60°C [~5°C below the T_d (see 2.3.11) of the primers) for 30 seconds, and DNA extension at 68°C for 1 minute followed (exceptions to this protocol are detailed elsewhere]. Reactions requiring a proofreading enzyme were performed using Deep $Vent_R$ DNA polymerase (New England Biolabs), and were conducted as above, differing only in the buffer conditions, which comprised 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl (pH 8.8), 2 mM $MgSO_4$, 0.1% Triton X-100, with no added $MgCl_2$, and in the amount of polymerase added (1-2 U Deep $Vent_R$).

Table 2.4. Oligonucleotide Primer Sequences*

Primer	Sequence 5'-3'	Region of homology	Use
Reverse	AACAGCTATGACCATG	downstream of polylinker in pUC and M13-based vectors	sequencing; PCR; S1 mapping
Universal	GTAAAACGACGGCCAGT	upstream of polylinker in pUC and M13-based vectors	sequencing; PCR
BKL37	CGAGCTGGCGGACTTCT	within *bldD* coding sequence	sequencing; gene disruption
BKL41	CGCCGTCATCTACGACC	within predicted HTH of *bldD*	sequencing
BKL47	CTCGTTGCGCCGCGAGT	downstream from the *bldD* coding sequence	sequencing
BKL50	CCACGACGGCCTTCCAG	within *bldD* coding sequence	sequencing; PCR; S1 mapping
BKL51	**GCGC**<u>**GAATTC**</u>GGCGTTCGACGATCTCG	upstream from *bldD* promoter	PCR; S1 mapping
BKL54	CCGCCTTCGCCACCGGT	complementary to *Streptomyces* 16S rRNA	Northern analysis for RNA loading
BKL68	**GAGG**<u>**TCTAGA**</u>GGTCGTAGATGACGGCG	within *bldD* coding sequence	gene disruption
BKL69	**GCGC**<u>**TCTAGA**</u>CGAGCTGGCGGACTTCT	within *bldD* coding sequence	gene disruption
BKL87	GTGCCGGTGGCGACGAC	within *bldG* promoter region	PCR
BKL88	ATGCTCCTGGACCGGCTC	within *bldG* promoter region	PCR
MAE1	GGAAGAGTCGGTGCGGA	upstream from *bldD* promoter	PCR; emsa; footprinting assays; sequencing
MAE2	GGTCGTAGATGACGGCG	complementary to BKL41; within predicted HTH of *bldD*	PCR; gene disruption
MAE3	GGGCAGTTCGCGGTGGC	upstream from *bldD*	sequencing
MAE4	<u>**TCTAGA**</u>GCGGCAGGCTGTGTTGTC	downstream from the *bldD* tsp	PCR; emsa; footprinting assays; sequencing
MAE5	**GGTA**<u>**AGCTT**</u>TCAGAGCTCGTCGTGGGAC	C-terminal end of *bldD* coding sequence	sequencing; PCR; protein overexpression; emsa
MAE6	**CGC**<u>**GGATCC**</u>TCCAGCGAATACGCCAAAC	N-terminal end of *bldD* coding sequence	sequencing; PCR; protein overexpression
MAE8	CATTACTGGATCTATCAAC	downstream from pQE9 polylilnker	sequencing
MAE9	GAATTCATTAAAGAGGAG	upstream from pQE9 polylinker	sequencing
MAE10	**GGC**<u>**GAGCTC**</u>AGGCGAATACTTCATATG	within *tsr*	PCR
MAE11	CGGTAGCAGGCTCACAG	upstream from *bldD* promoter	sequencing; PCR; emsa; footprinting assays

Primer	Sequence 5'-3'	Region of homology	Use
MAE12	GAGCTGTTTGGCGTATTCG	downstream from *bldD* translation start site	sequencing; PCR; emsa; footprinting assays
MAE15	TACGCAGCGTTACTCTGCTGCGT	within *bldD* promoter region	PCR; emsa
MAE16	ACGCAGCAGAGTAACGCTGCGTA	complementary to MAE15; within *bldD* promoter	PCR; emsa
MAE17	CTGGTAACTCACTGTGAGGTTACGCA	extends over the *bldD* tsp	emsa
MAE18	TGCGTAACCTCACAGTGAGTTACCAG	complementary to MAE17; extends over *bldD* tsp	emsa
MAE21	GTAACGCTGCGTAACCTCACAGTGAGTTACCAGCCG CG	extends over the *bldD* tsp	emsa
MAE22	GCGCGGCTGGTAACTCACTGTGAGGTTACGCAGCGT TAC	complementary to MAE21; extends over *bldD* tsp	emsa
MAE26	CGATGACTTTGATGACTGC	downstream from *ftsZ* translation start site	PCR; emsa; S1 mapping
MAE27	GGCACTTCGACGTGAGTG	upstream from *ftsZ* promoter	PCR; emsa
MAE33	**GCGCTCTAGA**GGCACTTCGACGTGAGTG	upstream from *ftsZ* promoter	PCR; S1 mapping
MAE35	ACTGTGAGGTTACGCAGC	within *bldD* promoter region	PCR;emsa
MAE36	ACAGTGAGTTACCAGCCG	immediately downstream from *bldD* tsp	PCR; emsa
MAE42	CCTACTGTACTATGGTAGC	nonspecific linker for SELEX	PCR; selection of BldD targets
MAE43	**X-GATC**GCTAACCATAGTACAGTAGG#	complementary to MAE42; SELEX linker	selection of BldD targets
MAE44	GGATTTCTGGCAGTTGAGG	upstream from *whiG* tsp	sequencing; PCR; S1 mapping; emsa; footprinting assays
MAE45	TACGACCGCCACAGCTCG	downstream from *whiG* translation start site	sequencing; PCR; S1 mapping; emsa; footprinting assays
MAE46	ACGCAGCAGCGTAACGCTGCG	mutagenized *bldD* promoter/BldD binding site	PCR; emsa
MAE47	ACGCAGCAGAGTTACGCTGCG	mutagenized *bldD* promoter/BldD binding site	PCR; esma
MAE51	**CGACCCGGG**CCTTCCGGTGACTCTTCG	downstream from SCE68, ORF 26.c tsp	PCR; emsa
MAE52	**GGTTCTAGA**GCGCTCGGACCGGTAGG	significantly downstream from SCE68, ORF 26.c	PCR; gene disruption
MAE53	**CGACTGCAG**TGAGCAGCCGGATCTGC	downstream from SCE68, ORF 26.c	PCR; gene disruption
MAE54	**CAGGAATTC**GTAGGGGTGGTTTCTCG	significantly uptream from SCE68, ORF 26.c	PCR; gene disruption
MAE65	GCATCACCGAGGCGACC	within SCE68, 26.c coding sequence	sequencing; PCR; S1 mapping; emsa; footprinting assays
MAE67	CTGGCGCATCTGATCGAG	upstream from bldN promoter region	PCR; sequencing

Primer	Sequence 5'-3'	Region of homology	Use
MAE68	CGTAGCCAGGCCCGAGG	downstream from *bldN* translation start site	sequencing; PCR; S1 mapping; emsa; footprinting assays
MAE69	CTGGGACACCCTCACCTCC	overlapping *sig1* translation start site	PCR; emsa
MAE70	GATTTGGGCCCGCCGACC	upstream from *sig1* promoter region	PCR; emsa
MAE71	GTCAATCGGGCACAGAAGC	just down from MAE67, upstream of *bldN* promoter	sequencing; PCR; S1 mapping; emsa; footprinting assays
MAE72	**GCA**<u>GAATTC</u>CGTGTCCGTCGCCGTTCC	upstream from SCE68, ORF 26.c tsp	PCR; sequencing; emsa; footprinting assays
MAE76	**GGTTACGTCGAACGACACG**	upstream from *whiG* tsp	PCR; sequencing; footprinting assays

* Nonhomologous sequences are shown in bold, and restriction enzyme sites are underlined

#X=phosphate

2.3.3 DNA analysis by agarose gel electrophoresis

DNA fragments greater than 300 bp in size were visualized using agarose gel electrophoresis. Fragments between 0.7 and 8 kb in size were electrophoresed at 96 V, on a 1% agarose, 1 × TBE (90 mM Tris, 89 mM boric acid, and 2.5 mM Na_2EDTA) gel, in a 1 × TBE buffer system. Lambda DNA (Roche) digested with *Pst*I was typically used as a molecular weight marker. Smaller DNA fragments (0.3-0.7 kb) were electrophoresed, on 2% agarose gels, using Marker V (Roche; pBR322 DNA cleaved with *Hae*II) as a molecular weight standard. To each sample, 1/10 volume of loading dye was added (0.25% bromophenol blue, 40% w/w sucrose) to provide a visual determinant for DNA progression. DNA bands were examined by staining in 1 × TBE containing ethidium bromide (EtBr), and viewing on a UV transilluminator.

2.3.4 DNA Purification from Agarose gels

DNA fragments larger than ~0.7 kb were purified using an agarose gel-based protocol (Zhen and Swank, 1993). Agarose gels of 0.7-2% were made using 1 × TAE buffer, and were poured to a thickness of 0.5-0.75 cm using flat plates with raised, taped edges to form the mold. The gels were electrophoresed at 84-96 V in an EtBr-containing 1 × TAE buffer system, until the blue dye had progressed to ~2/3 the length of the gel. The gel was then placed on a UV transilluminator, and a trough was cut, immediately below the desired fragment of DNA. The gel was replaced in the electrophoresis tank, and buffer was removed until it reached a level below the top of the gel, not covering the surface. The trough was filled with a solution of 15% PEG-8000 and 0.5 μg/mL EtBr in 1 × TAE, and electrophoresis of the DNA was continued at a voltage of ~133 V.

Movement of the DNA was followed using a hand-held UV illuminator (Mineralight® Lamp UVSL-25; Ultra-Violet Products, Inc.), and when the band of interest had moved into the centre of the trough, electrophoresis was stopped, and the DNA-containing PEG-TAE solution was removed from the trough. Phenol/chloroform and chloroform extractions were performed, and the DNA was precipitated by the addition of 1/10 volume of sodium acetate, and 2 volumes of ethanol.

2.3.5 DNA analysis by polyacrylamide gel electrophoresis

DNA fragments of 50-700 bp were examined using polyacrylamide gel electrophoresis. The fragments were electrophoresed on 5-8% polyacrylamide gels (29:1, acrylamide:N,N'-methylene bisacrylamide), at 150-200 V for 1-3 hours, using a 1 × TBE buffer system. Marker V (Roche) or λ-*Pst*I was used as a molecular weight standards. Loading dye was added to the samples (as above), and the gels were stained with ethidium bromide, and subjected to UV transillumination for visualization of the DNA bands.

2.3.6 DNA purification from polyacrylamide gels

Polyacrylamide gels were used for the purification of DNA fragments smaller than ~0.7 kb according to the "Crush and Soak" procedure outlined in Sambrook *et al.* (1989). The DNA fragments were electrophoresed, and visualized as described in 2.3.5. The DNA band of interest was excised from the gel with a scalpel, transferred to a 1.5 mL Eppendorf tube, where the DNA-containing gel was crushed. The DNA was eluted overnight at 37°C, in 2 volumes of elution buffer [0.5 M ammonium acetate, 1 mM

EDTA (pH 8.0)]. The buffer was then collected, the remaining gel fragments were washed with 1/2 volume of elution buffer, and the buffer fractions were pooled. One microlitre of glycogen (Roche) was added, and the DNA was ethanol precipitated, without the addition of salt, and washed twice with 70% ethanol before being dissolved in 5-20 μL milli-Q H_2O.

2.3.7 DNA sequencing

Cycle sequencing for DNA sequence analysis was conducted using the chain termination method of Sanger *et al.* (1977), as modified for use with Thermo Sequenase™ (Amersham). Reactions were carried out using purified plasmid DNA as template (50-500 ng) and 0.5-2 pmol of primer in a buffer consisting of 26 mM Tris-HCl (pH 9.0) and 6.5 mM $MgCl_2$, in a total volume of 16.5 μL. Eight units of Thermo Sequenase™ were added to the reactions, which were then dispensed as 4.5 μL aliquots into 4 × 0.6 μL or 0.2 μL tubes containing 2 μL master mix, plus 0.5 μL of each of α^{33}P-labeled ddGTP, ddATP, ddTTP or ddCTP (Amersham). The reactions were subjected to PCR amplification as outlined in 2.3.2, only the extension reaction was performed at 72°C rather than 68°C. At the conclusion of the thirty cycles, 4 μL of loading dye (Amersham) was added to each tube to stop the reactions.

The sequencing reactions were separated on a 6% denaturing polyacrylamide gel (19:1, acrylamide:N,N'-methylene bisacrylamide; 8.3 M urea), using a 1 × TBE buffer system and electrophoresis at constant power (35 W) for 1.5-2 hours. Following electrophoresis, the gels were transferred to 3MM Whatman No.1 filter paper, and were dried on a Bio-Rad gel dryer with Savant gel pump, for 1-2 hours. The sequencing

ladders were visualized by exposure to a phosphorscreen, scanned by a Molecular Dynamics Model 445 SI phosphorimager, and analyzed using Imagequant™ software.

2.3.8 Colony blot hybridization

Colony blot hybridizations were conducted for screening of large numbers of colonies for the presence of a particular plasmid insert. For *E. coli* colony blots, individual colonies were picked with a sterile toothpick, and streaked onto two LB plates supplemented with the appropriate antibiotic, one of which was overlaid with a Hybond™-N (Amersham) membrane, such that 50-100 colonies were streaked on each plate. The plates were incubated at 37°C for 9-17 hours, after which the master plates were refrigerated. The membranes were then removed from the second plate, and placed on 3MM Whatman paper soaked in denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 5 minutes. Neutralization of the filters was accomplished by placement of the filters on Whatman paper soaked in neutralizing solution [0.5 M Tris-HCl (pH 7.5), 3 M NaCl] for two intervals of 5 minutes. The membrane filters were then transferred to a dish containing 2 × SSC (0.3 M NaCl, 0.03 M trisodium citrate), and excess colony debris was removed using a gloved hand. Finally, the membranes were dried briefly, and UV cross-linked at 150 mJoules in a Bio-Rad GS Gene Linker.

Streptomyces colony hybridizations involved streaking of individual colonies onto Whatman 541 filters that were overlaid on R2YE plates, supplemented with the appropriate antibiotic. The colonies were allowed to grow for 2-4 days (during which time the mycelia grow into the filter). The filters were then soaked in TE buffer containing 4 mg/mL lysozyme for 10 minutes at 37°C. They were then transferred into a

solution of boiling 0.5M NaOH and 1% SDS for 5 minutes, followed by 5 minutes at room temperature in 0.5 M Tris-HCl (pH 7.5). Finally, the filters were immersed in 90% ethanol, and allowed to dry.

2.3.9 Southern blot analysis

Southern blot analysis was performed as described in Hopwood *et al.* (1985), as a modification of the Southern procedure (Southern, 1975). DNA was separated on a 1% agarose gel, and was stained as outlined in 2.3.3. The stained gel was photographed with a ruler, to permit size determination of DNA bands visualized after hybridization. The gel was then trimmed to remove the unused portions, and treated with 0.25 M HCl for 2 × 10 minutes, with gentle rocking, to aid in the efficiency of DNA transfer. This was followed by gentle rocking of the gel in denaturing solution (see 2.3.8) for 2 × 15 minutes, rinsing three times with water, and finally neutralizing it through treatment with 3 M NaCl and 0.5 M Tris-HCl (pH 7.5) while agitating gently for 20 minutes. In order to transfer the DNA from the gel to a nylon membrane (Hybond™-N; Amersham), the gel was placed well-side down on two pieces of 3MM Whatman No. 1 filter paper, which had been wrapped over a glass plate, with the ends submersed in 20 × SSC to form a wick. The membrane, cut to the same size as the gel, was placed on the gel, followed by two pieces of Whatman filter paper, again equivalent in size to the gel. Paper towels were then placed on the top (~5-10 cm of paper towels), and the entire apparatus was weighted down with a glass plate, and a 500 mL bottle of solution (~0.5 kg). Following overnight transfer, the apparatus was disassembled, the lanes were marked, and the membrane was UV cross-linked.

2.3.10 DNA labeling

Random primer labeling (Feinberg and Vogelstein, 1983; modified by Roche) involves the internal labeling of a DNA fragment, and is used to label probes for DNA hybridization (colony blots and Southern blots), and for RNA detection (northern blots; see below). Nine microlitres of DNA were denatured by heating to 95°C for 5 minutes, followed by immediate placement on ice. Two microlitres of hexanucleotide mix (Roche), 3 μL of 0.125 mM dNTP's (dATP, dGTP and dTTP), 5 μL (50 μCi) [α^{32}P]-dCTP (Amersham), and 1 μL (2 units) Klenow (Roche) were added to the denatured DNA and the reaction was incubated for 1 hour at 37°C, or overnight at room temperature. The labeled DNA was purified away from the unincorporated nucleotides using either a Nuc-Trap® probe purification column (Stratagene) or Micro Biospin® 6 chromatography column (Bio-Rad), after which 1 μL of the labeled probe was Cerenkov counted in a Beckman LS 3801 scintillation counter.

Oligonucleotide probes used for hybridization to northern blots (see below), or probes needed for DNA detection in other experimental situations (see below) were generated by end-labeling of the DNA (Chaconas and van de Sande, 1980; modified by Roche). Typically, 1-3 μL of DNA (10-50 pmol of oligonucleotide primer) were combined with 4-5 μL [γ^{32}P]-ATP (ICN) in a buffer consisting of 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 5 mM DTT, and 0.1 mM spermidine. A 1 μL aliquot of 1/10 diluted T4 polynucleotide kinase (10 U/μL; Roche) was added, the reaction was incubated at 37°C for 15 minutes, at which point a second 1 μL aliquot of kinase was added, and the incubation was continued for another 15 minutes. Oligonucleotide probes were then

separated from the unincorporated nucleotides, as outlined above; however, all longer probes (generated by PCR or restriction digest) were precipitated, with the addition of 1 μL glycogen, and were then redissolved in an appropriate volume of sterile milli-Q H_2O.

2.3.11 DNA Hybridization

Colony hybridizations were performed in deep glass petri dishes, using 10-20 mL of hybridization solution [3 × SSC (0.45 M NaCl, 0.045 M trisodium citrate), 4 × Denhardt's Solution {0.08% w/v Ficoll (MW 400,000), 0.08% w/v bovine serum albumin (Fraction V; Roche), 0.08% polyvinyl pyrrolidone (MW 360,000)}, and 100 μg/mL denatured salmon sperm DNA]. Prehybridization was carried out for more than one hour in a shaking water bath (Hot Shaker; Bellco Biotechnology), before addition of the labeled DNA probe.

Southern blot and northern blot hybridizations were conducted in glass hybridization tubes, in the same hybridization solution outlined above, only deionized formamide was added when necessary. The blots were prehybridized for at least 4 hours in a hybridization incubator (Robbins Scientific), before the labeled probe (2 million cpm/10 mL) was added.

Hybridization of colony blots, Southern blots and northern blots using labeled DNA fragments as probes, was carried out at a temperature approximately 25°C below that of the melting temperature of the probe. The hybridization temperature was optimized for each probe, and was calculated using the equation: $T_m = 81.5°C + 16.6 \log M + 0.41$ (%G+C) – 500/n – 0.61 (% formamide), where M is the ionic strength (0.45 for 3 × SSC), and n is the length of the shortest duplex segment (Hopwood *et al.*, 1985). For

oligonucleotide probes, hybridizations were carried out at 5°C below T_d, where the denaturation temperature was attained using the formula: $T_d = 4\ (G+C) + 2\ (A+T)$ (Hopwood *et al.*, 1985). Formamide was added to lower the T_m when the hybridization temperature was calculated to exceed ~65°C.

After overnight hybridization, the membranes were washed with 2 × SSC, 0.1% SDS for 2 × 30 minutes, and then with 0.2 × SSC, 0.1% SDS for an additional 2 × 30 minutes. Following these washes, the filters were wrapped in Saran Wrap, and exposed to X-ray film (Kodak X-OMAT AT film) at –80°C, and then developed in a FUJI RGII X-ray film processor, or alternatively, were exposed to a phosphorscreen , visualized using a phosphorimager, and analyzed using Imagequant™ software.

2.4 RNA analysis

2.4.1 Isolation of RNA

Streptomyces RNA was isolated using a modified version of the Kirby *et al.* (1967) procedure, detailed in Hopwood *et al.* (1985), with a few adjustments. Cultures were grown on cellophane discs placed on the surface of R2YE agar plates. Harvesting of the cultures involved scraping of the cell biomass from the cellophane discs using a sterile spatula, and transferring them to 20 mL Universal screw cap bottles containing 4 mL modified Kirby mixture and ~2 cm of glass balls. The cell suspensions were vortex mixed for 4 × 30 second intervals, and were kept on ice between mixings. Upon the addition of 5 mL phenol/chloroform, the bottles were again mixed, for 2 × 30 second intervals. The homogenates were transferred from the Universal bottles into polypropylene tubes using baked 5 mL pipets, and were centrifuged at 4°C and 8,500 rpm

for 5 minutes in the Beckman J2-H5 centrifuge, with the JA20 rotor. The aqueous phases were transferred to phenol/chloroform-containing polypropylene tubes with 100-1000 μL micropipettor tips, and the mixtures were again vortexed and centrifuged to separate the aqueous phases from the phenol layers. This process was repeated until there was no interface visible between the two layers. The nucleic acids were then precipitated using 1/10 volume sodium acetate and 1 volume isopropanol, and were kept on ice for at least 20 minutes before spinning for 10 minutes at 8000 rpm. The pellets were washed with 95% ethanol, redissolved in 450 μL DEPC-treated milli-Q H_2O, and transferred to 1.5 mL Eppendorf tubes. Treatments with RNase-free DNaseI (Roche) then followed, with 2 × 5 μL aliquots (containing 10 U/μL) being added to the tubes for a 30 minute incubation at room temperature per aliquot. Two phenol/chloroform extractions were performed after the DNaseI digestion, after which the RNA was precipitated and redissolved in 100 μL DEPC-treated milli-Q H_2O. Prior to storage at –80°C, 10 μL sodium acetate and 110 μL isopropanol were added to the RNA solutions.

2.4.2 Northern blot analysis

Northern blot analysis was conducted as described by Williams and Mason (1985). Forty micrograms of each RNA sample (10 μg if only analyzing the 16S rRNA), were dissolved in 2.5 μL DEPC-treated milli-Q H_2O. The RNA was denatured by adding 1 M glyoxyl, 50% DMSO, and 10 mM Na_2HPO_4-NaH_2PO_4 (pH 7.0) (final concentrations), and heating to 50°C for 1 hour. Molecular weight markers III and V (625 ng; Roche; Marker III: lambda DNA cleaved with *Eco*RI and *Hind*III) served as size markers, and were treated exactly as the RNA. The glyoxylated RNA and DNA markers

were size fractionated by electrophoresis at 4 V/cm on a 1.25% agarose gel, using a 10 mM Na_2HPO_4-NaH_2PO_4 (pH 7.0) recirculating buffer system. The gel was then trimmed, and the RNA was transferred to Hybond™-N (Amersham) membrane by overnight capillary blot transfer in 20 × SSC as described in 2.3.9. Following overnight transfer, the membrane was UV cross-linked and baked for 1 hour at 80°C under vacuum to remove the glyoxyl. The lanes corresponding to the two markers were removed at this stage, stained using 0.2% methylene blue in 0.2 M sodium acetate (pH 4.7) (Miller *et al.*, 1987), and washed with water to remove excess stain.

2.4.3 S1 nuclease mapping

S1 nuclease mapping of mRNA 5' ends was conducted not only to determine the transcription start site for a particular transcript, but also to assess the level of expression of an ORF over a given period of time. The probes used for S1 nuclease mapping were designed to span the anticipated transcription start site region, and were end-labeled in such a way that after digestion with S1 nuclease, only the end internal to the transcription start site would be labeled. This was accomplished either by restriction nuclease digestion of the probe after labeling, to remove the external label (used only in instances where the transcription start site had been previously determined), or incorporation of a nonhomologous tail onto the end of the external primer, to ensure that it would remain single stranded during RNA-DNA annealing, and thus would be removed during the S1 nuclease digestion. The importance of including a nonhomologous tail when examining the expression of genes whose transcription start site had not been previously mapped, was to allow the identification of full length protection of the probe, which would appear

to be slightly smaller (having had the nonhomologous end digested) than the product resulting from probe-probe reannealing. The removal of the external label, through restriction nuclease or S1 nuclease digestion, was also important for ease of data interpretation, as it served to eliminate any effects seen as a result of divergent transcript detection.

For each S1 nuclease reaction, 40-50 μg of RNA were precipitated and redissolved in 2 μL of probe solution (~25,000 cpm/μL in milli-Q H_2O). The RNA-probe mixture was then dried in a SpeedVac Concentrator (Savant), and resuspended in 20 μL of formamide hybridization buffer (3.2 mM PIPES buffer (pH 6.4), 0.4 M NaCl, 1 mM EDTA, and 80% v/v formamide). The reaction tubes were placed in an 80°C water bath and after 20 minutes were vortexed and briefly centrifuged, before being replaced at 80°C for an additional 10 minutes. The temperature of the water bath was then adjusted to ~5°C above the estimated melting temperature of the double stranded DNA probe (to minimize probe reannealing) (see 2.3.11 for melting temperature calculations; $M = 0.4$ for S1 hybridization buffer), and the water bath was allowed to slowly cool to this set temperature, where it was left to sit for 3-4 hours, or overnight. After this annealing period, the tubes were placed on ice, and either 200 U of Sigma S1 nuclease, or 150 U of Roche S1 nuclease, were added in digestion buffer [0.28 M NaCl, 30 mM CH_3COONa (pH 4.4), 4.5 mM $(CH_3CO_2)_2$ Zn, 20 μg partially-cleaved denatured calf thymus DNA] to give a total volume of 320 μL. The reactions were incubated at 37°C for 45 minutes, before the reactions were stopped with 2.5 M ammonium acetate and 0.05 M EDTA. The remaining DNA was precipitated by the addition of 1/10 volume sodium acetate, 1 volume of isopropanol and 1-2 μL of glycogen (Roche), and was chilled at –20°C for at

least 30 minutes. After centrifugation of the precipitated DNA for 10 minutes, the resulting pellet was washed with 80% ethanol, and dissolved in 4 μL of loading dye [98% deionized formamide, 10 mM EDTA (pH 8.0), 0.025% xylene cyanol, 0.025% bromophenol blue]. The mixture was heated to 95°C for 5-10 minutes, with frequent vortexing, prior to loading onto a 6% denaturing polyacrylamide gel. Sequencing ladders were generated by the dideoxy chain termination method (Sanger *et al.*, 1977), using the primer predicted to be downstream from the transcription start site (see 2.3.7).

2.4.4 Primer extension analysis

Primer extension analyses were performed using the procedure outlined in Penfold *et al.* (1996), to identify the transcription start site for *bldD* and the pattern of transcript expression for *ftsZ*. Primers internal to the coding sequence for each gene were used for the extension reactions. Fifty picomoles of primers were end-labeled (2.3.10) and ~6×10^5 cpm of the primers were then incubated with 30 μg of RNA in hybridization buffer (1 M NaCl; 167 mM Hepes; 0.3 mM EDTA, pH 8.0). The RNA-primer mixture was incubated at 80°C for 5 minutes before being placed at 37°C for 1 hour. The nucleic acid mixture was then precipitated by the addition of 95% ethanol, and incubation on ice for 30 minutes. A 10 minute centrifugation step served to pellet the RNA/DNA mixture, and the resulting pellet was washed with 95% ethanol. Twenty five microlitres of reverse transcriptase mix [55 μM dNTPs; 50 mM Tris-HCl, pH 8.0; 5 mM $MgCl_2$; 5 mM DTT; 50 mM KCl; 50 μg/mL bovine serum albumin (Fraction V); and 0.5 μL RNAguard® (Amersham)] was added to the primer-annealed RNA, followed by the addition of 1 μL (25 U) of AMV reverse transcriptase. The reactions were then incubated at 42°C for 1

hour. Following the extension period, the reactions volumes were increased to 300 μL by the addition of 1 × TE, and they were ethanol precipitated, together with 1/10 volume of sodium acetate, and 1 μL of glycogen per reaction tube. The primer extension products were resuspended in 10 μL of loading dye (see 2.4.3), heated to 90°C for 5 minutes, and 2-5 μL were loaded onto a 6% sequencing gel. Sequencing ladders were generated as above (2.4.3), using the same primer as used for the primer extension reactions.

2.5 Protein overexpression and purification

2.5.1 BldD overexpression

BldD was overexpressed in *E. coli* as a 6 × histidine-tagged fusion protein, as described in Elliot and Leskiw (1999). In order to generate the His-tagged fusion, the *bldD* coding sequence was cloned into the *E. coli* overexpression plasmid, pQE9 (Qiagen). The 520 bp *bldD* coding sequence was amplified using chromosomal DNA (1 μg) from both *S. coelicolor* strains J1501 (wild type) and HU66 (*bldD* mutant) as template. The oligonucleotide primers used in the amplification were MAE6 and MAE5 (Table 2.4), where MAE6 contained a *Bam*HI site engineered onto the 5' end, replacing the ATG at the beginning of the *bldD* coding sequence, and MAE5 possessed a *Hin*dIII site immediately following the *bldD* TGA stop codon at its 5' end. After purification of the amplification products (see 2.3.6), they were digested with *Bam*HI and *Hin*dIII, and were cloned into the *Bam*HI-*Hin*dIII digested vector, pQE9. The ligation products were transformed into *E. coli* JM109, and ampicillin resistant transformants were selected. Positive transformants were identified after growth of individual colonies in 3 mL LB broth + ampicillin, harvesting the plasmid DNA, digesting with *Bam*HI and *Hin*dIII, and

electrophoresing the resulting restriction fragments on an agarose gel. Glycerol stocks were prepared for the *E. coli* JM109 cells containing pQE9BldD$^+$ and pQE9BldD$^-$. The integrity of the two positive recombinant plasmids was verified by DNA sequence analysis, using MAE8, MAE9, BKL37 and BKL50 as primers (see Table 2.4).

To investigate whether BldD was overexpressed in its wild type and mutant form using this system, small scale induction studies were undertaken. Two millilitre LB + ampicillin cultures were grown on a rotating shaker overnight at 37°C, and 500 μL of the cultures were used the next morning to inoculate 2.5 mL fresh LB + ampicillin. The cultures were then grown for ~45 minutes (to an OD_{600} of 0.7-0.9), induced using 0.5 mM IPTG, and allowed to continue growing for an additional 1-2 hours. One millilitre of culture was then transferred to a 1.5 mL Eppendorf tube, and the cells were pelleted by centrifugation at 14,000 rpm in an Eppendorf 5415C centrifuge for 2 minutes. The culture supernatant was removed, and the cell pellet was resuspended in 100 μL protein loading dye [0.125 M Tris (pH 6.8), 6% SDS, 30% glycerol, 15% 2-mercaptoethanol, 0.003% bromophenol blue]. The protein mixture was then heated to 80°C for 10 minutes, after which 10 μL were removed for loading onto a mini 15% SDS-PAG (Bio-Rad, Mini ProteanII) with a 3.2% polyacrylamide (37.5:1 acrylamide:bisacrylamide), 0.068% SDS stacking gel, with a buffer system of 0.05 M Tris, 0.38 M glycine, and 0.1% SDS. The gel was electrophoresed for 0.75-1 hour at 120 V, and was then stained for ~2 hours using Coomassie Brilliant Blue Protein Stain (10% glacial acetic acid, 50% methanol, 0.001-0.002% w/v Coomassie Brilliant Blue). Destaining of the gel was accomplished using a solution of 45% methanol and 10% glacial acetic acid, with KimWipes or a foam plug being used to absorb excess stain.

To determine whether the overexpressed protein was present in a soluble form, the above procedure was repeated; however, after the cells were pelleted, they were frozen at –80°C overnight. The cells were thawed on ice, and resuspended in 2-5 volumes sonication buffer [50 mM sodium phosphate (pH 7.8), 300 mM NaCl], to which 1 mg/mL of lysozyme was added, per gram of wet weight. The cell suspension was incubated on ice for 30 minutes before sonication (on ice) for 4 × 15 second intervals using the Branson Sonifier (VWR Scientific) at a setting of 2 with the 2.5 mm diameter probe. Cell debris and insoluble matter were removed by centrifugation for 15 minutes at 14,000 rpm at 4°C, and the supernatant was transferred to a fresh 1.5 mL Eppendorf tube. Ten percent of the culture supernatant (soluble protein) and ~2% of the culture pellet were electrophorsed on a 15% SDS-PAG, as described above, to determine whether BldD was predominantly present in a soluble state.

Large scale induction was achieved using a 10 mL overnight culture, which was added to 1L fresh LB+ampicillin. The cells were grown for 3-4 hours at 37°C on a platform shaker, until mid-logarithmic phase (OD_{600} = 0.7-0.9), at which point IPTG was added to 0.5 mM. The culture was then grown for an additional 5-6 hours, after which the cells were pelleted in 250 mL centrifuge bottles at 4000 × g for 10 minutes (at 4°C) in a Beckman J2-H5 centrifuge with a JA14 rotor (done twice per bottle to get cells from 500 mL of culture/bottle). The bottles containing the cell pellets were then frozen at –80°C. The pellet was later thawed on ice, and treated as described above. The resulting cell suspension was divided between two 25 mL glass Corex tubes, and sonicated on ice for 8 × 30 seconds. The cell free extracts were combined into a 30 mL plastic centrifuge

tube, and the soluble protein was separated from the cell debris by centrifugation at 4°C for 10 minutes at 10,000 × g using the JA20 rotor.

Expression of BldD as a GST-fusion protein followed a procedure similar to the one detailed for the His-tagged fusion. The 520 bp *bldD* coding sequence was amplified using the primers MAE6 and MAE5, as described above, only VENT DNA polymerase was used for the amplification, to reduce the incidence of sequence error incorporation. After purification, the amplified fragment was blunt-ended, using 1 μL Klenow (2 U), in a buffer of 5 mM Tris-HCl (pH 7.8), 0.5 mM $MgCl_2$, 1 mM β-mercaptoethanol, and 5 μg/mL BSA, for 8 minutes at 37°C. This was followed by a 10 minute incubation in the presence of 8 nM dNTPs, to fill in the resulting 5' overhang. After removal of the enzyme with phenol/chloroform extraction, the DNA was precipitated, the product was digested with *Bam*HI, and then cloned into pGEX-2T, which had been digested with *Bam*HI and *Sma*I. Transformation, selection, and confirmation of insert integrity were all carried out as described above, only inserts were visualized by digestion with *Bam*HI and *Eco*RI, rather than *Bam*HI and *Hin*dIII.

Confirmation of GST-BldD overexpression and existence in a soluble form were accomplished as outlined above, only using 2 × YT (1.6% w/v tryptone, 1% w/v yeast extract, 1% w/v NaCl) in the place of LB, 1 × PBS [140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4 (pH 7.3)] in the place of sonication buffer, and 10% gels as opposed to 15% for protein electrophoresis.

2.5.2 BldD purification

His-tagged BldD was purified from the cell free extract using Ni-affinity chromatography. The supernatant, to which 10 mM imidizole had been added (to inhibit nonspecific protein interaction with the column resin), was applied to a disposable Bio-Rad column containing a 4 mL bed of Ni-NTA resin (Qiagen), that had been previously equilibrated with 10 mL sonication buffer containing 10 mM imidizole. The column was then washed with sonication buffer, containing increasing concentrations of imidizole: 1 × 10 mL of 10 mM imidizole; 1 × 10 mL 30 mM imidizole; and 1 × 10 mL 40 mM imidizole. Purified BldD was eluted using 5-6 mL of sonication buffer with 350 mM imidizole, which effectively competed with the His-tagged BldD protein for binding to the Ni-NTA resin. Two hundred fifty microlitre aliquots of the unbound fraction, each of the wash fractions, and the final eluted protein were dried in a Speed Vac for ~1.5 hours, before being resuspended in 35 μL protein loading dye, heated to 80°C for 10 minutes, and loaded onto a mini 15% SDS-PAG (see 2.5.1). The gel was stained to assess protein size and purity. Once protein purity had been established, the remaining eluant was dialyzed overnight at 4°C against 4 L of buffer [20 mM Tris-HCl (pH 8.5), 150 mM NaCl]. Pefabloc and glycerol were added to 1 mM and 10% v/v final concentrations, respectively. The dialysate was then divided into 25 μL aliquots, and stored at –80°C. Protein concentrations were determined using the Bradford protein microassay (Bradford, 1976; adapted by Bio-Rad), with bovine gamma globulin as standard.

Purification of GST-BldD utilized the same disposable Bio-Rad columns as above, only the bed resin was made up of ~4 mL of glutathione agarose (Sigma). The supernatant, described in 2.5.1, was applied to the column, and was passaged through by

gravity flow. The column was washed with 80 mL of 1 × PBS, before elution with 8 mL of reduced gluathione (10 mM; Sigma) in 1 × PBS. One millilitre fractions were collected during elution, and 30 µL of each fraction was electrophoresed on a mini 10% SDS-PAG to examine the purity of the GST-BldD fusion protein. In the place of dialysis, the fractions were concentrated using a Millipore Ultrafree® -4 Centrifugal filter, and the buffer was replaced with 20 mM Tris-HCl (pH 8.5), and 150 mM NaCl. Protein concentrations were determined as outlined above.

2.5.3 Cell-free extract preparation

Cell free extracts of *S. coelicolor* were prepared as outlined in Hong *et al.* (1993). Spores or mycelia were grown for 15-48 hours on cellophane discs overlaying R2YE plates. The culture biomass was then scraped from the plates with a sterile spatula, and transferred to sterile 1.5 mL Eppendorf tubes, where it was suspended in up to 1 mL of lysing buffer [0.1 M HEPES buffer, 1 mM Pefabloc (Roche), 1 mM PMSF, 1 µM Pepstatin A, 0.5 mg/mL lysozyme (Sigma)]. The cell suspension was then incubated for 10 minutes in a 37°C water bath, before being subjected to sonication (same settings as described in 2.5.1) for 5 × 15 seconds (on ice), with cooling intervals of 15-30 seconds. The soluble protein fraction was separated from the cell debris by centrifugation at 4°C, for 10 minutes at a speed of 14,000 rpm. The resulting supernatant was aliquoted into 50 µL fractions and stored at –80°C.

2.6 Protein analysis

2.6.1 Antibody preparation

To generate antibodies specific for BldD, purified His-tagged BldD (~1.5 mg) was concentrated by flash freezing, and dried in a Speed Vac before redissolution in protein loading dye. The concentrated protein was loaded into three adjacent lanes of a 10% SDS-PAG, which was then electrophoresed for 6 hours at 35-40 mA. The gel was stained with 0.05% Coomassie blue dissolved in milli-Q H_2O, and destained/washed with milli-Q H_2O. The light green-appearing BldD band was excised from the gel, the gel slice was cut into small pieces, frozen at –20°C, and then ground using a mortar and pestle. The ground gel was then mixed with 1 × PBS and Freund's complete adjuvant (for a total volume of 1-2 mL), and passaged repeatedly through an 18 gauge needle attatched to a 5 mL syringe, and then through a 23 gauge needle, before injection into a rabbit. The rabbit was booster injected three times, at 4 week intervals, each time using an additional 1.5 mg of BldD in PBS and Freund's incomplete adjuvant. After each booster, the rabbit was bled, and Western blots (see below) were performed with the resulting antiserum to test for antibody specificity. After the third boost, the final bleed was performed, and the antiserum was aliquoted and stored at –80°C.

2.6.2 Western blot analysis

Ten micrograms of cell free extract protein samples were heat denatured at 80°C for 10 minutes, in a water-filled temperature block, and then separated on a 10-15% SDS-PAG by electrophoresis at 35-40 mA for 5-6 hours. Transfer of the separated proteins to a PVDF membrane (Immobilon-P^{SQ}; Millipore) was carried out in a Bio-Rad Transblot

apparatus [transfer buffer: 19.2 mM Tris-HCl (pH 8.0), 0.192 M glycine, 0.015% SDS, 20% methanol] overnight at 58 V and 4°C. The membrane was then washed with milli-Q H_2O for 3 × 5 minutes, followed by a 5 minute wash with wash buffer [0.02 M Tris-HCl (pH 7.6), 0.14 M NaCl, 0.1% Tween 20], before being blocked for 1 hour at room temperature in 40 mL blocking buffer [wash buffer + 4-5% BSA (Boehringer Mannheim)]. Twenty millilitres of the blocking solution were then removed, and saved for use with the secondary antibody. The primary antibody was added to the remaining 20 mL of blocking buffer [α-BldD required a 1/50,000 dilution, while α-phospho-tyrosine required a dilution of 1/1000 (gift from H. Ostergaard)], and incubated with the membrane on a rocking platform (Bellco Biotechnology). After one hour, the blot was rinsed twice with wash buffer, and then subjected to 3 longer washes of 2 × 5 minutes and 1 × 15 minutes, on an orbital shaker (New Brunswick Scientific Co.). Reaction of the membrane with the secondary antibody was allowed to proceed for 30-60 minutes, using either donkey α-rabbit in a 1:5000 dilution (for BldD; Amersham) or goat α-mouse, diluted 1:20,000(for phospho-tyrosine; gift from Dr. H. Ostergaard). Again, the blot was rinsed twice with wash buffer, and then washed extensively for 4 × 5 minutes and 1 × 15 minutes. To allow for the detection of protein through ECL (enhanced chemiluminescence), the membrane was transferred to a fresh dish, to which 6 mL of each Enhanced Luminol Reagent and Oxidizing Reagent (DuPont NEN) solution were added. After agitation for one minute, the blot was removed, wrapped in Saran Wrap, and exposed to film (Reflection™ NEF; DuPont NEN) for 5-10 minutes.

2.6.3 Oligomerization of BldD in solution

The oligomeric state of BldD in solution was determined using fast protein liquid chromatography (FPLC). His-tagged BldD was applied to a Pharmacia MonoQ HR 5/5 column equilibrated with 20 mM Tris-HCl (pH 8.5) at 4°C. It was eluted using a 67.5 mL linear gradient from 0-0.5 M NaCl. The column fractions were analyzed by SDS-PAGE, and it was observed that BldD eluted in two separate peaks, offset from each other by about five fractions. The fractions corresponding to each of these peaks were pooled and dialyzed overnight against 20 mM Tris-HCl (pH 8.5). Each of these fraction groups was then chromatographed on a Pharmacia Superose 12 HR 10.30 column, equilibrated with 20 mM Tris-HCl (pH 8.5), and the resulting profiles were compared with those of protein standards run under the same conditions [bovine serum albumin (66 kDa), β-lactamase (29.5 kDa), and cytochrome C (12 kDa)]. Confirmation that the protein was BldD was accomplished by SDS-PAGE.

2.7 DNA-protein interaction

2.7.1 Electrophoretic mobility shift assays

To assess the ability of BldD to bind DNA, electrophoretic mobility shift assays were conducted. DNA fragments of various lengths were ^{32}P end-labeled, and 1-2 ng (1-20 fmol) of each labeled fragment was incubated with 0-80 pmol of purified His_6-BldD in a buffer consisting of 10 mM Tris-HCl (pH 7.8), 150 mM NaCl, 2 mM DTT, 1 μg poly d(I-C), and 10% glycerol, in a total volume of 20 μL, for 20 minutes at 30°C. Protein bound and free DNA were separated on a 1 × TBE, 1.5% glycerol-containing 8% polyacrylamide gel. Before loading of the samples onto the running gel, the gel was pre-

run at 175-185 V for 15-20 minutes. After electrophoresis for ~1.5 hours, the gel was dried for 45 minutes-1 hour, and placed on a phosphorscreen for visualization and analysis using a phosphorimager and Imagequant™ software. Competition mobility shift assays were conducted as described above, however, the binding reactions were supplemented with either ~500 ng of unlabeled probe, for specific competition, or ~500 ng of unlabeled, nonspecific DNA (BKL41-MAE5; a 118 bp fragment internal to the *bldD* coding sequence, and amplified using the oligonucleotide primers BKL41 and MAE5, from pAU171 as template; see Table 2.3 and 2.4) for nonspecific competition. Supershift assays were carried out with the BldD antibody included in the binding reactions (both undiluted, and with dilutions of 1/100 and 1/5), to confirm that the shifted DNA resulted from DNA binding by BldD. Oligomerization studies were carried out as above, only three different BldD protein mixtures were used: His_6-BldD; GST-BldD; or His_6-BldD and GST-BldD in a 1:1 mixture.

2.7.2 DNaseI footprinting assays

DNaseI footprinting was carried out to ascertain the sequence recognized and bound by BldD. Various fragments were subjected to DNaseI footprinting assays, and in each case, these probes were generated by PCR amplification using one labeled oligonucleotide primer, end-labeled as described in 2.3.10, and one unlabeled oligonucleotide primer. To assess the ability of BldD to protect both strands of the DNA, two labeled fragments were created for each footprinting investigation, one labeled on the coding strand, and the other on the noncoding strand. All amplification products were purified from 5-8% polyacrylamide gels as discussed in 2.3.6. Footprinting reaction

conditions were carried out under similar conditions as for the gel retardation assays, with minor modifications: the binding buffer contained 50 mM KCl instead of 150 mM NaCl, and 5 mM $MgCl_2$ and 2.5 mM $CaCl_2$ were added to the reactions; and protein concentrations were varied between 0 and 80 pmol per reaction. DNaseI was diluted in 1 × binding buffer to 0.05 U/μL (0.025 U/μL for the *whiG* template), for reaction with DNA and DNA+protein in all instances except for the *bldD* promoter, where 0.06 U/μL was required for reaction with DNA in the presence of BldD. One microlitre of the diluted DNaseI was added to each sample tube, which had been incubating at 30°C for 20 minutes, and was allowed to react for 10 seconds before being stopped by the addition of 50 μL of neutral phenol. Water was added to make the aqueous solution up to 50 μL, and this was followed by phenol/chloroform and choloroform extractions. The samples were precipitated in silanized 0.5 mL Eppendorf tubes, with the addition of glycogen and ethanol, resuspended in 4 μL sequencing stop solution (Amersham), and heated to 90°C for 5 minutes, with frequent vortexing, before loading onto a 6% sequencing gel for separation of the cleavage products. The footprinting ladders were visualized using the phosphorimager, as for the electrophoretic mobility shift assays. Determination of protected residues was accomplished using sequencing reactions, generated with an appropriate oligonucleotide, that were electrophoresed alongside the footprinting reactions.

