

Library

FUNCTIONAL INTERACTIONS OF BLDB OF *STREPTOMYCES*  
*COELICOLOR*

**A DISSECTION OF THE FUNCTIONAL INTERACTIONS OF THE  
MORPHOGENETIC PROTEIN BLDB OF *STREPTOMYCES COELICOLOR***

BY

REEM SAMIR AHMED ALI, B.SC. HONS.

A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfillment of the Requirements

for the Degree

Master of Science

McMaster University

© Copyright by Reem Samir Ahmed Ali, June 2002

MASTER OF SCIENCE (2002)  
(Biochemistry)

McMaster University  
Hamilton, Ontario

TITLE: A dissection of the functional interactions of the morphogenetic protein BldB of *Streptomyces coelicolor*.

AUTHOR: Reem Samir Ahmed Ali, B.Sc. Hons. (Trent University)

SUPERVISOR: Dr. Justin R. Nodwell

NUMBER OF PAGES: vii, 35

## ※ABSTRACT

*Streptomyces* initiate a complex developmental program during their 5-day life cycle, consisting of aerial mycelium formation and antibiotic production. Several developmental genes are involved in regulating these events, one of which is *bldB*. I have complemented a *bldB* null mutant, which demonstrated the loss of aerial mycelium formation and antibiotic production, restoring both characteristics. This demonstrated that *bldB* is essential for *S. coelicolor* morphogenesis but not viability. Using a bacterial two-hybrid system devised, I screened the *S. coelicolor* genome using BldB as “bait” for binding partners of BldB. The two most compelling candidates were *bbp1*, a homologue of UspA in *E. coli*, and *bbp2*, a homologue of SrmR in *S. ambofaciens*. Furthermore, I have investigated these interactions biochemically by affinity chromatography to further elucidate the details involved in these interactions. Preliminary results showed three protein bands obtained at approximately 68kDa, 55kDa and 35kDa, respectively.

## ※ACKNOWLEDGMENTS

I would like to thank *Dr. Justin Nodwell* for granting me the opportunity of working in his lab and for his guidance throughout my time at McMaster. Thanks to all *my lab mates* for all their help; my presentations were a success because of you! It was a pleasure working with everyone. I would like to dedicate this thesis to my family, *my wonderful parents*, and *my sisters Ruba, Rabab and Aameena*. Thanks for all your support during my masters and for believing in me no matter what. I know that I will always reach for higher goals because of you. To *everyone in 3H35 and 3H42*; thanks for always putting a smile on my face and being there for me. My experience throughout my masters was much more enjoyable because of all of you. I always knew where to find advice when I needed it. To *my friends Mandy, Nilesh and Henk* who kept me going when they knew things weren't going well. I am thankful for having attended McMaster, because I had the privilege of meeting the three of you. I hope that we will continue to stay in touch. To *my dear friend Joanne* whose long phone conversations were a true blessing, and to *my dearest Sonali and Milouni* for all the good times we spent together. Finally, to *my wonderful friend Prameet* whom I owe being here to. Thanks for always pushing me, thanks for always bringing me back to reality, thanks for believing in me more than I even believed in myself and thanks for always being there for me; I truly couldn't have done it without you and I will forever be grateful to have had you brought into my life and honored to have had shared my experience at McMaster with you.

## ※TABLE OF CONTENTS:

<i>Section</i>	<i>Page Number</i>
<b>CHAPTER 1: GENERAL INTRODUCTION</b>	1
<b>CHAPTER 2: PROTEIN-PROTEIN INTERACTIONS INVOLVING BLDB</b>	8
<b>A) TWO-HYBRID SCREEN</b>	8
Introduction	8
Materials & Methods	10
Plasmids & Competent Cells	11
Results	12
Candidates	14
Effects of BldB Mutations on Binding	19
Discussion	20
<b>B) AFFINITY CHROMATOGRAPHY</b>	23
Introduction	23
Materials & Methods	23
BldB Purification	24
Preparation of <i>S. coelicolor</i> Whole Cell Extract	25
Affinity Chromatography	25
Results	26
Discussion	28
<b>CHAPTER 3: FUTURE DIRECTIONS</b>	30
BldB-Binding Partners	30
Localization of <i>bldB</i> Expression	30
Future Perspectives	31
<b>REFERENCES</b>	30