2.7.3 Hydroxyl radical footprinting assays

Hydroxy radical footprinting was conducted according to the method of Tullius and Dombroski (1986), with minor modifications. Binding reactions were carried out as

in 2.7.1, with the omission of glycerol from the binding buffer, and the addition of 5-6 fold more probe (5-12 ng or 75-180 fmol). Three microlitres each of 0.5 mM $Fe(EDTA)^{2-}$, 10 mM Na-ascorbate and 0.3% H_2O_2 were mixed together and were added to every sample after the 20 minute incubation at 30°C. The reactions were carried out for 2 minutes and were stopped using 5 μL thiourea and 3 μL 100% glycerol. The reaction products were loaded directly onto an 8% nondenaturing, 1 × TBE-buffered polyacrylamide gel, and were separated into free and protein-bound DNA. The gel was exposed to X-ray film (Kodak) for 30 minutes to detect the desired DNA and protein-bound DNA bands, which were then excised from the gel. Elution of the DNA from the gel was accomplished as described in 2.3.6, and was followed by dissolution of the pellets in 4 μL sequencing stop solution, resolution of the bands on a 6% sequencing gel, and analysis using the phosphorimager. Determination of protected residues was accomplished using sequencing reactions, generated with an appropriate oligonucleotide, that were electrophoresed alongside the footprinting reactions.

2.8 Systematic evolution of ligands by exponential enrichment (SELEX)

To isolate potential chromosomal targets for BldD binding, a modified SELEX (Ochsner and Vasil, 1996) protocol was employed. *S. coelicolor* J1501 chromosomal DNA was partially digested using 0.21 U/μg of *Sau*3AI. The partial digests were separated by electrophoresis on a 5% polyacrylamide gel, and fragments of ~300-600 bp in size were excised from the gel to be purified by crushing and soaking. Complementary oligonucleotides MAE42 and P-MAE43 (Table 2.4), with MAE43 possessing a 4 bp *Sau*3AI complementary overhang, were annealed together by incubation of 300 pmol of

each oligonucleotide in annealing buffer [20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 50 mM NaCl, (Ref: from Methods in Molecular Biology)], in a total volume of 300 µL. The annealing mixture was heated to 100°C for 5-10 minutes, and was then slowly cooled over 45 minutes to 30-37°C. The reactions were precipitated and redissolved in 100 µL milli-Q H_2O. Four micrograms of the linkers were then ligated to 2.5 µg of the *Sau*3AI-digested chromosomal DNA in a 300 µL reaction volume. After overnight incubation at 15°C, the ligation reaction was precipitated and electrophoresed on a 5% polyacrylamide gel to remove free linkers. The ligation products were excised either all together, or in 3 sections of 300-450 bp; 450-550 bp and 550-650 bp, and were purified as above, redissolving to give a 20-30 µL final volume.

The library of chromosomal fragments was end-labeled as described in 2.3.10. Precipitation of the labeled DNA was followed by counting of the DNA pellet using a scintillation counter, and redissolution of the DNA fragments in milli-Q H_2O to give ~1-2 ng/µL (50,000 - 200,000 cpm/µL). Gel retardation assays were then carried out using the conditions outlined in 2.7.1, except that 10-20 ng of DNA was included in each reaction tube, only 20 pmol of protein was used per reaction, and the total reaction volume was adjusted to 25 µL. The gel was electrophoresed for 3-4 hours at 180 V, after which the gel was wrapped in Saran Wrap, and was exposed to X-ray film for 48 hours. The developed film was used as a template to cut the shifted bands from the gel, and the shifted DNA was then eluted from the gel by crushing and soaking (2.3.6). Up to half of the resulting purified DNA was used as a template for subsequent PCR amplification. Amplification was accomplished using 80 pmol of MAE42 as primer, 0.125 mM dNTPs, and commercial PCR buffer with detergents (Roche EXPAND buffer 3) in conjunction

with EXPAND DNA polymerase, in a total reaction volume of 100 μL. After an initial 5 minute denaturation, the reactions were subjected to 30 cycles of denaturing (95°C for 45 seconds), annealing (52°C for 45 seconds), and extension for 1 minute at 68°C. The PCR reactions were precipitated and purified on a 5% polyacrylamide gel, as described above. The purified products were end-labeled and the electrophoretic mobility shift-PCR cycle was repeated. After at least 3 rounds of selection, the PCR-amplified targets were either blunt-ended by Klenow-fill in, and ligated to *Sma*I-digested pUC119 before being transformed into *E. coli* DH5α, or ligated without blunt-ending into pTOPO10 and transformed into OneShot™ TOP10. Target-containing transformants were selected using ampicillin and IPTG+X-gal. The presence of an insert was confirmed by digesting the DNA of individual clones with *Xba*I and *Eco*RI, and running the digestion products on a 2% agarose gel.

Chapter 3

Results

The *S. coelicolor bldD* gene was initially identified through a mutagenic screen, where mutants were observed to be deficient in both the erection of aerial hyphae and the production of antibiotics (Merrick, 1976). The *bldD* locus was cloned by Dr. B. K. Leskiw as a 3.5 kb DNA fragment, which was sequenced by F. Damji, (1995). The most likely *bldD* candidate corresponded to an open reading frame that would encode a 167 aa protein (including 22 basic and 21 acidic residues), with a predicted molecular weight of 18,167 Da. Complementation of the *bldD53* mutation in strain HU66 using this open reading frame successfully restored aerial hyphae formation and antibiotic production, confirming that it was in fact the *bldD* gene. The BldD amino acid sequence was compared with the sequences of known proteins, and was found to share no significant sequence similarity with any proteins in the databases. Motif searches also failed to identify any meaningful functional motifs in BldD; however, a manual comparison of the BldD amino acid sequence to previously determined helix-turn-helix consensus sequences of known prokaryotic DNA-binding proteins, revealed a weak match to the signature sequence of the LysR family of transcription regulators, with 24 out of 26 amino acids fitting the loosely defined criteria (Fig. 3.1).

This study was initiated to characterize the role that BldD plays in the regulation of antibiotic production and morphological differentiation in *S. coelicolor*. The major goals were to determine the means by which BldD exerts its regulatory effects, and how this in turn influences the ability of the colony to form aerial hyphae and produce antibiotics.

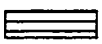
Fig. 3.1 Alignment of the putative helix-turn-helix in BldD with the LysR family helix-turn-helix signature sequence (Prosite; PS00044). Amino acid positions (aa) within BldD are indicated, and the signature sequence is shown below, with all possible amino acids for each position. Amino acids matching the signature sequence are indicated with an asterisk (*), mismatches are marked with a number symbol (#), and the dashes (-) illustrate positions where any amino acid is permitted.

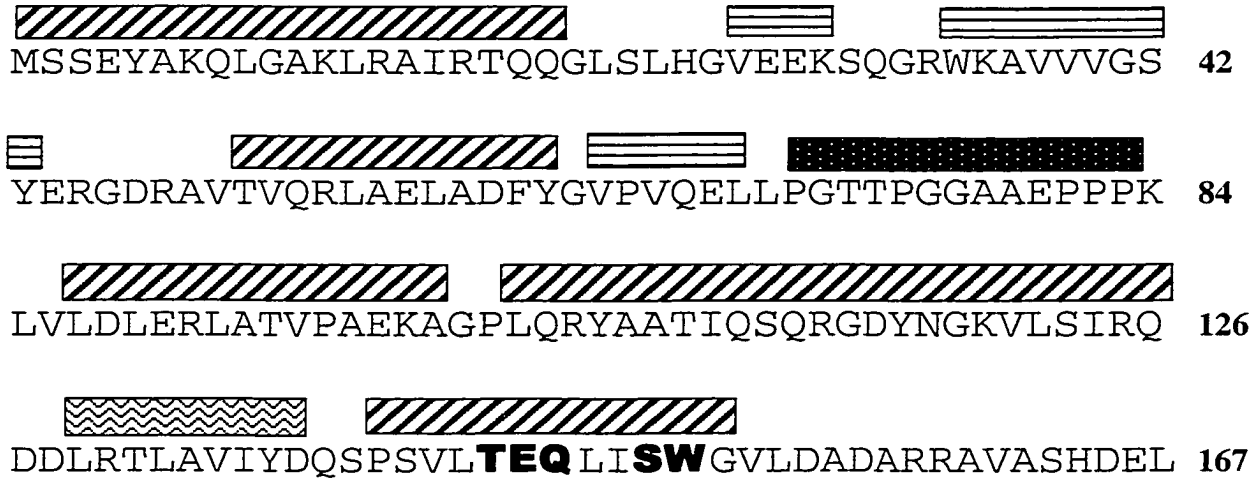
BldD	aa123-	SIRQDDLRTLAVIYDQSPSVLTEQLISWGVLDADARR						-159
		*--**------**--**--*--*#--#						
LysR family		L	SS	PP	LS	L	LR	L
HTH consensus		I	TT	SN	IT	I	IK	I
Sequence		V	GA	TQ	VA	V	VE	V
		M	A	AH	M	M	MQ	M
		F	L	K	A	F	FA	F
		Y	V	R		W	W	Y
		T						N
								T

3.1 Analysis of *bldD* sequence

3.1.1 Prediction of BldD structure

The existence of a potential helix-turn-helix motif (HTH) in the C-terminal portion of BldD (spanning amino acids 128-154), predicted based on its similarity to the LysR signature sequence, suggested that *bldD* may encode a DNA-binding protein. In an attempt to authenticate the existence of this DNA-binding motif, the inverse folding procedure of Bowie *et al.* (1991) was used to identify structural or functional domains within BldD. The BldD sequence was threaded (analysis kindly provided by Gregory Petsko of Brandeis University) onto the 3-D structure of proteins whose fold had been determined by nuclear magnetic resonance or X-ray diffraction. While there were no proteins identified as sharing an overall fold structure similar to BldD, the closest match being to flavodoxin, certain segments of BldD were found to correspond with known fold elements (Fig. 3.1.1). Based on these results, BldD appears to be a 2 domain protein, with the amino-terminal domain being separated from the carboxy-terminal domain by a very random and unstructured region, rich in proline and glycine residues (from amino acid position 70-83). The amino-terminal domain was found to be a mixture of alpha helices and beta sheets, while the carboxy-terminal domain was dominated by a long helix-turn-helix motif. Interestingly, the second helix (from amino acid position 140-151), overlapped the predicted LysR helix-turn-helix motif (amino acid positions 129-154). Given the identification of this potential DNA-binding motif, it was of interest to determine whether the *bldD* mutation fell within the helix-turn-helix region.

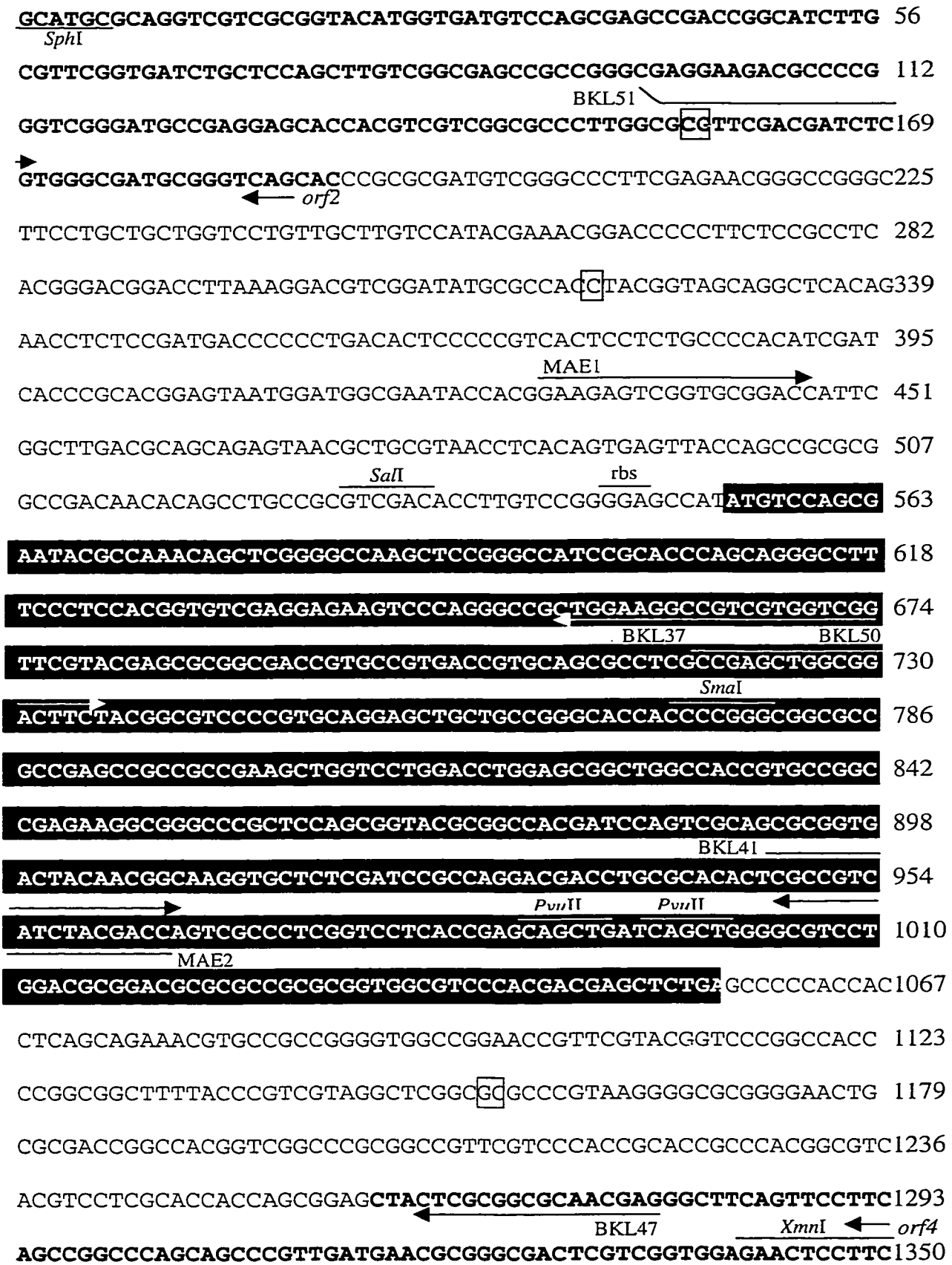
Fig. 3.1.1 Prediction of BldD secondary structure, using the inverse folding procedure (Bowie *et al.*, 1991). The BldD sequence was analyzed using a database of proteins whose 3-dimensional structure had been determined previously. A number of different structural elements were identified in BldD, and are indicated above the amino acid sequence:  represents an alpha helix;  represents a beta sheet;  identifies a disordered and unstructured region, and  indicates a turn. The amino acids predicted to serve as direct contact points with DNA are shown in bold. The numbers shown on the right refer to the amino acid positions at the end of each line



3.1.2 Confirmation of *bldD* sequence

Before determining the nature of the *bldD53* mutation, it was important to ensure that there were no errors in the sequence of the wild type *bldD* and its flanking regions, which could hamper identification of the mutation, and be detrimental to oligonucleotide design and future experimental investigations. The sequence of the *bldD* gene was determined to be accurate, as analysis of the sequencing reactions exposed to X-ray film revealed no discrepancies between both strands of the DNA, and thus there was no need to resequence it. Examination of the *bldD* flanking regions, however, did reveal some discrepancies, and consequently, approximately 500 bp upstream and 300 bp downstream from the *bldD* gene were resequenced by the Department of Biological Sciences sequencing service. The upstream flanking sequence, was examined using pAU184 as the plasmid template and MAE3 (Table 2.4) as the oligonucleotide primer, and was found to have 2 separate sequencing errors. The first was found at a site 395 bp upstream from the *bldD* translation start site, where two bases were determined to be missing from the original sequence, changing the sequence from TTGGCGTTCGA to TTGGCG**C**G**T**TCGA (Fig. 3.1.2). The second error was determined to be 233 bp upstream from the *bldD* translation start site, where a C-G base pair was missing; correction of the sequence changed it from CGCCACTACGG to CGCCAC**C**TACGG (Fig. 3.1.2). The sequence downstream from *bldD* was determined using pAU183 plasmid DNA as template, and BKL45 (Table 2.4) as the oligonucleotide primer. A single error was revealed, 98 bp downstream from the TGA stop codon of the *bldD* coding sequence, and resulted in the changing of the sequence from TCGGCCGGCCCG to TCGGC**G**CGCCCG (Fig. 3.1.2).

Fig. 3.1.2 Corrected nucleotide sequence of a 1.3 kb *Sph*I-*Xmn*I fragment containing *bldD*. The *bldD* coding sequence is shown in white, enclosed within a black box, and two partial ORFs, *orf2* and *orf4* are also indicated, encased in white boxes. Arrowheads indicate the direction of transcription. The *bldD* ribosome binding site is marked, as are a number of restriction endonuclease sites (*Sal*I, *Sma*I, and *Pvu*II) that are important in later sections of the thesis. Grey boxes mark nucleotides that had been incorrectly determined prior to this study. Primers used in the sequence determination are shown (BKL51, MAE1, BKL37, BKL41, MAE2, and BKL47); the arrows indicate the approximate location of the primers and the direction of primer binding (5'-3'), while the curved tail on BKL51 indicates a nonhomologous sequence addition to the primer.



3.1.3 Determining the nature of the *bldD* mutation

Once the sequence of the *bldD* gene and its flanking regions had been confirmed, it was possible to sequence the *bldD* gene in *S. coelicolor* HU66, the *bldD* mutant strain, to determine the nature of the *bldD* mutation. It was hoped that identifying the mutation would help shed light on the function of BldD in the cell; for instance, if the mutation happened to fall in the putative HTH motif, a DNA-binding role would be supported. Chromosomal DNA was isolated from HU66, and used as template for a polymerase chain reaction (PCR) in which the oligonucleotide primers BKL51 and BKL47 were used to amplify the *bldD* gene. Amplification was carried out twice, once using Taq DNA polymerase, and once using EXPAND DNA polymerase, to ensure that any mutation observed was not the result of an amplification-induced sequence error. The resulting amplification products were sequenced by the Biological Sciences sequencing service, using the primers MAE1, MAE2, BKL37, BKL41, BKL47, BKL50, and BKL51 (see Fig. 3.1.2; Table 2.4). For both the Taq-amplified fragment, and the EXPAND-amplified product, a single A-G point mutation was found within the *bldD* coding sequence. This base change resulted in the conversion of a tyrosine residue to a cysteine residue within the N-terminal domain of BldD, at amino acid position 62, a position far from the predicted HTH motif in the C-terminal domain (Fig. 3.1.3).

3.1.4 Attempted disruption of the *bldD* gene

Given that the *bldD* mutation was found to be a single point mutation, it was conceivable that the mutant protein retained some function, and that the observed *bldD* mutant phenotype did not accurately reflect that which would result from the complete

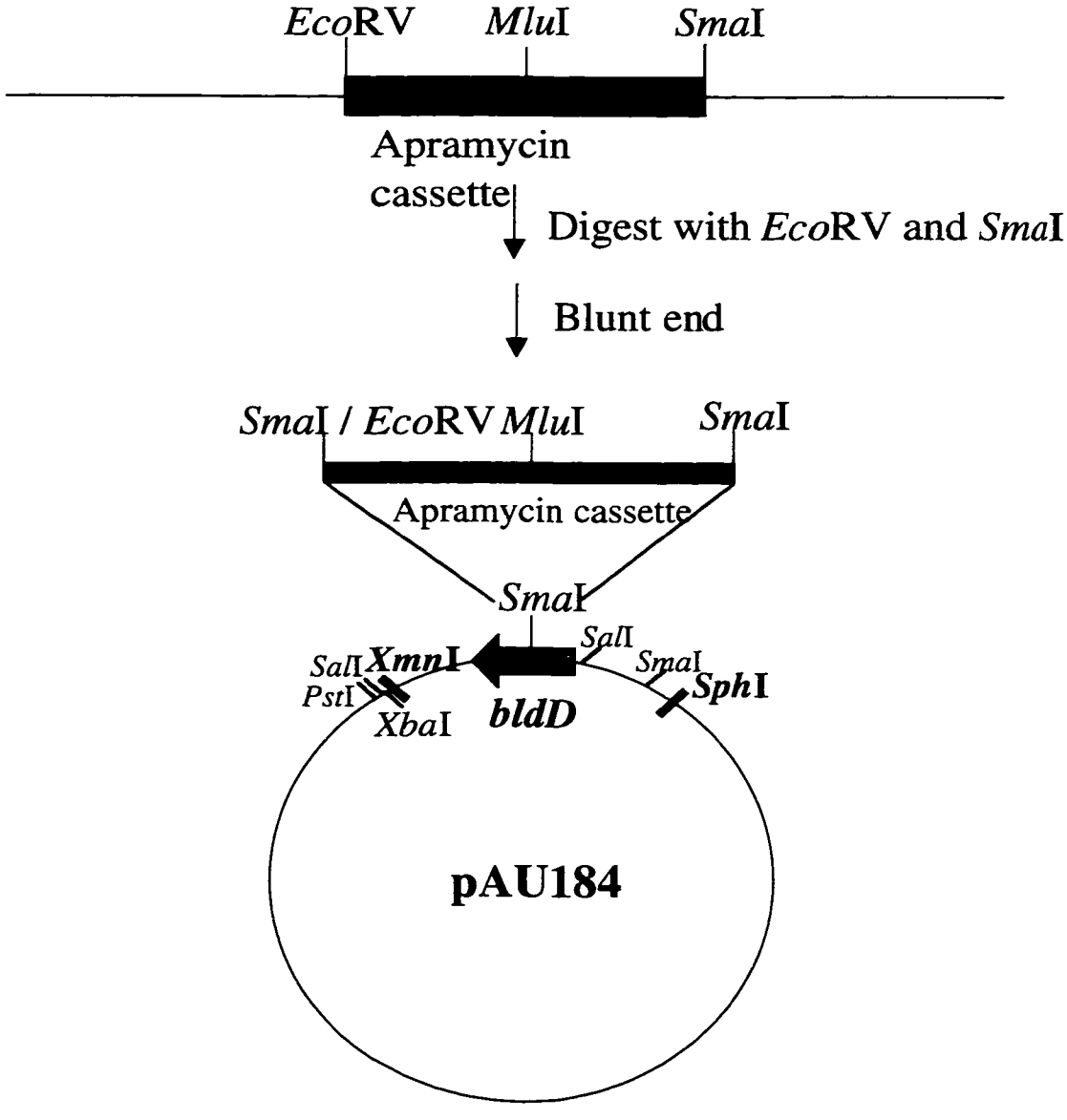
Fig. 3.1.3 Identification of the *bldD* mutation in *S. coelicolor* HU66. Chromosomal DNA was isolated from *S. coelicolor* HU66, and the mutant *bldD* gene was amplified by PCR and sequenced. The double stranded DNA sequence is shown, with the translated amino acid sequence given above. The numbers shown on the right refer to the amino acid positions at the end of each line. The site of the single point mutation, changing an A-T base pair to an G-C base pair, is shown, along with the resulting altered amino acid sequence where a tyrosine is changed to a cysteine residue at amino acid position 62 (shown in red).

```
fM  S  S  E  Y  A  K  Q  L  G  A  K  L  R  A  I  R  T  Q  19
ATGTCCAGCGAATACGCCAAACAGCTCGGGGCCAAGCTCCGGGCCATCCGCACCCA
TACAGGTCGCTTATGCGGTTTGTCGAGCCCCGGTTCGAGGCCCGGTAGGCGTGGGT

  Q  G  L  S  L  H  G  V  E  E  K  S  Q  G  R  W  K  A   37
GCAGGGCCTTTCCCTCCACGGTGTCGAGGAGAAGTCCCAGGGCCGCTGGAAGGCCG
CGTCCCGGAAAGGGAGGTGCCACAGCTCCTCTTCAGGGTCCCGGCGACCTTCCGGC

V  V  V  G  S  Y  E  R  G  D  R  A  V  T  V  Q  R  L  A  56
TCGTGGTCGGTTCGTACGAGCGCGGCGACCGTGCCGTGACCGTGCAGCGCCTCGCC
AGCACCAGCCAAGCATGCTCGCGCCGCTGGCACGGCACTGGCACGTCGCGGAGCGG
                  C
 E  L  A  D  F  Y  G  V  P  V  Q  E  L  L  P  G  T  T  P  75
GAGCTGGCGGACTTCTACGGCGTCCCCGTGCAGGAGCTGCTGCCGGGCACCACCCC
CTCGACCGCCTGAAGATGCCGCAGGGGCACGTCCTCGACGACGGCCCGTGGTGGGG

  G  G  A  A  E  P  P  P  K  L  V  L  D  L  E  R  L  A   93
GGGCGGCGCCGCCGAGCCGCCGCCGAAGCTGGTCCTGGACCTGGAGCGGCTGGCCA
CCCGCCGCGGCGGCTCGGCGGCGGCTTCGACCAGGACCTGGACCTCGCCGACCGGT

T  V  P  A  E  K  A  G  P  L  Q  R  Y  A  A  T  I  Q  S  112
CCGTGCCGGCCGAGAAGGCGGGCCCGCTCCAGCGGTACGCGGCCACGATCCAGTCG
GGCACGGCCGGCTCTTCCGCCCGGGCGAGGTCGCCATGCGCCGGTGCTAGGTCAGC

 Q  R  G  D  Y  N  G  K  V  L  S  I  R  Q  D  D  L  R  T  131
CAGCGCGGTGACTACAACGGCAAGGTGCTCTCGATCCGCCAGGACGACCTGCGCAC
GTCGCGCCACTGATGTTGCCGTTCCACGAGAGCTAGGCGGTCCTGCTGGACGCGTG

  L  A  V  I  Y  D  Q  S  P  S  V  L  T  E  Q  L  I  S   149
ACTCGCCGTCATCTACGACCAGTCGCCCTCGGTCCTCACCGAGCAGCTGATCAGCT
TGAGCGGCAGTAGATGCTGGTCAGCGGGAGCCAGGAGTGGCTCGTCGACTAGTCGA

W  G  V  L  D  A  D  A  R  R  A  V  A  S  H  D  E  L  *  167
GGGGCGTCCTGGACGCGGACGCGCGCCGCGCGGTGGCGTCCCACGACGAGCTCTGA
CCCCGCAGGACCTGCGCCTGCGCGCGGCGCGCCACCGCAGGGTGCTGCTCGAGACT
```

absence of BldD. To determine whether the phenotypic effect resulting from the point mutation differed from that observed in a *bldD* null mutant strain, attempts were made to both disrupt *bldD*, and replace it with an antibiotic resistance cassette.

The first attempt at disrupting *bldD* involved insertion of an apramycin resistance cassette into a unique *Sma*I site within the *bldD* coding sequence, and subsequent introduction of this recombinant construct into *S. coelicolor* J1501 for cross-over into the chromosome (Fig. 3.1.4). The apramycin resistance cassette was removed from pBSApR (see Table 2.1) by digestion with *Eco*RV and *Sma*I, and was blunt-ended using the Klenow fragment of DNA polymerase. This DNA fragment was to be inserted into a unique *Sma*I site, in the centre of the *bldD* coding sequence, which was present on a 1.3 kb insert in the pUC119 derivative, pAU184. Unfortunately, a second *Sma*I site had been overlooked in the insert (see Fig. 3.1.4), so a *Sma*I partial digest of pAU184 was performed, and the single site-digested DNA was purified away from the fully digested plasmid on an agarose gel. The apramycin resistance cassette was then ligated into the partially digested pAU184, and the ligation mixture was transformed into *E. coli* ET12567. To confirm that the apramycin resistance gene was present within *bldD*, and had not inserted into the second *Sma*I site, outside of the *bldD* gene, plasmids were isolated from apramycin resistant transformants. The recombinant plasmids were then digested with *Sal*I, and the size of the resulting insert-containing fragments were assessed. One representative plasmid, designated pAU193, was confirmed, by the presence of a 2.3 kb band rather than a 0.8 kb band, to have the apramycin resistance gene within *bldD*, and was used to transform *S. coelicolor* J1501 protoplasts. Apramycin resistant transformants were then selected. As pAU184 did not possess a *Streptomyces*

Fig. 3.1.4 Subcloning of the apramycin resistance cassette into pAU184, the pUC119-derivative containing *bldD* on a 1.3 kb insert, for the purpose of *bldD* disruption. The 1.3 kb insert is demarcated by the *Xmn*I and *Sph*I sites, indicated in bold lettering, and the *bldD* gene within this insert is shown in green, with the arrowhead indicating the direction of transcription. The apramycin resistance gene cassette was purified from pBSApR, by digestion with *Eco*RV and *Sma*I, and is shown in blue. The apramycin cassette was then blunt ended and ligated into pAU184, which had been partially digested with *Sma*I. Ligation into the *Sma*I site within *bldD* was confirmed by digestion with *Sal*I. The *Mlu*I site, within the apramycin resistance gene is shown, as are the *Pst*I and *Xba*I sites within the polylinker of pAU184.



origin of replication, any colonies observed to grow after selection with apramycin should have had the disruption construct integrated into the chromosome. A number of apparent positive transformants were detected, and remained apramycin resistant when restreaked onto R2YE containing 50 μg/mL apramycin. When the resistance phenotype was further checked by restreaking on minimal medium with glucose as a carbon source, the transformants were found to be apramycin sensitive, suggesting that they were false positives. To verify that the apramycin resistance was not present in the chromosome of these colonies, the chromosomal DNA was isolated from 5 representatives, was digested with *Sph*I and subjected to Southern blot analysis using a random primer labeled fragment internal to *bldD* as probe. Since all products were the same size as in the wild type control, rather than being increased by the ~1.5 kb size of the apramycin resistance cassette, it was concluded that the apramycin resistance cassette was not present. Colony hybridization was then carried out for the other 64 potential positive transformants, using the random primer labeled apramycin resistance gene as probe, and all were found to be negative.

Due to the problem of a false apramycin resistance phenotype, it was decided that a thiostrepton resistance marker would be inserted within the apramycin gene in pAU193. The thiostrepton resistance gene was chosen as a marker because thiostrepton provides a strong selection marker in *Streptomyces*, with the only drawback being the inability to select for thiostrepton resistance in *E. coli* during recombinant plasmid construction. The *tsr* gene was isolated by digestion of pAU5 with *Bss*HII, and was blunt-ended to permit ligation into the blunted *Mlu*I site in the apramycin resistance gene in pAU193. The ligation mixture was transformed into *E. coli* ET12567. Colony hybridizations were

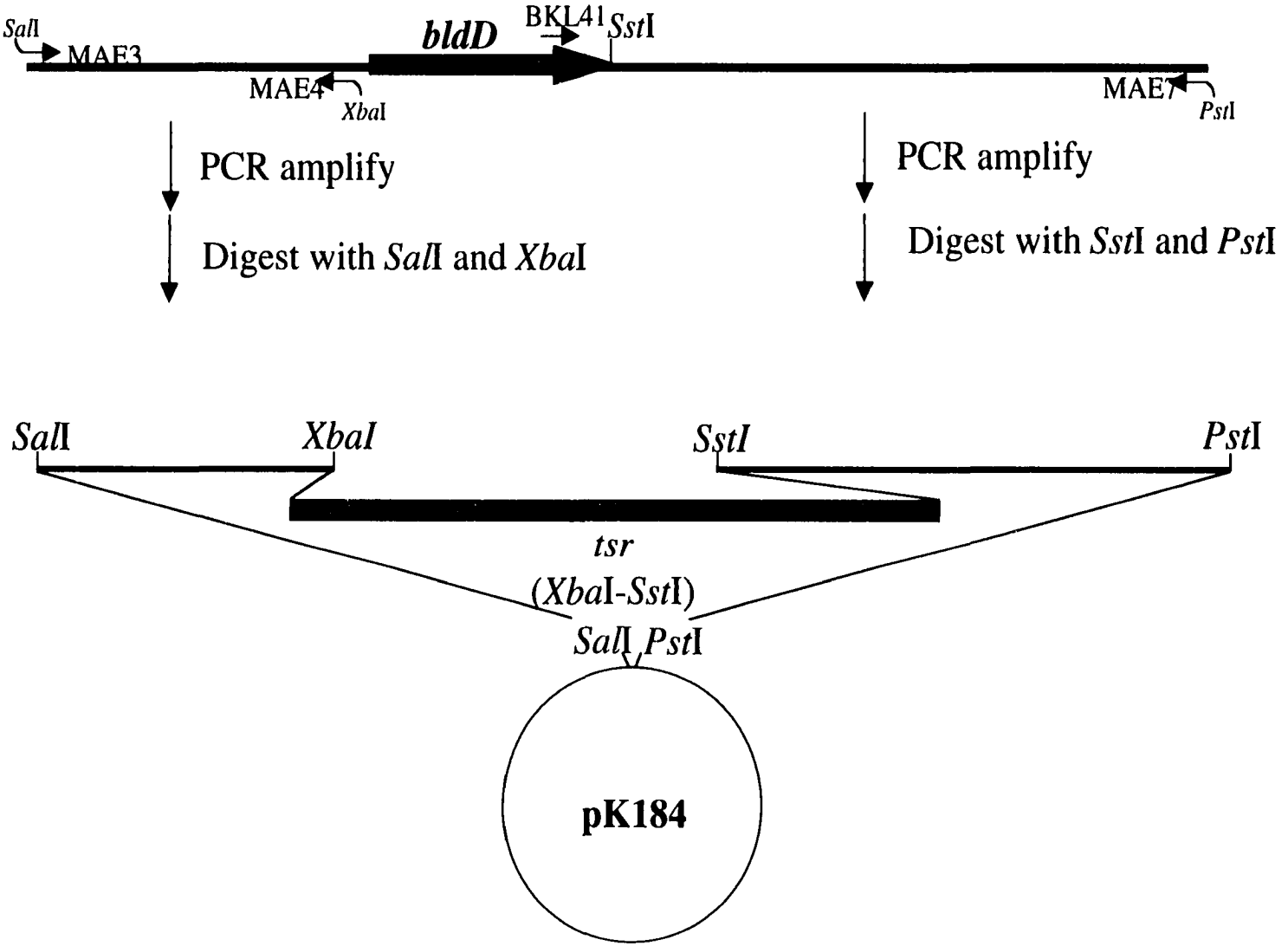
performed immediately, using random primer labeled *tsr* as the probe. A single positive transformant was identified, however, digestion with *Sal*I (*tsr* contained a single *Sal*I site) revealed that half of the construct had been lost, presumably by recombination because of the presence of multiple copies of the polylinker sites flanking *bldD*, the apramycin resistance cassette, and the thiostrepton resistance cassette. The duplicated polylinker regions would have provided sequences for recombination, especially in *E. coli* ET12567, which is notoriously recombinogenic (MacNeil *et al.*, 1992). In hindsight, the initial transformation should have been into *E. coli* DH5α, rather than directly into *E. coli* ET12567, due to its recombinogenic nature.

The next attempt to disrupt *bldD* focussed on simply inserting *tsr* into the blunt *Sma*I site located within *bldD* in pAU184. In this instance, the polylinker of pAU184 had been altered to remove a *Sal*I site, by digestion with *Xba*I and *Pst*I, followed by blunt ending and religation. This was necessary because *Sal*I was to be used to confirm the presence and orientation of the *tsr* insert. If a *Sal*I site was present in the polylinker, it would not be possible to distinguish between insert-containing and insert-free plasmid, as the resulting fragments would be the same size. The *Sal*I deleted pAU184 containing *bldD* was partially digested with *Sma*I, purified from the fully digested plasmid, and ligated with the blunted *tsr* fragment. The ligation mixture was introduced into *E. coli* DH5α, putative positive transformants were examined by digestion with *Sal*I, and a number of *tsr*-containing transformants were found to be present. The representative disruption plasmid, designated pAU194, was then passaged through *E. coli* ET12567, and transformed into *S. coelicolor* J1501. A single thiostrepton resistant, potential *bldD* disruptant was observed, and was cultured for the purpose of chromosomal DNA

isolation. The isolated DNA, as well as HU66 DNA as a negative control, was then digested with *Sph*I, *Xmn*I, and *Sph*I + *Xmn*I, and subjected to Southern blot analysis. Hybridization with random primer labeled *bldD* revealed that *bldD* was not disrupted, and confirmed that a single crossover elsewhere in the chromosome did not take place. All of the hybridizing fragments were equivalent in size to the negative control, and not 1.5 kb larger, which would have been the result of integration of the *tsr*-containing construct. To determine if there was a problem with the disruption construct, a scheme was designed to test the disruption plasmid for its ability to integrate into the chromosome. A second copy of *bldD* was introduced into the *bldD* mutant strain, HU66, using pAU181 (pSET152 containing the *bldD* gene), which integrated into the *att* site in the chromosome. It was expected that introduction of the *bldD* disruption construct into this strain would result in disruption at either site. Introduction of the disruption plasmid did not result in any thiostrepton resistant transformants, indicating that there was likely a problem with the disruption construct. If construct stability could be improved, this method could serve as a means of determining whether *bldD* is an essential gene (Vögtli and Cohen, 1992).

The generation of a *bldD* null mutation was then attempted using a complete gene replacement, rather than a gene disruption strategy (Fig. 3.1.5). DNA flanking the *bldD* gene was amplified, to give an approximately 600 bp upstream fragment (MAE3-4) and a fragment of about 1150 bp downstream (BKL41-MAE7). *tsr* was purified from pAU5 (see Table 2.1) using *Xba*I and *Sst*I. The flanking fragments were digested: MAE3-4 with *Sal*I and *Xba*I and BKL41-MAE7 with *Sst*I and *Pst*I, and these, together with the *Xba*I- and *Sst*I-digested *tsr* fragment, were ligated simultaneously into pK184 digested

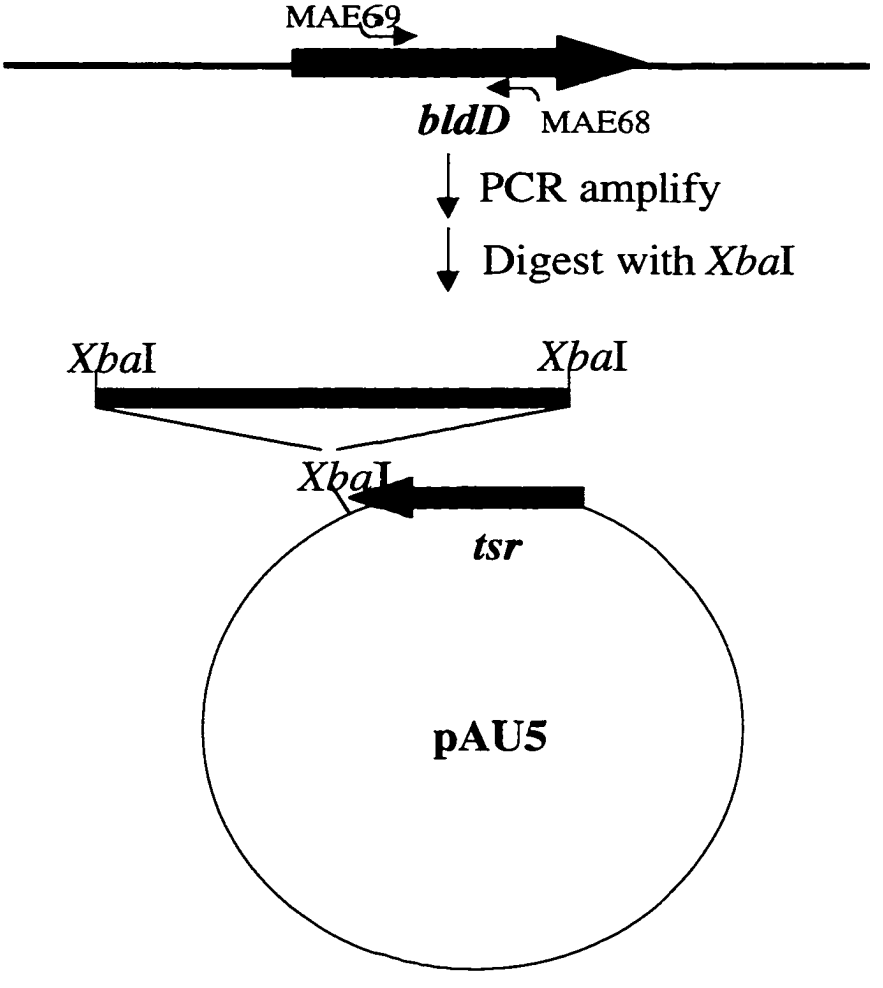
Fig. 3.1.5 Subcloning strategy for the replacement of *bldD* with the thiostrepton resistance gene cassette (*tsr*). The sequence upstream from *bldD* was amplified by PCR, using pAU171 as template and the oligonucleotide primers MAE3, which had a nonhomologous tail with a 5' *Sal*I site, and MAE4, which possessed a nonhomologous tail containing a 5' *Xba*I site. The arrows indicate the approximate location of the primers and the direction of primer binding (5'-3'), and the curved ends indicate the existence of a nonhomologous tail engineered to include a restriction enzyme site. The product was gel purified and digested with *Xba*I and *Sal*I. The sequence downstream from *bldD* was amplified by PCR using pAU171 as template and the oligonucleotide primers BKL41 and MAE7 (having a *Pst*I site engineered onto its 5' end). The gel purified DNA was then digested with *Sst*I and *Pst*I. *tsr* was purified from pAU5 by digestion with *Xba*I and *Sst*I, and is shown in blue, while *bldD* is shown as green, with an arrowhead indicating the orientation of the *bldD* gene. The *bldD* flanking sequences and the thiostrepton resistance gene cassette (*tsr*) were ligated simultaneously into the *E. coli* plasmid pK184, to generate the gene replacement construct.



with *Sal*I and *Pst*I. This should have resulted in the ligation of *tsr* between the two *bldD* flanking regions. The ligation mixture was introduced into *E. coli* DH5α, and was plated onto LB agar containing kanamycin for selection of plasmid containing transformants, and X-gal and IPTG for blue/white selection of insert-containing plasmids. A number of white transformants were observed; however, upon examination, by digestion with *Sal*I and *Pst*I, of the plasmid DNA isolated from 18 of these positive transformants, none were found to contain the correct insert. In retrospect, no transformants were obtained because the MAE3-4 fragment, which was digested with *Sal*I and *Xba*I, did not contain a cleavable *Xba*I site. An *Xba*I site had been engineered onto the 5' end of MAE4, however, no additional 5' nucleotides were included to allow for recognition of the site by the restriction enzyme.

The final attempt to create a *bldD* null mutant utilized a mutational cloning strategy (Rodicio and Chater, 1982) (Fig. 3.1.6). This strategy involved the PCR amplification of a central portion of the *bldD* coding sequence, which was to be ligated into a plasmid adjacent to *tsr*. The oligonucleotide primers BKL68 and BKL69, each with *Xba*I sites engineered onto their 5' ends, along with the requisite 3 extra 5' bases to allow for proper digestion of the *Xba*I sites, were used to amplify a 245 bp fragment, internal to *bldD*. This product, after digestion with *Xba*I, was ligated into *Xba*I-digested pAU5. Positive transformants were selected using ampicillin, and, after isolation of plasmid DNA, were examined by digestion with *Xba*I to confirm the presence of insert. This was followed by passage through *E. coli* ET12567, where construct integrity was again examined. Upon introduction into *S. coelicolor* J1501, and selection for thiostrepton resistant transformants, colonies with three different phenotypes were

Fig. 3.1.6 Subcloning of an internal *bldD* fragment into the thiostrepton resistance gene-containing plasmid pAU5, for disruption of *bldD*. The 264 bp internal *bldD* fragment was amplified using the oligonucleotide primers BKL68 and BKL69, whose location and binding directions are indicated by arrows, with the curved ends representing nonhomologous *Xba*I sites engineered onto the ends. To create the *bldD* disruption construct, the fragment was purified and digested with *Xba*I before ligation downstream from *tsr*, in the *Xba*I site of pAU5. *bldD*, is shown in green, and *tsr* is shown in light blue, with arrowheads indicating the direction of transcription in each case.



observed: a *bldD* phenotype, lacking hyphae and pigment; a wild type phenotype; and an intermediate one, where blue pigment was produced, and sparse hyphae were seen. Chromosomal DNA was isolated from representatives of each of these phenotypic categories, and along with J1501 chromosomal DNA, was digested with *Nco*I for Southern blot analysis. If a single crossover had occurred, the resulting *bldD*-hybridizing *Nco*I fragment would increase significantly in size, relative to the wild type, as the entire plasmid would be integrated into the chromosome within *bldD*. Upon hybridization with the internal *bldD* fragment used to create the mutagenic plasmid, it was found that none of the thiostrepton resistant colonies were *bldD* disruptants. They all had hybridizing fragments appearing to be the same size as the wild type, rather than the significantly larger fragment that would result from a single cross-over.

Given all of these failed attempts to disrupt *bldD*, some of which were due to difficulties with the disruption vector construction, while others were the result of unknown circumstances that prevented *bldD* disruption, it seems possible that *bldD* serves an essential role, and it might not be possible to disrupt it in the absence of a second copy. If this were true, it would suggest that the BldD Y62C mutant protein must retain partial function.

3.1.5 Examination of potential *bldD* gene mutations that had been isolated using a general mutagenic screen

While all attempts to disrupt *bldD* failed, the opportunity to examine three potential *bldD* mutants was presented by Justin Nodwell. He wished to isolate additional *bld* genes, through a mutagenic screen, and was grouping these "new" mutants on the

basis of their extracellular complementation profile. Each complementation group was defined by a particular *bld* gene, and once assigned to a particular group, he tried to genetically complement the new mutants in each category, using a cloned copy of the representative *bld* gene. For example, in an effort to identify mutants that represented alleles of *bldD*, mutants categorized as being in the *bldD* complementation group were transformed by Dr. Nodwell, with plasmid or phage DNA containing the *bldD* gene, to see if they could be genetically complemented. Three mutant strains in the *bldD* extracellular complementation group, designated MY262, MY405, and MY486, all appeared to be complemented by the *bldD* gene, and were provided, along with their corresponding complemented strains, to us by Dr. Nodwell. Of the three, only MY262 appeared to be more pleiotropic than the *bldD53* point mutant, as it was not only unable to erect aerial hyphae, but was also completely lacking in all pigment production when grown on rich R2YE medium. On this same medium, the *bldD53* mutant was observed to produce red pigment. Dr. Nodwell had observed that this particular mutant strain did not grow well in liquid culture, and the mycelia that did develop appeared to be very "twisty". We did not observe these characteristics, as the mutant strain grew well under our liquid culture conditions, and no differences were seen when comparing the mycelia of the MY262 mutant strain and its complemented strain using a phase contrast microscope. In our hands, the other two mutant strains, MY405 and MY486, did not appear to possess significant phenotypic differences relative to that of their *bldD* complemented strain (i.e. a wild type phenotype) either in liquid culture or when grown on the surface of agar plates.

Since MY262 had appeared to have the most severe phenotype, and was therefore of the greatest interest, we prepared chromosomal DNA from MY262 for the purpose of determining the *bldD* sequence. The *bldD* gene was PCR amplified using EXPAND high fidelity DNA polymerase, and primers BKL51 and BKL47, and the resulting product was purified from an agarose gel and sequenced by the Biological Sciences sequencing service, using the oligonucleotide primers BKL51, MAE1, BKL37, BKL41, BKL47, MAE2, and BKL50. Surprisingly, we found no mutation in the *bldD* gene, or in its flanking sequences (400 bp upstream), indicating that MY262 does not represent a *bldD* mutant strain. It was later determined, by Dr. Nodwell, that MY262 is an unstable strain, and the apparent complementation by *bldD* was simply a reversion of the mutant phenotype, and not a true complementation.

MY405 was also sequenced, but again, was found to harbour no mutation; MY468 was not sequenced, given its wild type appearance. Therefore, to date, there remains a single *bldD* mutant representative.

3.1.6 Pleiotropic effects of high copy *bldD* in the cell

Given the failed attempts to generate an *S. coelicolor bldD* null mutant strain, it was of interest to determine whether the other extreme situation, the presence of multiple copies of *bldD*, would be as unfavorable. The high copy plasmid pAU181, a pIJ486 derivative containing the *bldD* gene, was introduced into the wild type strain, J1501, and the *bldD* mutant strain, HU66, to examine the effects of high copy *bldD*. Relative to the general growth pattern observed for the nontransformed parent strains, the resulting transformants grew very poorly on R2YE agar. Typically, for the *S. coelicolor* J1501

strain, aerial hyphae are formed and red pigmented antibiotics are visible after 24 hours; aerial hyphae are abundant by 36 hours; and by 48 hours, copious amounts of blue and red pigment are produced, and sporulation is nearly complete. In the *bldD* mutant, substrate mycelial growth is often less vigorous than in the wild type strain (i.e. less biomass is present for the mutant strain than the wild type strain, given the same amount of innoculum and identical growth conditions), aerial hyphae are absent at all times, and red pigment is usually produced by ~36 hours. The pAU181 containing HU66 strain appeared very much like its *bldD* mutant parental strain up until ~24 hours, with poor growth, no hyphae, and no red pigment. The slight appearance of aerial hyphae occurred by 36 hours, and the colonies were completely covered with aerial hyphae by 66 hours; however, blue pigment was only slightly visible by 66 hours of solid phase culture. As seen with the mutant strain, multiple copies of *bldD* adversely affected growth of the wild type strain, with only sparse aerial hyphae and virtually no pigment production seen at 36 hours. By 48 hours, however, red and blue pigments were starting to appear, and hyphae had become more abundant. Growth of J1501 and HU66 strains containing pIJ486 without insert was not examined.

It was difficult to generate spore stocks for these strains, given their poor growth, and their far from abundant sporulation, and even when enough biomass was present to harvest spores from, the spores germinated poorly, failing to grow upon subculturing.

It may therefore be concluded from these investigations, that overexpression of *bldD* on a multicopy plasmid leads to delayed differentiation, which likely results from poor growth of the substrate mycelia.

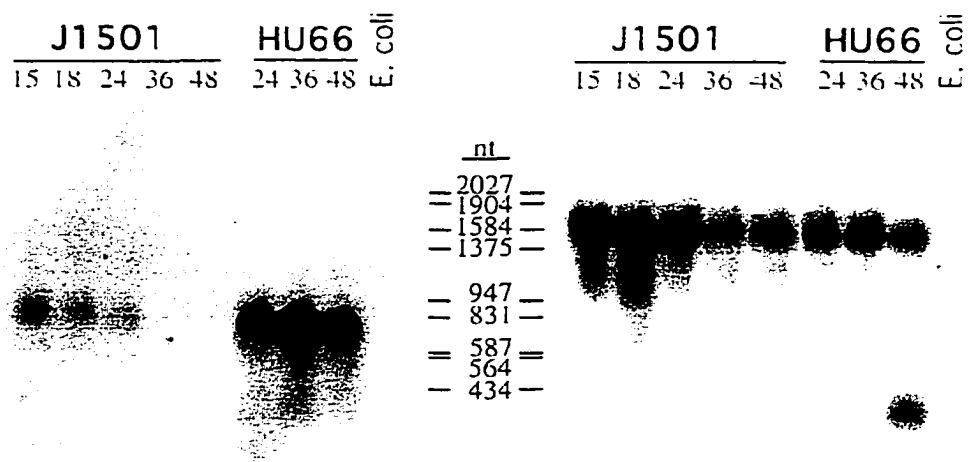
3.2 *bldD* transcript analysis

While the studies into different mutations in *bldD* did not help to elucidate the function of BldD, it seems that BldD abundance must be tightly controlled, as evidenced by the inability to generate a *bldD* null mutation (at least in our hands), and by the detrimental effects of *bldD* overexpression. To better understand the regulation of *bldD* expression, transcription analyses were undertaken. These studies were initiated to examine the timing of *bldD* expression, to determine the *bldD* transcription start site, to reveal its expression as a monocistronic versus polycistronic transcript, and to investigate differences between wild type and mutant *bldD* expression levels.

3.2.1 Northern blot analysis of *bldD*

Northern blot analyses were performed to determine both the approximate size of the *bldD* transcript, and the presence of *bldD* transcript. The RNA used for this study was harvested over a 48 hour period, from surface grown cultures of J1501 and HU66. RNA from at least three different time courses was used for *bldD* northern blot analysis. Forty micrograms of the resulting RNA, together with *E. coli* RNA as a negative control, were denatured by treatment with glyoxyl, and were electrophoresed along with DNA markers that had been subjected to the same treatment. After capillary transfer of the RNA to a nylon membrane, it was probed with a random primer labeled, 214 bp fragment internal to the *bldD* coding sequence (*Sma*I-*Pvu*II; see Fig. 3.1.2), a single transcript of ~600-750 nt was seen (representative results are seen in Fig. 3.2.1). The size of the transcript would be consistent with *bldD* expression as a monocistronic transcript, as the *bldD* coding sequence was 504 bp in length.

Fig. 3.2.1 Northern blot analysis of *bldD* transcripts in RNA samples (40 μg), isolated from surface grown *S. coelicolor* J1501 and HU66. RNA was isolated over a 48 hour period, at the timepoints indicated (hours post-inoculation). The RNA was denatured with glyoxyl and was separated on a 1.25% agarose gel, before transfer to a nylon membrane. The blot was probed with a random primer labeled, 214 bp *Sma*I-*Pvu*II fragment internal to *bldD* (see Fig. 3.1.2). *E. coli* RNA was used as a negative control. Transcript size was determined using molecular weight markers III and V (Roche). Hybridization with the *bldD* fragment was carried out at 50°C in 50% formamide. Washes were also performed at 50°C. RNA loading levels were assessed by probing the same blot, after having been stripped of the *bldD* hybridizing fragments, with an end-labeled oligonucleotide specific for 16S rRNA. Hybridization and washes were performed at 55°C in the absence of formamide. The blot, when hybridized with the internal *bldD* fragment, was exposed to Kodak X-ray film with an intensifying screen for 2 weeks at –80°C, and was exposed to X-ray film, without an intensifying screen, for 4 hours, when hybridized with the 16S rRNA probe.



bldD probe

16S rRNA probe

Examination of the hybridizing transcripts also revealed that the expression of *bldD* was temporally regulated (Fig. 3.2.1). Strongest expression was observed early in colony development (between 15 and 24 hours). Since under these growth conditions, slight aerial hyphae were observed by 18 hours post-inoculation, the 15 hour sample likely represents the transition period between vegetative growth and the onset of differentiation. After 24 hours, which corresponded to the appearance of abundant aerial hyphae and pigmented antibiotics, the *bldD* transcript was observed to decrease to virtually undetectable levels by 48 hours when sporulation was almost complete. In contrast, this temporal regulation was lost in the *bldD* mutant strain, where a very high level of transcript was seen at all timepoints examined. This suggested that the *bldD* mutant had lost the ability to control *bldD* transcription, and implicated the BldD protein in the negative regulation of its own expression, or alternatively, indicated increased *bldD* transcript stability in the *bldD* mutant. After stripping of the membrane, RNA loading was assessed using an oligonucleotide probe specific for 16S rRNA (BKL54). While the RNA levels were not exactly equivalent in all lanes, the variation observed could not account for the differences seen at the level of *bldD* transcript accumulation (Fig. 3.2.1).

3.2.2 S1 nuclease protection assays for the *bldD* transcript

To confirm the pattern of *bldD* expression and to determine the transcription start site for *bldD*, S1 nuclease protection assays were conducted. The RNA samples were hybridized overnight at 55°C to a 527 bp probe (MAE51-50; see Table 2.4), extending 399 bp upstream from the translation start site. Given the *bldD* coding sequence length of 504 bp, and the predicted transcript size of 600-750 nt, it was expected that the

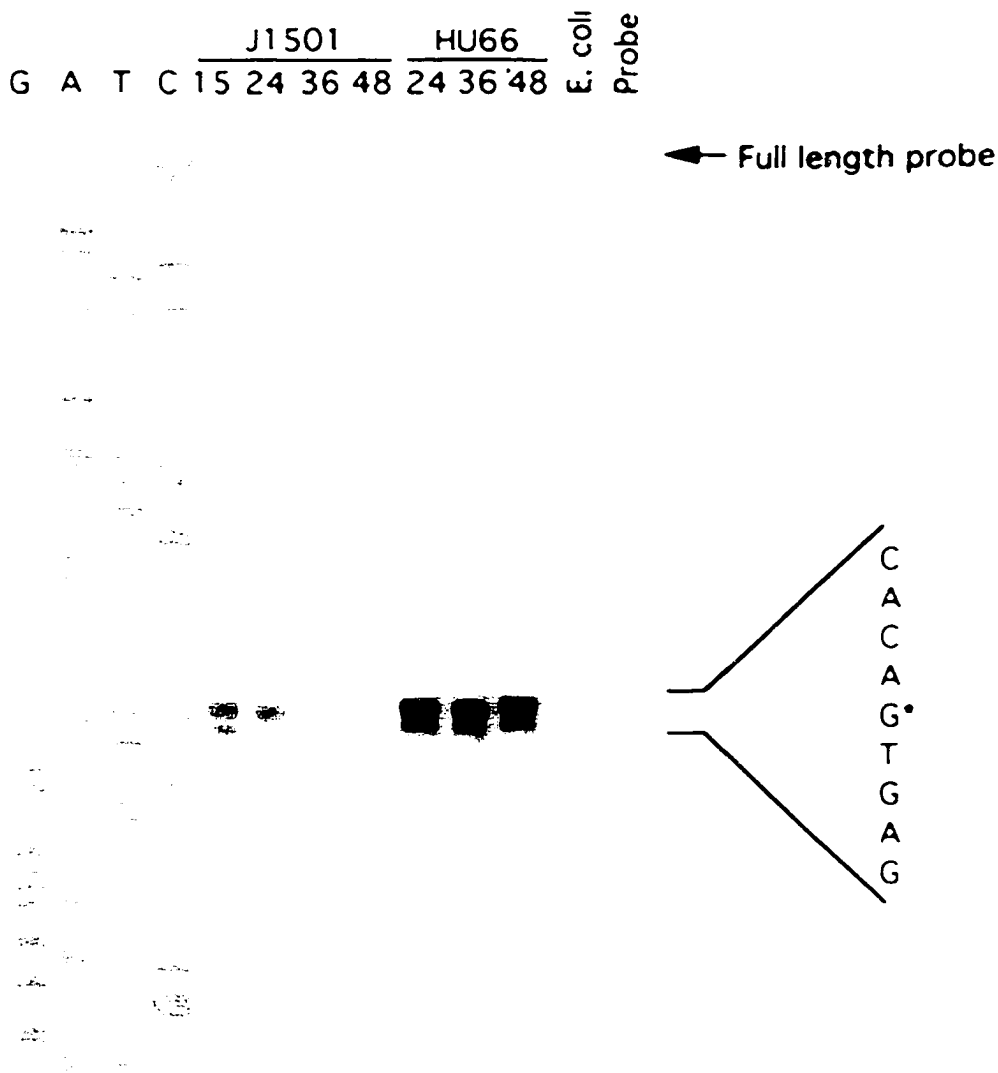
transcription start site would lie within these 399 upstream bases. In the unlikely event that it was located further upstream, the probe was designed to include a 10 nt nonhomologous extension on the upstream end, to allow differentiation between full length protection and probe-probe reannealing. The nucleic acid samples were treated with S1 nuclease, and the results obtained clearly demonstrated temporal occurrence of the *bldD* transcript in the wild type strain, and constitutive overexpression of the transcript in the mutant strain (Fig. 3.2.2), corroborating the northern blot observations. The S1 procedure was repeated twice more using the same RNA stock, and was performed using RNA isolated during two additional time courses, with identical results obtained each time.

Mapping of the transcription start site revealed one major protected band, 181 nt in length. A product of this size corresponded to a transcription start site, beginning with a G residue, 63 nt upstream from the *bldD* translation start site, immediately downstream from putative –10 and –35 elements separated by 18 nt and sharing substantial similarity with typical streptomycete *E. coli*-like promoters. A shorter protection product was also seen at 176 nt; however, it was not apparent whether it represented a second transcription start site initiating at a T residue 58 nt upstream from the ATG *bldD* start codon, or was merely an artifact of the S1 mapping procedure. To distinguish between the two possibilities, the RNA samples were subjected to primer extension analysis.

3.2.3 Primer extension analysis of the *bldD* transcript

In order to authenticate the S1 nuclease mapping results, and determine whether *bldD* was transcribed from one or two different promoters, primer extension analyses

Fig. 3.2.2 High resolution S1 nuclease mapping of *bldD* transcripts using RNA (50 μg) isolated at various timepoints (indicated as hours post-inoculation) from surface grown cultures of *S. coelicolor* J1501 and HU66. The RNA samples were incubated with a ^{32}P end-labeled probe (BKL51-50) at 55°C, and the resulting RNA-DNA duplex was treated with S1 nuclease and the labeled products were separated on a 6% sequencing gel. The gel was exposed to X-ray film without an intensifying screen for 72 hours. The *bldD* sequence ladder, indicated by G A T C, was generated using the oligonucleotide primer BKL50, which was also used in probe preparation. The asterisk (*) indicates the most probable transcription start site in the sequence corresponding to the template strand. The lane labeled *E. coli* represents the control lane using RNA isolated from *E. coli*, while the lane labeled Probe represents the control lane containing probe alone.



were carried out using the end-labeled oligonucleotide MAE12 (Table 2.4). Thirty micrograms samples of RNA were annealed after denaturation at 80°C, to MAE12 for 1 hour at 37°C. The reverse transcription reactions were conducted, and upon separation of the products on a 6% sequencing gel, a single product of 89 nt was observed (Fig. 3.2.3). This corresponds to the larger 181 nt fragment seen in the S1 mapping experiments, suggesting that the smaller 176 nt fragment seen with the S1 protection assay was an S1 nuclease artifact that presumably resulted from breathing at the end of the RNA-DNA duplex during treatment with the S1 nuclease.

On the basis of northern blot, S1 nuclease protection and primer extension analyses, it could be concluded that *bldD* is transcribed from a single promoter, starting 63 nt from the translation start site. It appears that transcription proceeds through the *bldD* gene to a long inverted repeat (ΔG = -57.6 kcal) downstream from the translation stop codon, which would provide a reasonable signal for transcription termination (Fig. 3.2.4). This would generate a *bldD* transcript of 648 nt, which would be consistent with the northern blot estimation of a 600-750 nt transcript.

3.3 BldD overexpression and purification

Once transcript analyses were complete and the timing of *bldD* expression had been determined it became important to correlate transcription with the appearance of the BldD protein. In order to examine BldD expression levels, and conduct *in vitro* protein assays, it was essential to isolate large quantities of purified BldD.

Fig. 3.2.3 Primer extension analysis of *bldD* transcripts. RNA (30 μg) isolated from surface grown cultures of *S. coelicolor* J1501 and HU66, at various timepoints (shown as hours post-inoculation) was incubated with the end-labeled oligonucleotide MAE12 (internal to the *bldD* coding sequence). When annealed to the *bldD* transcript, MAE12 served as the primer for reverse transcriptase extension reactions, and the resulting products were separated on a 6% sequencing gel. The sequencing reactions, labeled as G A T C, were performed using MAE12 as the primer, and were used to determine the *bldD* transcription start site; shown on the right is the template sequence, with an asterisk (*) marking the most likely transcription start point.

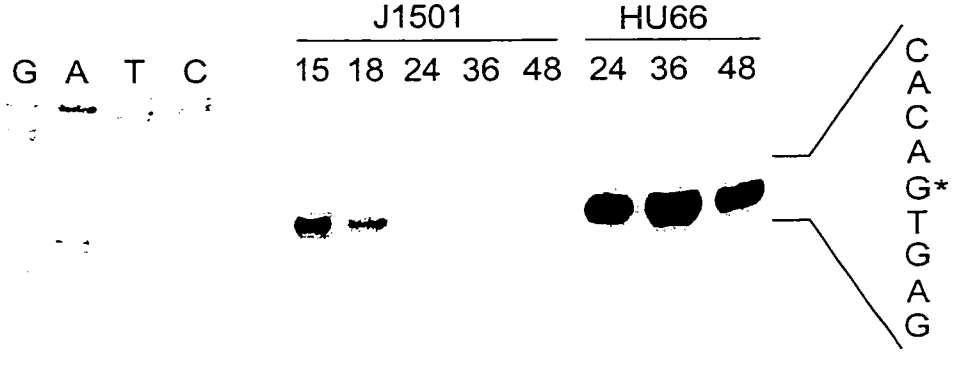
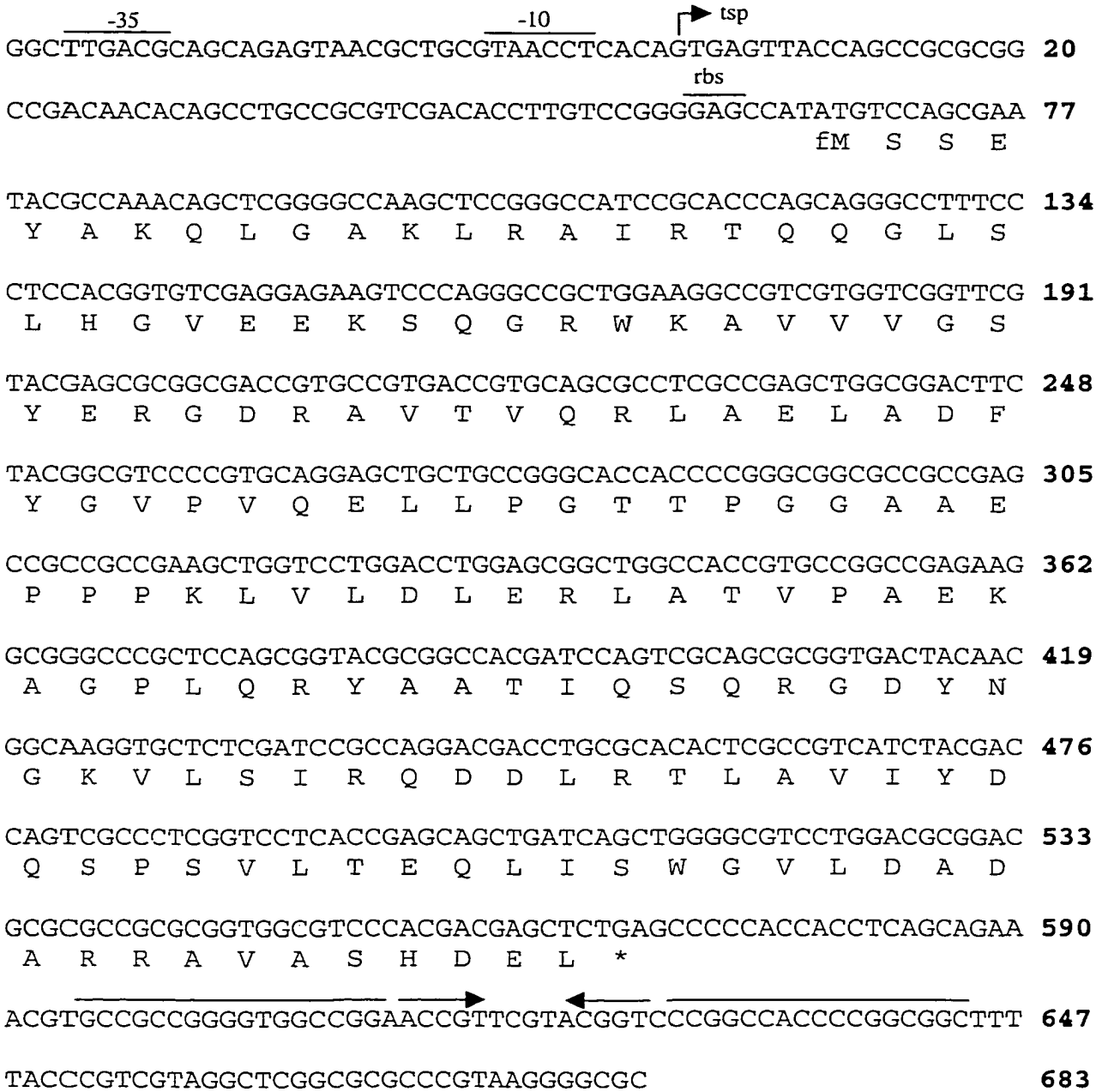


Fig. 3.2.4 Compilation of *bldD* transcription features. The proposed –10 and –35 promoter elements are indicated upstream from the transcription start point (tsp). The ribosome binding site (rbs) is shown, and the amino acid sequence for BldD is indicated below the nucleotide sequence. Downstream from the translation stop codon (*), is a long inverted repeat that appears to be a transcription termination signal (marked by arrows). The numbers to the right indicate the number of base pairs downstream from the transcription start site (tsp = +1).



3.3.1 His_6-BldD overexpression and purification

To generate purified BldD, the *bldD* coding sequence was amplified from both wild type and *bldD* mutant strains, and was initially cloned downstream of a 6-histidine tag in the *E. coli* plasmid pQE9. The cloning was accomplished by digestion of pQE9 with *Bam*HI and *Hin*dIII, into which the wild type (*bldD*$^+$) and mutant (*bldD*$^-$) coding sequences were ligated (amplified using the oligonucleotide primers MAE6 and MAE5, see Table 2.1, with MAE6 possessing a 5' *Bam*HI tail, and MAE5 having a 5' *Hin*dIII tail). Expression of the His_6-BldD fusion was driven by a T5 promoter containing 2 *lac* operator sequences, and was induced by the addition of 0.5 mM IPTG for 5-6 hours at 37°C. After induction, the cells were pelleted, frozen, treated with lysozyme, and sonicated to liberate the overexpressed protein. Induction of the wild type BldD-fusion protein resulted in the production of cytoplasmically localized protein, of which approximately 50% was soluble and 50% was insoluble protein (Fig. 3.3.1); only the soluble protein fraction was purified to avoid potential difficulties in protein renaturation.

The His-tagged BldD protein was purified using nickel-chelate affinity chromatography, and yielded ~2-5 mg protein/500 mL. When the purified protein was electrophoresed on a 15% SDS-polyacrylamide gel, it appeared to run at a molecular weight of approximately 21 kDa (Fig. 3.3.2), which was slightly larger than the calculated molecular weight of 19.3 kDa. This was not unexpected, however, as His_6-tagged proteins are known to move more slowly through the gel than would be predicted based upon their calculated molecular weight (Qiagen manual). Denaturing gel electrophoresis also served as a check for purity and stability of BldD; staining of the gel with Coomassie Blue showed slight degradation of BldD after storage at –80°C for

Fig. 3.3.1 BldD overexpression in *E. coli* JM109 as a His_6-tag fusion: examination of BldD induction with IPTG, and its presence in a soluble and insoluble form. The *bldD* coding sequence was ligated into the *E. coli* overexpression plasmid, pQE9, and was introduced into *E. coli* JM109. BldD expression was induced by the addition of 0.5 mM IPTG, and aliquots of the uninduced and induced cultures were compared to assess BldD overexpression. One millilitre of the uninduced and the induced cultures were pelleted, resuspended in 100 μL loading buffer, before removal of 10 μL samples to be electrophoresed on a 15% SDS-PAG, shown in the second and third lanes, respectively. The remaining induced culture was pelleted, and then incubated with lysozyme solution before sonication to disrupt the cells. Centrifugation separated the soluble protein from the insoluble protein and other cellular debris, and an aliquot of the supernatant (soluble protein), and a portion of the pellet resuspended in loading buffer (insoluble protein), were separated by electrophoresis, as shown in lanes 4 and 5, respectively. His_6-BldD is indicated with an arrow. Kaleidoscope markers (Bio-Rad) were used as protein size marker (lane 1).

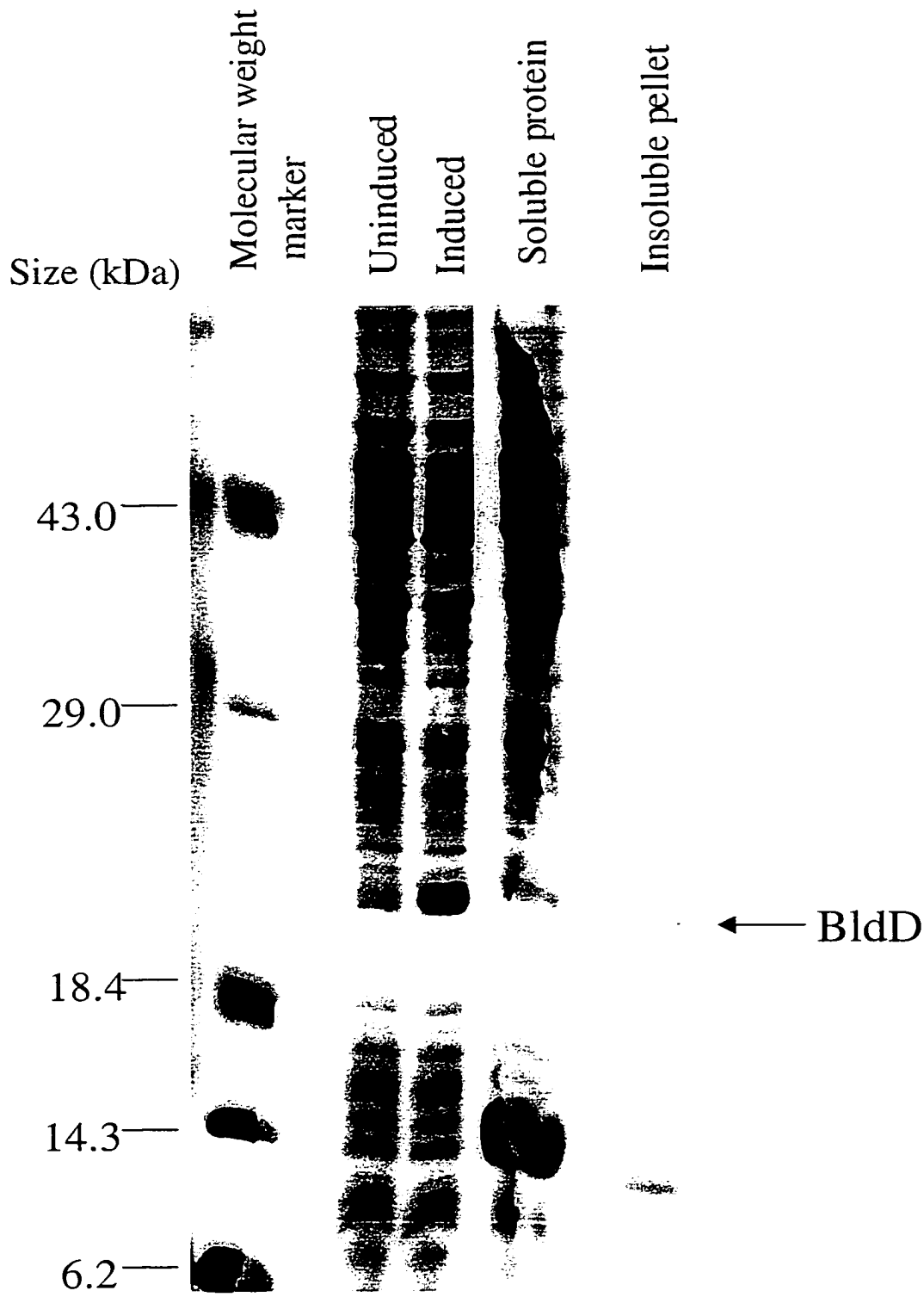
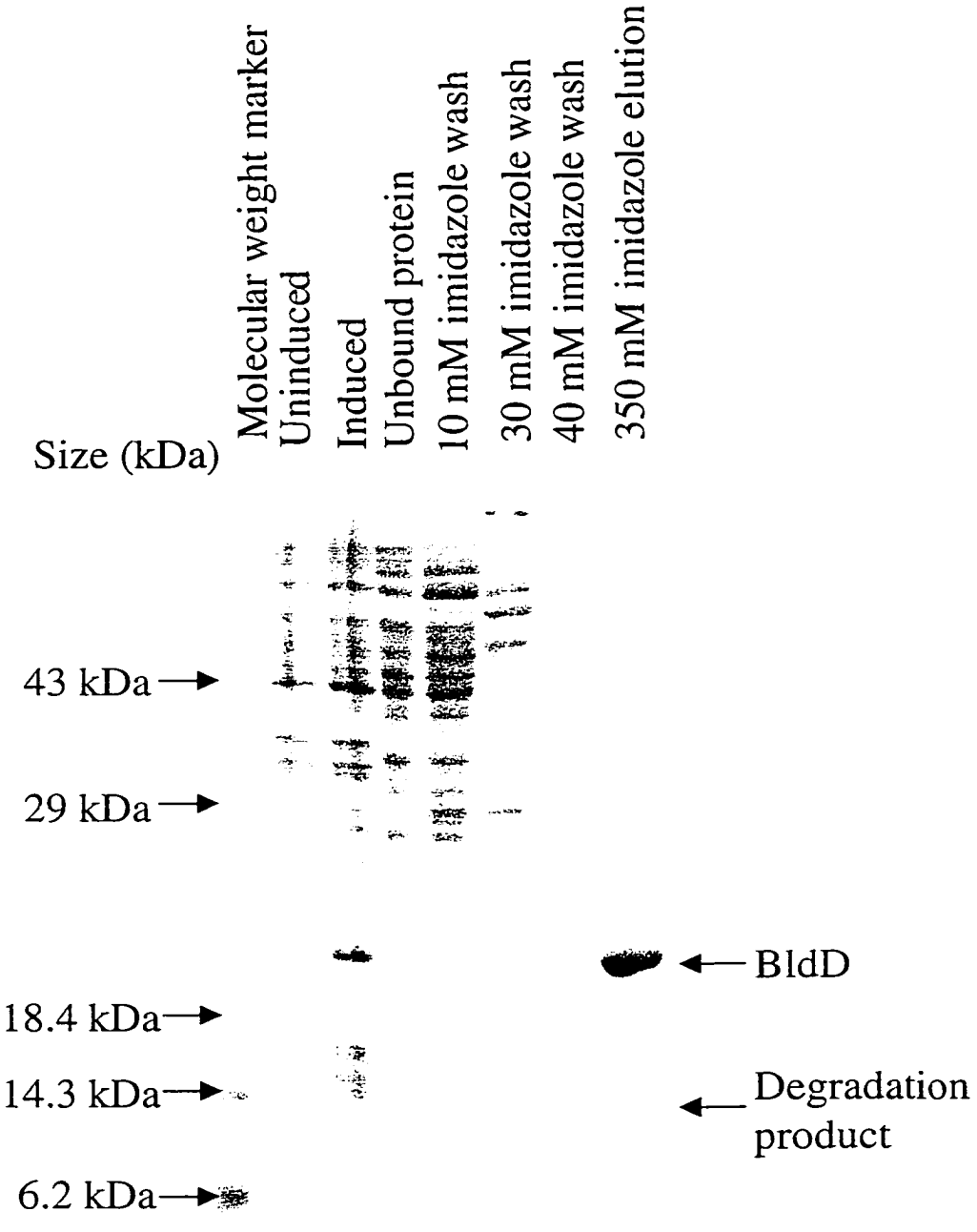


Fig. 3.3.2 Purification of His_6-BldD from *E. coli* JM109. BldD overexpression was induced by the addition of 0.5 mM IPTG, and the soluble protein was purified by nickel chelate affinity chromatography. Samples were collected from each wash with sonication buffer plus imidazole (10 mM, 30 mM, and 40 mM), and His_6-BldD was then eluted using 5 mL of 350 mM imidazole in sonication buffer. The eluted protein and wash samples (2 mL per sample), were dialyzed against 10 mM Tris-HCl (pH 8.5), and 150 mM NaCl, before 250 μL aliquots were removed, dried, resuspended in loading buffer, and electrophoresed on a 15% SDS-PAG. The gel was then stained with Coomassie Blue. Lanes: (1) Marker (Gibco BRL; low molecular weight standards); (2) uninduced cell free extract; (3) induced cell free extract. Lanes 4-8 represent elutions from the Ni-chelating column. Lanes: (4) unbound protein; (5) 10 mM imidazole wash; (6) 30 mM imidazole wash; (7) 40 mM imidazole wash; and (8) BldD elution with 350 mM imidazole. BldD is indicated with an arrow, as are putative BldD degradation products below.



extended periods (see Fig. 3.3.2). Attempts to overexpress the mutant BldD protein by cloning of the mutated gene from HU66 into the same pQE9 expression vector were unsuccessful. In this case, little increase in protein production was seen after induction with IPTG, suggesting that the T62C point mutation confers an inherent instability to the BldD protein that prevents its overexpression in *E. coli*. Attempts to purify the poorly overexpressed mutant BldD protein, yielded no product (Fig. 3.3.3).

3.3.2 Overexpression and purification of a GST-BldD fusion protein

As a control to ensure that any observations made with the His_6-tagged protein were due to the BldD portion of the fusion protein, and not to the N-terminal His_6 tag, wild type BldD was also overexpressed and purified as a glutathione S-transferase (GST) -fusion protein. The *bldD* coding sequence was amplified by PCR from J1501 chromosomal DNA, using the oligonucleotide primers MAE6 and MAE5 (see section 3.3.1. and Table 2.1), and was blunt-ended, and then digested with *Bam*H1 before being cloned downstream from the GST sequence in the *Bam*HI- and *Sma*I-digested *E. coli* overexpression plasmid, pGEX2-T. Cultures were grown for 3-4 hours at 37°C, before the addition of 1 mM IPTG to induce BldD overexpression. BldD was purified from the other soluble proteins by passage through a column containing glutathione agarose, which was bound by the GST protein tag. The BldD fusion protein was eluted using 8 mL of 10 mM reduced glutathione, and was collected in 1 mL aliquots, which were then separated on a 10% SDS-PAG, along with Broad Range molecular weight markers (Bio-Rad). After staining with Coomassie Blue (Fig. 3.3.4), it was found that BldD ran at a molecular weight of ~45 kDa, as expected from a fusion of the 18.2 kDa BldD to the ~25

Fig. 3.3.3 Purification of the mutant BldD protein from *E. coli* JM109. The mutant His_6-BldD protein from *E. coli* JM109 did not appear to be vastly overexpressed after induction using 0.5 mM IPTG; however, purification of the overexpressed protein, was attempted using nickel chelate affinity chromatography. After application of the soluble protein to the nickel affinity column, the column was washed with sonication buffer plus imidazole (10 mM, 30 mM and 40 mM washes), before elution with 350 mM imidazole. A two hundred and fifty microlitre aliquot of the eluted protein was dried, along with an analogous volume of the wild type protein, and was resuspended in loading buffer for electrophoresis on a 15% SDS-PAG. The gel was stained with Coomassie Blue. M: protein molecular weight standards (low range; Gibco BRL); $BldD^+$: wild type BldD; $BldD^-$: mutant BldD.

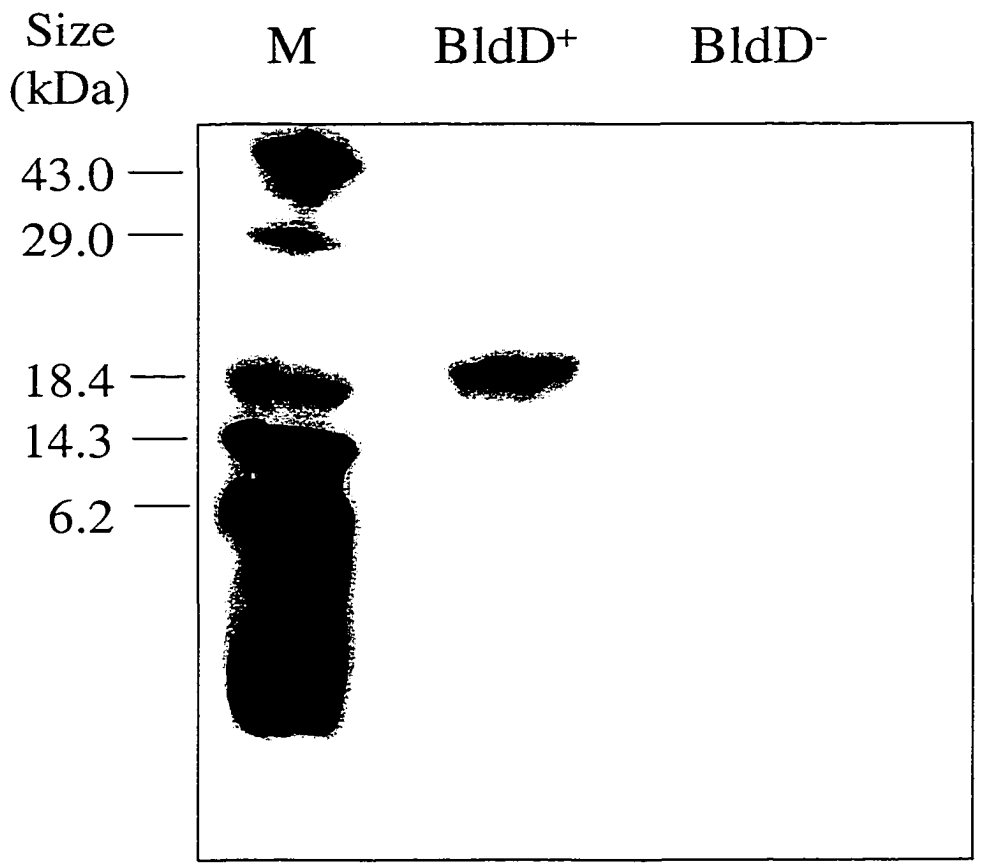
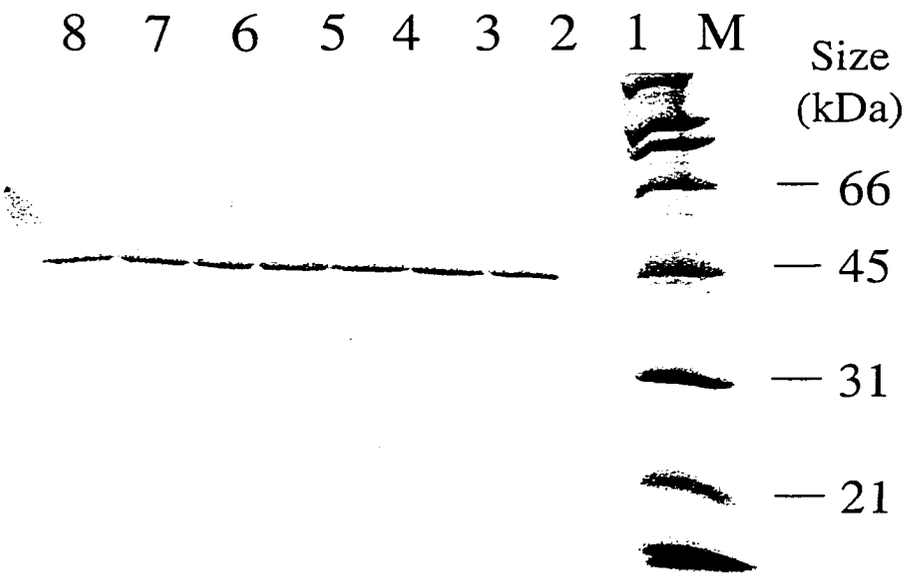


Fig. 3.3.4 Purification of the GST-BldD fusion protein from *E. coli* JM109. BldD overexpression was induced by the addition of 1 mM IPTG, and the soluble BldD fusion protein was purified using glutathione-agarose column chromatography. After application of the soluble protein fraction to the column, the column was washed with 20-30 column volumes of 1× phosphate buffered saline (PBS), and the remaining GST-BldD was then eluted with 10 mM reduced glutathione. One millilitre aliquots were collected, and 10 μL samples were removed from each fraction for separation on a 10% SDS-PAG. M: SDS-PAG molecular weight standards – broad range; 1-8: 10 μL samples from each of the first 8 fractions of eluted GST-BldD.



kDa GST. Purification yielded ~3-5 mg protein/500 mL, with no detectable contaminating proteins.

3.4 BldD analysis

3.4.1 Determination of the oligomeric state of BldD in solution

Once purified His_6-BldD had been isolated, it was of interest to determine how BldD existed in solution, whether in a monomeric form, or as a higher order complex. Approximately 5 mg of His-tagged BldD protein, purified as described above (3.3.1) using a Ni-chelating column, was first applied to a MonoQ ion exchange column. Two peaks were seen after elution of BldD from the column (Fig. 3.4.1) using a linear gradient (0-0.5 M) of NaCl in 20 mM Tris-HCl (pH 8.5), and it was confirmed that each of these peaks corresponded to BldD by separating the appropriate fractions (50-100 μL per 1 mL fraction, precipitated using 50% TCA, and redissolved in ~10 μL sample buffer) on a 15% mini SDS-polyacrylamide gel. Fractions 21-23 and 34-41 were combined, and were reapplied separately to a Superose gel filtration column. In each case, three peaks were seen to elute (Fig. 3.4.2) using 20 mM Tris-HCl (pH 8.5). Based on comparisons with protein standards run under identical conditions (bovine serum albumin, 66 kDa; β–lactamase, 29.5 kDa; and cytochrome C, 12 kDa), the first peak was found to correspond to BldD in a tetrameric form. The second peak contained the majority of protein eluted off the column, and comprised the dimeric form of BldD, while the third peak, which was significantly smaller than the others, represented monomeric BldD. It was estimated based on peak size, that the tetramer, dimer and monomer forms of BldD were present in a ratio of 4:8:0.5, suggesting an equilibrium between these forms, with

Fig. 3.4.1 Anion exchange column purification of His_6-BldD. The His-tagged BldD fusion protein, initially purified using nickel chelate affinity chromatography, was applied to a MonoQ anion exchange column. The protein was then eluted using a concentration gradient of NaCl, indicated by the dotted line, that increased from 0-500 mM NaCl over 70-75 fractions, before rapidly rising to 1 M, over 5 fractions, and then returning to 0 mM NaCl. Two major protein peaks were observed, at fractions 21-23, and 34-41.

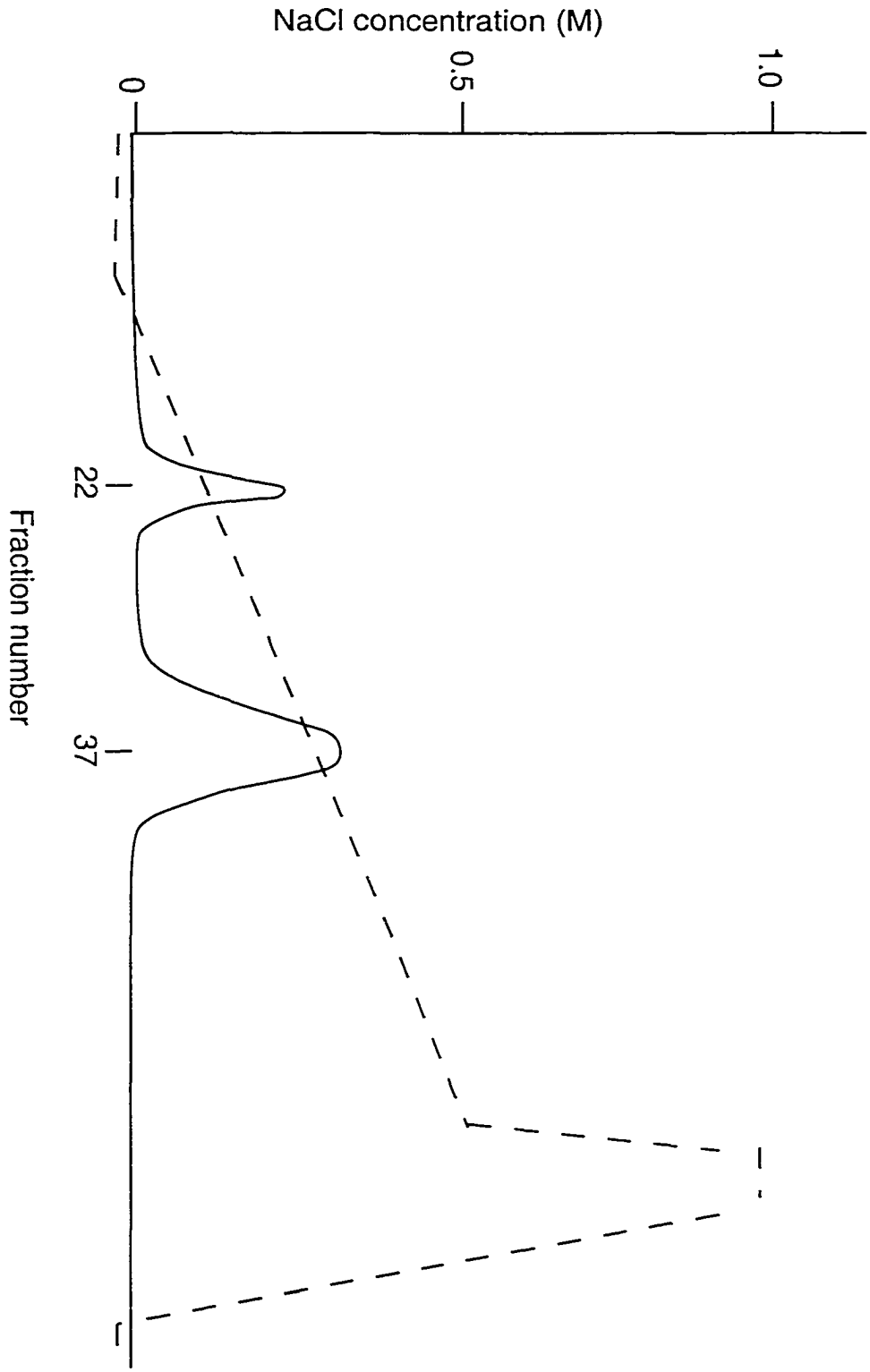
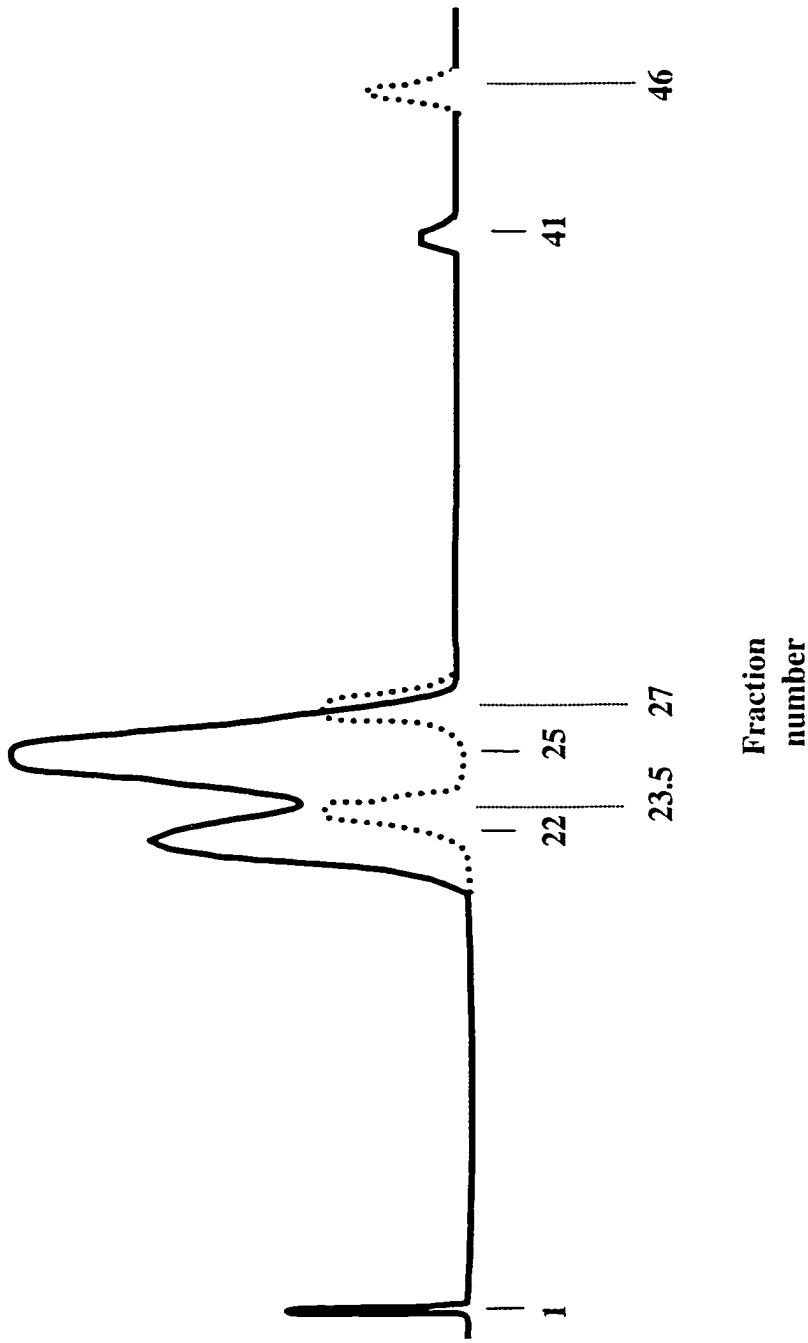


Fig. 3.4.2 Gel filtration column purification of His_6-BldD. The BldD fusion protein, purified by nickel chelate column chromatography, was applied to a Superose 12 column. The protein was eluted using a buffer system of 20 mM Tris (pH 8.5), and three main peaks were observed to elute: the first, at fractions 21-23, which corresponded to the tetrameric form of BldD (T); the second, between fractions 23 and 27, which represented the dimeric form of BldD (D), and the third, in fractions 41 and 42, which represented monomeric BldD (M). The void volume is seen in the first fraction. Size standards (BSA, 66 kDa; β-lactamase, 29.5 kDa; cytochrome c, 12.0 kDa), are shown as dotted lines, as are the fractions in which they were observed to elute.



the dimeric form being most favoured. This was confirmed by loading purified BldD directly onto the gel filtration column, where an elution pattern (4:8:0.5 tetramer:dimer:monomer ratio) identical to that obtained after running the combined MonoQ fractions on the gel filtration column, was observed.