## ✳LIST OF FIGURES:

<i>Figure Title</i>	<i>Page Number</i>
<b>Figure 1:</b> The Life Cycle of <i>Streptomyces coelicolor</i>	2
<b>Figure 2:</b> Complementation of the <i>bldB</i> Null Mutant	5
<b>Figure 3:</b> Mobility Shift Assay	7
<b>Figure 4:</b> The Two-Hybrid System	9
<b>Figure 5:</b> Results from the Two-Hybrid System Screen	16
<b>Figure 6:</b> Sequence Homology of <i>bbp1</i> with Usp	17
<b>Figure 7:</b> Sequence Homology of <i>bbp2</i> with SrmR	18
<b>Figure 8:</b> Results from the Tyrosine 21 Mutants	20
<b>Figure 9:</b> BldB Purification	27
<b>Figure 10:</b> Affinity Chromatography	28

**※LIST OF TABLES:**

*Table Title* \_\_\_\_\_ *Page Number*

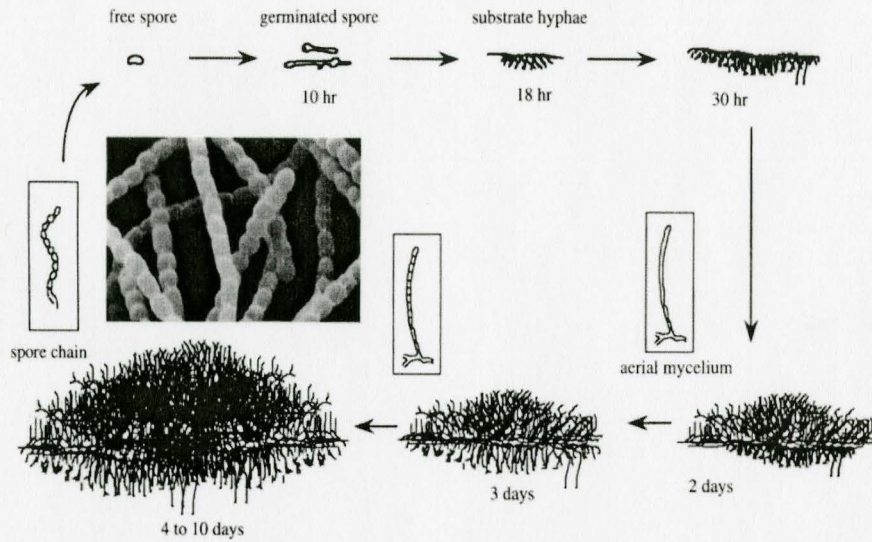
**Table 1:** A List of All the Candidates for BldB-Binding Partners  
Obtained from the Two-Hybrid System Screen 15



## ※ CHAPTER 1: GENERAL INTRODUCTION

*Streptomyces* are filamentous soil bacteria that initiate a complex morphogenetic program involving structural and biochemical changes during their 5-day life cycle (Fig. 1). Initially, these bacteria grow vegetatively on agar media as branching substrate hyphae. After 24-36 hours of growth, they produce aerial hyphae, which eventually coil and septate giving rise to spores, a developmental change accompanied almost simultaneously by the production of various antibiotics. Both antibiotic production and aerial mycelium formation are regulated by a well-coordinated network of genes (Bibb, 1996; Chater, 1998; Pope *et al.*, 1998; Kieser *et al.*, 2000) .

It is this morphological complexity and capability of antibiotic production that initiated the interest in genetic analysis of *Streptomyces* (Chater, 1998; Kieser *et al.*, 2000). The members of the genus *Streptomyces* are responsible for the production of almost half of all known microbial antibiotics (Bibb, 1996). *Streptomyces coelicolor* produces four different antibiotics using different regulatory mechanisms and biochemical pathways influenced by varying physiological and environmental factors (Hobbs *et al.*, 1992; Hood *et al.*, 1992). Exploring the different developmental genes involved in the tight regulation of the biochemical pathways leading to such morphological events will develop an understanding of controlling these mechanisms of differentiation.



**Figure 1: The life cycle of *Streptomyces coelicolor*. The vegetative, branching mycelial mass erects aerial hyphae, which in turn coil and septate into uninucleoid compartments giving rise to spores (Chater, 1998).**

There are several developmental and regulatory genes involved at the different levels of differentiation within the *S. coelicolor* life cycle. The *whi* genes (*whiA*, *B*, *G*, *H*, *I*, and *J*) (Hopwood *et al.*, 1970; Chater, 1972; Chater & Merrick, 1976; Chater *et al.*, 1989; Tan *et al.*, 1998), for instance, are known to be responsible for the initiation of cell division in aerial hyphae, giving rise to prespore compartments. Other events, such as sporulation, were found to be under the control of various sigma factors, such as sigma factor F ( $\sigma^F$ ) (Potuckova *et al.*, 1995).

Of these developmental genes, some of the more interesting ones are those that are involved in both differentiation and production of secondary metabolites. Mutations that block the early stages of morphogenesis, resulting in a failure to produce aerial mycelia and antibiotics, are called the *bld* (bald) mutants (Chater, 1998). Several *bld* genes have

been identified (*bldA*, *B*, *C*, *D*, *G*, *H*, *I*, *K*, *M* and *N*) (Champness, 1998; Chater & Merrick, 1976; Merrick, 1976; Nodwell *et al.*, 1996; Willey *et al.*, 1991, 1993), of which all mutants were found to be defective in the production of SapB, a small extracellular protein secreted by Bld<sup>+</sup> strains, which acts as a morphogen associated with aerial mycelia, facilitating their growth (Willey *et al.*, 1991; Chater, 1998). There have been some evidence suggesting a role for signaling amongst these mutants.

When some *bld* mutants are grown in close proximity to one another, the production of SapB and in turn the restoration of aerial mycelium formation in one mutant can be induced by another – a phenomenon called extracellular complementation (Willey *et al.*, 1993). Each mutant secretes signals up to its point of deficiency and responds to signals downstream of it. This was interpreted by Willey *et al.*, 1993, as evidence for a signaling cascade involving at least five extracellular signals, where each of the mutants is deficient in the production of one of these signals (Nodwell *et al.*, 1996, 1998 & 1999).

The aerial hyphae and antibiotic production in some *bld* mutants can also be restored upon growing on a poor carbon source. For instance, on mannitol, aerial mycelium formation is restored for *bldA*, remaining defective in antibiotic production (Champness, 1988), while the ability of *bldH* to produce both aerial mycelia as well as antibiotics is restored (Merrick, 1976). *bldB*, however, is the *bld* mutant that remains the most defective, failing to produce both aerial mycelia and antibiotics regardless of the carbon source (Champness, 1988; Merrick, 1976; Pope *et al.*, 1996).

Many *bld* mutants, such as *bldA*, *C*, *D*, *G* and *H*, have been shown to be defective in the regulation of carbon utilization, or the regulation of the galP1 promoter, a glucose

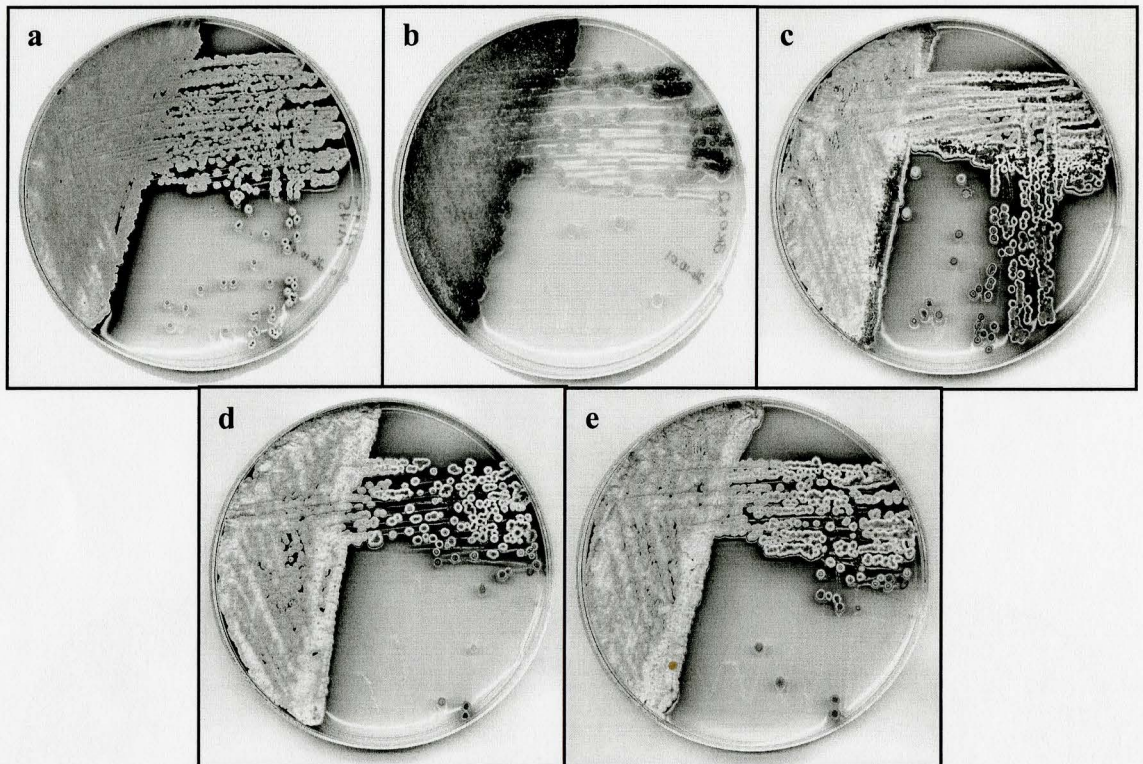
sensitive and galactose dependent promoter that directs the expression of the galactose utilization operon (Pope *et al.*, 1996). The *bldB* mutant, however, represented the *bld* mutant with the most defective phenotype. It is the only *bld* mutant resulting in the most severe consequences during the *S. coelicolor* life cycle, failing to produce antibiotics, failing to initiate morphogenesis, and remaining defective in the regulation of carbon utilization (Pope *et al.*, 1996).