The BldD oligomeric state in solution could be further authenticated using analytical ultracentrifugation. This would provide an accurate determination of molecular weights for different association states of BldD at defined conditions, and at equilibrium.

3.4.2 Western blot analysis of BldD

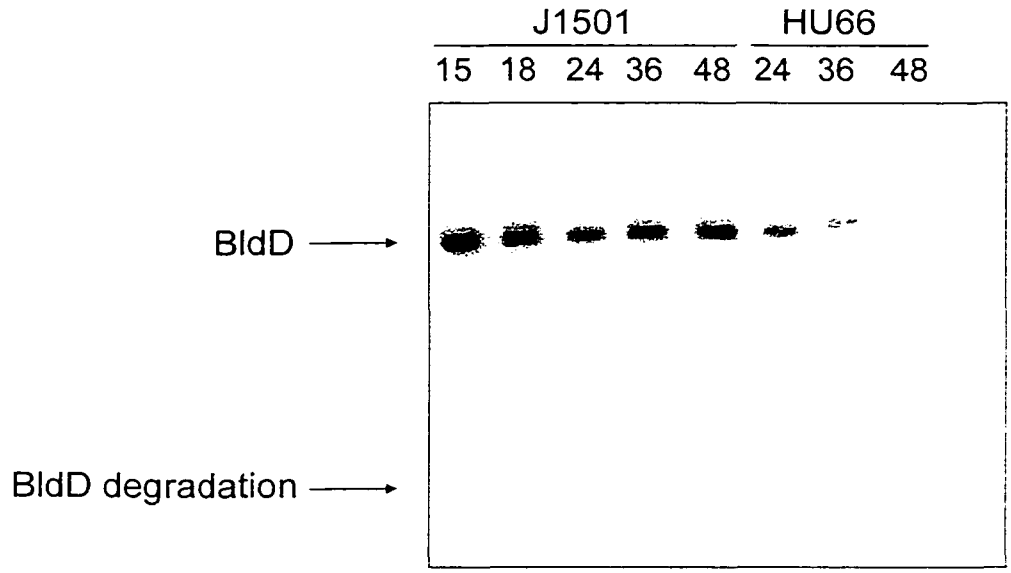
The isolation of purified BldD and the examination of BldD association with itself, suggested that BldD was a cytoplasmically localized protein that existed in one of three oligomeric forms. To further the general survey of BldD characteristics, purified BldD was used to generate antibodies specific for BldD. Purified His_6-BldD (approximately 1.5 mg) was separated on a 10% SDS-PAG, which was then stained with 0.05% Coomassie blue in water. The band corresponding to BldD was excised from the gel, ground into pieces, and mixed with 1 × PBS and Freund's complete adjuvant (or Freund's incomplete adjuvant for booster injections), before injection into the rabbit. The resulting BldD-specific polyclonal antibodies were then utilized in western blot analyses, to examine the BldD expression pattern in *S. coelicolor*, and to correlate the presence of BldD with the observed transcription pattern for *bldD*. Cell free extracts from both *bldD*$^+$J1501 and *bldD*$^-$ HU66 were harvested at various times over a 48 hour time course,

and the proteins were separated on a 15% SDS-PAG before transfer to a PVDF membrane.

The membrane was first incubated with the BldD polyclonal antibody preparation (1:50,000 dilution) and then horseradish peroxidase-conjugated, donkey anti-rabbit secondary antibodies (1:5000 dilution), before treatment with the chemiluminescence reagents. The treated membranes were exposed to film for 5-10 minutes. In contrast to the temporal regulation of *bldD* transcription, it was found that BldD was present at constant levels in all timepoints of colony development in the wild type strain (Fig. 3.4.3), suggesting that the wild type protein is very stable. Conversely, in the HU66 *bldD* mutant strain, where a vast overexpression of transcript was seen in RNA samples isolated from the same time course experiment, the protein was present at lower levels than in the wild type strain, and appeared to decrease with culture age (Fig. 3.4.3). Degradation products were visible below the BldD protein in extracts from the *bldD* mutant strain, possibly indicating protein instability. This potential instability may explain the inability to overexpress the mutant protein in *E. coli* in Section 3.3.1.

Overall, it appears that the wild type BldD protein is reasonably stable, as maximal transcription is attained at 15 hours, after which it decreases, while the protein remains at constant levels throughout colony development. Conversely, the mutant protein is transcribed at a high level at all times, and yet there is less of the mutant protein present than the wild type protein at all culture stages, suggesting that the mutant protein may indeed be unstable.

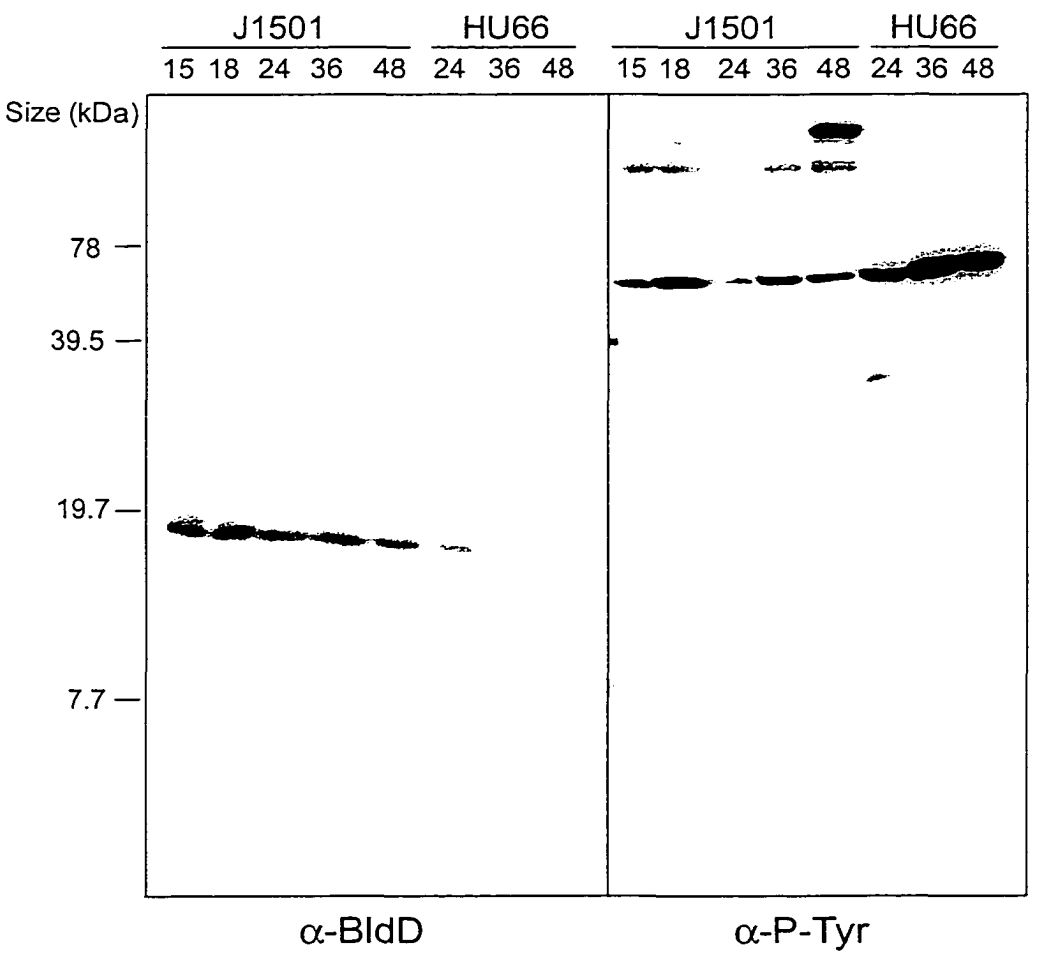
Fig. 3.4.3 Western blot analysis of BldD using cell-free extracts, isolated from surface grown cultures of *S. coelicolor* J5101 and HU66 (times indicated represent hours post-inoculation). Ten microgram samples of total cellular protein were separated on a 15% SDS-PAG and were transferred to a PVDF membrane. The membrane was first incubated with rabbit α-BldD polyclonal antibodies, and was followed by incubation with donkey α-rabbit secondary antibodies (Amersham), complexed with horseradish peroxidase. BldD was detected by treatment with ECL (enhanced chemiluminescent) reagents, and was exposed to X-ray film for 5-10 minutes. BldD, and potential BldD degradation products, are indicated with arrows. Kaleidoscope Prestained Standards (Bio-Rad) and Protein Molecular Weight Standards (Low Range; GibcoBRL) were used as size markers.



3.4.3 Phospho-tyrosine analysis of BldD

Tyrosine phosphorylation is a relatively unusual post-translational protein modification in prokaryotes, but has been observed to play a role in differentiation in *Caulobacter crescentus* (Wu *et al.*, 1999), and has also been demonstrated in *S. coelicolor* (Waters *et al.*, 1994). Given that the only known *bldD* mutation involved the changing of a tyrosine residue into a cysteine residue, it was possible that the mutant phenotype was due to a loss of tyrosine phosphorylation on Tyr62. To examine the possible tyrosine phosphorylation of BldD, cell free extracts, harvested over a 48 hour period from *S. coelicolor* J1501 and HU66, were run in parallel on 2 separate 15% SDS-PAGs, and were transferred to PVDF membranes. One membrane was probed with polyclonal antibodies specific for BldD (1:50,000 dilution), while the other was incubated with phospho-tyrosine specific antibodies (1:1000 dilution; kindly provided by H. Ostergaard). After treatment with the donkey anti-rabbit (1:5000 dilution) and the goat anti-mouse (1:20,000 dilution) secondary antibodies for the BldD and phosphotyrosine-specific antibodies respectively, followed by detection using chemiluminescence, the two blots were compared. It was found that after a 5 minute exposure to film, BldD was visible, and was present in approximately equivalent levels through all timepoints in the wild type strain, and was observed to decrease in abundance in the *bldD* mutant strain. There was, however, very little tyrosine phosphorylation evident for any proteins in the 15-25 kDa range in the wild type strain or the *bldD* mutant strain (Fig. 3.4.4). Given that the BldD protein is ~18.2 kDa, and was clearly visible with the BldD antibodies, these results suggest that BldD is not phosphorylated on a tyrosine residue. Prolonged overnight exposure to the X-ray film did reveal a faint anti-tyrosine

Fig. 3.4.4 Western blot analyses of BldD, using cell-free extracts isolated from surface grown cultures of *S. coelicolor* J1501 and HU66 (times are indicated as hours post-inoculation), comparing BldD expression levels with the incidence of tyrosine phosphorylation. Ten microgram (for BldD) and fifty microgram (for tyrosine phosphorylation) protein samples were separated by electrophoresis on 15% SDS-PAGs run simultaneously, and transferred to PVDF membranes. For detection of BldD, the membrane was incubated with α-BldD antibodies, before reaction with donkey α-rabbit secondary antibodies (Amersham) conjugated to horseradish peroxidase. Tyrosine phosphorylated proteins were detected by incubation of the membrane with α-phosphotyrosine specific antibodies (α-P-Tyr; kindly provided by Dr. H. Ostergaard), followed by incubation with rabbit α-mouse secondary antibodies (generously provided by Dr. H. Ostergaard), again conjugated to horseradish peroxidase. The proteins were visualized by treatment of the antibody-labeled membranes with ECL reagents, and exposure of the membranes to X-ray film for 5-10 minutes. Kaleidoscope Prestained Standards (Bio-Rad) were used as molecular weight markers.



reactive band in the BldD size range, however, even stronger bands were observed in the lanes corresponding to the BldD mutant extracts, suggesting that the bands do not represent BldD. Interestingly, a larger molecular weight protein of ~50 kDa was observed to be hyperphosphorylated in the *bldD* mutant strain, relative to the wild type. This may suggest that BldD is involved in the regulation of a kinase or a phosphatase that interacts with this hyperphosphorylated protein, although differences in protein loading levels could also account for this observation. This was not pursued further in this study.

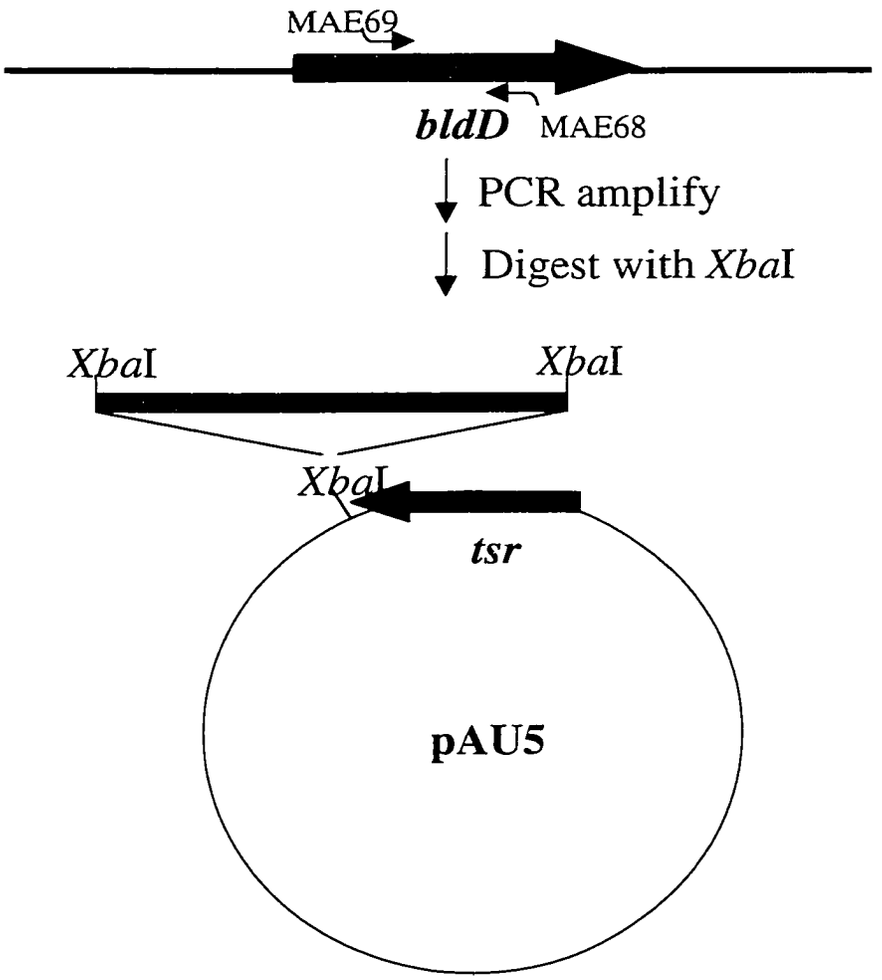
3.5 BldD-DNA interactions

Since the analysis of BldD secondary structure had predicted the existence of a C-terminal helix-turn-helix domain, implying a role for BldD as a DNA-binding protein, the examination of this possibility was the first reasonable step in beginning to address the mechanism by which BldD exerts its effects. The isolation of purified BldD protein made this investigation feasible, and based on the observations from the transcript analyses that suggested BldD may negatively regulate its own expression, the *bldD* promoter region was chosen as the target to examine DNA binding. A variety of DNA fragments were used in the assessment of BldD binding, and they are illustrated in Fig. 3.5.1.

3.5.1 Electrophoretic mobility shift assays

As a first step in addressing the DNA binding ability of BldD, purified BldD was incubated with a variety of DNA fragments spanning its promoter region. To assess the ability of BldD to bind to these promoter fragments, electrophoretic mobility shift assays

Fig. 3.5.1 Probes for electrophoretic mobility shift assays of the *bldD* promoter region. The primers used to amplify the probes are indicated by small arrows and are represented in the name of each fragment. The nonhomologous tail on MAE4 is represented by a curved tail on the arrow. The positions of the probes are shown relative to the translation start point (ATG), transcription start site (tsp), and –10 and –35 promoter elements. The size of each probe is indicated to the right of its name, and to the far right of is the number of shifted fragments observed when incubated with BldD (N/A indicates that the number of shifted bands was difficult to ascertain; 1? indicates weak binding). *bldD* is shown in green, with the arrowhead indicating the direction of transcription.



were conducted. Initially, a 257 base pair probe (MAE11-12; Table 2.4; Fig. 3.5.1), extending from 91 bp downstream of the *bldD* transcription start site, to 166 bp upstream, was amplified by PCR, end-labeled, and used to assay the binding of BldD (0-80 pmol) in the presence of excess, nonspecific competitor DNA (salmon sperm DNA). The presence or absence of a protein-DNA complex was determined by running the reactions on a 6% polyacrylamide gel, and this resulted in the detection of a shifted BldD-DNA product (Fig. 3.5.2). The shifted DNA did not appear as a distinct band, but rather, appeared as a shifted smear, indicating that the conditions were not optimal for BldD binding, and that under these conditions the affinity of BldD for the *bldD* promoter was not strong. While investigating conditions that improved the BldD binding affinity, attempts were also made to delimit the site recognized and bound by BldD, using a shorter DNA fragment in the assay for BldD binding. MAE1-4, a 100 bp probe encompassing a region 63 bp upstream from the transcription start site, to 37 bp downstream, was incubated with 0-80 pmol of BldD, in the presence of poly d(I/C), rather than salmon sperm DNA. It was possible that BldD binding sites were present in the salmon sperm DNA, given the more random nature of the salmon sperm sequence relative to that of the poly d(I-C), and that these sites specifically competed with the labeled probe for BldD binding. The reactions were separated on a 6% polyacrylamide gel, and a distinct band representing protein-bound DNA was observed (Fig. 3.5.3), suggesting that the change in nonspecific DNA added to the reactions promoted higher BldD binding affinity for its promoter DNA. This shorter MAE1-4 probe fragment was then subdivided into two segments overlapping by 22 bp: a 52 bp probe (MAE1-15, extending from –63 to –12, relative to the transcription start point), and a 77 bp probe

Fig. 3.5.2 Electrophoretic mobility shift of BldD binding to a 257 bp fragment spanning the *bldD* promoter region and transcription start site. One to two nanograms (6-12 fmol) of the ^{32}P end-labeled MAE11-12 probe fragment, was incubated with increasing amounts (0-80 pmol, as indicated) of purified His_6-BldD fusion protein in binding buffer (10 mM Tris-HCl, pH 7.8; 150 mM NaCl; 2 mM DTT; 1 μg salmon sperm DNA; 10% glycerol) in a total volume of 20 μL, at 30°C. Samples were loaded onto a continuously running, nondenaturing 6% polyacrylamide gel, and were electrophoresed in 1 x TBE. Free DNA and BldD–DNA complexes are indicated.

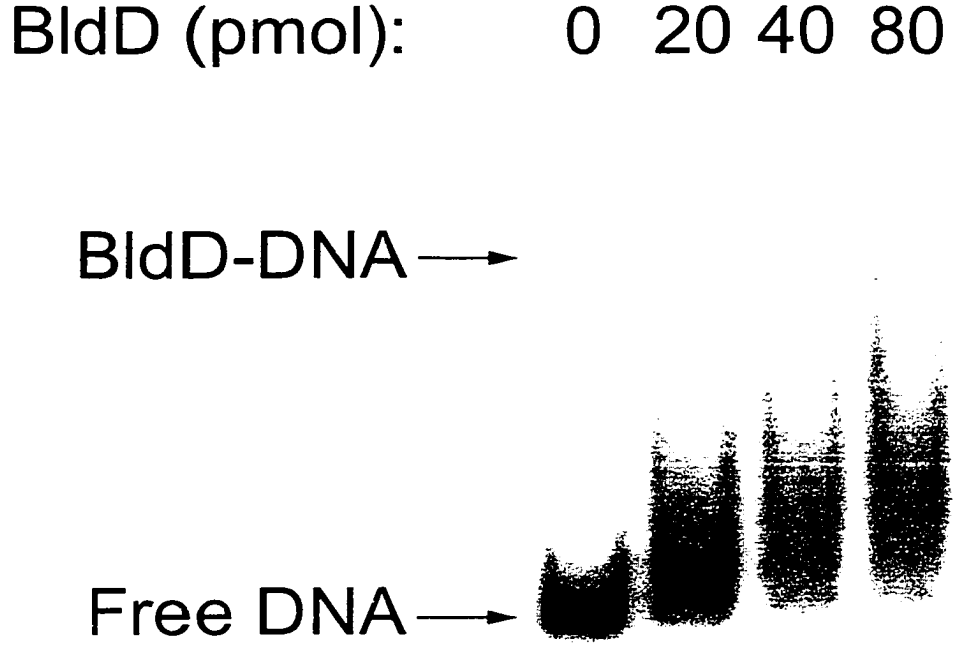
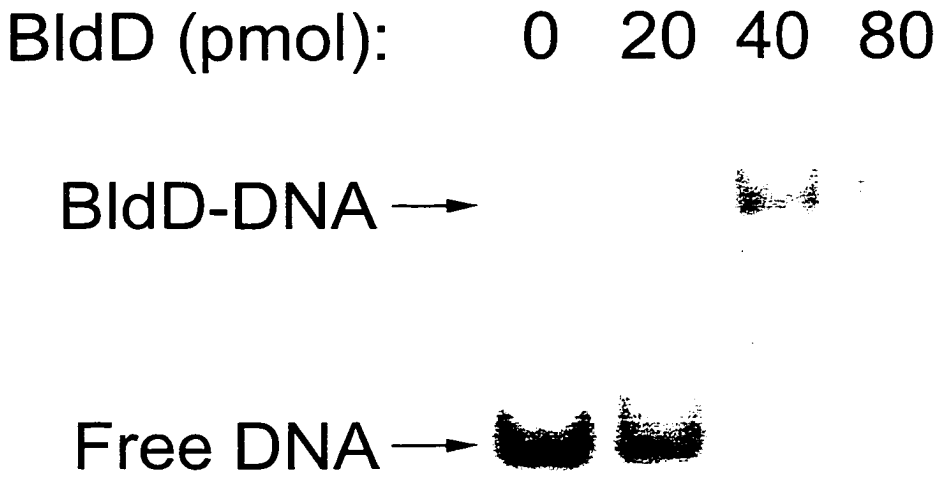
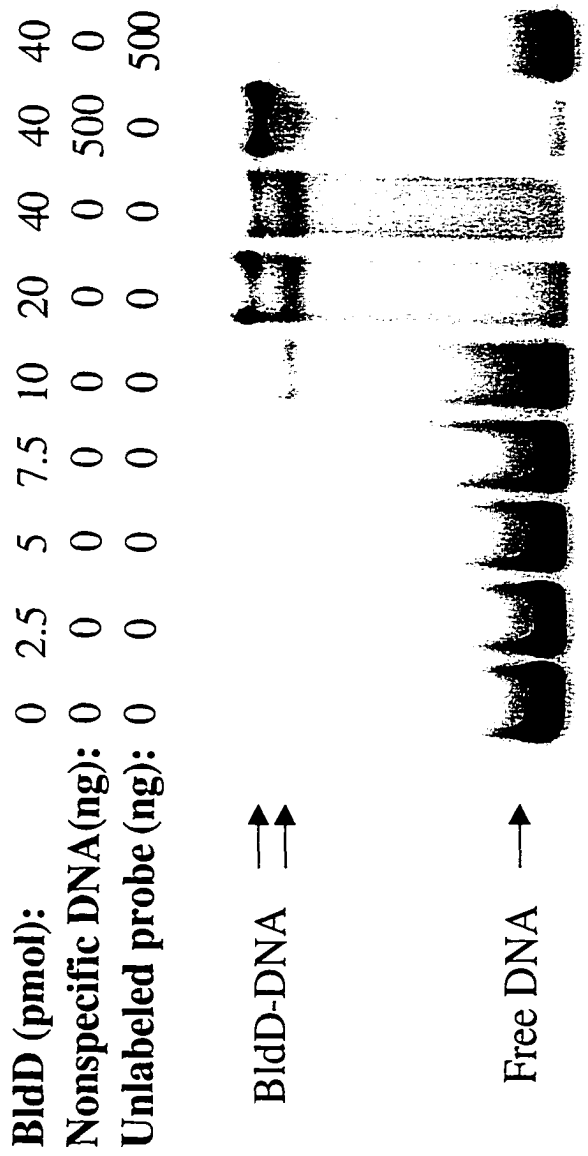


Fig. 3.5.3 Electrophoretic mobility shift of the 100 bp MAE1-4 probe when bound by BldD. End-labeled, 100 bp probe (MAE1-4; 1-2 ng or 15-30 fmol) spanning the *bldD* promoter region, was incubated with 0-80 pmol of purified His_6-BldD, as indicated, in the presence of excess nonspecific DNA (poly dI/dC), for 20 minutes at 30°C, in a total reaction volume of 20 μL. Each reaction was loaded onto a continuously running, nondenaturing 6% polyacrylamide gel, and electrophoresed in 1 × TBE. The locations of free DNA and BldD complexed to DNA are marked with arrows.



(MAE16-4, extending from –33 to +37), in an attempt to better define the BldD binding sequence. After end-labeling, both of these fragments were used as targets for BldD binding. The reactions were again carried out using poly d(I/C) as the nonspecific competitor, and the free DNA was separated from the BldD-DNA complexes, this time, on an 8% polyacrylamide gel. The higher gel concentration helped to maintain the components of the protein-DNA complex in close proximity during their inevitable disassociation during electrophoresis, thereby promoting their reassociation, and hopefully leading to clearer banding patterns for the shifted fragments. Different buffering conditions were also used to determine the best conditions for BldD binding, and it was found that 1 × TBE with 1.5% glycerol, was most effective in generating clear, shifted bands. It was found that BldD was capable of binding to, and shifting MAE 16-4 (Fig. 3.5.4), to give two retarded fragments, likely visualized due to the higher resolution of the 8% gel, rather than the 6% gel. This was repeated multiple times, and representative results are shown in Fig. 3.5.4. In each case, the two fragments were present in approximately equivalent amounts when maximal binding was attained, suggesting that there may be some equilibration between the two forms. The absence of a complete shift to the higher molecular weight complex suggested that there was only a single site for BldD binding, as opposed to two different sites. Specificity of BldD binding was demonstrated through competition mobility shift assays, which involved the addition of ~500-fold excess unlabeled probe (MAE16-4) to the binding reactions, and resulted in nearly complete abolition of protein binding to labeled fragments (Fig. 3.5.4). Excess unlabeled, nonspecific DNA (BKL41-MAE5) from within the BldD coding sequence was also added to the binding reactions, and this addition did not affect binding

Fig. 3.5.4 Electrophoretic mobility shift of BldD binding to a 77 bp region (MAE16-4) of the *bldD* promoter. One to two nanograms (20-40 fmol) of ^{32}P end-labeled DNA was incubated with different amounts of BldD (0-40 pmol, as indicated). Excess nonspecific DNA (poly dI/dC) was included in each reaction, for a total volume of 20 μL, and the reactions were incubated at 30°C for 20 minutes before being loaded onto a continuously running nondenaturing 8% polyacrylamide gel containing 1.5% glycerol. The free DNA was separated from the BldD-bound DNA (both indicated with arrows) by electrophoresis in 1 × TBE. Specificity of binding was illustrated by the addition of ~500 ng (10 pmol) of unlabeled competitor DNA (MAE16-4) to the reaction loaded into the final reaction lane, and by the addition of ~500 ng (6 pmol) of nonspecific competitor DNA (BKL41-MAE5; a 118 bp fragment internal to the *bldD* coding sequence) to the reaction run in the penultimate lane.



of the labeled fragments by BldD (Fig. 3.5.4). Supershift assays using BldD-specific antibodies were also conducted to confirm that BldD was solely responsible for the mobility shift of the *bldD* promoter (using MAE 16-4). The addition of 1 μL of undiluted antibody was observed to result in a complete supershift of all BldD-complexed DNA; this supershift was not observed in the absence of BldD (Fig. 3.5.5), confirming that BldD was responsible for binding and shifting the labeled *bldD* fragment.

When the shorter MAE1-15 was used as the binding target, there was absolutely no shifted DNA seen (Fig. 3.5.6). Interestingly, the addition of 9 bases to the downstream end of MAE1-15, giving MAE1-35 (extending from –63 to –3), resulted in a shift essentially equivalent to that of MAE16-4 when incubated with BldD (Fig. 3.5.6). These 9 bases were therefore important for BldD binding. However, while being required, they were shown not to be sufficient for complete BldD binding to take place, using MAE18-4 as a binding target. MAE18-4 was a 60 bp DNA fragment that spanned the promoter from –16 to +37, thereby including the 9 bp between –12 and –3. While defined binding as a single shifted fragment was exhibited using this probe (Fig. 3.5.7), lower BldD binding affinity was observed relative to that seen with MAE16-4 and MAE1-35 (see Fig. 3.5.4 and 3.5.5), judging by the proportion of free probe still present upon addition of 40 pmol of BldD. To confirm the importance of the bases between –12 and –3, relative to the transcription start site, in BldD binding, electrophoretic mobility shift experiments were carried out using a 46 bp fragment (MAE36-4) stretching from –3 to +37, and therefore lacking these 9 bases. This fragment showed virtually no shift (Fig. 3.5.7), verifying that these bases are essential for BldD binding to its own promoter.

Fig. 3.5.5 Electrophoretic mobility supershift assay of BldD binding to the end-labeled probe MAE16-4 in the presence of BldD-specific antibodies. One to two nanograms (20-40 fmol) of probe were added to each reaction tube, with either 0, 20 or 40 pmol of BldD. α-BldD antibodies were added to reactions containing 40 pmol of BldD, and as a control, were added to protein free reactions, with dilutions as indicated. Free DNA, BldD-DNA complexes, and Ab-BldD-DNA complexes are marked by arrows.

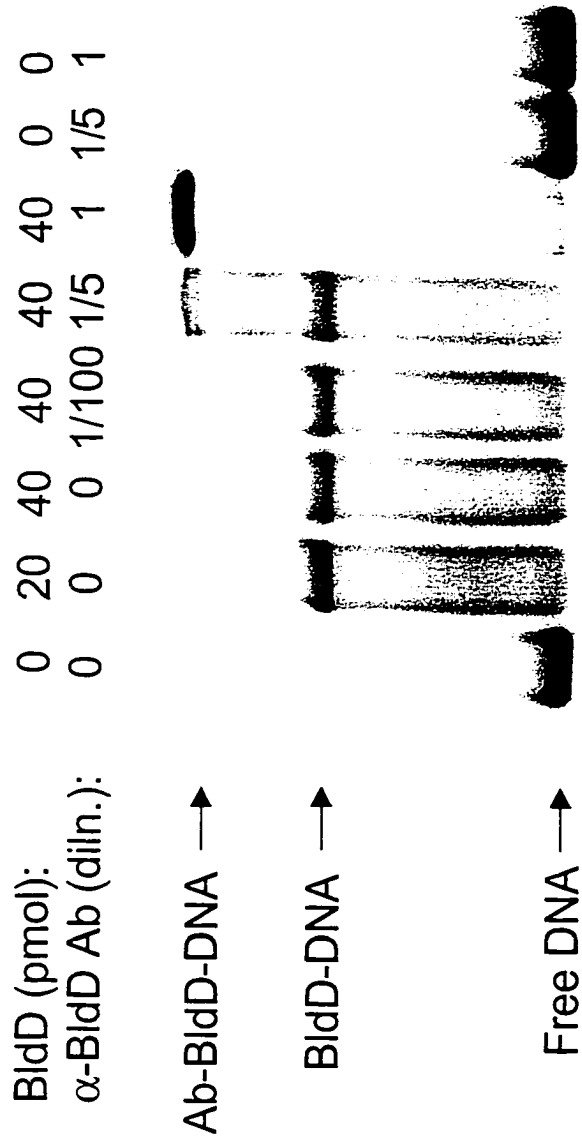


Fig. 3.5.6 Electrophoretic mobility shift assay of BldD binding to a 52 bp fragment, MAE1-15, and a 61 bp fragment, MAE1-35. End labeled MAE1-15 probe, or MAE1-35 probe (1-2 ng; 29-59 fmol for MAE1-15, and 25-50 fmol for MAE1-35) was incubated with increasing amounts of BldD (0-40 pmol) in a 20 μL reaction volume, at 30°C for 20 minutes. The reactions were loaded onto a continuously running nondenaturing 8% polyacrylamide, 1.5% glycerol-containing gel, and were electrophoresed in 1 × TBE. The locations of free DNA and DNA bound by BldD are marked by arrows.

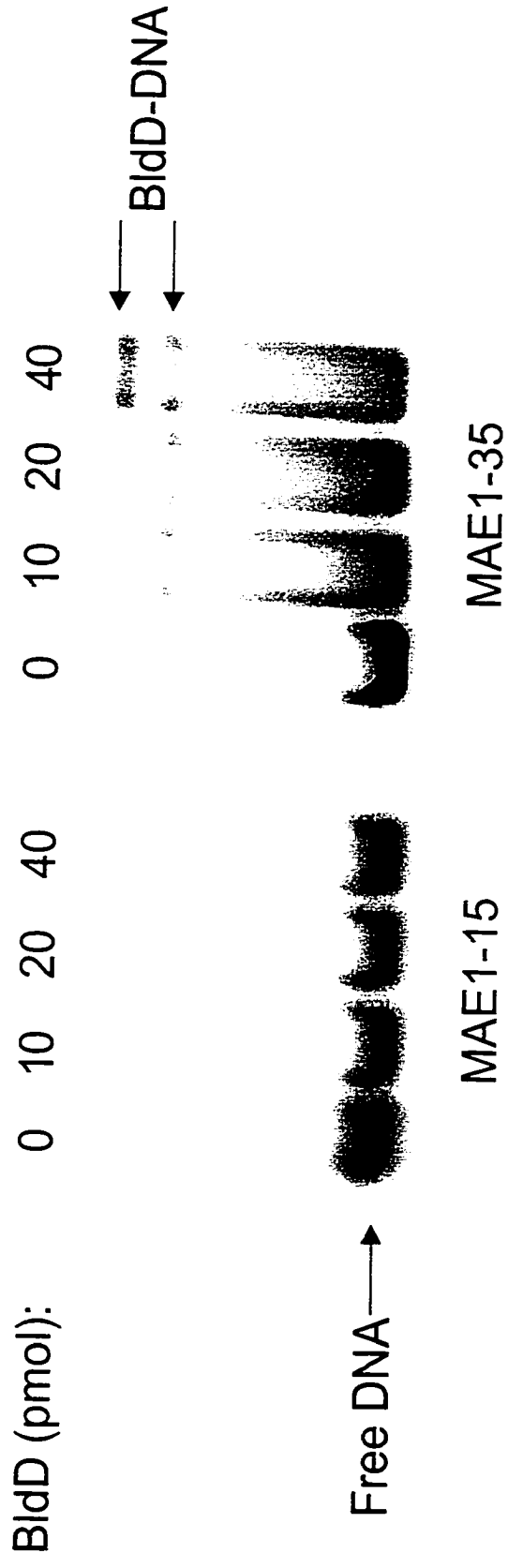
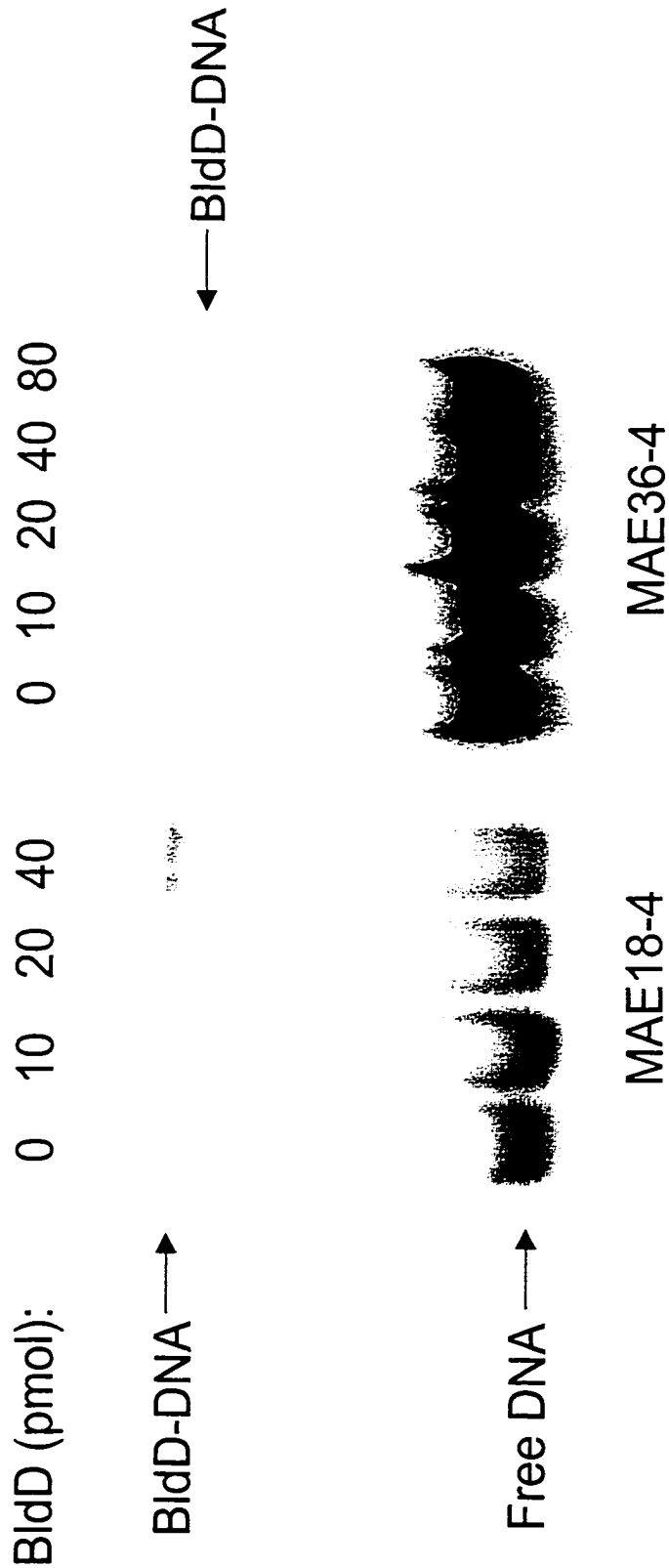


Fig. 3.5.7 Electrophoretic mobility shift assay of BldD binding to MAE18-4 or MAE36-4. One to two nanograms (25-50 fmol of MAE18-4 or 31-62 fmol of MAE36-4) of the ^{32}P end-labeled, 60 bp probe MAE18-4, or the 49 bp probe MAE36-4, was incubated with 0-40 pmol of purified His_6-BldD, as indicated, in a 20 μL reaction volume for 20 minutes at 30°C. Free DNA and BldD-bound DNA (indicated by arrows) were separated by electrophoresis on a 1 × TBE buffered, 1.5% glycerol-containing 8% polyacrylamide gel.



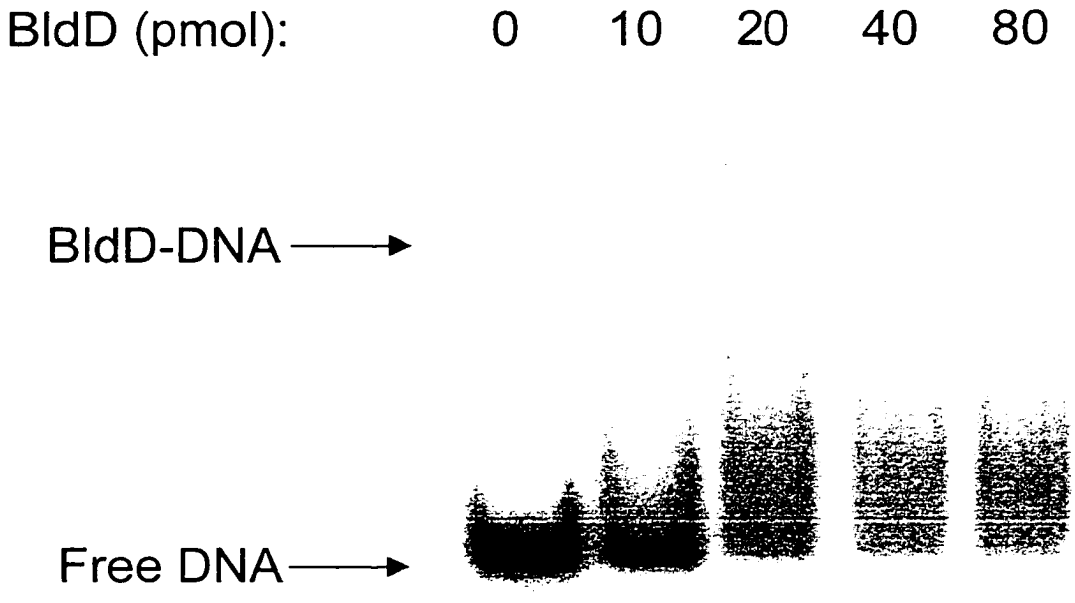
It could therefore be concluded that BldD binds specifically to its own promoter. BldD binding was observed when using fragments (see Fig. 3.5.1) MAE11-12, MAE1-4, MAE16-4, MAE1-35, and to a lesser extent, MAE18-4, as probe. No binding was seen for MAE1-15, and very little binding was observed when using MAE36-4 as probe. It was found that binding of BldD to the *bldD* promoter required bases from –12 to –3, relative to the transcription start site. Binding by BldD at such a position would support a role for the direct repression of its own transcription.

The ability of BldD to bind and shift the various promoter fragments in the mobility shift assays appeared to be concentration dependent, where little binding was seen prior to the addition of 10 pmol of protein. Maximal binding occurred upon the addition of 40 pmol of BldD (0.38 μg), and the addition of more protein, up to 80 pmol, did not result in any increase in fragment retardation (as seen in Fig. 3.5.3). The range of protein concentrations used for the binding assays allowed for the determination of a dissociation constant of approximately 400 nM. There also appeared to be positive cooperativity involved in BldD binding, suggested by comparing the amount of BldD required to bind 10% of the labeled probe, versus the amount needed to bind 90%. Maximal binding of BldD (90%) was attained in less than the 1.81 log units of protein concentration needed in non-cooperative binding (Carey, 1991), with maximal BldD binding to its promoter region attained using 40 pmol of protein (corresponding to a change in protein concentration of 1.60 log units). This suggested that oligomerization may be required for BldD binding to occur, with the binding of the initial BldD protein increasing the affinity with which additional protein can bind.

3.5.2 DNaseI footprinting of the *bldD* promoter

Since the electrophoretic mobility shift experiments had suggested that BldD bound DNA within the *bldD* promoter region, it was of interest to more precisely localize the site(s) of BldD interaction with its promoter, through footprinting experiments. DNaseI footprinting was conducted using the 257 bp *bldD* promoter fragment (MAE11-12) that had been specifically end-labeled on either its template or nontemplate strand, and was repeated at least three times (representative results are shown). Since DNaseI requires divalent cations for its activity, the binding buffer used for the mobility shift experiments had to be modified to include 5 mM $MgCl_2$ and 2.5 mM $CaCl_2$. The salt concentration was also altered from 150 mM NaCl to 50 mM KCl, as all attempts to footprint the promoter fragment using the 150 mM NaCl-containing buffer failed. It is not known why the NaCl buffer negatively impacted the appearance of BldD footprints, although it is conceivable that the NaCl itself adversely affected the DNaseI. The ability of BldD to bind its target DNA in a buffer containing 150 mM NaCl was compared with that in a 50 mM KCl-containing buffer, using electrophoretic mobility shift assays. It was found that BldD dissociated from its target sequence much more readily in the 50 mM KCl buffer, than in the 150 mM NaCl-containing buffer, with a smear, rather than a shifted band visible (Fig. 3.5.8). To account for this, a greater range in protein concentration was used for the footprinting experiments than had been used for most of the electrophoretic mobility shift assays: 0, 20, 40, 60 and 80 pmol of BldD were added to the footprinting reactions, while typically, a maximum of 40 pmol was used for the mobility shift experiments. The products resulting from the DNaseI cleavage were electrophoresed on a 6% sequencing gel, and were visualized using a phosphorimager

Fig. 3.5.8 Electrophoretic mobility shift of BldD binding to the 100 bp MAE16-4 probe in a KCl-containing binding buffer. One to two nanograms (15-30 fmol) of ^{32}P end-labeled DNA was incubated in a 20 μL reaction volume with different amounts of BldD (0-80 pmol, as indicated), at 30°C for 20 minutes. The binding buffer contained 50 mM KCl, rather than the150 mM NaCl that had been used for all other BldD binding reactions. Each reaction was loaded onto a continuously running, nondenaturing 6% polyacrylamide gel, and electrophoresed in 1 × TBE. The locations of free DNA and BldD complexed to DNA are marked with arrows.