Several *bldB* point mutants, all of which resulted in a bald phenotype, have been described (Pope *et al.*, 1998). These all suggested that *bldB* plays a significant role in *S. coelicolor* morphogenesis involving aerial mycelium formation and antibiotic production.

To confirm the significance of *bldB* in *S. coelicolor*, a null mutant of *bldB* was constructed (Eccleston *et al.*, accepted for publication in the “Journal of Bacteriology”, 2002) where the *bldB* gene was completely replaced with a neomycin resistance marker. The null mutant resulted in a loss of aerial mycelium formation and antibiotic production, thereby giving a phenotype that lacked the fuzzy white, or gray, appearance to the colonies and produced dark pigmentation of the media at a much delayed rate.

I constructed the complementation plasmid, pRA1, containing the *bldB* gene and its promoter, and transformed it into the null mutant to reintroduce *bldB* back into the *S. coelicolor* chromosome (Eccleston *et al.*, accepted for publication in the “Journal of Bacteriology”, 2002). The complemented mutant displayed wild type phenotype shown by wild type M145, consisting of restored aerial mycelium formation and regular antibiotic production (Fig. 2). This confirms the observations in the initial point mutants

and suggests that the role of BldB in *S. coelicolor* is significant and linked to such morphogenetic changes.

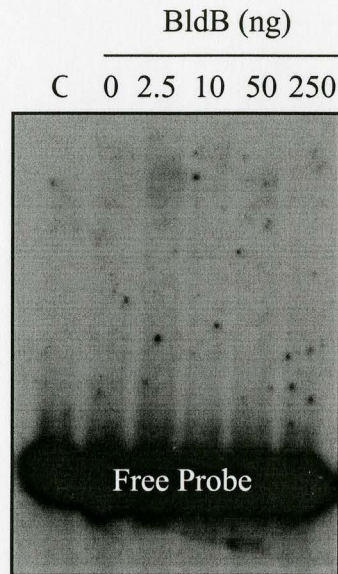


**Figure 2: Complementation of the *bldB* null mutant where (a) is wild type M145, (b) is the *bldB* null mutant, defective in aerial mycelium formation and delayed in antibiotic production, (c) is the control plasmid pSET152 transformed into wild type M145, (d) is the complementation plasmid, pRA1, transformed into wild type M145 and (e) is the complemented null mutant, with aerial mycelium formation and regulated antibiotic production restored.**