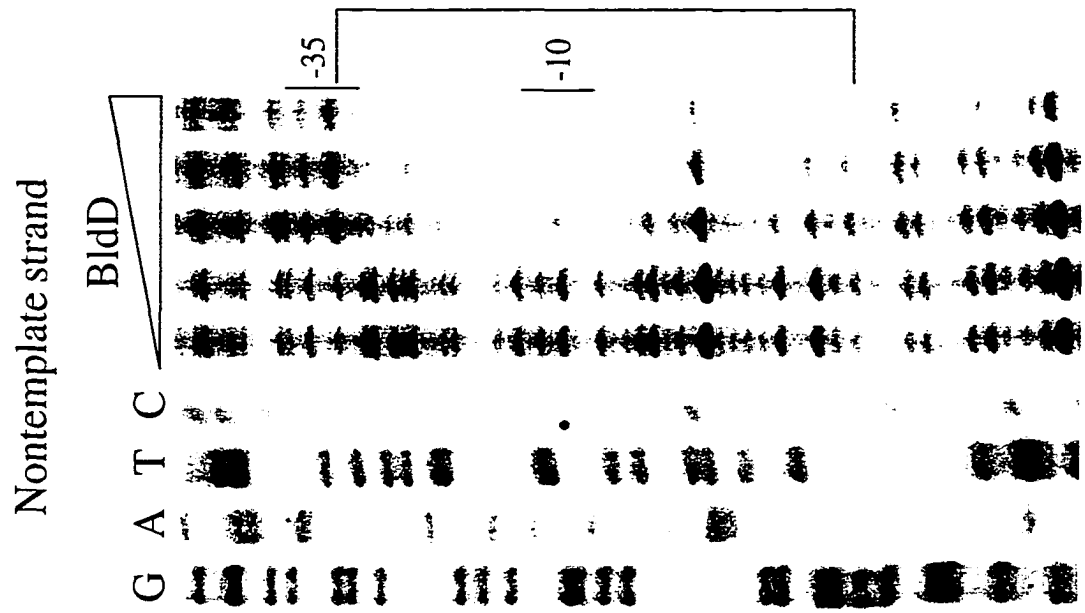


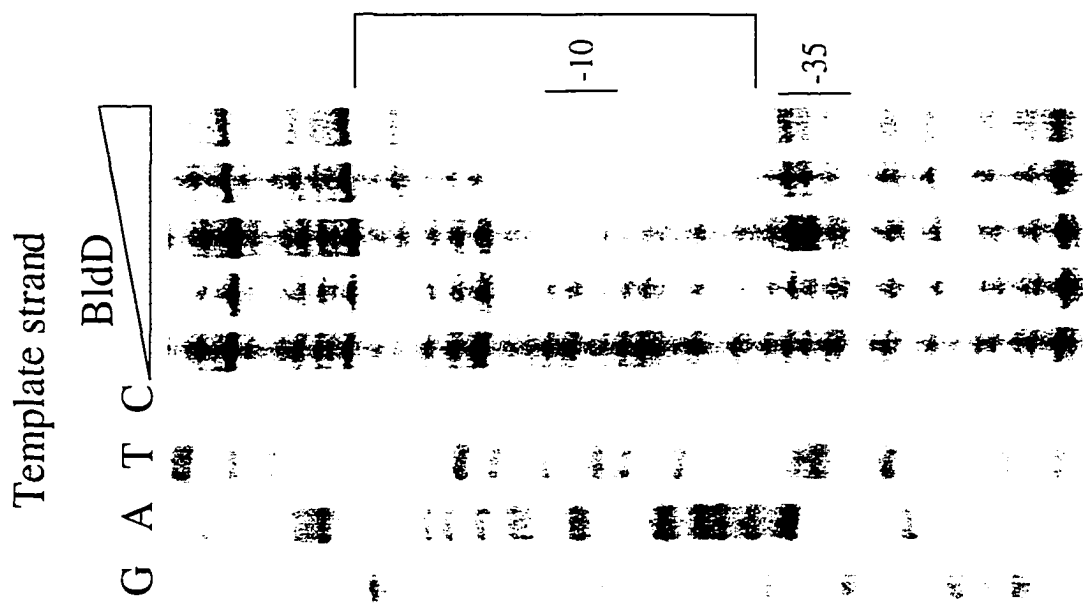
(Molecular Dynamics Model 4551 SI) and Imagequant™ software. The protected bases were compared with a sequencing ladder run simultaneously with the footprinting reactions, generated using the MAE11-12 DNA fragment as template, together with MAE11 and MAE12 as oligonucleotide primers for the template strand and nontemplate strands, respectively. In the presence of BldD, a single area spanning a 43 base segment of the DNA was found to be protected from DNaseI cleavage, with the protected region on the lower strand (-29 to +13) being offset by 2-3 bases from the protected region on the upper strand (-27 to +16) (Fig. 3.5.9). It is of note that the area of protection encompassed the –10 region of the *bldD* promoter, as well as the transcription start site.

3.5.3 Hydroxyl radical footprinting of the *bldD* promoter

Since DNaseI constitutes a large molecule probe, and DNaseI footprinting typically generates a footprint larger than the size of the binding protein, a finer analysis of the BldD binding site was undertaken using hydroxyl radical footprinting, where the hydroxyl radical can provide information to single base resolution. Attack of the deoxyribose sugars of the DNA backbone by the hydroxyl radical is sequence independent, and occurs in the minor groove of the DNA helix, allowing for identification of individual bases that are protected from cleavage and thus indicating bases important, in this case, for BldD binding. The template and nontemplate strands of the 100 bp promoter fragment (MAE1-4) were independently end-labeled, and were incubated in binding buffer, both alone and with 80 pmol of BldD, in the absence of glycerol. Glycerol has been shown to inhibit the cleavage attack by the hydroxyl radical. Electrophoretic mobility shift assays revealed that BldD binding was somewhat

Fig. 3.5.9 DNaseI footprinting of the *bldD* promoter region with His_6-BldD. One to two nanograms (6-12 fmol) of the 257 bp probe, MAE11-12, labeled on either its template or nontemplate strand, were incubated with increasing amounts of BldD (0, 20, 40, 60, and 80 pmol), in binding buffer with 50 mM KCl, 5 mM $MgCl_2$ and 2.5 mM $CaCl_2$. The 20 µL reactions were left at 30°C for 20 minutes, before 0.05 U of DNaseI was added and allowed to react for 10 seconds. The reaction products were separated on a 6% sequencing gel. Areas of BldD protection are shown with brackets, and the –10 and –35 promoter elements are indicated. The sequencing reactions (G A T C) were generated using either MAE11 or MAE12 as primers, for the template and nontemplate strands, respectively.

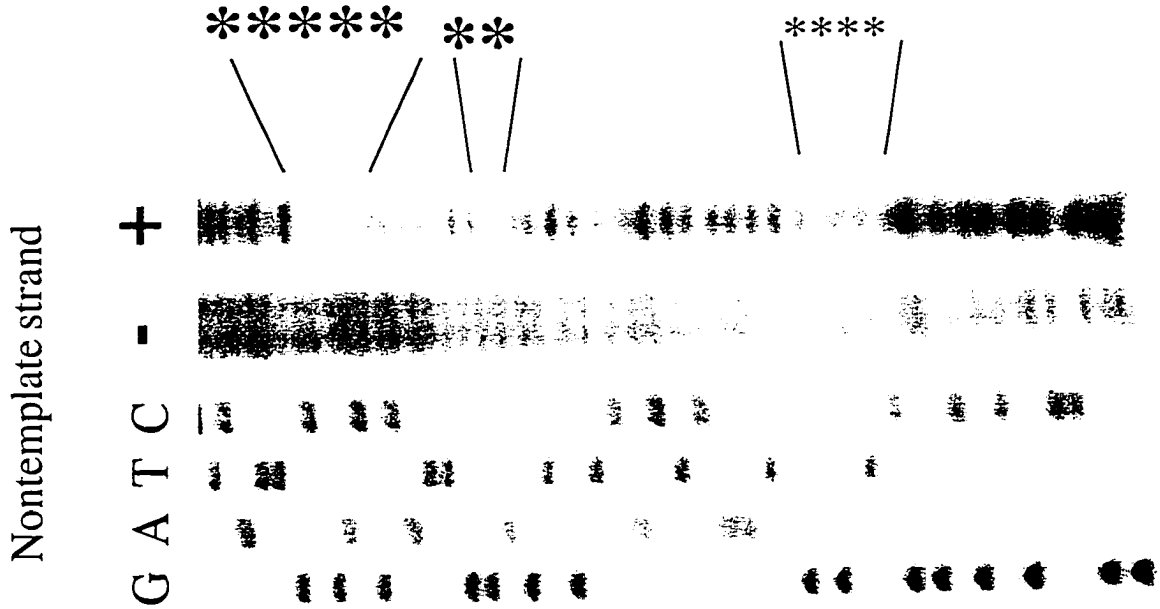


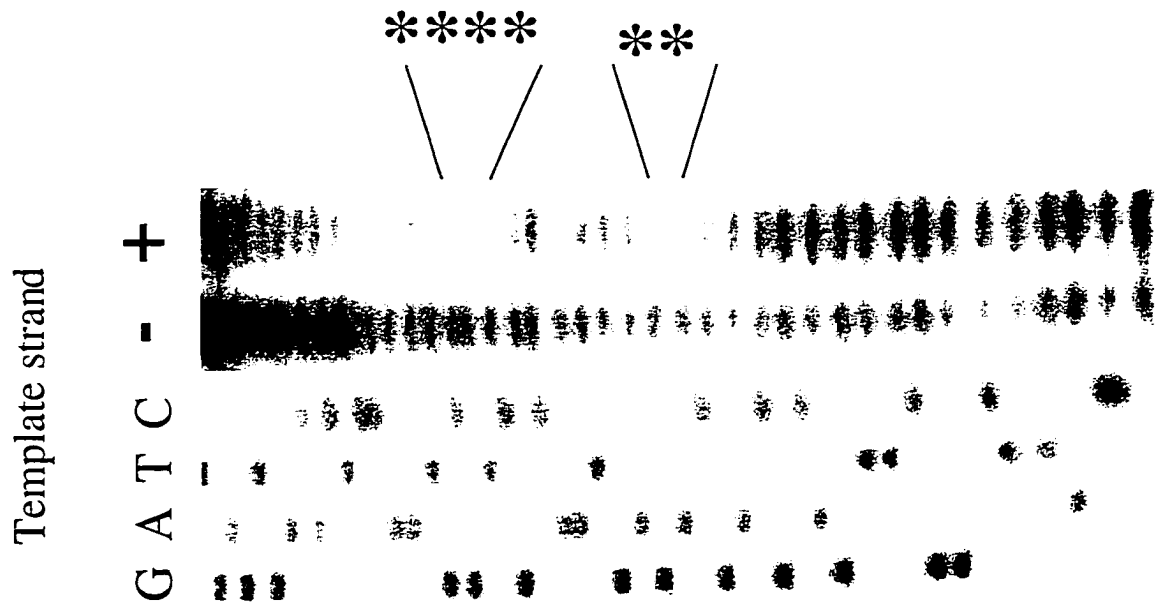


diminished in the absence of glycerol, so, as for the DNaseI footprinting experiments, 80 pmol of BldD was determined to be the minimal protein level that would result in sufficient binding to generate a footprint. After treatment of the promoter fragments with the hydroxyl radical ion, in the presence and absence of BldD, the products were separated on an 8% polyacrylamide gel. This served to separate any unbound probe from that bound by BldD. Excision and purification of only the bound DNA helped to reduce the background that would result from the cleavage of unbound probe. The protein-free DNA was also electrophoresed on the 8% polyacrylamide gel, and excised and purified, as for the bound DNA, to ensure equivalent treatment for the reactions with and without BldD. As DNA is invariably lost during the purification process, 5-12 ng (75-180 fmol) of DNA was used in the binding reactions, compared with the 1-2 ng (15-30 fmol) than had been used for previous binding assays, to ensure that a signal was detectable after separation on a 6% sequencing gel. The procedure was repeated at least three times, with representative results shown here (Fig. 3.5.10). To determine the bases protected by BldD, sequencing ladders, generated using MAE1-4 as template, and MAE1, and MAE4 as the oligonucleotide primers for the template and nontemplate strands, respectively, were run concomitantly with the footprinting products. BldD was determined to afford strong protection to template strand bases at positions –14 to –11, and –23 to –24. On the lower, nontemplate strand, BldD was seen to confer strong protection at positions: -18 to –14 and –5 to –4, and much weaker protection from +9 to +12 (Fig. 3.5.10)

Taken together, the DNaseI and hydroxyl radical footprinting results seemed to suggest a site for BldD binding centered just upstream from the –10 region of the promoter. Examination of the sequences flanking this site revealed the presence of both

Fig. 3.5.10 Hydroxyl radical footprinting of the *bldD* promoter with His_6-BldD. The 100 bp probe MAE1-4, was end-labeled on either its template or nontemplate strand, and 5-12 ng (75-180fmol) of this labeled probe was incubated with (+) or without (-) BldD (80 pmol), in the absence of glycerol, for 20 minutes at 30°C. The 20 μL reactions were then treated with a mixture of 50 μM $Fe(EDTA)^{2-}$, 1 mM Na-ascorbate, and 0.03% H_2O_2, for 2 minutes, after which the products were loaded onto an 8% nondenaturing, 1 × TBE-buffered polyacrylamide gel. Free DNA and BldD bound DNA were eluted from the gel, and the cleavage products were resolved on a 6% sequencing gel. Bold asterisks (*) indicate strong protection by BldD, while lighter asterisks (*) show sites of weaker protection. The sequencing reactions (G A T C) for the template and nontemplate strands were generated using the oligonucleotide primers MAE1 and MAE4, respectively.





an imperfect inverted repeat and an imperfect direct repeat, either of which may represent the site recognized and bound by BldD (Fig. 3.5.11).

3.5.4 Electrophoretic mobility shift of the mutated *bldD* promoter region

An attempt was made to differentiate between BldD binding to the inverted repeat, or the direct repeat, using mutagenic oligonucleotides, since both of the repeat types have a single mismatch. Mobility shift probes with either a perfect direct repeat, or a perfect indirect repeat, were generated. MAE47 and MAE4 (Table 2.4), were used to create a 70 bp fragment (extending from –33 to +37, relative to the transcription start point) that contained a perfect indirect repeat, while MAE46 and MAE4 (Table 2.4) were used in the amplification of a 70 bp fragment, that had a perfect direct repeat (Fig. 3.5.12 A.). Mobility shift assays revealed that both fragments were bound well by BldD (Fig. 3.5.12 B.), with the product profile in each case bearing strong similarity to that obtained when using MAE16-4 as the binding target (compare with 3.5.4); although, MAE47-4 may be bound slightly better than MAE46-6, judging from the percentage of free DNA remaining after the addition of 40 pmol of BldD. From these results it was appeared that BldD preferentially bound the inverted repeat rather than the direct repeat.

Fig. 3.5.11 Summary of DNaseI protection, hydroxyl radical protection, and potential BldD recognition sequences. The *bldD* promoter region is shown, with the –10 and –35 elements, and the transcription start site (tsp) indicated. DNaseI footprints are shown with brackets, while sites of BldD protection from the hydroxyl radical are shown as asterisks, with bold asterisks (*) illustrating stronger protection, and lighter asterisks (*) representing less-well protected areas. Potential BldD binding sites are indicated above the sequence figure, with the top set of arrows illustrating a direct repeat, and the bottom set, indicating an inverted repeat. Downstream of the transcription start point is a sequence that corresponds to half of the inverted repeat.

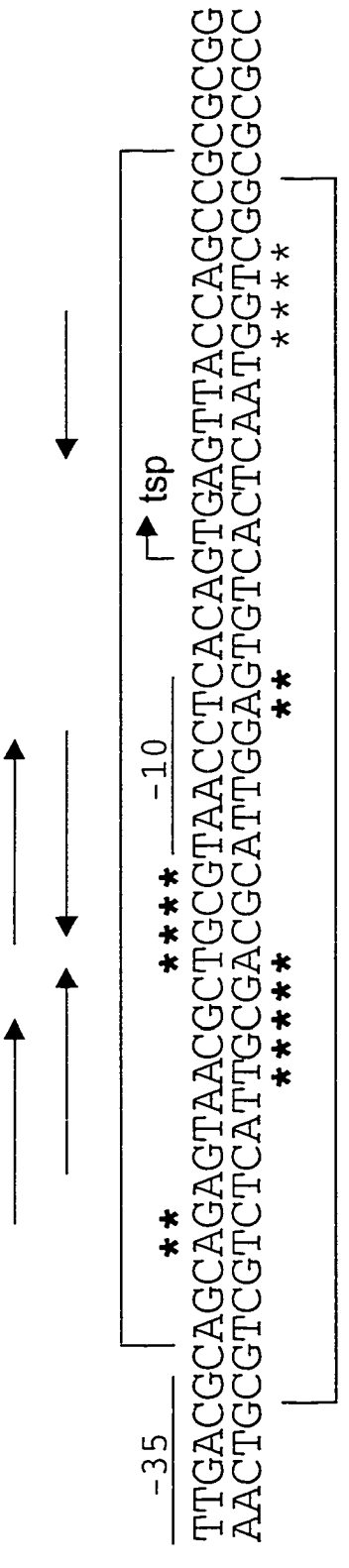
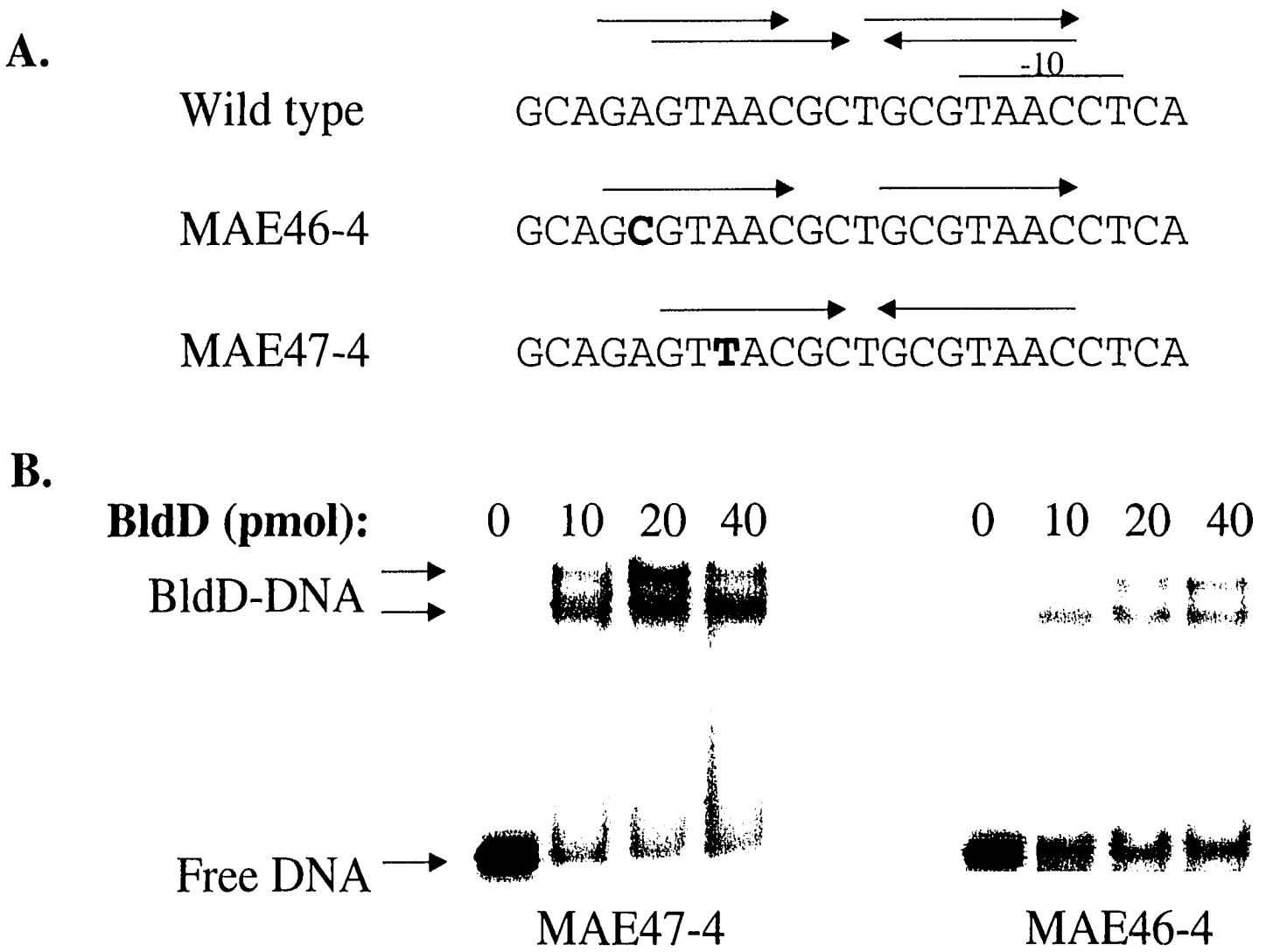


Fig. 3.5.12 Electrophoretic mobility shift of BldD binding to mutant *bldD* promoter region fragments. One to two nanograms (20-40 fmol) of end-labeled MAE47-4, or MAE46-4 were incubated with 0-40 pmol of His_6-BldD in a total volume of 20 μL, for 20 minutes at 30°C. The sequence of the two probe fragments had been altered, relative to the wild type (changed bases are indicated in bold), to generate a perfect indirect repeat for MAE47-4, or a perfect direct repeat for MAE46-4 (**A**). The reaction products were loaded onto a continuously running, 1.5% glycerol-containing, nondenaturing 8% polyacrylamide gel, and were electrophoresed in 1 × TBE. Free DNA and BldD-DNA complexes are indicated with arrows in (**B**).

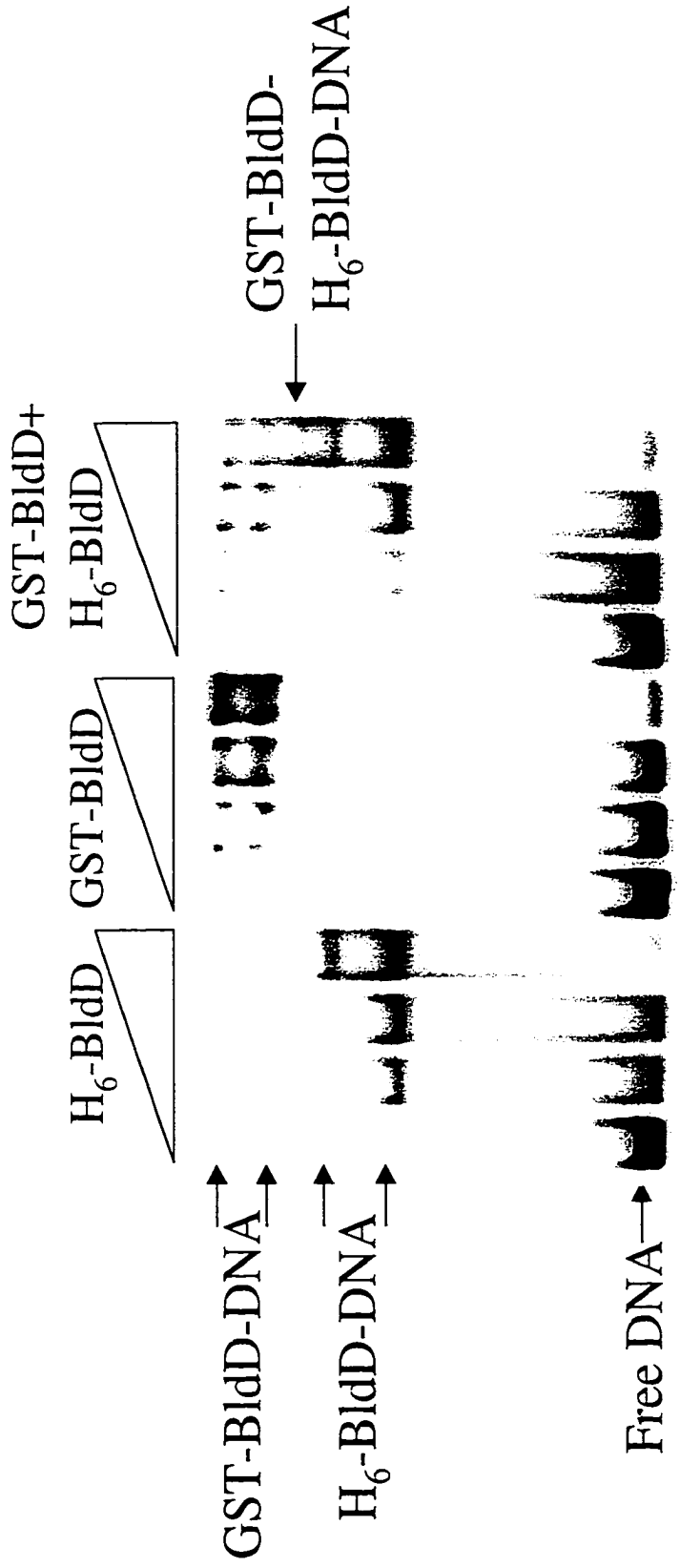


3.6 BldD oligomerization studies when bound to DNA

3.6.1 Oligomeric state of BldD when bound to DNA as determined using electrophoretic mobility shift assays

As BldD appears to bind to a repeated unit, and the electrophoretic mobility shift assays (see section 3.5.1) suggested that binding by BldD may require some form of positive cooperativity, studies were undertaken to determine the oligomeric state of BldD when bound to DNA (Hope and Struhl, 1987). Electrophoretic mobility shift assays were conducted using end-labeled MAE16-4 (section 3.5.1) and His_6-BldD, GST-BldD, or a mixture of His_6-BldD and GST-BldD. Based on the observation that BldD could exist in one of three oligomeric forms in solution (see Fig. 3.4.2), and that there appeared to be some equilibration between the three forms, it was conceivable that the GST-tagged BldD could associate with the His-tagged BldD to form a heterodimeric complex, or even a tetrameric complex, made up of any combination of the two protein forms. By comparing the results obtained from the mobility shift assays using His_6-BldD and GST-BldD, with those generated using the protein mixture, it would be possible to differentiate between monomer, dimer and tetramer binding. Zero to forty picomoles of BldD were added to each reaction tube, along with 1-2 ng (20-40 fmol) of end-labeled MAE16-4 probe, and after incubation at 30°C, the DNA and protein products were separated on an 8% polyacrylamide, 1.5% glycerol-containing gel. Two shifted bands were observed both in the presence of His_6-BldD and GST-BldD, as was expected given the results obtained in section 3.5.1; however, the protein-bound DNA migrated to different positions depending on the protein added, as the GST-tagged BldD was significantly larger than the His-tagged BldD (Fig. 3.6.1). When equal amounts of the two fusion

Fig. 3.6.1 Oligomeric state of BldD when bound to DNA, as determined by electrophoretic mobility shift. The ability of BldD to bind to 1-2 ng (15-30 fmol) of end-labeled MAE1-4 was compared using 0, 10, 20 and 40 pmol of His_6-tagged BldD, GST-tagged BldD, or an equal mixture of the two. The twenty microlitre reactions were incubated for 20 minutes at 30°C, before being separated on a continuously running 8% nondenaturing polyacrylamide gel containing 1.5% glycerol, buffered by 1 × TBE. Free DNA and DNA complexed with His_6-BldD, GST-BldD and GST-BldD-His_6-BldD are indicated with arrows.



proteins were mixed together and incubated with the end-labeled probe, a number of different products were observed: two large shifted fragments were seen, corresponding to the GST-BldD-bound DNA; two smaller shifted fragments were seen, corresponding to the His_6-BldD; and an intermediate fragment was observed. The intermediate fragment most likely represented binding by a GST-His_6-BldD dimer, as there would be no intermediate fragment if BldD bound as a monomer, and more than one intermediate fragment would be expected if BldD bound as a tetramer (for example, intermediates would include $[His_6][GST]_3$; $[His_6]_2[GST]_2$; $[His_6]_3[GST]$). This confirmed that BldD bound its own promoter as a dimer.

3.7 BldD targets

While extensive study of BldD binding to the *bldD* promoter had revealed much about BldD in terms of its DNA binding abilities, including the sequence it recognized, the location of binding, and the oligomeric state of the binding protein; the ultimate goal of this project was to determine how BldD exerted its regulatory effects. It appears to act as a repressor of its own expression, however, this could not represent the sum total of BldD effects in the cell, since aerial hyphae formation and antibiotic production are also defective in the *bldD* mutant. The following section outlines the search for additional BldD targets.

3.7.1 Sequence scanning for targets of BldD

The first approach to the identification of other BldD targets involved scanning of the *S. coelicolor* genome for the putative BldD recognition site identified in Section 3.5.

The sequence search focused on the promoter regions of genes known to be involved in differentiation, including other *bld* genes, *whi* genes and antibiotic regulatory genes. Sequences sharing similarity with the proposed BldD binding motif were discovered in the promoter region of *whiG,* which encodes a sigma factor required after aerial hyphae formation for the development of mature spores (Chater *et al.*, 1989), and also upstream from *ftsZ,* which specifies a tubulin-like protein required for cell division (Fig. 3.7.1). In *Streptomyces*, *ftsZ* is not required during vegetative growth, as only occasional crosswalls are laid down in the substrate mycelia, but is essential for the subdivision of the aerial hyphae into the unigenomic spore compartments (McCormick *et al.*, 1994).

3.7.2 Analysis of BldD-*whiG* interactions

3.7.2a Electrophoretic mobility shift assay for BldD binding to the *whiG* promoter region

To determine whether the sequence similarity between the BldD binding site within the *bldD* promoter region and the sequence within the *whiG* promoter region, was significant, electrophoretic mobility shift assays were conducted using a 354 bp probe (MAE44-45; Table 2.4) that spanned the *whiG* promoter region, extending from +210 to −144, relative to the transcription start site. His-tagged BldD (0-40 pmol) was incubated with 1-2 ng (4-8 fmol) of this end-labeled probe in the presence of excess, nonspecific competitor DNA (poly dI/dC), and the free DNA was separated from any resulting BldD-DNA complexes, on an 8% polyacrylamide gel. The *whiG* promoter region probe fragment was observed to shift completely upon the addition of 40 pmol of protein (Fig. 3.7.2), indicating that BldD binds strongly to this DNA fragment (K_d ~250

Fig. 3.7.1 Alignment of the proposed BldD binding site with similar sequences in the upstream regions of *whiG* and *ftsZ*. The imperfect inverted repeat in each sequence is indicated with arrows. The numbers to the right of the sequences show the position of each sequence relative to the transcription start site (+1) for each gene (for *ftsZ*, the distance is to the sporulation transcription start site).

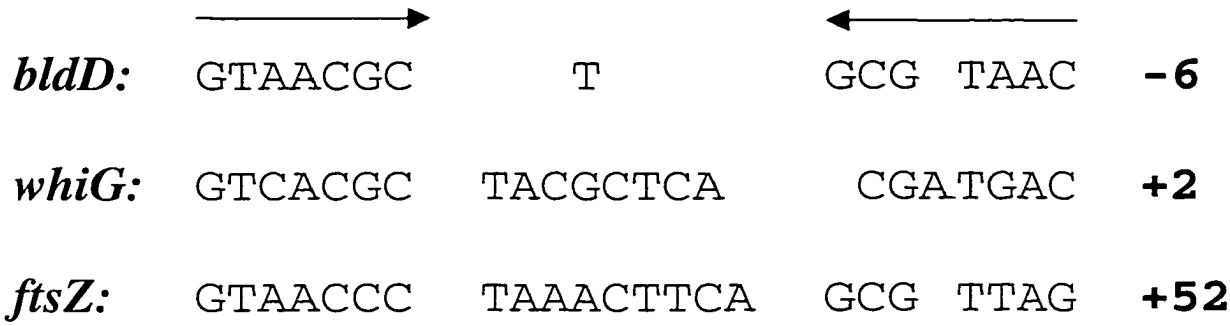


Fig. 3.7.2 Electrophoretic mobility shift assay of BldD binding to a 354 bp region of the *whiG* promoter. One to two nanograms (4-8 fmol) of ^{32}P end-labeled probe (MAE44-45; Table 2.4) were incubated with increasing concentrations of BldD (0-40 pmol, as indicated). The twenty microlitre reaction volumes were incubated at 30°C for 20 minutes, before separation of the free DNA from the BldD-complexed DNA (indicated with arrows). BldD binding specificity was demonstrated by the addition of ~500 ng/6 pmol of unlabeled, nonspecific competitor DNA (BKL41-MAE5; Table 2.4) to the reactions loaded into the fifth reaction lane, and ~500 ng/2pmol of unlabeled probe (specific competitor; MAE44-45) to the reactions separated in the final reaction lane.

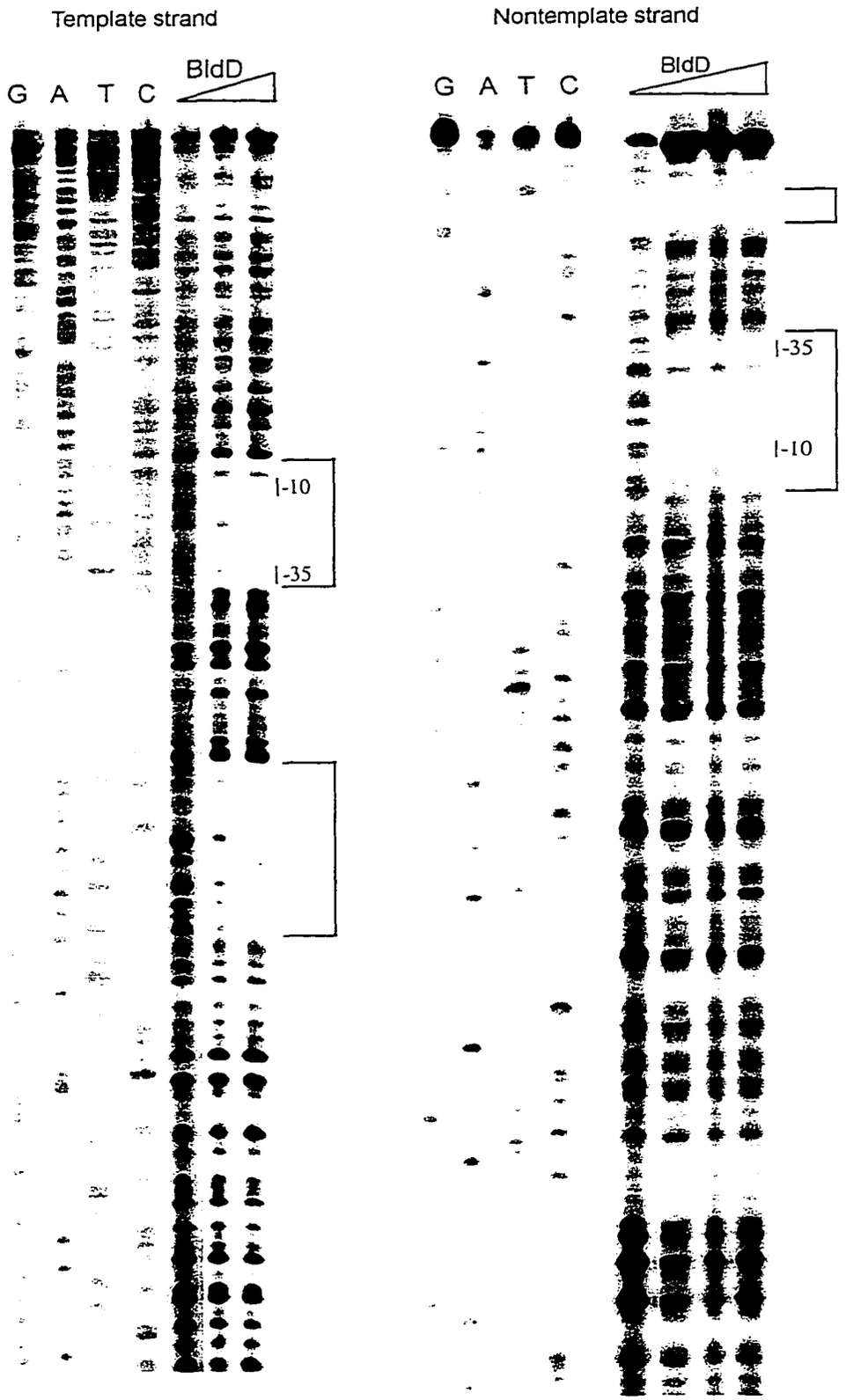
nM) (this experiment was performed at least three times, with representative results shown).

Specificity of binding was evaluated using competition mobility shift assays with both specific and nonspecific BldD binding targets. The addition of 500 fold excess nonspecific DNA (BKL41-MAE5; a 118 bp fragment, internal to the *bldD* coding sequence) to a reaction tube containing 40 pmol of BldD, along with the 1-2 ng (4-8 fmol) of labeled probe typically used in mobility shift assays, had no influence on the ability of BldD to bind to the labeled probe; however, the addition of 500 fold excess unlabeled probe (specific target) served to abolish BldD binding to the labeled fragment (Fig. 3.7.2).

3.7.2b DNaseI footprinting for *whiG* promoter

Binding of BldD to the *whiG* promoter was then examined in greater detail, using DNaseI footprinting assays. The same 354 bp fragment used for the mobility shift assays was end-labeled on the nontemplate strand, while a slightly longer 478 bp fragment was end-labeled on the template strand, and both were incubated with 0, 40 or 80 pmol of BldD before exposure to 0.025 U of DNaseI. The resulting cleavage ladders were separated on a 6% sequencing gel, and it was found that, in the presence of BldD, two different sites were protected on both strands. This experiment was repeated at least three times, with representative results illustrated in Fig. 3.7.3. The first site encompassed the known –10 and –35 promoter elements, stretching from +13 to –45, relative to the transcription start site, while the second site was found significantly upstream, between nucleotides –132 and –92. The sites of protection were determined by

Fig. 3.7.3 DNaseI footprinting of the template strand and nontemplate strand of the *whiG* probe. The 354 bp (MAE44-45) probe DNA was end-labeled specifically on the nontemplate strand, while the 478 bp (MAE76-45) probe DNA was end-labeled specifically on the template strand. One to two nanograms (4-8 fmol for MAE44-45 and 3-6 fmol for MAE76-45) of each probe was incubated with BldD (0 pmol, 40 pmol and 80 pmol, respectively) in binding buffer modified to contain 50 mM KCl, 5 mM $MgCl_2$, and 2.5 mM $CaCl_2$ for 20 minutes at 30°C. DNaseI (0.025U) was added to each reaction tube, and was allowed to react for 10 seconds. The resulting cleavage products were separated on a 6% sequencing gel. Areas protected by BldD are bracketed, and the –10 and –35 promoter elements are indicated. The sequencing ladders, as indicated by G A T C, were generated using the oligonucleotide primers MAE76 or MAE 45, for examination of the template or nontemplate strands, respectively.



comparison to a sequencing ladder, generated using MAE44-45 as template, with MAE45 as the oligonucleotide primer for the nontemplate strand and MAE76-45 as template with MAE76 as the oligonucleotide primer for the template strand.

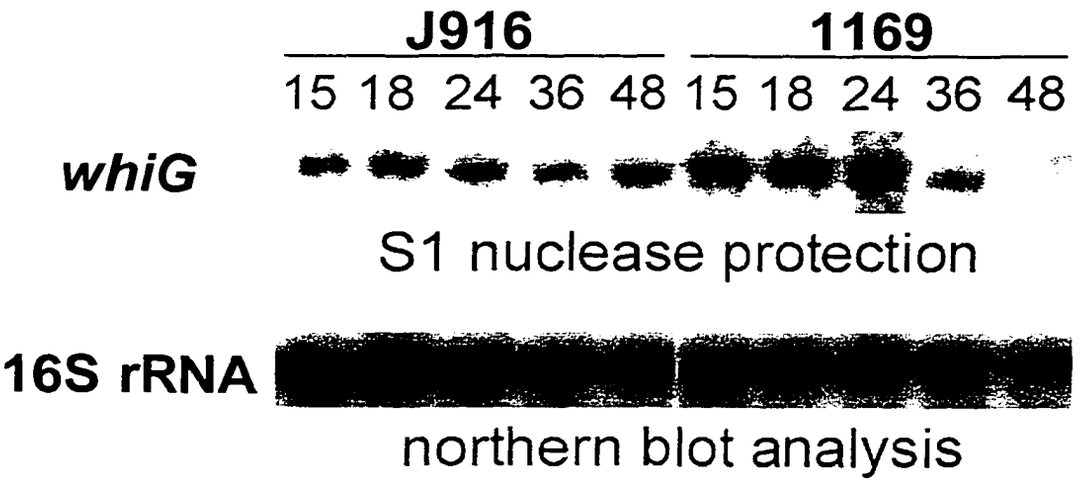
3.7.2c S1 nuclease mapping of *whiG* in wild type and *bldD* mutant strains of *S. coelicolor*

While BldD appeared to be capable of specifically binding to the *whiG* promoter under *in vitro* conditions, it was not known what effect, if any, this would have on the *in vivo* expression levels of *whiG*. To assess this, S1 nuclease mapping studies were undertaken, using RNA isolated from both wild type *S. coelicolor* strain J916 and its congenic *bldD* mutant strain, 1169, at various timepoints. Initially, the S1 mapping studies were conducted using RNA isolated from *S. coelicolor* strains J1501 and HU66; however, no discernable difference was seen in *whiG* expression when comparing the wild type and *bldD* mutant strains. J1501 and HU66 differ only in plasmid content, but do grow differently, as HU66 grows less vigorously than J1501. In the event that either of these factors would hinder the detection of a difference in *whiG* expression between the two strains, RNA was isolated from a truly congenic pair, J916 and its *bldD* mutant derivative, 1169. In solid culture experiments on R2YE agar, *S. coelicolor* J916 grew in an identical manner to J1501 (described in Section 3.1.6); however, *S. coelicolor* 1169 grew much better than HU66, with reasonable growth observed at 15 hours, and red pigment production evident by 24 hours. Forty microgram samples of RNA were mixed with a single end-labeled 317 bp *whiG*-specific probe (generated by digestion of the 354 bp mobility shift fragment with *Bcl*I after end-labeling), and this nucleic acid mixture was

subjected to denaturation for 30 minutes at 80°C, before being cooled to 58°C for 3-4 hours. After treatment with S1 nuclease, the remaining labeled DNA fragments were separated on a 6% sequencing gel. It was found that in J916, the *whiG* transcript was present at relatively constant levels in all samples examined, as had been observed previously in studies using wild type *S. coelicolor* A3(2) (Kelemen *et al.*, 1996) (Fig. 3.7.4). Unlike J916, constant expression was not observed in the *bldD* mutant strain, 1169. Up until the timepoint corresponding to the formation of aerial hyphae in J916 (i.e. from 15-24 hours post-inoculation), strong *whiG* expression was seen in strain 1169 at a ~2-3 fold higher concentration than in J916. However, by the 36 and 48 hour timepoints, when aerial hyphae were abundant and spore formation had begun in the wild type strain, the level of *whiG* transcripts had dropped off significantly in the *bldD* mutant, to barely detectable levels in the 48 hour sample. To verify these results, the S1 nuclease protection experiments were repeated, using RNA isolated from a separate time course, and the results were identical to those seen in Fig. 3.7.4.

To confirm that the observed pattern of *whiG* expression seen for both mutant and wild type strains was not simply due to differing RNA amounts present for each sample, relative RNA abundance was compared for each timepoint, using northern blot analysis. Ten micrograms of RNA from the same RNA preparations used for the S1 nuclease protection studies, were hybridized with a labeled oligonucleotide (BKL54) specific for the constitutively expressed 16S rRNA, and revealed that the RNA levels used in each sample aliquot were essentially equivalent (Fig. 3.7.4).

Fig. 3.7.4 S1 nuclease protection of *whiG* transcripts from *S. coelicolor* J1501 and HU66. Forty micrograms of RNA, isolated at various times (indicated as hours post-inoculation) from surface grown cultures of wild type *S. coelicolor* strain J916 and the congenic *bldD* mutant strain, 1169, were annealed to a 317 bp end-labeled probe spanning the *whiG* transcription start site. The RNA-DNA hybrid was treated with S1 nuclease, and the labeled products were separated on a 6% sequencing gel. To demonstrate that equivalent RNA levels were present in all RNA samples used for the S1 nuclease protection experiments, northern blot analysis of the same RNA samples (10 μg), shown below the S1 nuclease protection, was performed using a probe specific for the consitutively expressed 16S rRNA (BKL54).



The finding of a sequence similar to the previously determined BldD binding site in the *whiG* promoter region, the observance of BldD-bound DNA in electrophoretic mobility shift assays using the *whiG* promoter region as a probe, the identification of specifically bound regions of the *whiG* promoter using DNaseI footprinting, and the determination of an *in vivo* effect of BldD binding to *whiG*, all suggest that BldD is involved in regulating *whiG* expression *in vivo*.