BldB is a protein of unknown function, with some homologues in *S. coelicolor*, including WhiJ and AbaA, but no known homologues in nonactinomycetes (Gehring *et al.*, 2000). Investigating the levels of *bldB*-specific reverse transcripts, Pope *et al.*, 1998, observed the presence of low levels of *bldB* RNA during vegetative growth and their

increase at the onset of morphogenesis. Furthermore, an increase in *bldB* expression in strains containing a *bldB* mutation was observed by the same group, suggesting the *bldB* gene product, a 99 amino acid protein, might regulate *bldB* transcription. Protein threading studies suggested the presence of a helix-turn-helix motif in the carboxyl terminus of the predicted BldB protein structure, common to many DNA-binding proteins (Pope *et al.*, 1998). This preliminary data, suggested the similarity of *bldB* to *arbB* in *Bacillus Subtilis*, where the ArbB protein plays a key role in gene expression involved in the transition between vegetative and stationary-phase growth, binds its own promoter and represses its own synthesis during vegetative growth (Strauch *et al.*, 1989). Combining these theories with results obtained from RNA analysis, Pope *et al.*, 1998, suggested that BldB is a DNA-binding protein, and that the *bldB* promoter may be a target for its activity and for the control of its own transcription.

We tested this theory using a mobility shift assay with the *bldB* promoter as a probe for binding BldB protein. The promoter fragment of *bldB* was divided into two segments, 100bp each with an overlapping region of 20bp, both amplified, radioactively labeled, and used to probe samples of increasing BldB concentrations for DNA-binding (Fig. 3). Results observed showed no shift with either probes (data for probe 2 not shown). The possibility of DNA as a BldB-binding partner was therefore not supported. Experiments by other investigators have also failed to detect a direct interaction of BldB with DNA (private communication from “Dr. Janet Westpheling”).



**Figure 3: Mobility Shift Assay (10-day exposure).** The first lane represents the control sample where no BldB was added (C); the remaining lanes represent samples of increasing BldB concentration, resulting in no shift as observed with the control.

In the absence of DNA-binding there was no obvious hypothesis to explain BldB's mechanism of action. Furthermore, studies in the Nodwell lab have shown evidence for BldB dimerization (Eccleston *et al.*, accepted for publication in the "Journal of Bacteriology", 2002). My goal was therefore to try to identify proteins that interact with BldB using a two-hybrid system and further investigate these proteins biochemically by affinity chromatography.

## **※CHAPTER 2: PROTEIN-PROTEIN INTERACTIONS INVOLVING BLDB**

### **PART A: TWO-HYBRID SCREEN**

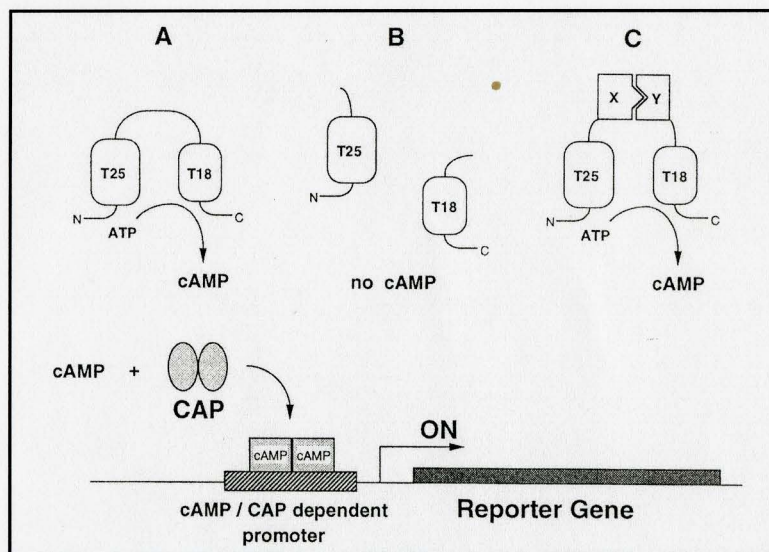
#### **Introduction:**

The two-hybrid system that we used for this work was devised by Ladant and colleagues based on the activation of adenylate cyclase in a *cya* null mutant strain of *Escherichia coli* (Karimova *et al.*, 1998). In wild type *E. coli*, cAMP produced by adenylate cyclase is required for the activation of sugar metabolism. The catalytic domain of *Bordetella pertussis* adenylate cyclase can be divided into two fragments; T18 and T25 encoding the amino- and carboxy-terminal halves of the domain. When expressed in a *cya* strain of *E. coli*, the two fragments are incapable of interacting and therefore have no activity. However, when fused to peptides that interact with each other, the two fragments are brought together producing the native enzyme which restores cAMP synthesis (Fig. 4). This in turn activates the expression of genes involved in sugar metabolism, such as the maltose regulon, where the utilization of maltose can be assayed using MacConkey media supplemented with maltose.

This particular two-hybrid system was chosen for the various advantages it presented. This screen represented a technique utilizing specific clones for identifying protein-protein interactions. It relies on the generation of a regulatory molecule in *E. coli*,



cAMP, through the interaction of two polypeptides, which unlike the yeast two-hybrid system, do not need to be in the vicinity of the activation pathway in which cAMP is involved (Karimova *et al.*, 1998). This will allow the identification of interacting proteins present anywhere in the cytosol or inner-membrane, and analyze their colocalization (Karimova *et al.*, 1998). The use of *E. coli* in this screen presents a higher efficiency for transformations, allowing the use of this system in library screens, such as the one conducted in this case, using a specific protein as “bait” (Karimova *et al.*, 1998). Furthermore, the screen allows the fusion of any gene of interest to the cAMP/CAP-dependent promoter, where the detection of the protein-protein interaction is represented phenotypically on MacConkey media supplemented with the appropriate sugar.



**Figure 4: The Two-Hybrid System devised by Ladant and colleagues, 1998. A) The two segments forming the adenylate cyclase protein bind to form cAMP. This in turn binds CAP which activates the reporter gene. B) In *cya* deficient *E. coli* strains, the two segments are unable to bind, preventing cAMP production. C) When these segments are fused to peptides that bind each other, they become active, therefore enabling cAMP production (Karimova *et al.*, 1998).**

For the purposes of screening the *S. coelicolor* chromosomal DNA library for any BldB-binding partners, the *bldB* gene was fused to the T18 fragment of the *cya* gene to produce plasmid T18-BldB. This was used as “bait” to screen a library of the *S. coelicolor* genome genetically fused to the T25 fragment. Any interaction resulting from the binding of BldB to any polypeptide sequence encoded by the library was expected to cause the activation of adenylate cyclase, and consequently the production of cAMP, in turn causing CAP and activate the maltose regulon involved in the catabolism of maltose. Hence, in a *cya*<sup>-</sup> *E. coli* strain, maltose fermentation can be restored through the complementation of the T18 and T25 fragments via BldB and any of its possible binding partners.

This system had been previously utilized in the Nodwell Lab for the purposes of demonstrating dimerization properties of BldB (Eccleston *et al.*, accepted for publication in the “Journal of Bacteriology”, 2002). In this case, the *bldB* gene was fused to both the T18 and T25 fragments, respectively, resulting in a pink phenotype indicating an interaction on MacConkey media supplemented with maltose. This interaction was, therefore used as a positive control throughout the screen against all possible candidates.

## **Materials & Methods:**

The *S. coelicolor* chromosomal DNA library, consisting of approximately 90,000 clones, was constructed by Nancy Sheeler. Chromosomal M145 DNA was digested with *DpnII* and gel purified. The pT25 plasmid was digested with *PstI* and *KpnI* to remove the *BamHI* site present following the T25 fragment. An in-frame *BamHI* site was introduced

by annealing two oligonucleotides containing a *PstI* site and a *KpnI* site at their respective ends. Ligating the purified chromosomal DNA into the newly formed pT25 plasmid allowed the introduction of this DNA into the in-frame *BamHI* site, which biases for the correct reading frame. The *bldB* plasmid and the tyrosine mutants were constructed by Marcus Eccleston (Eccleston *et al.*, accepted for publication in the “Journal of Bacteriology”, 2002). The *cya<sup>-</sup>* *E. coli* strain DHP-1, was obtained from Ladant and colleagues (The Pasteur Institute). MacConkey agar and maltose were obtained from Difco (Mississauga ON). Ampicillin was obtained from Sigma (Oakville ON), and a 50mg/mL stock was filter-sterilized and stored at 4<sup>0</sup>C. Chloramphenicol was obtained from Sigma (Oakville ON), and a 34mg/mL stock was stored at 4<sup>0</sup>C.