3.7.3 Analysis of BldD-*ftsZ* interaction

3.7.3a Electrophoretic mobility shift assays for *ftsZ*

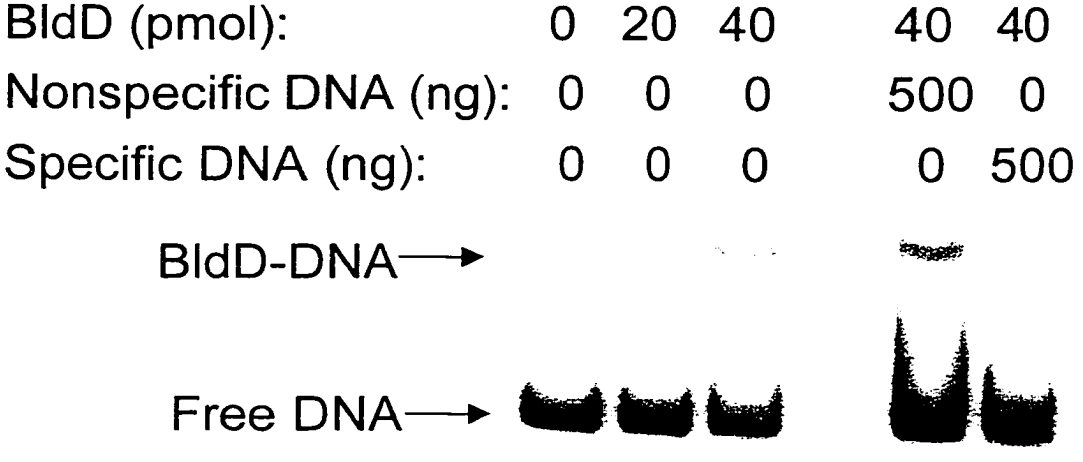
As mentioned above, a sequence bearing some similarity to the predicted BldD binding sequence was identified upstream from the coding sequence of *ftsZ*. Electrophoretic mobility shift assays were initiated, as for *whiG*, to ascertain whether the similarities seen between the BldD binding site at the *bldD* promoter and the sequence upstream from *ftsZ* had any biological significance. The proposed BldD binding site was located upstream from the translation start codon (112 bp upstream), but downstream from the sporulation (28 bp downstream), and vegetative (83 bp downstream) transcription start sites that had been previously identified in *S. coelicolor* (as defined by Flärdh and Chater, 1998). Therefore, a 382 bp probe (MAE 27-26; Table 2.4) was designed with the putative binding site in a central position and the flanking regions encompassing the translation start point and both promoter elements, extending from +43 to –339, relative to the translation start site. Following PCR amplification, the probe was end-labeled and incubated with increasing concentrations of BldD (0-40 pmol) in the presence of excess nonspecific DNA (poly dI/dC). The BldD-bound fragments were then

separated from the free DNA on an 8% polyacrylamide gel (Fig. 3.7.5). Specificity controls were also conducted, whereby ~500 fold excess unlabeled, nonspecific DNA (BKL41-MAE5, a fragment internal to the *bldD* coding sequence) and ~500 fold excess unlabeled, specific competitor DNA (MAE26-27) were added to binding reactions. The nonspecific DNA had no effect on the ability of BldD to bind the *ftsZ* fragment, while the specific competitor virtually abolished all binding. While BldD binding appears to be specific, the proportion of labeled fragment bound did not increase significantly with the addition of more BldD protein beyond 20 pmol, with the majority of labeled DNA remaining unbound. This observation is reproducible, seen on at least 3 separate occasions. This may suggest that the binding of BldD upstream from the *ftsZ* coding sequence may represent strong, nonspecific binding, or may indicate suboptimal binding conditions, perhaps missing a stabilizing cofactor of some sort.

3.7.3b Primer extension analysis of *ftsZ*

In an effort to authenticate whether or not the *in vitro* binding of BldD to the *ftsZ* fragment was specific, primer extension analyses were undertaken to examine whether there was an *in vivo* effect on *ftsZ* expression in the *bldD* mutant strain, Thirty microgram samples of RNA isolated over a 48 hour period, from *S. coelicolor* J1501 and HU66, were incubated with the end-labeled oligonucleotide primer MAE26 (Table 2.4), which was internal to the *ftsZ* coding sequence. Following the extension reactions, the resulting products were separated on a 6% sequencing gel. It appeared as though the transcript originating from the vegetative *ftsZ* promoter, which was present at a relatively constant

Fig. 3.7.5 Electrophoretic mobility shift for BldD bound to the *ftsZ* promoter region. One to two nanograms (4-8 fmol) of MAE27-26, a 382 bp, end-labeled probe spanning the *ftsZ* promoter region, were incubated with 0-40 pmol of His_6-BldD (as indicated) for 20 minutes at 30°C. Specificity of binding was examined by the addition of ~500 ng/2 pmol of unlabeled competitor DNA (MAE27-26) to the reaction loaded into the final lane, and by the addition of ~500 ng/6 pmol of nonspecific competitor DNA (BKL41-MAE5; a 118 bp fragment internal to the *bldD* coding sequence) to the reaction run in the penultimate lane. The twenty microlitre reactions were loaded onto a continuously running 8% polyacrylamide, 1.5% glycerol-containing gel, and were electrophoresed in 1 × TBE. The free DNA and BldD-DNA complexes are indicated.



level throughout colony development in the wild type strain, was unaffected by the *bldD* mutation, with no discernable differences seen when comparing the wild type and *bldD* mutant strains (Fig. 3.7.6). Transcription from the sporulation promoter was temporally regulated in the wild type strain, with slight upregulation seen in the 36 and 48 hour samples, timepoints during colony development when aerial hyphae were abundantly present and spore formation was commencing. In the *bldD* mutant strain, however, transcripts were barely discernable, and no upregulation was seen (Fig. 3.7.6). This was observed at least three times, using RNA from different time courses (representative results are shown).

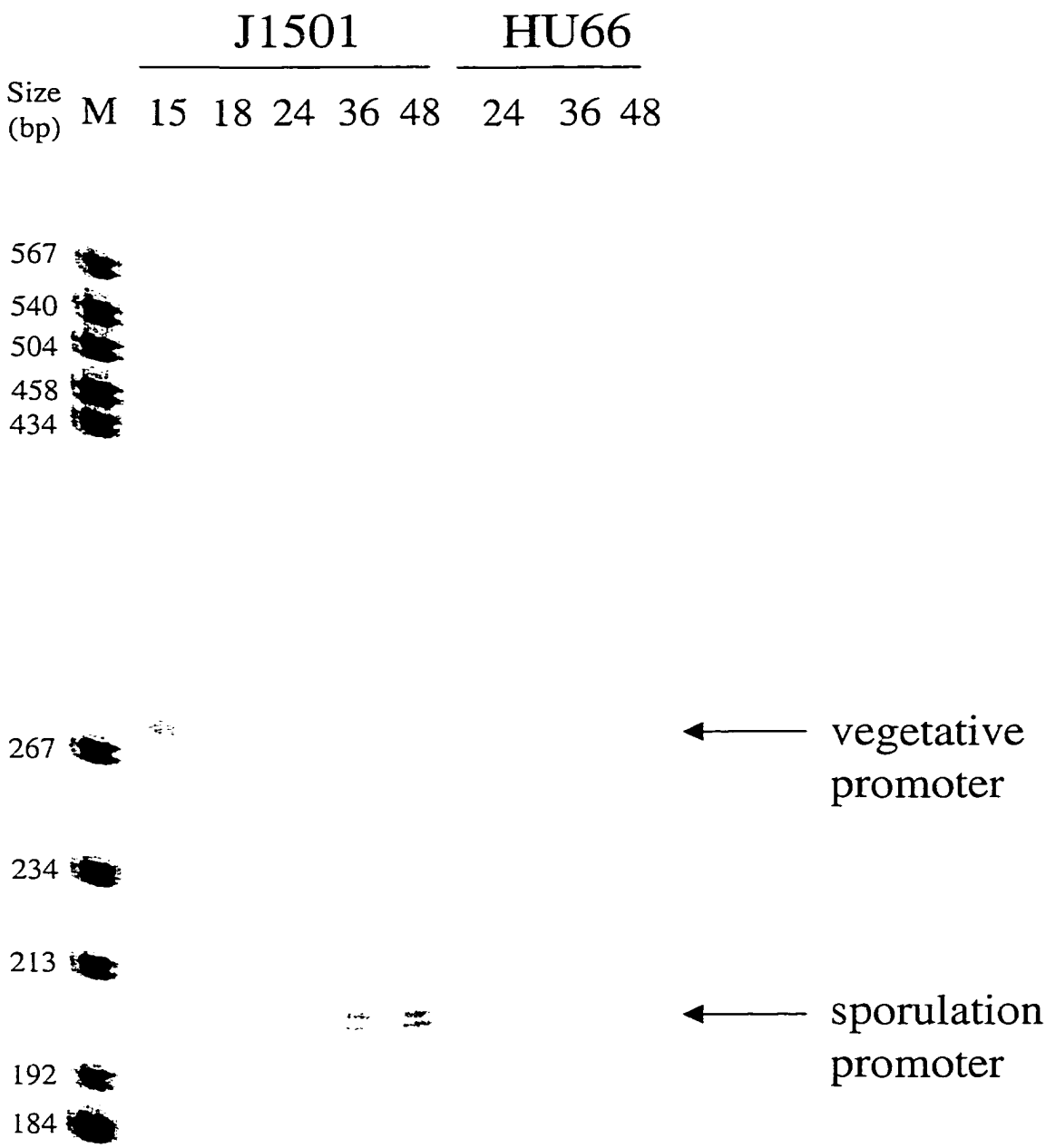
While BldD appears to bind *ftsZ* weakly, as judged by the electrophoretic mobility shift assays, there does appear to be an *in vivo* BldD influence on expression from the sporulation promoter. The conclusion that *ftsZ* is a direct target of BldD cannot be made, however, without considering the influence of other BldD targets on *ftsZ* expression, since it is known that in the *whiG* mutant, the same expression pattern is seen for *ftsZ* as observed in the *bldD* mutant (Flärdh and Chater, 1998). Regardless, it is obvious that the *bldD* mutation affects the *in vivo* expression of *ftsZ*; however, whether this effect is a direct or an indirect one awaits further experimentation.

3.7.4 Dependence of *bldN* expression on BldD

3.7.4a Transcript analysis of *bldN* in *S. coelicolor* J916 and 1169

Another potential *bldD* target was brought to our attention by Dr. Mark Buttner, whose lab had been working to characterize the newly discovered *bldN* gene, which encodes a sigma factor required for aerial hyphae formation. In examining the effects of

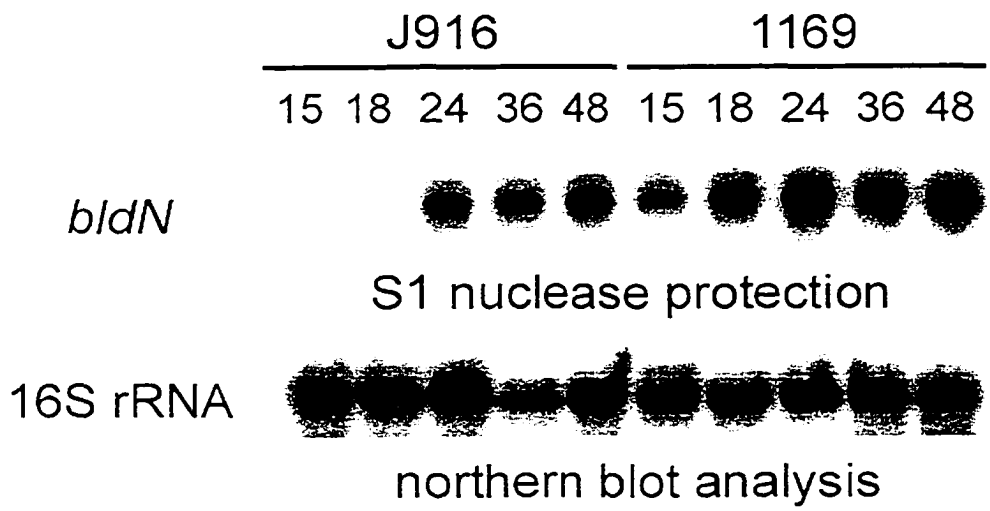
Fig. 3.7.6 Primer extension analysis of *ftsZ* transcripts using RNA (30 μg) isolated from surface grown cultures of *S. coelicolor* J1501 and HU66 (times are indicated as hours post-inoculation). The RNA samples were incubated with the end-labeled oligonucleotide MAE26 (internal to the *ftsZ* coding sequence), extension reactions were performed, and the resulting products were separated on a 6% sequencing gel. Transcripts originating from the vegetative promoter and the sporulation promoter are indicated. End-labeled molecular weight marker V (M; Roche) served as size marker.



different *bld* mutations on the expression of *bldN*, it appeared that BldD influenced *bldN* transcription (M.J. Bibb and M.J. Buttner, personal communication). To validate the Buttner lab results, S1 nuclease protection assays were performed using 40 μg samples of RNA isolated from *S. coelicolor* J916 (wild type) and 1169 (*bldD* mutant) during two different time courses, one of which was used previously for the *whiG* experiments. The RNA samples were annealed at 59°C, to a single end-labeled 181 nt probe, which was generated by PCR amplification of MAE71-68 (Table 2.4), followed by end-labeling and digestion with *Cla*I. It was found, after separation of the S1 nuclease-digested products, that *bldN* expression in the *bldD* mutant strain, 1169, was significantly different from that seen in the J916 wild type strain (Fig. 3.7.7). Transcription of *bldN* in *S. coelicolor* J916 (wild type) was observed to be temporally regulated, with very low levels of transcript present throughout the transition phase of growth (virtually undetectable at 15 hours and low in the 18 hour sample), and increasing levels seen upon the initiation of differentiation at ~24 hours. In the *bldD* mutant, however, abundant transcript was observed at the earliest examinable timepoint (15 hours), before increasing to a maximum at 24 hours, where it remained steady in the samples from the 36 and 48 hour cultures. At all timepoints in the *bldD* mutant strain, *bldN* expression was greater than in the wild type strain, ranging from a >10 fold difference seen in the early 15 and 18 hour samples, to a two fold difference observed at the later times (determined using Imagequant™ software). This confirms the results obtained by Bibb and Buttner, and suggests that BldD controls *bldN* expression, most likely as a repressor.

As a control for RNA loading levels, northern blot analysis was performed using 10 μg samples of RNA from the same stock as used for the S1 nuclease protection

Fig. 3.7.7 S1 nuclease protection assay for *bldN* transcripts using RNA isolated from surface grown cultures of the *S. coelicolor* strains J916 and 1169. The RNA samples were isolated at five different timepoints over a 48 hour period of colony development (indicated as hours post-inoculation). Forty microgram samples of RNA were annealed to the gel purified 181 bp probe, labeled at one end (internal to *bldN*). The probe was generated by PCR amplification of MAE71-68, end-labeling, and digestion with *Cla*I to remove the label from the non-protected end. Treatment with S1 nuclease was followed by separation of the labeled products on a 6% sequencing gel. To ensure that the RNA amounts in each sample were equivalent, northern blot analysis of 10 μg RNA samples, shown below the S1 nuclease protection, was conducted using an end-labeled oligonucleotide probe (BKL54), specific for 16S rRNA.



experiment. The membrane was hybridized with an end-labeled oligonucleotide primer (BKL54) specific for 16S rRNA, and it was found that the amount of RNA present in each sample was relatively equivalent.

3.7.4b Electrophoretic mobility shift assays for *bldN*

In an attempt to ascertain whether the differing expression levels of *bldN* in the wild type and *bldD* mutant *S. coelicolor* strains were the result of a direct effect of BldD binding to the *bldN* promoter, electrophoretic mobility shift assays were conducted using a DNA fragment encompassing the *bldN* promoter region as a target for binding by the purified BldD protein. The *bldN* probe was a 208 bp DNA fragment (MAE71-68; Table 2.4), extending from –83 to +126, relative to the transcription start site. When 1-2 ng (7-14 fmol) of this end-labeled probe were incubated with His_6-BldD (0-40 pmol) in the presence of excess poly d(I-C), separation of the products on an 8% polyacrylamide gel revealed three shifted fragments (Fig. 3.7.8). Almost all of the BldD-complexed DNA was found in the upper band upon the addition of 40 pmol of BldD. The appearance of the two minor bands, may suggest more than one BldD binding site in the *bldN* promoter region, or may simply represent a small proportion of non-specific binding by BldD to this DNA fragment. Identical results were obtained upon repetition of this electrophoretic mobility shift experiment (performed at least three times).

Competition mobility shift assays were also conducted in order to assess the specificity of BldD binding. Five hundred nanograms of nonspecific BldD target DNA (BKL41-MAE5, a 118 bp DNA fragment internal to the *bldD* coding sequence) were

Fig. 3.7.8 Electrophoretic mobility shift assay of BldD binding to a 208 bp DNA fragment spanning the *bldN* promoter region. End-labeled MAE71-68 was incubated in a 20 μL reaction, with increasing amounts of BldD (0-40 pmol, as indicated). After twenty minutes of incubation, the reactions were loaded onto a continuously running 1.5% glycerol-containing, 8% polyacrylamide gel, with 1 × TBE buffer. The separated products, free DNA and BldD-bound DNA, are indicated with arrows. Binding specificity controls included the addition of ~500 ng/6 pmol of unlabeled nonspecific DNA (BKL41-MAE5; internal to the *bldD* coding sequence), shown in the penultimate lane, and the inclusion of ~500 ng/4 pmol of unlabeled specific probe (MAE71-68) in the reaction loaded in the final lane.

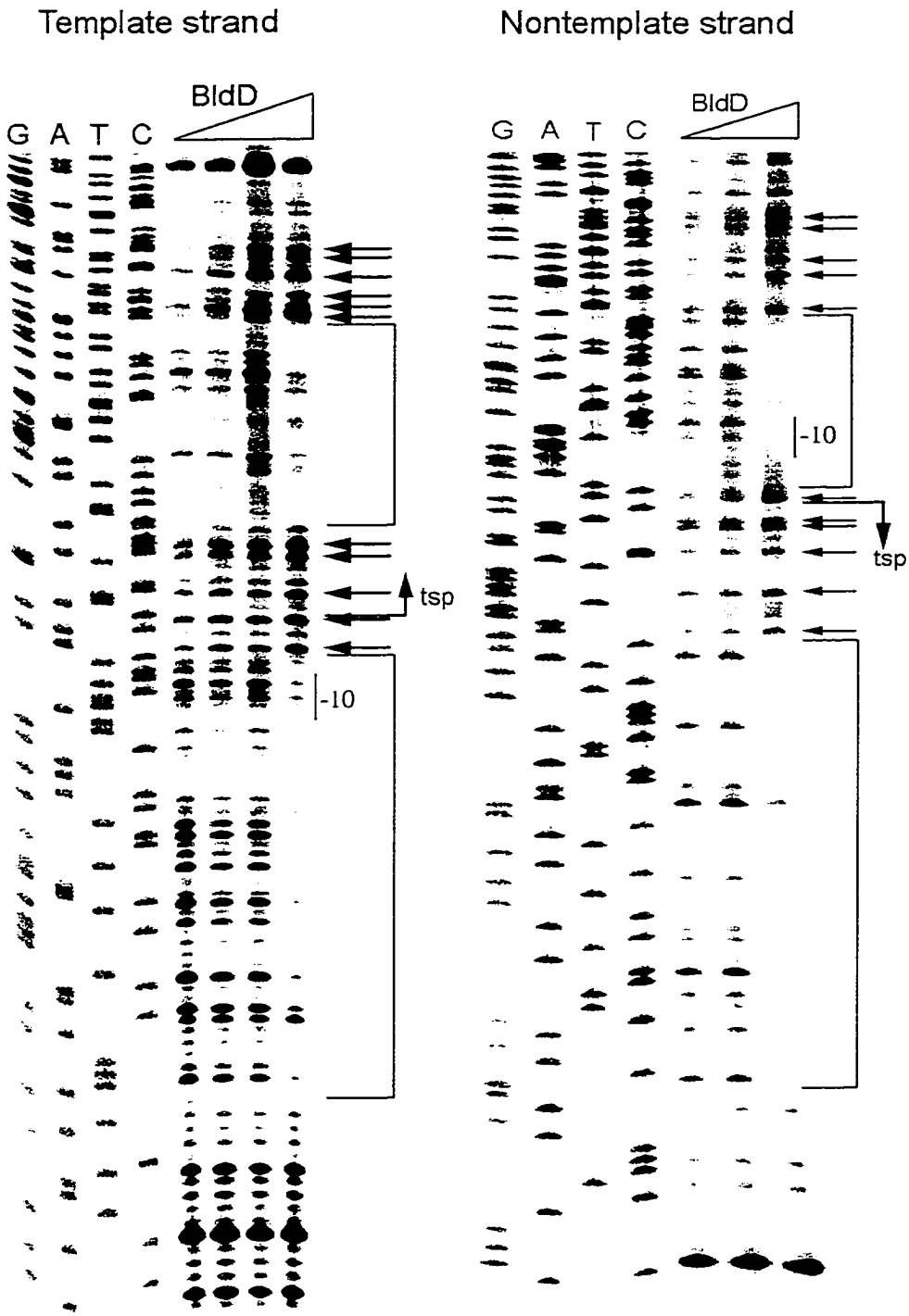
added to the mobility shift reactions, and were seen to have no effect on the ability of BldD to bind to the labeled *bldN* promoter fragment. BldD binding to the labeled probe was abolished, however, when 500 fold excess unlabeled *bldN* probe was added to the binding reactions, demonstrating specificity in the binding of BldD to *bldN*. An approximate K_d was determined to be 200 nM.

3.7.4c DNaseI footprinting of the *bldN* promoter region

To further delineate the BldD binding site(s) in the *bldN* promoter region, DNaseI footprinting assays were conducted using the 208 bp mobility shift probe fragment, labeled either on the template or nontemplate strand. One to two nanograms of this probe were incubated with increasing concentrations of BldD (0-80 pmol) before treatment with 0.05U of DNaseI for 10 seconds. Separation of the DNaseI cleavage products on a 6% sequencing gel revealed that, as expected from the electrophoretic mobility shift analyses, BldD conferred protection from DNaseI at two separate sites: from –3 to –49 and from +19 to +61, relative to the transcription start site (Fig. 3.7.9). DNaseI hypersensitive sites were observed between the two binding sites on both the template and nontemplate strands; upstream from the promoter localized site (-3 to –49) on the nontemplate strand; and downstream from the +19 to +60 site, into the translated portion of the gene, on the template strand. It therefore appears that BldD binding to the *bldN* fragment occurs at two sites, one upstream from the transcription start site, and the other downstream.

Furthermore, it appears that BldD binding alters the DNA structure of the *bldD* promoter, as suggested by the observed regions of DNaseI hypersensitivity. Identical results were obtained on at least three separate occasions.

Fig. 3.7.9 DNaseI footprinting of the *bldN* promoter region in the presence of increasing amounts of BldD. The 208 base pair gel shift probe fragment, MAE71-68, was specifically labeled on either the template or nontemplate strand, and was incubated with increasing amounts of BldD (0, 20 40 and 80 pmol for the template strand, and 0, 40 and 80 pmol for the nontemplate strand) for 20 minutes before a 10 second exposure to 0.05 U of DNaseI. The cleavage products were separated on a 6% sequencing gel. Footprints resulting from BldD protection of the DNA are bracketed, DNaseI hypersensitive sites are indicated with arrows, the transcription start point (tsp) is marked, as are the –10 and –35 promoter elements. The sequencing reactions, labeled G A T C, were performed using the oligonucleotide primers MAE71 or MAE68 for analysis of the template and nontemplate strands, respectively.



3.7.5 Selection of BldD targets from *S. coelicolor* total chromosomal DNA

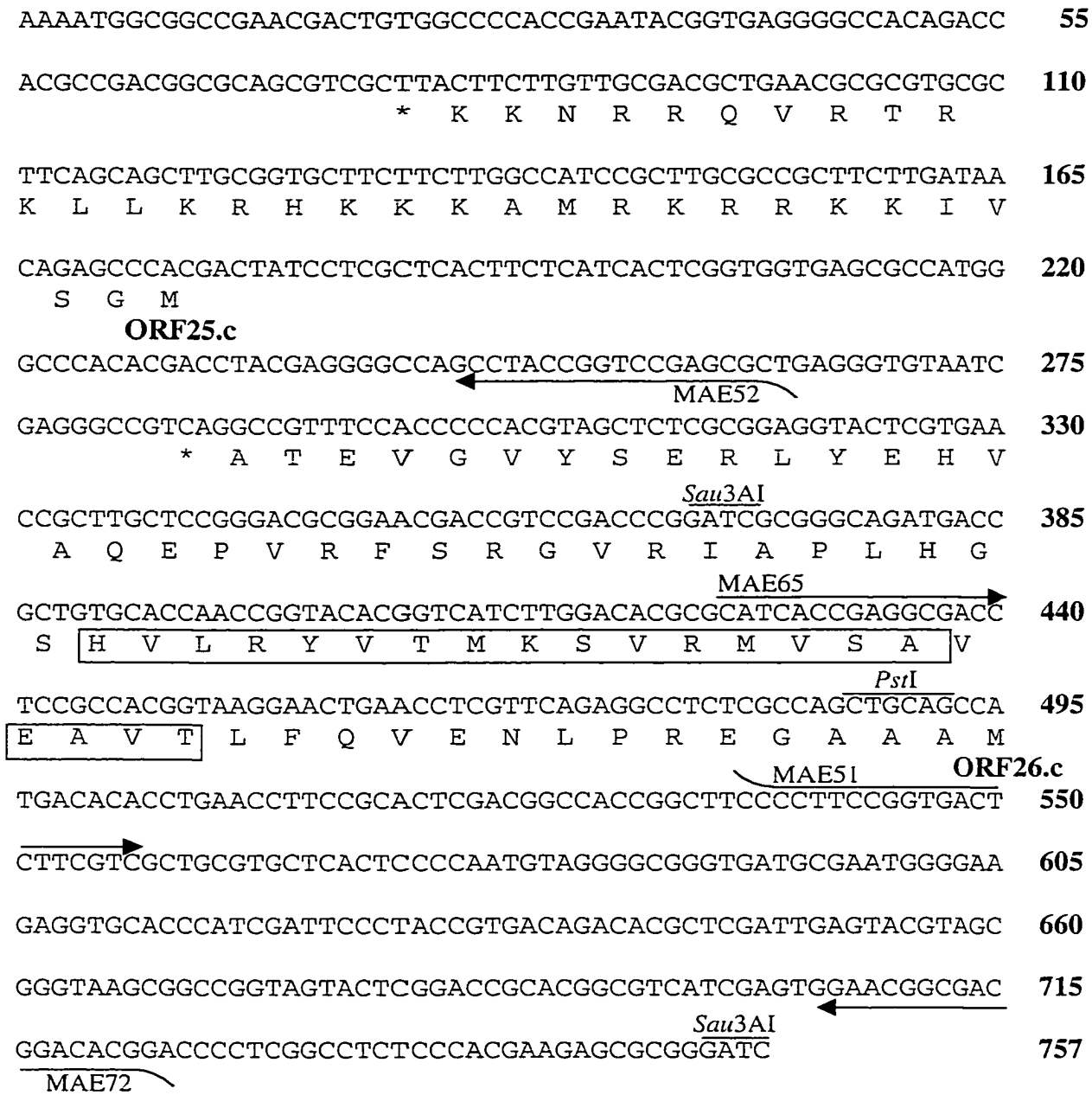
The final procedure used to isolate BldD targets involved a modified SELEX (systematic evolution of ligands by exponential enrichment) approach. The SELEX technique has been used successfully to isolate targets of DNA-binding proteins. Typically, selection involves the use of a random library of oligonucleotides or DNA/RNA fragments, which are incubated with the protein of interest (Tuerk and Gold, 1990). The protein-bound DNA is separated from the unbound portion, and is amplified by PCR. The binding-separation-amplification cycle is repeated a number of times, to ensure the isolation of specific targets. For the purpose of this study, a library of *S. coelicolor* J1501 chromosomal DNA was generated by partial digestion with *Sau*3AI. Fragments ranging in size from 300-600 bp were purified, and linkers of known sequence were ligated onto the ends. The linkers consisted of two annealed complementary oligonucleotides (MAE42 and MAE43; Table 2.4), one with a *Sau*3AI overhang (MAE43) to aid in ligation to the *Sau*3AI digested chromosomal DNA. Electrophoretic mobility shift assays were carried out using 10-20 ng of end-labeled, linker-containing library DNA fragments, in the presence of 20 pmol of His_6-BldD. The concentration of BldD added to each reaction was maintained at a low level, so as to minimize the binding of nonspecific targets by BldD. After electrophoresis for 3-4 hours on an 8% polyacrylamide gel, any shifted DNA fragments were excised from the gel, purified, and used as template for subsequent PCR amplification, with oligonucleotide MAE42 as a primer (representing one of the oligonucleotides used in the creation of the linkers; Table 2.4). After gel purification, the amplified products were then end-labeled, and cycled through the mobility shift selection procedure at least twice more. The final products

were blunt-ended and cloned into the *Sma*I site of pUC119, or were cloned without blunt-ending into pCR®TOPO (Invitrogen). Positive clones were determined by harvesting the plasmid DNA from individual transformants, and digesting the plasmid DNA with *Xba*I and *Eco*RI, to identify clones that contained an insert of approximately 300-600 bp in size. The insert DNA from positive clones was sequenced, and the resulting sequences were compared with the *Streptomyces* sequence database (http://www.sanger.ac.uk/Projects/S.coelicolor) to identify regions resembling the known BldD binding sequences.

The entire SELEX procedure was repeated 6 times, and so far, five different clones have been identified. One particular fragment appeared with overwhelming frequency (more than 50% of all positive clones), and it corresponded to a region upstream from a small open reading frame on the *S. coelicolor* cosmid, SCE68. This open reading frame, designated ORF 26.c, was predicted to encode a protein of 70 amino acids, and was found to be 68.3% identical, over 63 of its 70 amino acids, to an 87 amino acid protein from *Mycobacterium leprae*. While a function has not been assigned to either of these proteins, it appears that the *S. coelicolor* SCE68 ORF 26.c contains a probable helix-turn-helix motif, stretching from amino acids 16-37, suggesting DNA binding potential (Fig. 3.7.10).

Several additional targets appeared with varying frequencies: fragments corresponded to regions from cosmids SCE2 (position 18151-18555; upstream from a potential transposase; ~5% of isolated fragments), SCD78 (2063-2524; within a putative plasmid replication initiator protein; ~20% of isolated fragments), SC66T3 (7121-7613; within a hypothetical protein; ~5% of isolated fragments) and St6F2 (cosmid sequencing

Fig. 3.7.10 Nucleotide sequence of a 757 nucleotide DNA fragment containing ORF 26.c from cosmid SCE68. The single stranded nucleotide sequence is shown, with the numbers indicating the nucleotide position at the end of each line. The amino acid sequence for the proposed ORF 26.c and ORF 25.c gene products is shown below the nucleotide sequence. Within the ORF 26.c coding sequence, a putative helix-turn-helix is boxed. Oligonucleotide primers important in probe generation, and disruption attempts, outlined later, are marked by arrows; the curved ends of MAE 52, MAE 51 and MAE72 indicate nonhomologous tails, containing restriction enzyme sites for *Xba*I, *Sma*I, and *Eco*RI, respectively. Restriction enzyme sites for *Sau*3AI and *Pst*I are shown.



unfinished; within cytochrome P_{450}; ~20% of isolated fragments). Use of these fragments as probes for electrophoretic mobility shift assays revealed that they shifted poorly (Fig. 3.7.11), with less than ~10% of the labeled DNA present in the shifted band, even after the addition of 40 pmol of BldD, making it likely that these were nonspecific targets. The St6F2 fragment shifted slightly better than the other nonspecific targets, as determined by the proportion of free DNA remaining in the presence of 40 pmol BldD, although it did not shift as well as the other previously determined targets (see Fig 3.5.4, Fig. 3.7.2, and Fig. 3.7.8). The location of the St6F2 fragment, within the coding sequence of the cytochrome P_{450}, made it unlikely to be a specific BldD target. *bldN* and *whiG* were not identified in any of the cloned fragments, indicating that the conditions were not optimal for BldD target selection.

Since ORF 26.c was present in such abundance in the final selected fragments, attempts were made to prevent the ligation of this fragment into the cloning vector and thereby increase the likelihood of cloning other BldD target DNAs. Since a unique *Pst*I site was located immediately down from the proposed ORF 26.c translation start site, a portion of the SELEX-amplified DNA was digested with *Pst*I before ligation into either pUC119 or pCR®TOPO. Transformation of the resulting DNA mixture into pUC119 allowed the isolation of a clone containing the *bldD* promoter target. As it had been previously determined that BldD was capable of binding to its own promoter, this served as a positive internal control for the SELEX procedure. Although clones corresponding to *whiG,* and *bldN* were still not identified, indicating that the conditions for the SELEX procedure were still not optimal, the *bldN* promoter region was found to be present in the final, amplified selection mix (Fig. 3.7.12) for at least 3 out of the 6 SELEX procedures

Fig. 3.7.11 Electrophoretic mobility shifts for selected, potential BldD targets. One to two nanograms (~4-8 fmol) of each end-labeled fragment (SCD78, SCE2, and St6F2) were incubated with increasing amounts of BldD (as indicated) in a 20 μL reaction volume for 20 minutes at 30°C. The products were loaded onto a continuously running 8% polyacrylamide, 1.5% glycerol-containing gel, and were separated by electrophoresis in 1 × TBE. Free DNA and BldD-bound DNA are indicated.

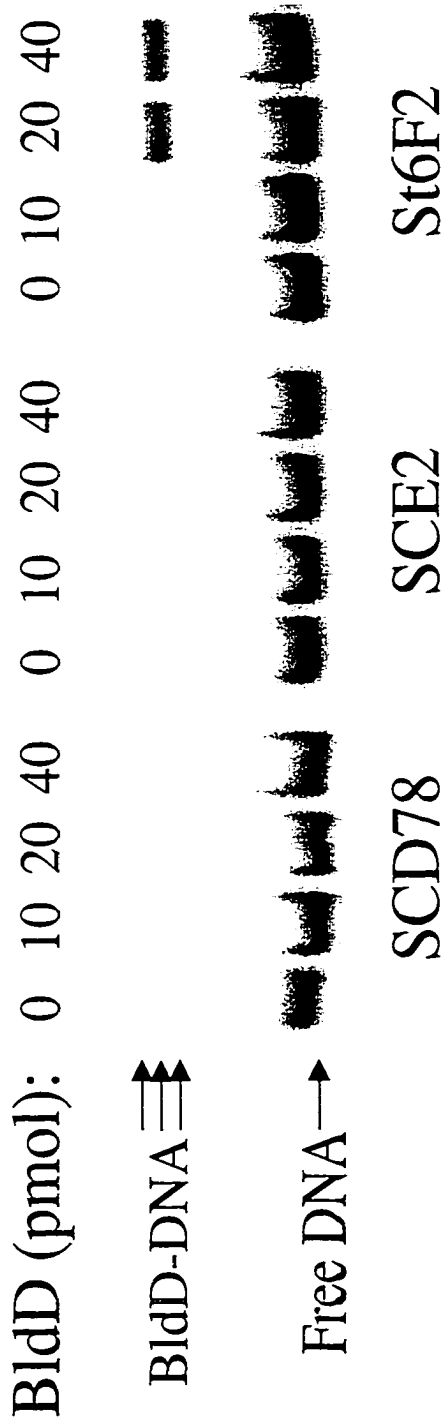
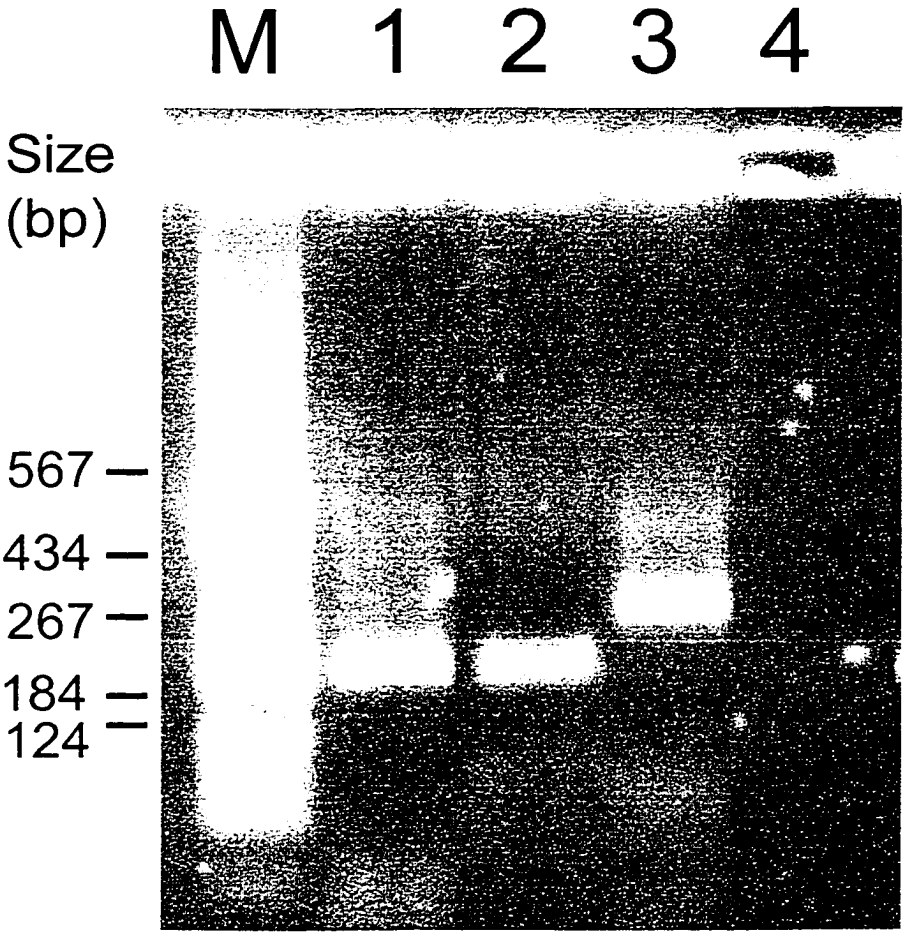


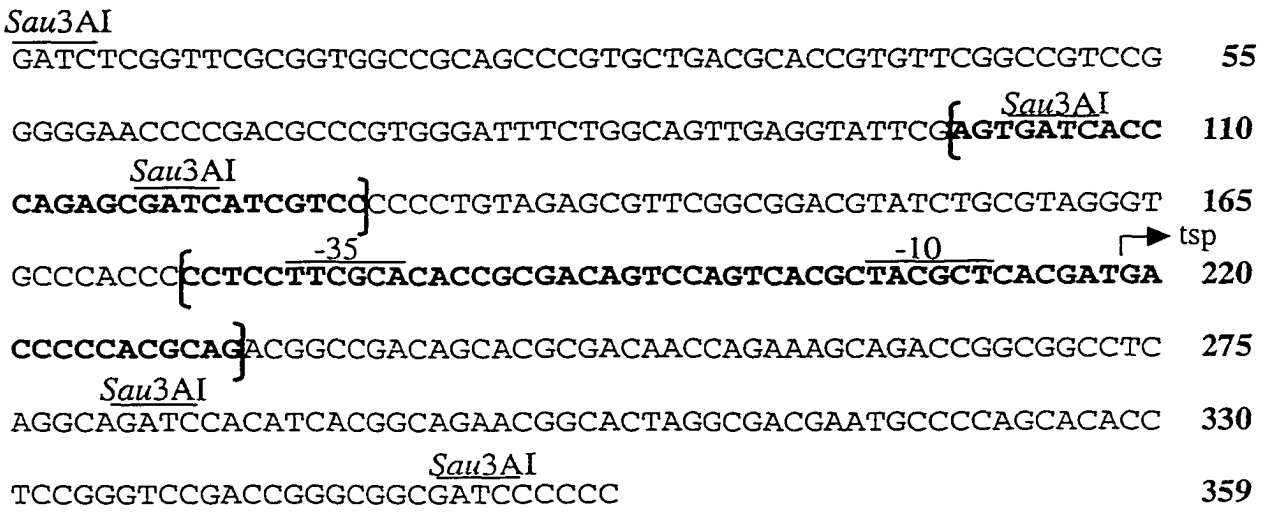
Fig. 3.7.12 PCR amplification of BldD targets from the final SELEX ligation mixture. MAE69 and MAE70 were used to amplify a 196 bp fragment of *sigI*, shown in lane 1; MAE71 and MAE68 were used to amplify a 208 bp fragment of *bldN*, shown in lane 2; MAE11 and MAE12 were used to amplify a 257 bp fragment of *bldD*, seen in lane 3; and as a negative control, BKL87 and BKL88 were used to amplify a 230 bp fragment of *bldG*, shown in lane 4. Molecular weight marker V (Roche) is indicated with an M, and the marker sizes, in base pairs, are shown to the left.



carried out. This was determined by PCR amplification of *bldN* using MAE71 and MAE68 (corresponding to nt –83 to +125, relative to the transcription start site) as primers, and the SELEX ligation mixture as template. This same approach was used to confirm the presence of the *S. coelicolor sig1* gene in the amplified DNA. *sig1* encodes a stress response sigma factor, with homology to the *Bacillus subtilis sigF* gene (GenBank accession number: AJ249450), and has recently been identified as a BldD target through work done in the lab of C. Thompson (personal communication). They had been working to determine regulators of the *sig1*/anti-sigma factor (the two genes appear to be translationally coupled) promoter region, by immobilizing the fragments of DNA containing the promoter in a column, and passing cell free extracts through, to isolate bound proteins. One protein was isolated, and sequencing of its amino-terminus revealed it to be BldD.

To ensure that any chromosomal fragment could not be amplified from the SELEX ligation mixture, BKL87 and 88 were used in an attempt to amplify a fragment of similar size from the *S. coelicolor bldG* promoter, using the SELEX ligation mixture as template. This promoter region is known not to be bound by BldD, and was not amplified from the SELEX ligation mixture (Fig 3.7.12). While *whiG* had been shown to be a BldD target, it was not amplified from the ligation mixture, presumably because of a large number of *Sau*3AI sites between the primers used for the amplification. In retrospect, it is possible that due to the numerous restriction sites in the *whiG* promoter region shown to be bound by BldD (Fig. 3.7.13), that the *whiG* fragment(s) may have been smaller than the 300-600 bp *Sau*3AI fragments that were purified, and as a result would not have been present in the original selection mixture.

Fig. 3.7.13 *Sau*3AI sites within the *whiG* promoter region. The nucleotide sequence for a 359 bp region of the *whiG* promoter region is shown, with the numbers on the right indicating the nucleotide number at the end of each line. *Sau*3AI sites are shown, as are the –10 and –35 promoter elements, and the transcription start site (tsp). The regions determined to be protected by BldD during DNaseI footprinting are bracketed, with the protected sequences shown in bold.



A final attempt was made to isolate the known BldD targets (*bldN, sigI,* and *whiG)* by digesting the original linker-containing chromosomal library with *Pst*I, prior to initiation of the selection procedure, and then using this as probe for the SELEX procedure. No new targets were identified.

To address the inability to clone known BldD targets, a new SELEX library should be generated, by digesting the chromosome with *Hpa*II. *Hpa*II sites are actually more prevalent in the *S. coelicolor* chromosome than *Sau*3AI sites, due to the G+C rich nature of its restriction site. By carrying out a partial digest using this restriction enzyme, the likelihood of generating a library of random fragments would be increased. Alternatively, a library of random oligonucleotides could be used in lieu of a chromosomal DNA library.

3.7.6 Analysis of BldD-ORF 26.c interactions

3.7.6a Electrophoretic mobility shift assays for ORF 26.c

To better understand the relationship between BldD and the previously uncharacterized ORF 26.c, electrophoretic mobility shift assays were conducted using probe DNA spanning the translation start site of ORF 26.c (from +130 to –255). The 483 bp probe fragment was amplified using the forward and reverse primers and pUC119-SCE68 as template. pUC119-SCE68 is a pUC derivative, generated during the SELEX procedure, containing the 385 bp DNA fragment (described above) cloned into the *Sma*I site. One to two nanograms of end-labeled probe were added to reactions containing 0-80 pmol of BldD, in the presence of excess nonspecific DNA [poly d(I-C)]. Separation of the free DNA from the BldD-bound DNA on an 8% polyacrylamide gel revealed two

shifted fragments, with the labeled DNA found to be distributed evenly between these two products upon the addition of 40 pmol of BldD (Fig. 3.7.14A). This was reminiscent of the pattern observed when BldD bound the *bldD* promoter, only in this case, with the addition of 80 pmol of BldD, essentially all of the shifted DNA was found in the upper band position. A second probe was created, using the oligonucleotide primers MAE65 and MAE72 (Table 2.4) and pUC119-SCE68 as template, and use of this 308 bp probe (+74 to –226, relative to the translation start site) in the electrophoretic mobility shift assays (together with 0-40 pmol of BldD), gave results identical to those obtained when using the larger probe (Fig. 3.7.14B). Competition mobility shift assays were performed using 500 fold excess unlabeled specific competitor (MAE65-72), which completely abrogated BldD binding to the labeled probe, while the addition of 500 fold excess unlabeled nonspecific competitor (BKL41-MAE5; internal to the *bldD* coding sequence) did not influence binding of the labeled probe by BldD. An approximate K_d was determined to be 400 nM.

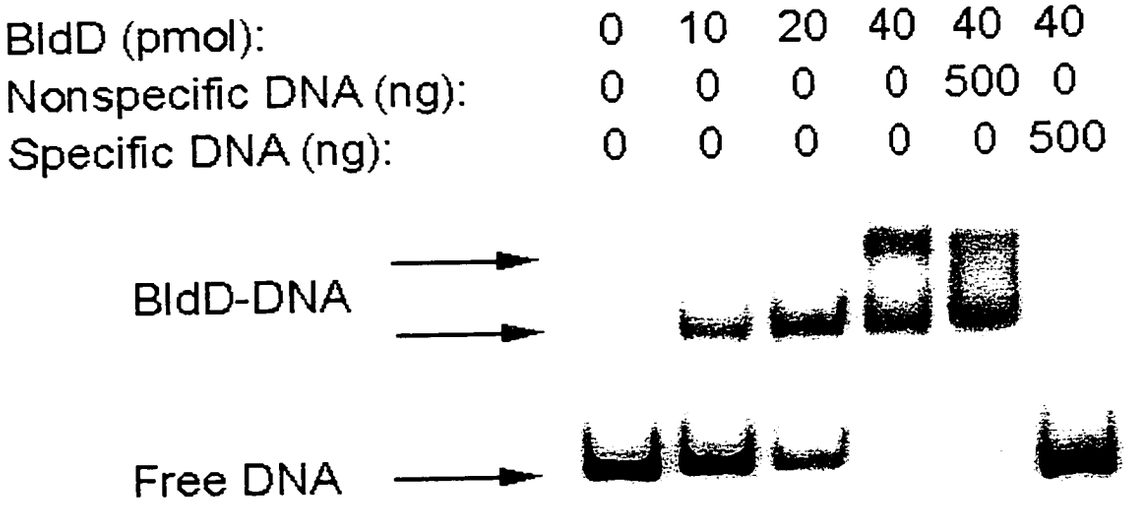
3.7.6b DNaseI footprinting assays for ORF 26.c

Since the electrophoretic mobility shift pattern generated by BldD binding to DNA upstream from the ORF 26.c translation start site was reminiscent of the pattern observed when BldD bound the *bldD* promoter region, it was possible that BldD bound the ORF 26.c fragment at a single site. To investigate how BldD binds upstream from the ORF 26.c translation start site, and to more precisely localize the BldD binding site(s), footprinting assays were carried out using the 308 bp mobility shift fragment, labeled either on the template or nontemplate strand, as probe. The addition of BldD (40 pmol

Fig. 3.7.14 Electrophoretic mobility shift analysis of BldD binding to the ORF 26.c promoter region. One to two nanograms (3-6 fmol) of a 483 bp fragment, spanning the ORF 26.c promoter region, generated using the forward and reverse primers, with pUC119-SCE68 as template (panel A), and an end labeled probe MAE65-72 (5-10 fmol; panel B), extending from within the ORF 26.c coding sequence to cover a 308 bp region, were incubated with 0-40 pmol of BldD in a total volume of 20 μL. After incubation at 30°C for 20 minutes, the reactions were loaded onto a continuously running 8% polyacrylamide gel, containing 1.5% glycerol, and were electrophoresed in 1 × TBE. The free probe and probe complexed with BldD are indicated. Binding specificity was established in reactions with MAE65-72 end-labeled probe (panel B), using unlabeled probe (~500 ng/2pmol of MAE65-72) as a competitor in a reaction containing 40 pmol of BldD, shown in the final (sixth) reaction lane, and also using unlabeled, nonspecific competitor DNA (~500 ng/6 pmol of BKL41-MAE5), seen in the fifth reaction lane.

A.

B.

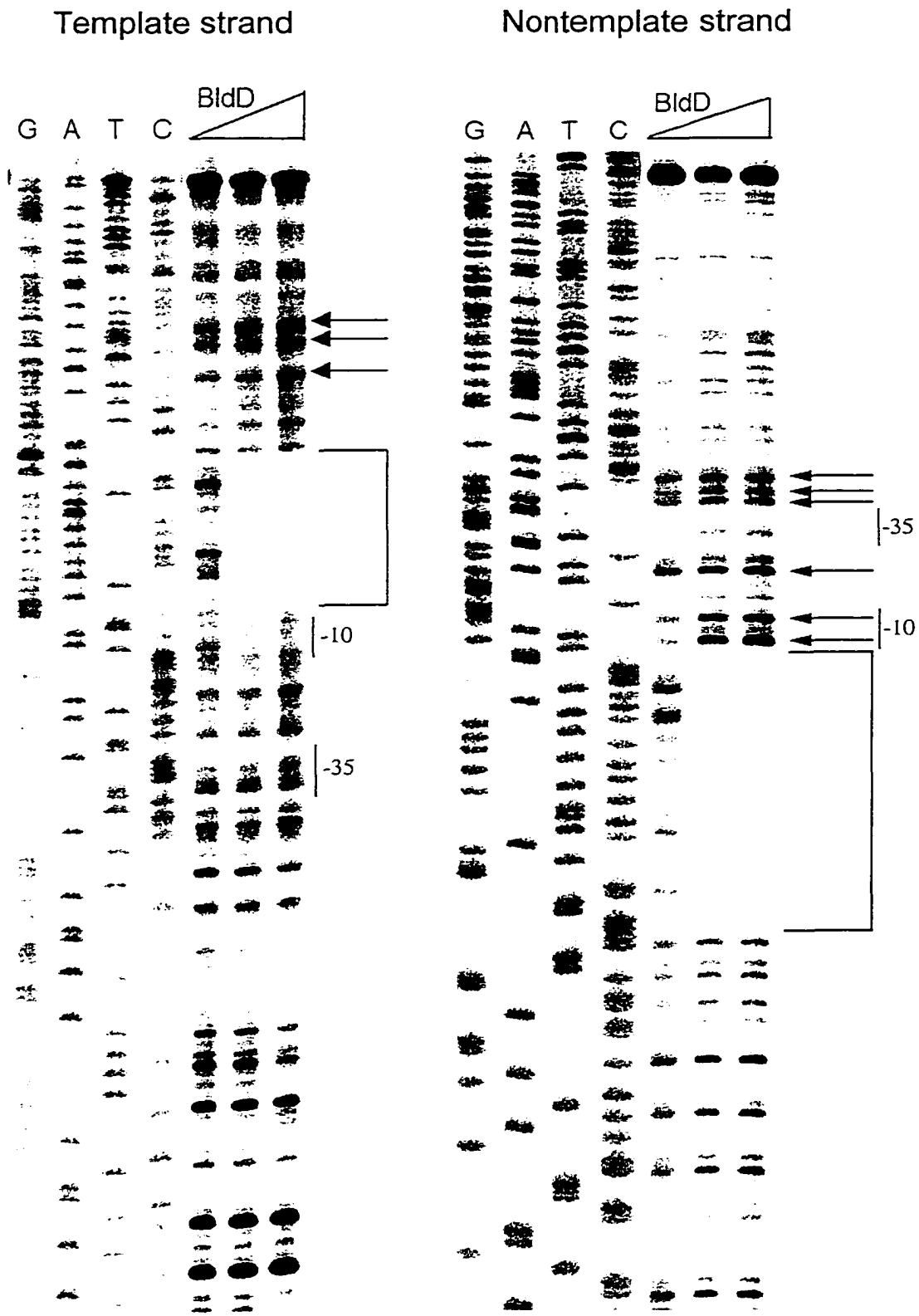


and 80 pmol) revealed only one binding site after separation of the DNaseI cleavage ladders on a 6% sequencing gel (Fig. 3.7.15), as opposed to the 2 sites suggested by the electrophoretic mobility shift assays. As discussed in section 3.5.1 and 3.5.2, the existence of 2 shifted bands in the electrophoretic mobility shift assays, but the observation of only a single binding site in the footprinting assays may suggest equilibration between two different conformational states. The protected region was seen to span 36 bp, extending from –44 to –80, relative to the translation start site. Upstream from this on the nontemplate strand, and downstream on the template strand, a number of DNaseI hypersensitive sites were observed, suggesting that binding by BldD induced conformational changes in the DNA. These conformational changes may have been sufficient to generate the appearance of the two shifted fragments observed in the electrophoretic mobility shift assays, however, a final conclusion cannot be made in the absence of further biochemical analyses. These hypersensitive sites are seen on the template strand, extending from +1 to +19 relative to the translation start site, and are seen on the nontemplate strand, from -80 to -116. In addition, as seen in Fig. 3.5.8, hypersensitive sites were not observed when BldD was bound to the *bldD* promoter region.

3.7.6c S1 nuclease protection of ORF 26.c in *S. coelicolor* strains J916 and 1169

While the *in vitro* ability of BldD to bind upstream from ORF 26.c identified it as a putative, specific BldD target, the effect that BldD binding had on its *in vivo* expression was not known. *In vivo* studies were undertaken to examine the transcription of ORF 26.c in both the parent *S. coelicolor* strain J916 and *bldD* mutant strain 1169. High

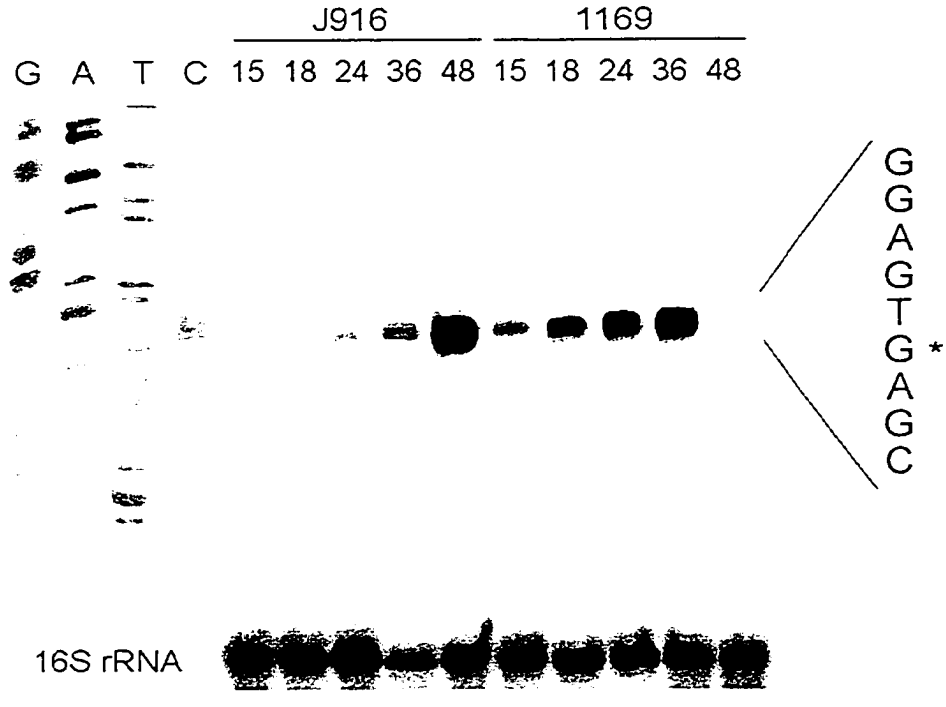
Fig. 3.7.15 DNaseI footprinting of BldD bound to ORF 26.c. The 308 bp probe, MAE65-72 (1-2 ng/5-10 fmol), was end-labeled on either the template or nontemplate strand, and was incubated with BldD (0, 40 or 80 pmol) in 20 μL binding reactions. Twenty minutes of incubation at 30°C was followed by a 10 second exposure to 0.05 U DNaseI, after which the cleavage products were separated on a 6% sequencing gel. The area protected from DNaseI cleavage by BldD binding is bracketed, DNaseI hypersensitive sites are indicated with arrows, and –10 and –35 elements are indicated. The sequencing reactions (G A T C) were generated using MAE65 or MAE72 as oligonucleotide primers, for examination of the template strand or nontemplate strand, respectively.



resolution S1 nuclease mapping was carried out to determine the pattern of ORF 26.c expression throughout colony development, and also to identify the ORF26.c transcription start site. A 391 bp probe was generated by PCR amplification using the MAE65 oligonucleotide primer and the reverse primer (which would provide a nonhomologous tail to allow differentiation between full length probe protection and probe-probe reannealing), together with the original pUC119-SCE68 clone (see 3.7.6a) as template. After purification, the DNA probe fragment was labeled at both ends, and annealed at 58°C with 40 μg total RNA samples. Following treatment with S1 nuclease, the products were separated on a 6% sequencing gel. A single protected fragment of 145 nt was detected (Fig. 3.7.16), implying a transcription start site at the G residue 72 nt upstream from the translation start site. The sequence upstream from the transcription initiation point was examined, and putative –10 and –35 sequences, separated by an 18 bp spacer, were identified (see below; Fig. 3.7.18). The position of the BldD binding site was then assessed relative to the mapped transcription start site, and it was found that protection conferred by BldD binding to the ORF 26.c operator extended from +30 to –6, and the DNaseI hypersensitive regions, indicative of DNA bending, were found to extend from the –10 region, to just upstream from the –35 region of the promoter on the nontemplate strand, and from +53 to +72 on the template strand.

Examination of transcript abundance during colony development revealed temporal regulation of ORF 26.c expression (Fig. 3.7.16). In the wild type strain, a low level of transcript was seen in the 15 hour sample, and increased gradually up until 36 hours; however, between 36 and 48 hours a greater than 6 fold increase in expression was seen. For these surface grown cultures, aerial mycelium were first visible at 24 hours,

Fig. 3.7.16 High resolution S1 nuclease mapping of the ORF 26.c transcription start site using RNA (40 μg) isolated, at various timepoints as indicated, from *S. coelicolor* strains J916 and 1169. The 391 bp end-labeled probe was generated using the oligonucleotides MAE65 and reverse primer, with pUC119-SCE68 as template. After annealing, the RNA-DNA duplexes were treated with S1 nuclease, and the products were separated on a 6% sequencing gel. MAE65 was used as the oligonucleotide primer to generate the sequence ladder, shown as G A T C. The asterisk (*) indicates the most probable transcription start site in the sequence corresponding to the template strand. RNA levels in the samples were assessed by northern blot analysis using 10 μg samples of RNA, from the same stock as used for the S1 mapping, hybridized to a probe specific for 16S rRNA (BKL54), and are shown below the S1 nuclease protection assay.



and very abundant by 36 hours. The cultures were fully sporulated by 48 hours. Pigmented antibiotics were first visible at 24 hours. This would be consistent with a role for ORF 26.c in differentiation of the aerial hyphae to form spores. This same expression pattern was not observed in the *bldD* mutant strain, where transcript levels were seen to increase from 15 to 36 hours, where the amount of transcript was more than 3 fold greater at each time point when compared with the wild type strain. Between 36 and 48 hours, when significant upregulation of ORF 26.c expression was observed in the wild type strain, transcription in the *bldD* mutant was seen to fall off significantly, to ~15 fold less than the amount present in the wild type strain.

To ensure that the differences seen in transcript were not the result of inconsistent levels of RNA present in each of the samples, northern blot analysis was performed on 10 μg samples of the same RNA stock solutions. Hybridization of the membrane with end-labeled BKL54, an oligonucleotide probe specific for 16S rRNA, revealed that approximately consistent amounts of RNA were present in each of the samples. The J916 36 hour sample was observed to contain less RNA than the others, suggesting that the 6 fold increase in expression between 36 and 48 hours in the S1 nuclease protection experiment is likely not as dramatic as it appears.

It therefore appears that BldD can specifically bind to DNA upstream from ORF 26.c, as demonstrated by the electrophoretic mobility shift assays and DNaseI footprinting assays. It also appears that BldD binding may alter the DNA structure in the areas surrounding its binding site, as suggested by the appearance of DNaseI hypersensitive sites. *In vivo* transcript analyses confirmed a role for BldD in influencing the appearance of the ORF 26.c transcript, and suggested a regulatory role that is not

simply repression or activation, given that the initial overabundance of transcript was followed by a significant decrease in transcript abundance (although the possibility that transcript instability is involved cannot be discounted).

3.7.6d Northern blot analysis of ORF 26.c

Although both 5' S1 nuclease protection and northern blot analyses provide information about the temporal appearance of transcripts, only northern blot analysis allows determination of the transcript size. Therefore, northern blot analyses were carried out with 30 μg samples of RNA isolated from *S. coelicolor* J1501 (unfortunately, the northern blot analysis was successful only once, despite attempts to repeat it, and as a result, it has not been successfully performed using J916 RNA samples). The RNA samples were denatured by treatment with glyoxyl, and were electrophoresed on a 1.25% agarose gel, along with glyoxylated DNA markers. A random primer labeled probe, equivalent to the original electrophoretic mobility shift probe that extended from +130 to –255 relative to the ORF 26.c translation start, was used for overnight hybridization to the membrane at 55°C, and revealed a single transcript, determined to be ~600 nt in length (Fig. 3.7.17). Since the ORF 26.c coding sequence comprised 210 nt, and the upstream untranslated portion of the transcript added only an additional 72 nt, to give 282 nt, it would have to extend for an additional 300 nt beyond ORF 26.c. Analysis of the sequence on cosmid SCE68, revealed a small open reading frame, immediately downstream from ORF 26.c. This ORF, designated ORF 25.c, is predicted to encode a lysine rich 32 amino acid protein of unknown function (Fig. 3.7.18). Like ORF 26.c, this putative ORF 25.c possessed similarity to an ORF located just downstream from the ORF

Fig. 3.7.17 Northern blot analysis of ORF 26.c transcripts using RNA isolated from surface grown cultures of *S. coelicolor* strain J1501 (15, 18, 24, 36, and 48 hours post-inoculation, as indicated). The RNA samples (30 μg) were denatured with glyoxyl, separated on a 1.25% agarose gel, and transferred to a nylon membrane. The RNA on the membrane was hybridized at 55°C, with a random primer labeled probe, extending 385 bp, generated by amplification from pUC119-SCE68 using the forward and reverse primers. Size markers, treated as for the RNA, were molecular weight markers III and V (Roche).

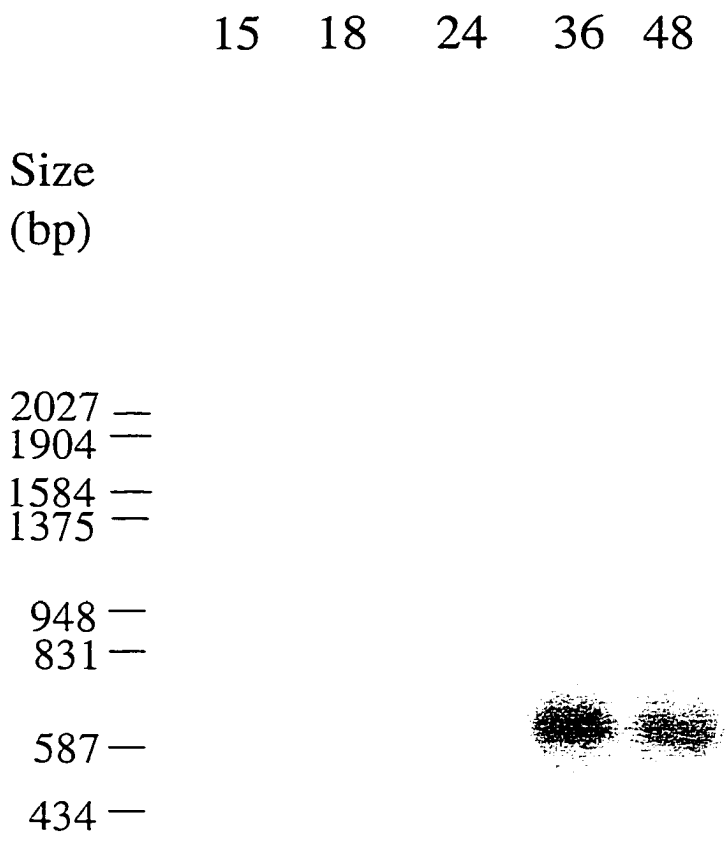
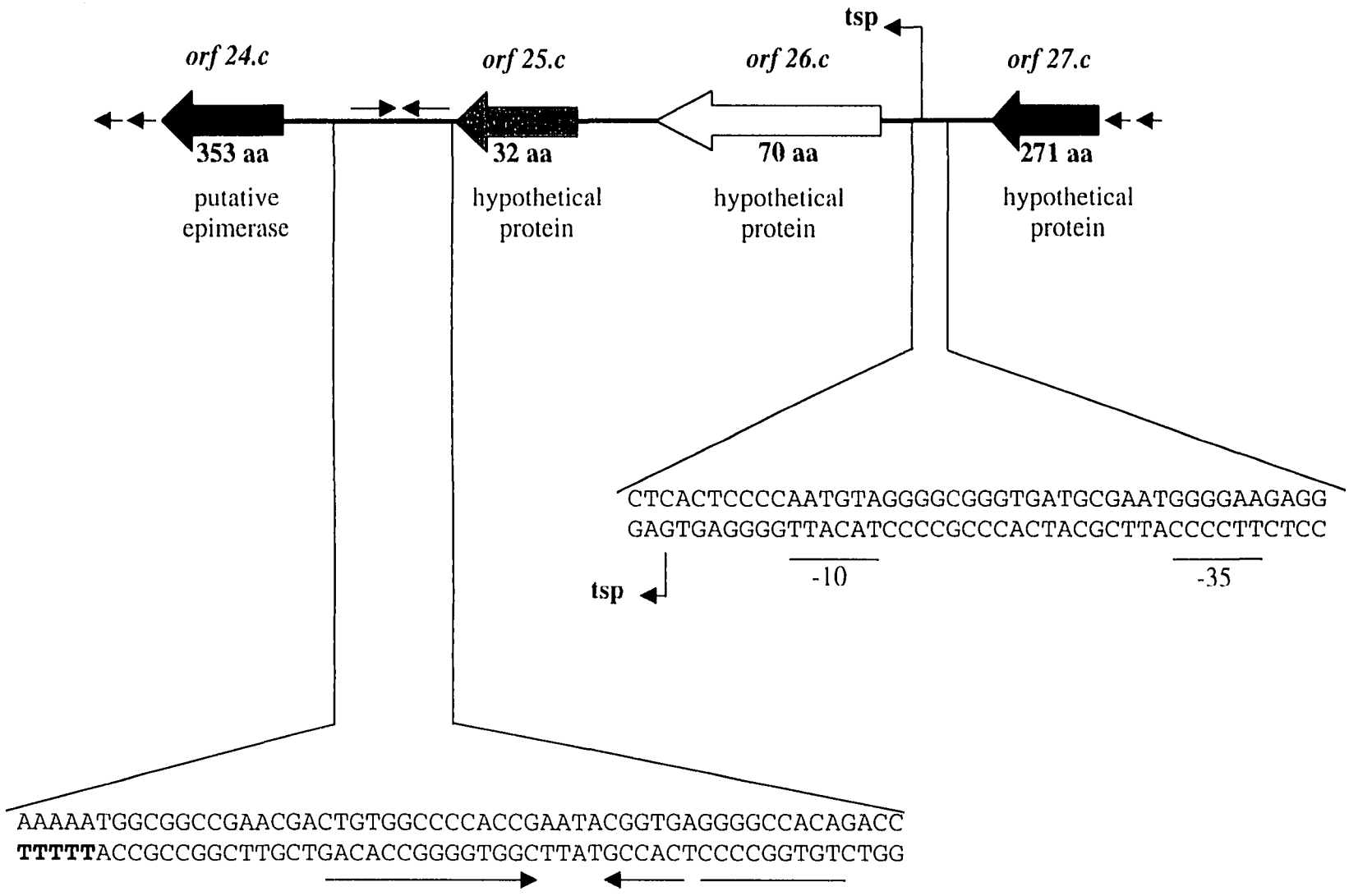


Fig. 3.7.18 Schematic diagram illustrating the location and orientation of ORF 26.c, shown in yellow, and ORF 25.c, shown in orange, and two partial ORFs, ORF 24.c and ORF 27.c. The transcription start site for the ORF 26.c and ORF 25.c polycistronic message is shown, as is its relation to putative –10 and –35 promoter elements. The long inverted repeat downstream from ORF 25.c is shown with arrows, and the poly-T region, where transcription may terminate, is bolded.

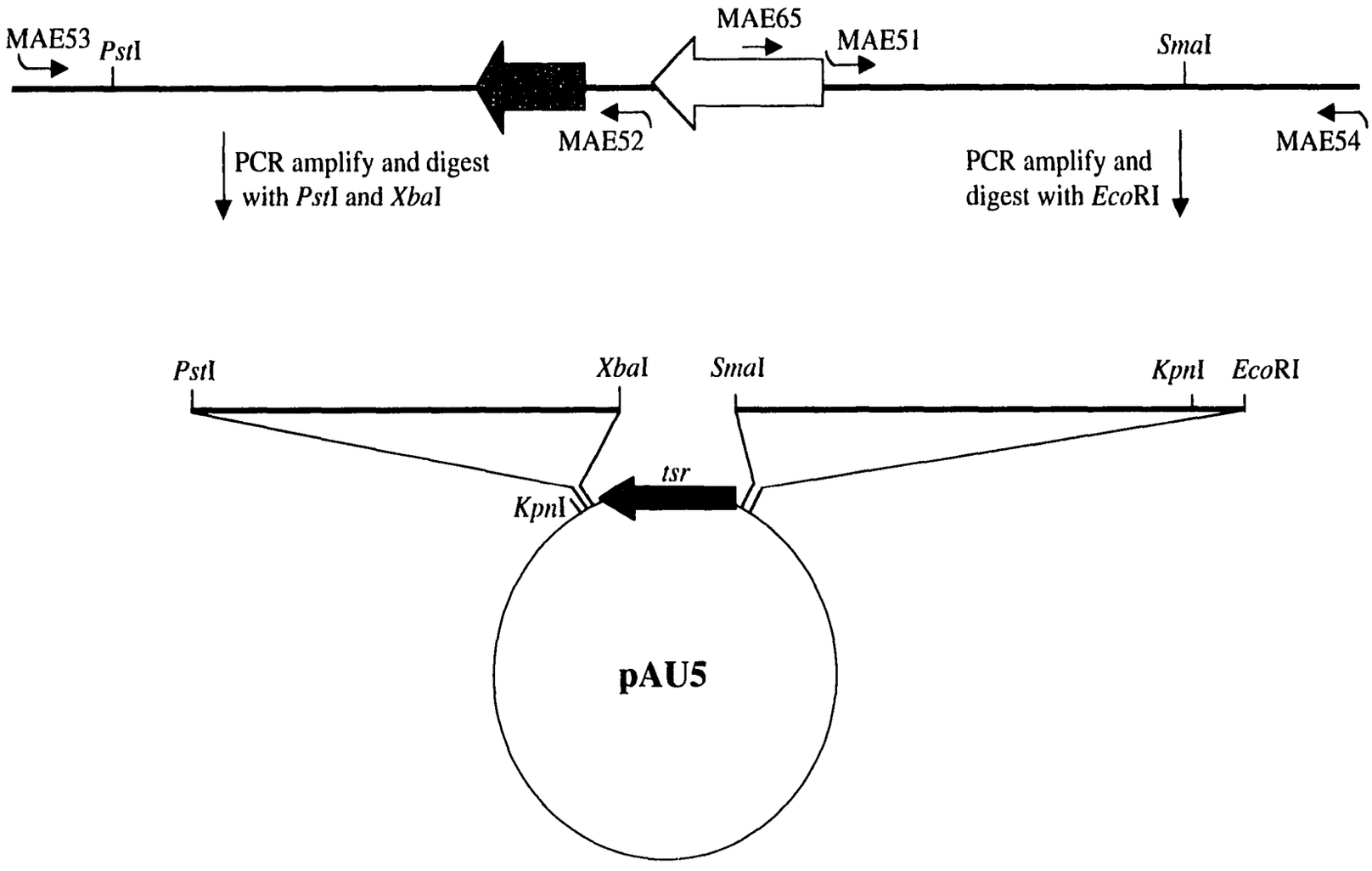


26.c homologue in *Mycobacterium leprae,* suggesting a conserved organization for these two open reading frames. The translation stop codon for ORF 26.c is separated from the translation start codon for ORF 25.c by 108 bp. A long inverted repeat, followed by a string of U's in the mRNA sequence that may serve as a transcription termination signal, was found downstream of ORF 25.c. Termination at this point would give a product of 581 nt, a size consistent with the transcript length of ~600 nt observed in the northern blot analysis.

3.7.6e Attempted gene replacement of ORF 26.c with *tsr*

Since the ORF 26.c promoter appeared to be a target of BldD, and since the appearance of the ORF 26.c transcript was temporally regulated to coincide with sporulation of the aerial mycelia, it seemed likely that the product of ORF 26.c and the co-transcribed ORF 25.c contributed to differentiation. To determine the role played by ORF 26.c, attempts were made to replace ORF 26.c in the chromosome of *S. coelicolor* J1501, with the thiostrepton resistance gene (Fig. 3.7.19). The gene replacement approach involved amplification of sequences flanking ORF 26.c, about 1000 bp on either side, followed by introduction of these flanking regions onto either side of *tsr* (the thiostrepton resistance marker) in pAU5. The downstream flanking sequence was amplified using MAE 52, found just downstream from the ORF 26.c translation stop codon with an *Xba*I site engineered onto its 5' end, and MAE53, positioned 1232 bp away with a *Pst*I site added on to its 5' end. Digestion of this downstream product with *Xba*I and *Pst*I resulted in a fragment of 990 bp, rather than the expected 1232, as a second *Pst*I site was found 242 bp away from MAE53. This fragment was then ligated into pAU5,

Fig. 3.7.19 Subcloning strategy for the replacement of ORF 26.c with the thiostrepton resistance gene. ORF 26.c is shown in yellow; ORF 25.c is shown in orange; and *tsr* is coloured blue. In each case, the arrows indicate the gene orientation. The ORF 26.c downstream flanking sequence was amplified using MAE53 (with a *Pst*I site engineered onto the 5' end) and MAE52 (with an *Xba*I site included on the 5' end). The resulting fragment was digested with *Pst*I and *Xba*I, and ligated into *Pst*I-*Xba*I-digested pAU5. The upstream fragment was amplified using MAE51 (possessing a 5' *Sma*I site) and MAE54 (having a 5' *Eco*RI site), after which it was blunt ended and digested with *Eco*RI. The fragment was ligated into pAU5+MAE53-52, which had been digested with *Sma*I and *Eco*RI, to generate the gene replacement construct. The *Kpn*I sites used to determine whether the gene disruption construct had integrated into the chromosome are indicated.



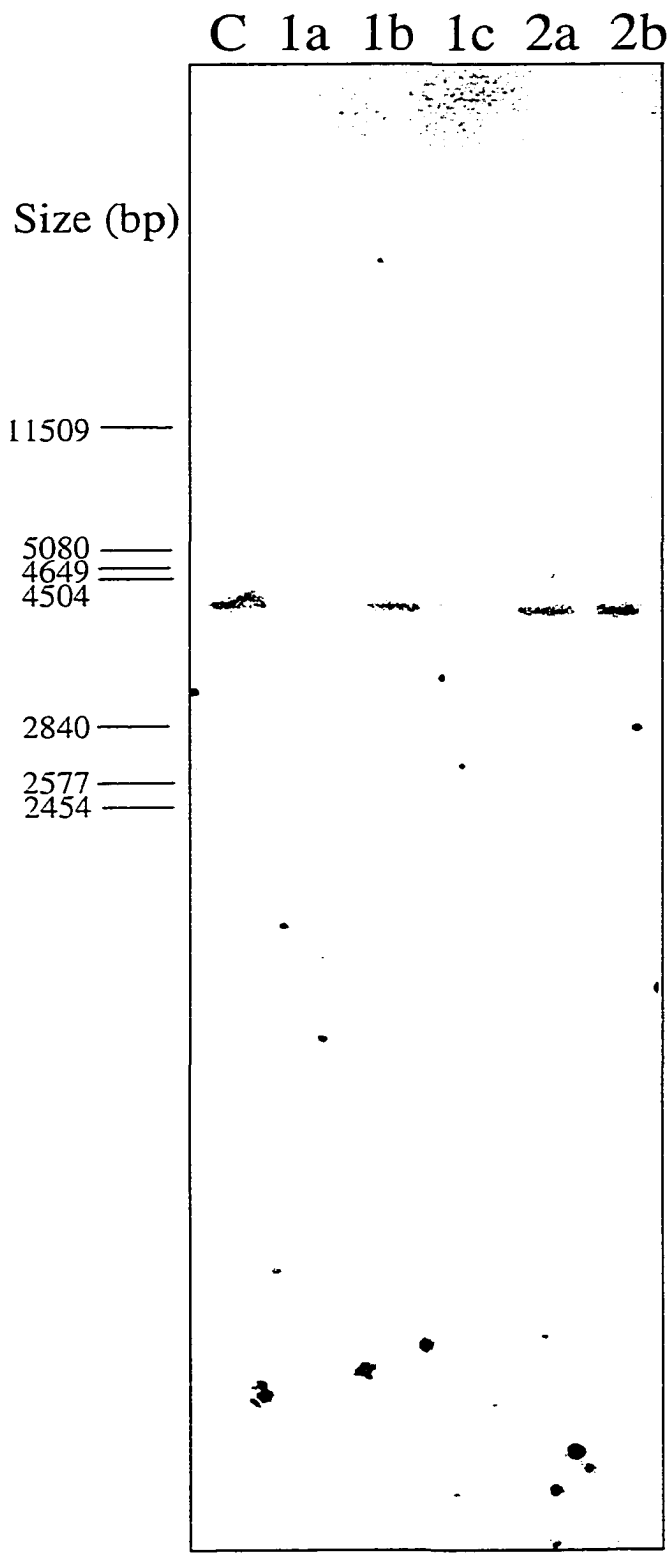
which had been digested with *Xba*I and *Pst*I. The upstream flanking sequence was amplified using MAE 51, which was located just down from the ORF 26.c transcription start site and had a *Sma*I site engineered onto its 5' end, and MAE 54, which had an *Eco*RI site added on to its 5' end, and was located 1201 bp away. After amplification, the MAE51-54 fragment was blunt-ended and digested only with *Eco*RI, not with *Sma*I, as a second *Sma*I site was found 786 bp away; the goal was to keep the flanking sequences as long as possible, as the longer the flanking sequence, the more likely recombination will occur. Attempts were made to ligate this fragment into *Sma*I and *Eco*RI digested pAU5+MAE52-53; however, after multiple failures, a different approach was initiated. The MAE51-54 fragment was digested with *Sma*I, to give a 786 bp fragment, and this was inserted into *Sma*I digested pUC119, and introduced into *E. coli* DH5α. Positive transformants were identified by blue/white selection, and were confirmed by digestion with *Xba*I and *Eco*RI, to liberate the ORF 26.c upstream flanking arm. This flanking sequence was then removed as an *Xba*I (which was then blunt-ended), and *Eco*RI segment, and was successfully ligated into pAU5+MAE52-53 cut with *Sma*I and *Eco*RI. The flanking arms of the resulting plasmid were sequenced to confirm the integrity of each of the inserts, and to ensure that no recombinational events had taken place.

This gene replacement construct was then introduced into *S. coelicolor* J1501 protoplasts, both in a native form, and after alkali denaturation (Oh and Chater, 1997) and thiostrepton resistant transformants were selected. Theoretically, any thiostrepton resistant colonies observed should have had the ORF 26.c-replacement construct integrated into the chromosome, either through a single crossover or a double crossover event, since pAU5-based vectors do not replicate in *Streptomyces*. A variety of colony

phenotypes were observed in the resulting transformants, including essentially wild type phenotype, along with a few primarily bald colonies (with hyphae in areas of dense growth) having very little in the way of antibiotic production. Chromosomal DNA was isolated from 2-3 colony representatives of each phenotype, and was digested with *Kpn*I (see Fig. 3.7.19), followed by Southern blot analysis, using one of the flanking arms as a probe. It was found that no replacement had taken place; a double cross-over would have given a fragment ~500 bp larger than the wild type, while a single cross-over would have resulted in a *Kpn*I fragment either ~240 bp (by cross-over within the upstream flanking sequence) or ~500 bp larger (by cross-over downstream from ORF 26.c) (Fig. 3.7.20). It was possible that this difference was too subtle to detect by Southern blotting, so PCR amplification of ORF 26.c was conducted to determine whether a gene replacement had occurred. MAE54, upstream from ORF 26.c, and MAE65, within the ORF 26.c coding sequence, were used in the ORF 26.c amplification from the chromosomal DNA isolated above. A product of ~1300 bp was observed in all instances, as would be expected if ORF 26.c were intact, confirming that replacement of ORF 26.c by *tsr* had not occurred, and that a single cross-over upstream from ORF 26.c had not taken place (as MAE54 was no longer present in the final gene replacement construct). Retransformation of the J1501 protoplasts with the replacement construct, and selection using a higher concentration of thiostrepton, to try and reduce the number of apparent spontaneous thiostrepton resistant mutants, had no effect, again, with only false positive colonies appearing.

Failure to create an ORF 26.c null mutant may be a result of a technical failure, or may indicate that the gene has an essential function. A different approach may be taken, with the most reasonable one being the creation of an in-frame-mutation/deletion, as it

Fig. 3.7.20 Southern blot analysis of chromosomal DNA isolated from potential ORF 26.c disruptant strains. The chromosomal DNA was digested with *Kpn*I, and was separated on a 1% agarose gel, before transfer to a nylon membrane for hybridization with random primer-labeled MAE52-53 (one of the flanking sequences used in the generation of the disruption construct). Lambda DNA, digested with *Pst*I, was run on the gel as a size marker. C indicates the J1501 control; 1a,b and c are different representatives of the *bld* phenotype seen in the thiostrepton-resistant colonies, while 2a and 2b represent the wild type phenotype observed for the thiostrepton-resistant colonies.



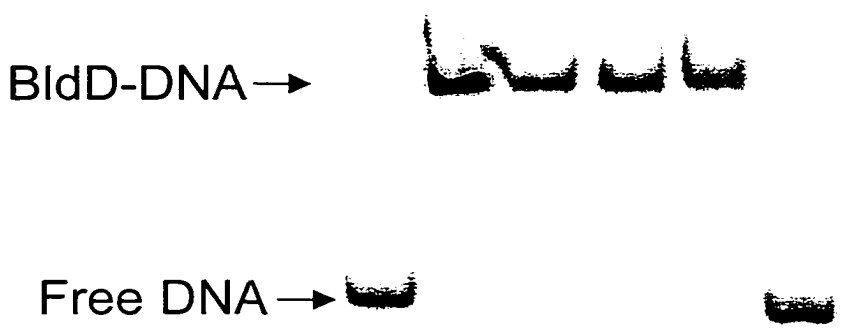
would affect only ORF 26.c, and not the downstream ORF 25.c. In the event that this does not succeed either, a second ORF 26.c gene could be introduced into the *att* site, and attempts made to disrupt/truncate either copy. If the gene disruption is possible when more than one copy of the gene is present, but not when a single copy exists, this would suggest that the gene is essential (Vögtli and Cohen, 1992).

3.7.7 Analysis of BldD-*sig1* interactions

sig1, and its proposed regulator, were identified as a BldD target through work in the lab of Charles Thompson. The gene encoding the proposed *sig1* anti-sigma factor is positioned before the *sig1* gene, however, as the two genes overlap, they are believed to be translationally coupled. We were informed of this target, and while we were unable to isolate it as a clone through the SELEX procedure, we were able to amplify it from the ligation mixtures of our final selected target DNAs used in the transformations into *E. coli*. To obtain further evidence that *sig1* is a target of BldD, electrophoretic mobility shift assays were carried out. The promoter region of the gene upstream of *sig1* was amplified using the oligonucleotides MAE70 and MAE69 (Table 2.4) as primers, and J1501 chromosomal DNA as template. The resulting 197 bp fragment, extending from −188 to +9, relative to the translation start site of the putative anti-sigma factor regulator of *sig1*, was end-labeled, and incubated with 0-40 pmol of BldD in the presence of excess nonspecific DNA [poly d(I-C)]. The unbound DNA and protein-bound DNA were separated on 8% polyacrylamide gels, and a single, shifted fragment was observed, with virtually all the labeled DNA bound after the addition of 20 pmol of BldD (Fig. 3.7.21). Competitive mobility shift assays revealed that the addition of 500 fold excess

Fig. 3.7.21 Electrophoretic mobility shift of BldD binding to the promoter region of *sigI*. End- labeled probe, MAE69-70 (1-2 ng/8-10 fmol), was incubated with 0-40 pmol of BldD (as indicated), for 20 minutes at 30°C. The resulting products were separated on a 1.5 % glycerol, 8% polyacrylamide gel, buffered with 1 × TBE, and the resulting free DNA and BldD-complexed DNA are indicated. Binding specificity was examined by adding ~500 ng/6 pmol of unlabeled, nonspecific competitor DNA (BKL41-MAE5, internal to the *bldD* coding sequence) to the fifth reaction lane, and ~500 ng/4 pmol of unlabeled, specific competitor DNA (MAE69-70) to the final reaction lane.

BldD (pmol):	0	10	20	40	40	40
Nonspecific DNA (ng):	0	0	0	0	500	0
Specific DNA (ng):	0	0	0	0	0	500

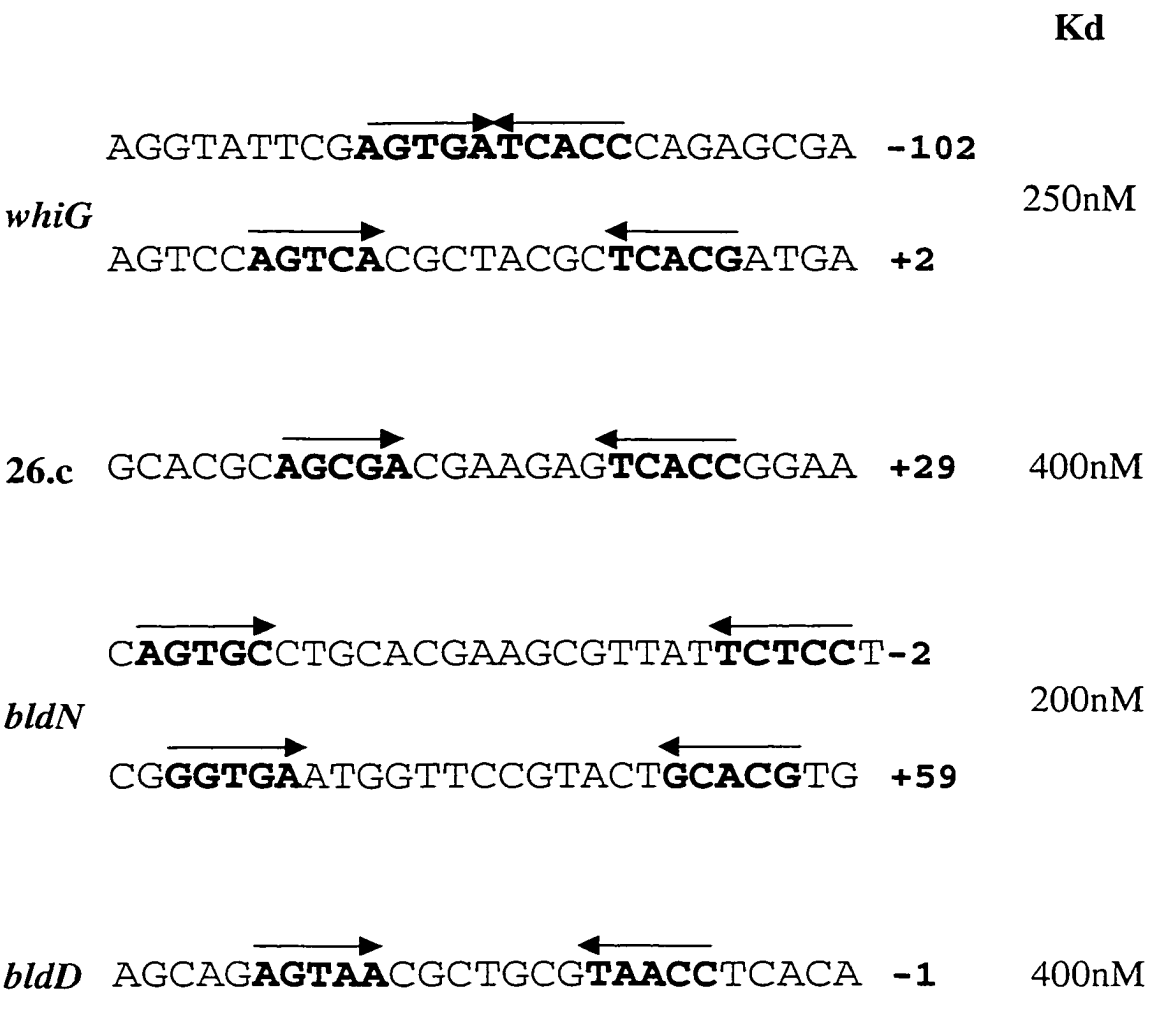


nonspecific DNA (BKL41-MAE5; a 118 bp DNA fragment found internal to the *bldD* coding sequence) had no effect on BldD binding to the labeled *sig1* fragment, and as expected, the addition of 500 fold excess specific target DNA (MAE70-69) virtually abolished BldDbinding to the labeled fragments. These results suggest that *sig1*, and its putative associated anti-sigma factor, are direct targets of BldD, and that BldD recognizes and binds a single site in the promoter region of the operon. An approximate K_d was determined to be 200 nM.

3.8 BldD consensus sequence alignment

The BldD binding sites in the various BldD targets that were identified in this study, were examined and compared, in order to determine whether BldD recognized a consensus binding sequence. The two BldD binding sites from *whiG* and *bldN*, and the single sites from *bldD* and ORF 26.c, were aligned by eye, and a BldD consensus binding sequence was assigned (Fig. 3.8.1). It appears that BldD recognizes targets possessing an imperfect palindrome, AGTgA (n) TCACc, whose repeats are separated by a spacer of variable distance and sequence. The putative BldD binding sequence identified in *ftsZ*, which may or may not represent a direct target of BldD, corresponded fairly well with this imperfect palindrome, with 7 out of 10 bases matching the proposed palindromic sequence. The implications of the variable spacing between the two halves of the palindrome will be discussed in detail in the Discussion.

Fig. 3.8.1 Compilation of 6 known BldD binding sites. The nucleotide sequences of 4 BldD targets are shown, with putative BldD recognition sequences bolded. The arrow heads indicate the inverted repeats within each sequence, corresponding to the proposed BldD recognition sequence for each BldD binding site. The nucleotide position shown to the right of each sequence, is relative to the transcription start site for the representative gene. Approximate K_d values are shown at the far right. A BldD binding consensus sequence is shown at the bottom, with capital letters representing extremely well conserved residues, while the lower case letters indicate residues with more variance associated at those positions. The (n) represents the intervening sequence between the consensus halves, and is of a variable length.



Consensus: **AGTgA (n) TCACc**

Chapter 4

Discussion

Prior to this work, it had been determined that mutation of *bldD* resulted in a block in both antibiotic production and morphological differentiation, indicating a global influence of *bldD* in *S. coelicolor*. This thesis has focussed on illuminating the nature of this *bldD* influence, shedding light on both the regulation of *bldD* itself, and the regulation by BldD in *S. coelicolor*.

4.1 Structural features of BldD

Sequencing of the *bldD* gene revealed that *bldD* encodes a small protein of 167 amino acids, showing no homology to known proteins, and possessing no obvious functional motifs. Its small size and lack of apparent functional domains suggested it may be a unique regulatory protein, possibly a DNA binding transcription factor. It was not unexpected that traditional database searches failed to reveal any matches to a DNA-binding motif, as *Streptomyces* transcription factors often do not conform to standard helix-turn-helix motifs, possibly due to unorthodox amino acid combinations, resulting from the high G+C content of *Streptomyces* DNA (Molnar and Murooka, 1993). A manual search revealed a potential helix-turn-helix motif in the C-terminal portion of the protein, similar to that seen for LysR-like transcription regulators; however, BldD did not share any additional homology with the LysR-like proteins. LysR-like proteins (LLP) have an N-terminal DNA-binding domain rather than a C-terminal domain; the LLP are much larger than BldD, 275-325 aa residues, compared with 167 aa residues for BldD; and the LLP are often transcribed from a promoter that overlaps the promoter of a divergently transcribed target gene (reviewed in Schell, 1993), while *bldD* is separated by

>200 bp from its upstream neighbor, which appeared to encode a pyrimidine biosynthesis gene, and as such, is presumed to be unrelated to *bldD*.

In an attempt to gain further insight into BldD structure and function, and to try to authenticate the proposed existence of a BldD helix-turn-helix motif, the BldD sequence was threaded onto the folds of proteins whose three dimensional structure had been determined previously. From this, it appeared that BldD possessed a C-terminal helix-turn-helix domain, overlapping the one predicted by comparison with the LysR signature sequence. This putative C-terminal DNA binding domain was separated from the amino terminal domain by a random and unstructured region. This two-domain protein organization is a common feature among transcription factors, with a DNA-binding domain joined by a flexible connector to a second domain, having signaling and/or oligomerization function (reviewed in Parkinson and Kofoid, 1992). This proposed two domain organization of BldD could be examined using proteolytic digestion of purified BldD, as it would be expected that the random, unstructured central portion of the protein would be cleaved more readily than either of the more globular domain regions. Interestingly, there have been correlations made between the function of a transcription factor, and the domain in which the DNA-binding motif is believed to reside. A survey of known transcription factors in *E. coli*, has suggested that N-terminally localized DNA-binding domains may be associated with any of repression, activation, or dual function regulation (both activation and repression activities), while C-terminal helix-turn-helix motifs are associated with activation or dual function regulation, but not repression (Prag *et al.*, 1997). Determining whether this generalization, that C-terminal helix-turn-helix motifs are not found in transcription factors whose sole function is that of repression,

applies to *Streptomyces* transcription factors, required a compilation of transcription factors whose function and DNA-binding domains had been elucidated (Table 4.1). Analysis of these *Streptomyces* transcription factors revealed that the majority of them did conform to this model, with 9/10 repressor proteins adhering to this standard. The only exception was CutR, part of the CutRS two-component regulator system. However, this may not represent an exception because although the phenotype of a *cutR* mutant suggested a role in repressing secondary metabolism (Chang *et al.*, 1996); it had been proposed that phosphorylated CutR may in fact activate the expression of a repressor (Chang *et al.*, 1996). Using these guidelines then, it would be expected that BldD functions as an activator, or as a dual-function regulator, given the C-terminal DNA-binding domain suggested by the comparisons with LysR-like proteins, and three-dimensional structural predictions. Since BldD appears to negatively regulate its own expression, based on transcriptional analyses in the wild type and *bldD* mutant strains and the ability of BldD to bind to its own promoter, that would eliminate the possibility that BldD acts soley as an activator. It is, therefore, most likely that BldD acts as a dual function regulator.

4.2 Control of BldD modification, stability, and expression

The only known *bldD* mutation was found outside of the C-terminal domain, at position 62 of the N-terminal domain. The positioning of this Y62C mutation in a domain separate from the one involved in DNA-binding, made it unlikely that Y62 had a direct influence on any DNA-binding capabilities of BldD. Unfortunately it was not possible to test whether the mutant BldD protein could bind DNA, as it was not overexpressed in *E.*

Table 4.1 Helix-turn-helix location and protein function of Streptomyces DNA-binding proteins

Protein	H-T-H location	Protein function	Reference
AbsA2		Repressor	(Brian et al., 1996)
ActII-ORF1	N-terminus	Repressor	(Fernández-Moreno et al., 1991)
ActII-ORF4	N-terminus	Activator*	(Wietzorrek and Bibb, 1997)
AdpA	C-terminus	Activator	(Ohnishi et al., 1999)
AmfR	C-terminus	Activator	(Kudo et al., 1995)
ArgR	N-terminus	Repressor*	(Rodriguez-Garcia et al., 1997)
ArpA	N-terminus	Repressor*	(Miyake et al., 1990)
BarA	N-terminus	Repressor*	(Nakano et al., 1998)
BldB	C-terminus	Repressor/Dual function?	(Pope et al., 1998)
BldD	C-terminus	Dual function	this work
CcaR	N-terminus	Activator*	(Perez-Llarena et al., 1997)
ClaR	C-terminus	Activator	(Paradkar et al., 1998)
CrpA	N-terminus	Activator	(Onaka et al., 1998)
CrpB	N-terminus	Repressor	(Onaka et al., 1998)
CutR	C-terminus	Repressor?	(Chang et al., 1996)
DnrI	N-terminus	Activator*	(Tang et al., 1996)
DnrN	C-terminus	Activator*	(Furuya and Hutchinson, 1996)
FarA	N-terminus	Repressor	(Waki et al., 1997)
GlnR	C-terminus	Activator	(Wray and Fisher, 1993)
ImpA	N-terminus	Repressor	(Shiffman and Cohen, 1993)
LipR	C-terminus	Activator	(Servin-Gonzalez et al., 1997)
MerR	middle	Dual function?	(Brunker et al., 1996)
NrsA	N-terminus	Repressor	(McCue et al., 1996)
RamR	C-terminus	Activator	(Ma and Kendall, 1994)
RedD	N-terminus	Activator	(Wietzorrek and Bibb, 1997)
RedZ	C-terminus	Activator	(Guthrie et al., 1998)
Reg1	N-terminus	Repressor	(Nguyen et al., 1997)
StrR	C-terminus	Activator	(Thamm and Distler, 1997)
TipAL	N-terminus	Activator*	(Holmes et al., 1993)
WhiH	N-terminus	Repressor	(Ryding et al., 1998)

* Demonstrated activity

coli. The importance of the Y62 residue in BldD could range from a role in protein stability or BldD oligomerization, to an involvement in ligand interaction, or even post-translational modification. In *E. coli*, a tyrosine residue has been shown to play a critical role in the signal transduction by CheY, the response regulator in the signal transduction pathway of bacterial chemotaxis (Zhu *et al.*, 1996). Tyrosine residues have also been deemed essential for the function of the prolipoprotein diacylglyceryl transferase (required for the proper modification of *E. coli* lipoproteins), likely contributing to protein stability, or to the stabilization of one of its catalytic states (Sankaran *et al.*, 1997). Tyrosine residues are critical for protein-dimerization, and other protein-protein interactions in both prokaryotes and eukaryotes. In *E. coli*, DNA gyrase (GyrB) requires a tyrosine residue for proper dimerization (Brino *et al.*, 2000), as does an *E. coli* pyrophosphatase (Salminen *et al.*, 1999). In eukaryotes, tyrosine residues have been demonstrated to be important for dimerization in the retinoic acid response elements (RAREs; Rachez *et al.*, 1996), and the NFκB p50 homodimer (Sengchanthalangsy *et al.*, 1999) in addition to a variety of other protein-protein interactions, and these are often stimulated by tyrosine phosphorylation, such as in the STATs (signal transducers and activators of transcription) (Haspel *et al.*, 1996). Tyrosine phosphorylation was originally believed to be an exclusively eukaryotic signaling control; however, it is increasingly being found to be important for bacterial pathogens, such as *Yersinia* (Zhang, 1996) and *E. coli* (Vincent *et al.*, 1999), as well as differentiating bacteria, especially *Streptomyces*, where numerous proteins appear to be tyrosine phosphorylated (Waters *et al.*, 1994).

The possibility that BldD was controlled by phosphorylation of Y62 was examined through western blot analyses, and comparing the results obtained using BldD-specific antibodies and phospho-tyrosine-specific antibodies. It appears that BldD is not tyrosine phosphorylated, given the absence of tyrosine phosphorylation of proteins in the 15-25 kDa range, where BldD would be found. BldD expression levels were then compared with the results from western blot analyses to further examine the wild type and mutant BldD protein. It was found that the wild type BldD protein was very stable, present at virtually constant levels throughout a 48 hour time course, while transcript levels revealed *bldD* expression in the wild type strain to peak at 15 hours, and then decrease to low levels by 36 and 48 hours. The constant protein levels in the absence of continued *bldD* transcription suggests that BldD is a stable protein *in vivo.* Conversely, in the *bldD* mutant strain, constitutive overexpression of the *bldD* transcript did not translate into an overabundance of mutant protein, as the BldD mutant protein was present at a lower level than in the wild type, and was seen to decrease over time, with degradation products visible on the western blot. This suggests that the Y62 residue may play a role in protein stability. Alternatively, since BldD appears to exist primarily as a dimer in solution (discussed in greater detail later on), it is possible that Y62 is involved in oligomerization of BldD. The loss of Y62 may result in an inability to dimerize, which may in turn lead to the exposure of sequences that target BldD for degradation. This possibility is supported by the observation that mutation of the dimerization domain in the eukaryotic protein, TBP (TATA-binding protein), obviated not only dimerization, but also stimulated protein degradation, based on correlation between dimer stability and levels of TBP *in vivo* (Jackson-Fisher *et al.*, 1999).

A number of different experiments may be conducted to help gain further insight into the role played by Y62 in BldD structure and function. UV spectra could be used to determine the location of aromatic amino acids on the protein surface, versus those that are buried. It would also be of interest to overexpress the Y62C mutant protein, and examine it for residual function, dimerization capabilities, and DNA binding ability. While this mutant protein was not overexpressed using the His_6-tag system, it is possible that adjusting culture conditions or using a different overexpression scheme would permit its overexpression and analysis. Alternatively, altering the cysteine residue to another amino acid may permit overexpression. Given the apparent instability associated with this mutation, however, this may prove to be impossible. Further insight may be gained from the mutagenesis of this residue, as changing it to a phenylalanine, serine, or threonine residue may reveal whether its hydroxyl group is needed for hydrogen bonding, or whether its aromatic ring is important for some type of stacking interaction. Overexpression of these mutant proteins would allow for the examination of their oligomeric states, and their ability to bind DNA. Introduction of these mutations into a wild type *S. coelicolor* strain would permit an assessment of *in vivo* function, and the phenotypic effects associated with that function.

To gain a more thorough understanding of the mode of action of BldD in *S. coelicolor*, including how it dimerizes, how it binds DNA, and how it influences the expression of its targets, it will be important to fully characterize the BldD protein. Alanine scanning mutagenesis could be very useful in mapping the different functional domains in BldD, determining the amino acids important in DNA-binding, dimerization, and activation and/or repression. The translation of the BldD amino acid sequence into a

corresponding function, would help to further the first objective of this thesis: the characterization of *bldD* regulation, and the BldD protein.

4.3 Evidence for DNA-binding activity

Gel shift assays were originally conducted to broadly define the BldD-binding site, beginning with a 257 bp fragment spanning the *bldD* promoter region. The binding site was then narrowed down to a 70 bp area overlapping the transcription start point (tsp) and the promoter region. There were typically 2 shifted fragments observed, and the addition of more protein did not result in shifting of the majority of the DNA to the more slowly migrating position. This banding pattern could indicate a single binding site for BldD, with the two observed bands representing different conformational states of the protein or the DNA. One possibility is that a BldD dimer binds to one half site of a repeated unit, and then bends the DNA, or alters its structure in some way to permit binding to the other half site. The two fragments could represent equilibration between these two DNA structural states. It is also plausible that the two bands represent differing amounts of protein bound to the DNA, perhaps representing a BldD monomer bound to one site, and then a second monomer binding to the other site, or even binding of two dimers to the two half sites. Alternatively, the two bands could represent two completely separate binding sites.

To differentiate between single or multiple BldD binding sites, the bound area was scrutinized using DNaseI and hydroxyl-radical footprinting. DNaseI footprinting studies revealed that BldD binding resulted in a single protected footprint, extending for 42 bp on both strands, encompassing the tsp, as well as the –10 and –35 regions of the

promoter. This strongly supported the proposal that BldD binds to a single site within the *bldD* promoter. Strong protection by BldD against the hydroxyl radical was seen between the –10 and –35 regions of the promoter, suggesting that the BldD binding site was located between these two hexamers, while a weaker zone of protection was seen downstream from the tsp on one strand of the DNA. In the area defined by the DNaseI footprints and the strong hydroxyl radical protection, there were two sequences that could be recognized by BldD: a 15 bp imperfect inverted repeat (7 bp per half site), or a direct repeat, covering 17 bp (7 bp per half site). These two sequences shared significant overlap with each other, making it difficult to discern the repeat-type recognized by BldD. An attempt was made to distinguish between these two repeats, by creating DNA fragments possessing a perfect inverted or a perfect direct repeat, and examining the ability of BldD to bind to them by gel mobility shift assays. It appeared that BldD had slightly higher affinity for the perfect inverted repeat than it did for the perfect direct repeat.

There were several additional factors that suggested BldD may recognize the inverted repeat rather than the direct repeat. The first was that the protection conferred by BldD was symmetrical, with strong protection seen equally on both strands of the DNA. This suggested binding by BldD to both strands of the DNA, which would occur with binding to the inverted repeat, but not the direct repeat. BldD recognition of the inverted repeat was strengthened by the observation that the hydroxyl radical footprint generated by BldD binding was reminiscent of the footprint that resulted from binding of the Trp repressor to its target sequence, an inverted repeat, positioned such that TrpR bound one face of the DNA, in the major groove (Carey, 1989). The similarity in BldD

and TrpR protection profiles, together with the spacing of the *bldD* promoter-localized half sites of the inverted repeat, suggested that BldD may recognize a similar target, and bind in a similar fashion, to TrpR. A second line of support for BldD binding to the inverted repeat came from the determination that the C-terminal helix-turn-helix domain of BldD was weakly similar to that of the LysR-like family of DNA-binding proteins. These LysR-like proteins recognize, and bind to, a consensus sequence containing a T-N_{11}-A motif, usually found within a 15 bp partially dyadic sequence. The inverted repeat in the *bldD* promoter region also constituted a 15 bp partially dyadic sequence, within which a T-N_{11}-A motif was found. As the BldD helix-turn-helix motif bore some resemblance to the LysR helix-turn-helix signature sequence, it is quite conceivable that these proteins would recognize similar sequences.

Downstream from the inverted repeat, was found an additional half site, corresponding to that of the inverted repeat. It is possible that BldD recognizes and binds weakly to this site, given that a very small proportion of DNA containing only this fragment as a target (MAE 36-4) was shifted using gel mobility shift assays, and a weak hydroxyl radical footprint was observed in this region. Since weak binding to this site may have resulted in the appearance of the larger of the two shifted fragments seen using MAE16-4 as a probe, gel mobility shift assays were conducted using MAE1-35 as a binding target. BldD binding to this probe fragment resulted in the appearance of two shifted fragments, despite the fact that MAE1-35 did not contain the single downstream half site. It may therefore be concluded, that the appearance of the two shifted fragments, when using MAE16-4, or MAE1-35 as probe, was a result of BldD binding to a single site, most likely the inverted repeat.

In an attempt to further elucidate how BldD bound its target sequence, the inverted repeat was segmented, and the resulting fragments were used as probes in gel shift assays. It was found that there was absolutely no binding by BldD when using the MAE1-15 fragment as a target, which contained the left half site and the majority of the right, while defined binding was seen with MAE18-4, which contained the entire right half site but not the left. These results suggest that BldD binding to its target is initiated by binding to the right half site. Interestingly, BldD binding to MAE18–4 generated a single shifted band, not the 2 bands that had been observed previously for MAE16-4. This perhaps suggests that in the presence of only the right half site, a single conformation existed for the BldD-DNA complex. We had originally believed that binding by BldD would occur most readily in a dimer form, with one monomer preferentially recognizing the right half site, and this binding then serving to position the other monomer, such that it could recognize the left half site. If this were true, the 2 shifted bands observed from the gel shifts could represent equilibration between the right half bound, and both sites occupied. Unfortunately, it was not possible to determine which of the 2 shifted bands was represented in the shift attained using MAE18-4 as probe, as it differed in size and binding site position within the fragment (at the end versus in the middle), when compared with MAE16-4.

To authenticate this theory of how BldD binds, the BldD oligomeric state was examined both in solution and when bound to DNA. Using gel filtration FPLC, His-tagged BldD was confirmed to exist primarily as a dimer in solution, as has been found for many transcription factors (reviewed in Pabo and Sauer, 1992). A note of caution must be added to this observation, however, as a study into the dimerization of His-

tagged proteins suggested that the His_6-tag itself may contribute to dimerization (Wu and Filutowicz, 1999). In order to clarify this, BldD dimerization could be tested by examining the gel filtration elution profile of GST-tagged BldD, or BldD with its purification tag removed.

Although the oligomeric state of BldD in solution could not be determined conclusively, due to the potential influence of the His_6-tag, analysis of the oligomeric state when bound to DNA confirmed that it existed as a dimer, at least when bound to its own promoter. Examination of the state of BldD when bound to DNA was achieved using a series of gel shift assays, that compared the mobility of two differently sized BldD proteins (His-tagged, versus GST-tagged) when bound to DNA. Their binding was examined both individually, and when mixed together, where a heterodimeric intermediate was observed, thereby substantiating the prediction that BldD bound as a dimer. The use of the 2 different protein types in this experiment alleviated concerns stemming from the use of the His_6-BldD fusion protein, as the GST-tag of GST-fusion proteins is not known to promote dimerization.

While BldD appears to exist predominantly as a dimer in solution, and has been shown to bind DNA as a dimer, it is not clear that BldD dimerizes before binding to its target DNA. Many transcription factors bind DNA to give a dimeric protein-DNA (2:1) complex. This binding may occur through one of two different mechanisms: (1) the dimer pathway, where the proteins dimerize first, and then associate with the DNA, or (2) the monomer pathway, where 2 monomers bind DNA sequentially, and assemble their dimerization interface when bound to DNA. As transcription factors are often in a dimeric form in solution, it had been assumed that they used the dimer pathway for

assembly onto DNA. A recent study by Kohler *et al.* (1999) has cast doubt on this assumption, however, as they have demonstrated that for certain transcription factor families, the rate of DNA binding was too rapid to be accounted for by association via the dimer pathway. The monomer pathway was shown to allow for more efficient assembly of protein-DNA complexes, as nonspecific DNA competitors were observed to slow the dimer pathway, but not the monomer pathway. This monomer pathway, was, however, only observed at low protein concentrations, which may in fact be more representative of the intracellular concentration of transcription factors than the higher protein concentrations used for most *in vitro* analyses. When the oligomeric state of the BldD protein was examined using gel filtration FPLC, the majority of the protein was present in a dimer form; however, a fraction of it was observed to exist as a monomer. It may therefore be possible that BldD exists as a monomer under *in vivo* conditions.

In the case of BldD binding, however, unless binding of the first BldD monomer significantly altered the DNA structure in that region, it seems unlikely that a second monomer could recognize the left half site, as absolutely no binding was observed using the left side as a target (MAE1-15) in gel shift assays. It is, however, possible that DNA binding helps BldD to dimerize *in vivo*, as has been observed for the Arc repressor (Rentzeperis *et al.*, 1999), where it first associates with DNA as an unstructured monomer, and this association then promotes proper folding and dimerization. Alternatively, it is possible that dimer binding (i.e. less efficient binding) by BldD to the *bldD* promoter region, is a means of delaying the repression *bldD* expression, in light of the fact that many negative autoregulators bind their targets with greater affinity than they bind their own gene (Maloy and Stewart, 1993). This does not, however, appear to

always be the case for BldD, as it binds at least two of its targets with an affinity similar to that with which it binds its own promoter (see below).

The dimer/monomer binding dichotomy could be resolved by examining BldD binding to the probe fragment MAE18-4 (see Fig. 3.5.1), which contains only half of the inverted repeat presumed to be recognized and bound by BldD, using the two different BldD fusion proteins. BldD binding to this probe fragment has been shown to yield a single band in gel mobility shift assays (see Fig. 3.5.7), although the band would be at a different position depending on whether the His_6-BldD or GST-BldD fusion protein was used. A mixture of the two proteins would be expected to give at least two bands, one corresponding to the probe bound to each protein type. The presence of an additional, intermediate band would most likely represent a heterodimeric intermediate, indicating that BldD does not bind as a monomer, and must instead bind the right repeat half as a dimer.

4.4 BldD target identification and consensus binding sequence determination

The second major objective of this thesis was to determine how BldD controlled the formation of aerial hyphae and production of antibiotics. In order to do this, it was necessary to identify targets of BldD binding. To define these chromosomal targets, a variety of search techniques were utilized, including scanning for putative BldD binding sequences in the chromosomal sequences of regulatory genes involved in differentiation, and using a gel shift-based selection procedure. A number of different BldD targets were identified, including *whiG*, *ftsZ*, and ORF 26.c-ORF 25.c. Personal communication from

the labs of Drs. Mark Buttner and Charles Thompson also alerted us to the *bldN* and *sigI* targets.

The alignment of BldD binding sequences, determined for each of the identified targets (2 sites for each of *whiG* and *bldN*, and 1 site for each of *bldD* and ORF 26.c-ORF 25.c), resulted in the definition of a consensus sequence for BldD binding: AGTgA (n) TCACc. The partially dyadic symmetry of the BldD binding sequence was consistent with previous observations that BldD bound DNA in a dimer form, with each monomer recognizing one half of the inverted repeat. Many known DNA binding proteins, such as Cro and λ repressor, that exist as homodimers, bind to an inverted repeat sequence, although most homodimeric transcription factors recognize a repeat sequence with a fixed spacer length between the repeats. Flexibility in site recognition is not unheard of, however, and has been demonstrated previously for AraC, an arabinose catabolism regulator in *E. coli* (Carra and Schleif, 1993), and for CytR, a LacI family member in *E. coli* (Jørgensen *et al.*, 1998), both of which possess a flexible connection between dimerization and DNA-binding domains, allowing them to bind operator half sites separated by varying distances. Predictions of BldD protein structure have suggested that BldD is a two domain protein, with a long, disordered linker region. It is conceivable that this flexible linker allows BldD to bind sequences with a relaxed spacer requirement. If true, the relaxed/flexible spacing between the halves of the inverted repeat, and the relatively loose sequence recognition by BldD, may also have contributed to the isolation of nonspecific target DNA sequences during the SELEX procedure. An alternative explanation for the isolation of nonspecific targets, is that the DNA fragments may have been selected as a result of nonspecific interactions with the positively charged N-

terminal His-tag. This was ruled out by the conduction of experiments in which the fusion tag was replaced, and in which the same nonspecific targets were identified.

This SELEX-based protocol used to isolate ORF 26.c-ORF 25.c differed from the typical procedure, in that a chromosomal library of fragments was used as a target for BldD binding, rather than a library of random oligonucleotides. While this selection method did allow for the identification of ORF 26.c as a target, the chromosomal library used may have been incomplete, as *whiG* was not present in the selection mixture. The second difficulty with the SELEX procedure was the inability to clone the *bldN*- and *sigI*- containing fragments. The reason for this is unknown, given that for at least half of the ligations performed, it was possible to amplify them from the ligation mix. The possibility therefore remains that there are additional BldD targets yet to be identified in the chromosome that were either not represented in the chromosomal library, or were simply not selected and cloned during the SELEX procedures. Despite these difficulties, the selection of BldD targets using a chromosomal library, as opposed to one of random oligonucleotides, was deemed to be more useful, as evidence had suggested that a consensus-binding sequence determined using an *in vitro* system, does not necessarily reflect natural binding sites (Shultzaberger and Schneider, 1999); given the variable spacer region between the halves of the degenerate inverted repeats recognized by BldD, it would have been difficult to assign a strict consensus sequence. The problems encountered with this SELEX procedure could likely be overcome using a more complete library. This could be accomplished by partial digestion of chromosomal DNA with *Hpa*II, as *Hpa*II sites are abundant in the G-C rich *S. coelicolor* chromosome, to permit a more randomized library to be produced. A *Hpa*II-based library would also eliminate the

potential problem of digestion within the BldD binding site, as the BldD consensus sequence appears to be relatively A-T rich. Annealing of the linkers to a *Hpa*II-based library would not be as easily accomplished as for a *Sau*3AI library, given the 2 bp overhang generated by *Hpa*II digestion, relative to the 4 bp overhang resulting from digestion with *Sau*3AI. This difficulty could be overcome by using an excess of linker in the ligation process, and the comprehensive nature of the library could be assessed following a trial amplification, by testing for the presence of a variety of DNA fragments. Preferential amplification of some DNA species, as was observed for the ORF 26.c promoter fragment, could be reduced by using low concentrations of dNTPs, fewer PCR reaction cycles, and a reduced amount of template DNA. Hopefully the use of this new library, and more optimal PCR conditions, would allow for a more complete screening of BldD targets, on the chance that this study had not detected them all. Genetic experiments could also be conducted to identify targets of BldD, for example, by suppression of engineered BldD mutations.

For each of the BldD targets that were isolated, attempts were made to understand how BldD binding controlled their expression. It was found that for each target gene, transcription differed when comparing their expression in wild type and *bldD* mutant strains. *whiG* was bound at 2 sites by BldD, and its transcript was present at constant levels in the wild type strain, but in the mutant strain, expression levels were initially higher, and then dropped to undetectable levels. *bldN* was also bound at 2 sites, and was temporally regulated in the wild type strain, but expressed at higher levels throughout culture development in the *bldD* mutant strain. *bldD* was bound at a single site, and while it was temporally regulated in the wild type strain, it was constitutively

overexpressed in the *bldD* mutant. ORF 26.c-ORF 25.c was bound at one site, and was temporally regulated in the wild type strain, but like for *whiG*, in the mutant strain, expression levels were initially higher than the wild type, but dropped to low levels later in growth.

How does BldD affect the expression of these targets? If one assumes that the final outcome of BldD control is the same for all targets, there exist two main options. The first possibility is that BldD acts as a repressor. In the absence of a functional, or fully functional, BldD protein, this repression would be relieved, resulting in greater transcription of its target genes. This seems to be a reasonable presumption for *bldD* and for *bldN*, given the increased transcript present for both genes, at all time points in the *bldD* mutant. The lower levels of transcript observed at the later timepoints for *whiG* and ORF 26.c could be the result of a reduction in transcript stability. Such an effect could possibly be mediated through a loss of BldD control (repression) of the developmentally regulated RNase ES, which has been shown to be associated with differentiation (Hagège and Cohen, 1997), although to date there is no evidence for any regulatory connection between BldD and RNase ES. To determine whether such a connection exists, the expression of RNase ES could be compared in wild type and *bldD* mutant strains. Alternatively, to simply assess whether the decrease in transcript abundance seen for *whiG* and ORF 26.c-25.c was a result of decreased transcription, or decreased transcript stability, a promoter probe construct could be used, with the *whiG* or ORF 26.c promoter fused to a reporter gene, such as *xylE*.

Given the C-terminal placement of its helix-turn-helix motif, which suggests that BldD could not act solely as a repressor, the second option is that BldD functions as an

activator of its target genes. Considering that the BldD protein is present at constant levels throughout colony development, and neither *bldN* nor ORF 26.c-25.c are transcribed to any great level before ~24 hours, if BldD were to function as an activator, then its activity must be modulated by some sort of inducing molecule. The *bldD* point mutation that converted a tyrosine residue into a cysteine residue in the N-terminal domain of BldD, may have altered BldD such that it no longer requires the inducer molecule, and consequently has constitutive activator activity. This would explain the observed early overexpression of target transcripts in the *bldD* mutant strain, for each of *whiG, bldN, bldD,* and ORF 26.c. Western blot analyses have revealed that unlike the constant level of BldD seen throughout growth in the wild type strain, the mutant BldD protein is present at lower levels, with the protein levels decreasing between 24 and 48 hours, apparently as a result of protein degradation. The decreased expression seen for *whiG* and ORF 26.c in the 36 and 48 hour samples could reflect the decreased abundance of the BldD activator; however, the gradual decrease in the abundance of the mutant BldD protein through 24, 36 and 48 hours could not be directly correlated with the sudden decrease in transcript expression seen for *whiG* and ORF 26.c. Direct comparisons between target transcripts and protein abundance may be misleading, however, as the protein samples used for western blot analysis were harvested from different strains than was the RNA used for the analysis of these two genes. The low protein levels in the mutant strain during the later timepoints, however, fails to explain the continued expression of *bldN* and *bldD* seen at these later times, although it could be the result of increased mRNA stability, rather than continued transcription. This could be tested in the same way as mentioned above, using a reporter construct to differentiate

between continued transcription, and increased transcript stability. Finally, it seems unlikely that BldD acts solely as an activator, as there is no evidence to suggest that BldD positively regulates its own expression, given that transcription was seen to peak at 15 hours, and then decrease.

If the BldD target genes are subject to differential regulation, then it is possible that BldD acts as a dual function regulator, having both activator and repressor capabilities. The most reasonable explanation for the early overexpression of transcripts in all BldD targets is that BldD represses all of its targets at an early stage of colony development. With respect to *bldN* and *bldD*, BldD likely maintains its repressing role, since for both genes we see higher levels of expression in the later stages of development in the *bldD* mutant than was observed in the wild type strain. For *whiG* and ORF 26.c-ORF 25.c, however, BldD appears to have an activation role in late colony development, given that in the *bldD* mutant strain, the transcript levels for these genes falls off in these later timepoints. This potential dual function regulation of *whiG* and ORF 26.c-ORF 25.c is not unprecedented, as an increasing number of proteins have been found to be capable of binding within the promoter region of their target genes, and acting both as repressors and activators when bound to the same sequence. MerR, which regulates the mercury-detoxification genes (Ansari *et al.*, 1995), is perhaps the best studied of these proteins; however, the same characteristics have been found to be exhibited by others, including PcaR in *Pseudomonas putida*, which controls the degradation of protocatachuate (Guo and Houghton, 1999), and Pip from *Streptomyces pristinaespiralis*, involved in the regulation of pristinamycin multidrug resistance (Salah-Bey and Thompson, 1995). These proteins all share similar binding positions, usually binding to a partially

palindromic sequence within the spacer between the –10 and –35 hexamers, although binding may also overlap the –10 or –35 sequences, or even extend downstream from the transcription start site. They bind initially as repressors, and are converted into activators by the binding of a small inducer molecule. The targets of these dual function regulators all appear to possess suboptimal spacing between the –10 and –35 sequences, and in many cases, it is believed that the activator function of these proteins serves to optimize the orientation of the –10 and –35 promoter elements, to enhance the formation of an open transcriptional complex.

In the case of *whiG*, BldD binds to two sites, one of which is localized to the *whiG* promoter, overlapping the –10 promoter sequence. Interestingly, the –10 and –35 promoter elements in the *whiG* promoter are separated by 22 bp, which would be considered suboptimal, given the optimum spacing of 17 bp (reviewed in Fassler and Gussin, 1996). These characteristics would both point to a MerR-like mechanism of regulation. MerR binds its target promoter initially as a repressor, and introduces a bend into the DNA. Binding of an inducer triggers a conformational change in MerR that results in the unbending and unwinding of the promoter region, serving to align the –10 and –35 promoter elements so that RNA polymerase can initiate transcription. Unlike the MerR-like proteins, however, *whiG* had a second BldD binding site, ~100 bp upstream from its transcription start site. It is possible that this upstream site helps to mediate the effects of BldD bound at the promoter-localized site. The initial repression of *whiG* could occur, like for MerR, by bending of the DNA; however, repression would not be complete in this instance, as *whiG* is still transcribed in the wild type strain. Binding of an inducer of some sort, may permit conversion of BldD to an activator form, with the

promoter-based site possibly helping to align the –10 and –35 sequences for RNA polymerase recognition, and the upstream site, perhaps acting to enhance RNA polymerase contacts with the nonconsensus –35 sequence. The only downside to this theory is that there was no indication of any DNA bending in the DNaseI footprinting assays, which would be evidenced as DNaseI hypersensitivity sites. To investigate whether the *whiG* promoter fragment is indeed bent by BldD binding, circular permutation assays could be conducted, whereby a series of identically sized DNA fragments could be generated, containing the binding site of interest situated at various locations within each fragment, and gel shift assays would then be used to compare the relative mobilities of the protein-bound fragments (Miller *et al.*, 1997).

An alternative theory to explain *whiG* regulation, would involve a completely different control mechanism, where repression is manifested through binding to the promoter localized site, while activation is mediated through the upstream binding site, or conversely, that repression was imposed by binding to the upstream site, and activation was controlled by binding to the promoter localized site. A better picture of the impact of BldD binding at each of these sites will await mutagenesis of each site, to determine the effect on *whiG* expression. Another option would be that BldD is simply a repressor of *whiG*, and that the absence of transcript at 36 and 48 hours is simply the result of the loss, or the absence, of a BldD-dependent sigma factor (or activator of some sort) required for *whiG* transcription at these later times.

The other BldD target that appears to be subject to dual regulation is ORF 26.c-ORF 25.c, as it is expressed prematurely in the *bldD* mutant strain, suggesting a loss of repression, but late in colony development, when transcription peaks in the wild type

strain, transcript levels fall off abruptly in the *bldD* mutant, suggesting a loss of activation. Conforming to the MerR-like characteristics of binding site placement and suboptimal spacing in the promoter, the ORF 26.c-25.c hexameric promoter elements are separated by 18 bp, rather than the ideal of 17 bp, and ORF 26.c-25.c is bound by BldD at a single site, downstream from its transcription start site. Interestingly, DNaseI footprinting data for ORF 26.c revealed regions of DNaseI hypersensitivity within the promoter region, upstream from the protected area, in the presence of BldD. This enhanced cleavage suggested that the DNA structure had been altered in this area, likely through bending of the DNA by BldD. DNA bending could, again, be confirmed by circular permutation assays. It is possible that when acting in a repressive capacity, BldD, like MerR, bends the DNA, preventing association of the RNA polymerase with the promoter region, and limiting its ability to initiate transcription. MerR requires an inducer molecule to stimulate its activator function, and a similar situation may exist for BldD, which may require such an inducer to generate a conformational shift that would then permit the initiation and progression of transcription. One possible scenario is that without the initial bending of the DNA, early expression of ORF 26.c would be seen. This is, in fact, what was observed experimentally. However, BldD must be involved in more than just the unbending of the DNA to permit activation, for if this were its sole activation function, then in the absence of functional BldD, transcription would still be seen in the later time points, and it is not. It may be, that upon binding of an inducer, not only does BldD unwind the DNA, but also interacts with the RNA polymerase, as has recently been demonstrated for MerR (Kulkarni and Summers, 1999). Potential inducers may include small peptide signals, ions, or A-factor-like compounds (γ-butyrolactones).

Alternatively, BldD may recruit a second activator to the ORF 26.c promoter, or may be required for the expression of a sigma factor, or some other activator, that is needed for ORF 26.c expression at 48 hours.

While BldD binds *bldN* at two sites, its effects must be exerted in a manner different from that for *whiG*, given the different positioning of the binding sites, one within the *bldN* promoter, and the other downstream from the *bldN* transcription start site, and the apparent absence of BldD activator function, as *bldN* is overexpressed at all times throughout development in the *bldD* mutant strain. Recall that as for ORF 26.c, DNaseI footprinting of the *bldN* promoter region in the presence of BldD revealed regions of hypersensitivity between the two BldD binding sites on both the template and nontemplate strand. This again suggests that DNA bending is mediated by BldD binding, and that this bending may result in repression of *bldN* expression.

The DNaseI hypersensitivity sites seen for BldD binding to *bldN* (and perhaps ORF 26.c) could be further examined for evidence of phasing, indicative of DNA loop formation (Hochschild, 1991), and the binding site placement could be altered to determine what, if any, effect is seen.

The final BldD target that was identified and investigated in this study was *ftsZ*. In *S. coelicolor*, *ftsZ* is not an essential gene (McCormick *et al.*, 1994), which is unusual, as it is required for viability in all other bacteria where it has been identified (Dai and Lutkenhaus, 1991; Beall and Lutkenhaus, 1991); however, it is needed for proper sporulation to take place, as synchronous septation is required for the formation of unigenomic spores (McCormick *et al.*, 1994). Gel mobility shift assays showed that while BldD did bind the *ftsZ* promoter region, it did not bind nearly as well as it did its

other identified targets, although binding appeared to be specific. Transcript analyses, however, suggested that while the BldD mutation had no impact on *ftsZ* expression from its vegetative promoter, it did negatively affect the expression of the "sporulation-specific" transcript, suggesting a possible activation role in *ftsZ* expression from its sporulation promoter. This BldD influence could be direct, although it seems unlikely given the poor binding observed with the gel shift assays, or it could be more indirect. It had been shown previously (Flärdh and Chater, 1998) that a number of *whi* genes, including *whiG*, affected the expression of the *ftsZ* sporulation transcript, so it is possible that the impact of the *bldD* mutation on the expression of *ftsZ*, was mediated through its effects on *whiG* rather than direct BldD control. Future experiments to determine whether the BldD regulation of *ftsZ* is direct or indirect might include DNaseI footprinting, which would allow determination of the site bound by BldD. As it is not clear, at this stage, whether BldD directly influences *ftsZ* expression, it will not be discussed in great detail.

Given all of the above observations, the question that begs addressing here, is what determines whether BldD acts as a repressor or an activator, and why is it that certain promoters, like *bldN*, remain unaffected by the induction of BldD activation? The lack of activator activity cannot be attributed to the number of BldD binding sites, as ORF 26.c and *bldD* have a single BldD binding site, and *whiG* and *bldN* have 2 binding sites, and yet for each pair, one appears to be repressed while the other appears to be dually regulated. The absence of activation also cannot be ascribed solely to the spacing of the half sites bound by BldD, as the *bldD* and ORF 26.c half sites share similar spacings, and yet again, appear to be regulated differently. The ultimate determinant is

probably a combination of binding site locations, and ability of BldD to recognize its inducer. For *bldN,* the two BldD binding sites are in close proximity. As BldD binding to these sites is believed to induce bends in the DNA, as evidenced by the DNaseI hypersensitivity, it is conceivable that the BldD dimers bound at each site bend the DNA to permit interaction with each other. This in turn would allow the dimers to form a tetrameric complex, which may then be incapable of recognizing an inducer molecule. BldD has been shown to exist in a tetrameric form in solution, although it predominantly exists as a dimer (this could be confirmed using analytical ultracentrifugation). To investigate the possibility that BldD binds *bldN* such that the BldD dimers bound at each of the two binding sites interact to form a tetrameric complex, protein-protein crosslinking could be used. Subsequent binding of another protein to *bldN* may relieve the repression by BldD under wild type circumstances. A candidate for this may be the *S. coelicolor adpA* homologue, since in *S. griseus*, AdpA activated the expression of *adsA/ bldN,* when bound in a position identical to the site bound by BldD, downstream from the transcription start site. It will be interesting in future experiments, to determine whether AdpA and BldD can bind the *bldN* promoter simultaneously, or whether there is competition between the two proteins for their binding sites. One caveat to the AdpA influence on *bldN* in *S. coelicolor*, is that the expression of the *adpA* homologue is dependent on *bldA*, as it contains a TTA codon, however, *bldN* expression does not depend on *bldA* (Bibb *et al.*, 2000).

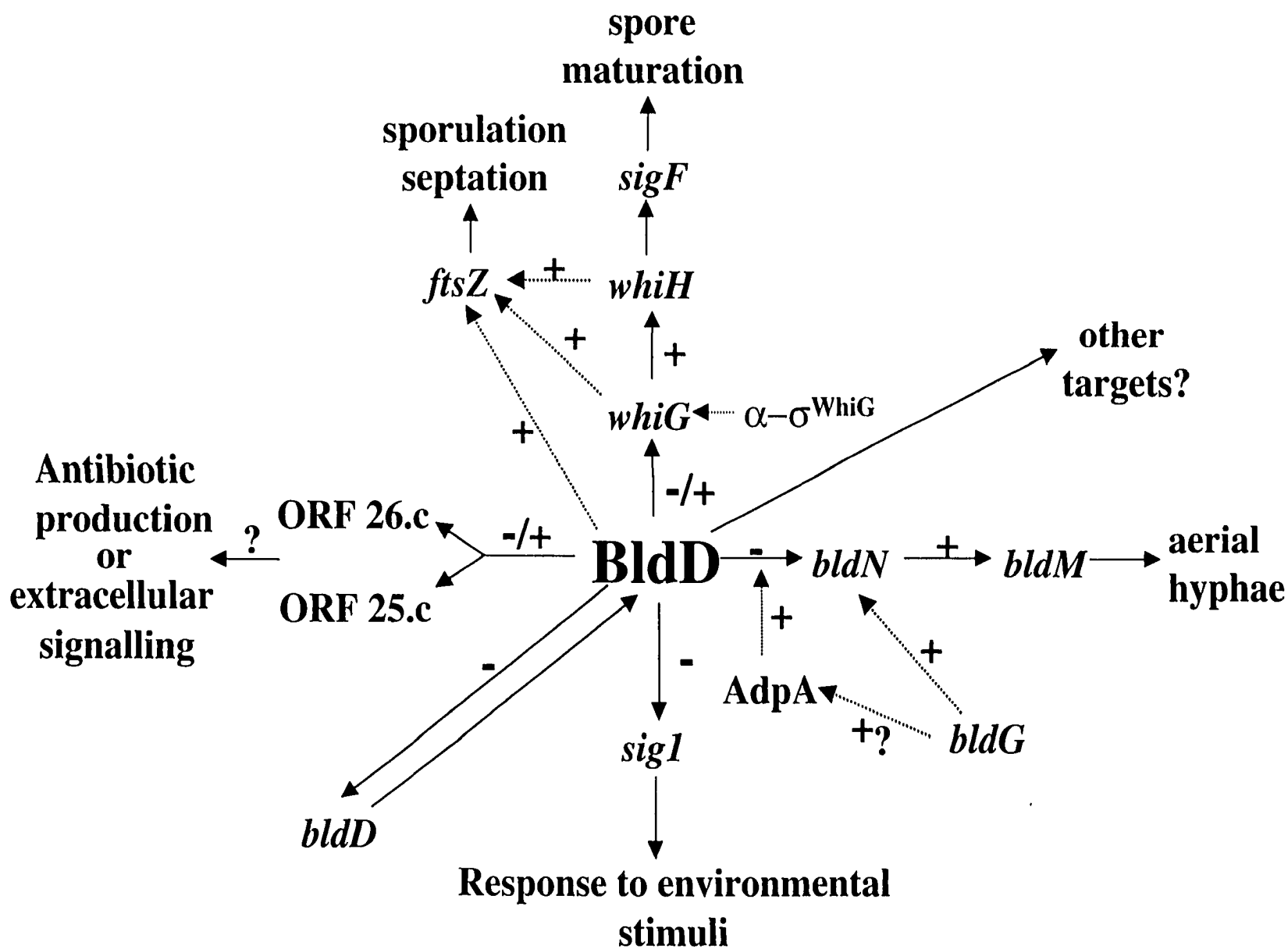
In the case of *bldD*, the other repressed BldD target, BldD binds its own promoter at a site directly overlapping the –10 element of the promoter. Even if BldD was in a conformation capable of recognizing an inducer, its binding position in the –10 region

would likely preclude the ability of the RNA polymerase to recognize the promoter and/or initiate transcription. The –35 promoter element is believed to be involved in promoter recognition by the RNA polymerase, while the –10 region is thought to be involved in promoter recognition, as well as open complex formation of the promoter, where DNA unwinding initiates (Fassler and Gussin, 1996). By binding to this site, BldD may prevent promoter recognition by the RNA polymerase, or may prevent open complex formation, regardless of whether it is in an active state due to association with an inducer molecule.

Given all of the observations concerning the expression of the BldD target and the location of the BldD binding sites, it seems most plausible that BldD is responsible for the repression of *bldN*, and *bldD*, and is somehow involved in repressing, and later activating, the expression of *whiG* and ORF 26.c. In each case, it will be necessary to do a number of mutagenesis experiments, involving mutation of each BldD binding site, and observing both the *in vitro* and *in vivo* effects of a reduction, abolition, or improvement of BldD binding. Also, in order to determine the mode of repression, it will be important to do binding assays using both purified BldD and purified RNA polymerase, to determine whether BldD repression is accomplished by occlusion of RNA polymerase binding, or whether it is achieved through some other means.

The final question that must be addressed is how BldD regulation of each of these genes impacts differentiation in the wild type strain, and how it influences the observed phenotype of the *bldD* mutant strain (see Fig. 4.1 for a summary). *whiG* is normally expressed at a constant level, and it was unexpected that it appeared to be differentially

Fig. 4.1 Compilation of all known BldD targets, and the proposed effects of BldD on their expression. Activation is indicated with a plus (+), while repression is shown as a minus (-). Solid lines indicate direct regulation, while dotted lines indicate indirect regulation, or direct regulation that has not yet been demonstrated. Question marks denote hypothesized pathways or effects. Sigma factors are denoted as σ, while the anti-sigma factor is shown as α–σ.



regulated both early and late in colony development. As the *whiG* sigma factor (σ^{WhiG}) is also believed to be subject to post-translational control via an anti (α) -sigma factor (Kelemen *et al.*, 1996), BldD may keep the *whiG* expression level such that the necessary equilibrium between σ^{WhiG} and the α–σ^{WhiG} can be maintained. In the absence of BldD, more σ^{WhiG} would be expected to be present early in colony development than would be expected in a wild type situation, and this may upset the balance between these two factors, leading to early *whi* gene expression, even in the absence of aerial hyphae. This hypothesis could be tested by examining the timing of *whiH* or *whiI* expression, as both are known to be directly dependent on σ^{WhiG} for their expression. In the later time points of colony development, *whiG* expression was seen to drop significantly, although whether this would have any impact on protein levels in these later times requires western blot analysis.

For *bldN*, it could be predicted from the transcript levels in the *bldD* mutant strain, that, like for *whiG*, *bldN* would be expressed much earlier in the *bldD* mutant than in the wild type strain. Loss of BldD repression may also abolish the need for the proposed *bldN* activator, AdpA (Yamazaki *et al.*, 2000). Interestingly, it has been found that *bldG*, encoding an anti-anti-sigma factor (Bignell *et al.*, 2000), is absolutely required for *bldN* expression (Bibb *et al.*, 2000). It is possible, that the *bldG*-regulated sigma factor is needed for *bldN* transcription, or alternatively, that it is needed for the expression of its putative activator, *adpA*. Examination of *adpA* expression in the *bldG* mutant may provide insight into the regulatory connection between these two genes. Overall, the loss of regulation by BldD would again, as for *whiG*, negatively impact the

timing of the *bldN* sigma factor expression, and consequently, the expression of its target genes, including *bldM*, and may stimulate the formation of aerial hyphae before the colony is ready to commit to the differentiation process.

As it is not known what role is played by the products of ORF 26.c and ORF 25.c, the only conclusion that may be drawn from these experiments is that they would be expressed earlier than normal, as for *whiG* and *bldN*. As ORF 26.c appears to possess a helix-turn-helix motif, it could presumably encode a transcription factor, and the timing of expression of its targets would then also be altered. ORF 25.c may encode an extracellular signalling peptide given its small size (32 aa; 4 kDa), and highly charged nature. As many exported proteins are cotranscribed with their transporters (Higgins, 1992), it is possible that ORF 26.c could serve as an activator for expression of the transporter, responsible for the export of the ORF 25.c product. This is an attractive hypothesis, in light of the proposed extracellular signalling cascade, where specific *bld* mutants are proposed to produce a factor capable of extracellular complemention of other *bld* mutants (Willey *et al.*, 1993). A number of new genes have recently been proposed to belong to the *bldD* complementation group (Nodwell *et al.*, 1999), and ORF 25.c, or ORF 26.c, may represent some of these newly classified genes. The first step in examining the function of these two genes, however, will be to ascertain the effect of their loss of function, through some form of gene disruption. Thus far, attempts to create such a disruptant strain have failed. A western blotting procedure, like the one used to detect the extracellular localization of SapB (Willey *et al.*, 1991), could also be used to ascertain whether the ORF 25.c product is found in an extracellular location.

In the case of *sigI*, it has been found that disruption of the operon-encoded anti-sigma factor located upstream from the *sigI* gene, results in a *bld* phenotype (C. Thompson, personal communication). However, we do not know what, if any, effects the mutation has on the ability of the mutant strain to produce antibiotics. We have been informed that *sigI* is repressed by BldD, so it would appear that, like for *whiG* and *bldN*, the timing of sigma factor expression might be inappropriate in a *bldD* mutant; however, as *sigI* and its corresponding anti-sigma factor are co-transcribed, what effect this would have on the expression of its target genes, is unknown.

Thus far, none of the identified BldD targets appear to have a role in antibiotic production, although the effects of ORF 26.c and ORF 25.c have not yet been revealed (and we are unaware of the effect of *sigI* on antibiotic production). It is possible that the loss of antibiotic production seen in the *bldD* mutant is simply a secondary effect of the mistimed expression of so many other genes, perhaps being affected by way of precursor availability. This has been demonstrated previously when *whiG* was placed at high copy number. Under these conditions, there was a reduction in the production of actinorhodin. Alternatively, there may be a separate global activator of antibiotic production that is regulated by BldD, perhaps ORF 26.c and/or ORF 25.c, or even an additional target not yet identified.

Overall, this project has succeeded in preliminary characterization of BldD and its effects in *S. coelicolor*. A number of BldD targets have been identified, and it appears that BldD is a major player in the timing of all differentiation processes. Despite these advances, however, a thorough understanding of the BldD regulatory cascade is still

lacking, and much work is required before full comprehension of this complex developmental cascade can be attained.

Chapter 5

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