### **Plasmids & Competent Cells:**

The *S. coelicolor* genomic library, consisting of approximately 90,000 independent clones, was inserted into the pT25 plasmid (containing *cat*) using different ligation reactions. This DNA, T25-CL, was transformed into Novagene super comp cells. The colonies produced were pooled and their DNA was prepared using a QIAGEN Midi-Kit. Each transformation resulted in one library aliquot, 33 aliquots in total, each consisting of approximately 2000 – 5000 clones and stored at –20<sup>0</sup>C. The *bldB* gene was cloned into the high-copy number pT18 plasmid (containing *bla*). This DNA, T18-BldB, was prepared as above. The BldB tyrosine 21 mutants and amino-terminal deletion mutants were constructed in the high-copy number pT18 plasmid.

Four types of competent cells were required for the two-hybrid system screen. The first group consisted of the *cya*<sup>-</sup> *E. coli* strain DHP-1 lacking the pT18-BldB plasmid, prepared for heat-shock transformations (DHP-1). The second group consisted of the *cya*<sup>-</sup> *E. coli* strain DHP-1 containing the pT18-BldB plasmid, prepared for eletrotransformations (T18-BldB/DHP-1<sup>E</sup>) (as per the manufacturer's instructions). The third group consisted of the same cells as the ones in the second group, prepared using the protocol for calcium chloride competent cells (T18-BldB/DHP-1<sup>C</sup>) (Sambrook *et al.*, 1989). The fourth group consisted of *E. coli* strain XL-1 Blue, prepared in the same manner as the first group of competent cells (XL-1).

For testing the tyrosine mutants, four additional groups of competent cells were prepared, each consisting of the *cya*<sup>-</sup> *E. coli* strain DHP-1 containing a T18-Mut plasmid, respectively (T18-A/DHP-1, T18-C/DHP-1, T18-L/DHP-1 and T18-F/DHP-1).

## **Results:**

The screening procedure included several measures taken to eliminate false positives and verify the credibility of the results observed. Initially, each library aliquot was transformed into T18-BldB/DHP-1<sup>E</sup> competent cells, plated on MacConkey medium supplemented with 1% maltose, 100µg/mL ampicillin and 30µg/mL chloramphenicol, and incubated for 48 hours at 30<sup>0</sup>C. Approximately 1500 colonies were obtained per plate of transformation, most of which were white while many were red. All pink or red colonies were restreaked on MacConkey medium supplemented with 1% maltose, 100µg/mL ampicillin, and 30µg/mL chloramphenicol, each with a positive control, T25-

BldB, and a negative control, DHP-1 competent cells. Most colonies exhibited no red phenotype upon interacting. Approximately 7% maintained their pink phenotype, which ensured that the initial screening step was indeed necessary for eliminating false candidates.

Each colony that maintained a pink phenotype was used to inoculate 2mL of LB broth supplemented with ampicillin and chloramphenicol, and incubated with shaking overnight at 37<sup>0</sup>C. DNA was prepared from these cultures, transformed into XL-1 competent cells, and plated on LB agar supplemented with chloramphenicol. One isolated colony was used to inoculate 2mL of LB broth supplemented with chloramphenicol and incubated with shaking overnight at 37<sup>0</sup>C. In parallel, the same colony was used to inoculate 2mL of LB broth supplemented with ampicillin and incubated with shaking overnight at 37<sup>0</sup>C. This step allowed the separation of the T25-CL plasmid containing the interacting candidate (which confers chloramphenicol resistance), from the T18-BldB plasmid (which confers ampicillin resistance). Cultures that demonstrated chloramphenicol resistance and ampicillin sensitivity were used to mini-prepare the respective DNA, where 1mL of the culture was stored at -20<sup>0</sup>C, while the other was used for the mini-preparation.

Each candidate clone was then transformed into T18-BldB/DHP-1<sup>C</sup> competent cells, plated on MacConkey medium supplemented with 1% maltose, 100µg/mL ampicillin and 30µg/mL chloramphenicol, and incubated for 48 hours at 30<sup>0</sup>C to retest its capacity to bind. By testing each candidate in DHP-1 without the BldB bait plasmid, I was able to identify clones of the *S. coelicolor* adenylate cyclase gene. False candidates at this point

in the screen dropped out at the rate of 1 out of every 3 candidates. By carrying out these controls therefore, I was able to verify the credibility of the screen and the candidates obtained. The DNA from colonies that again revealed an interaction with BldB was purified by a QIAGEN Midi-Kit and sent for sequencing analysis (Molecular Biology Central Facility, McMaster University, Hamilton ON).

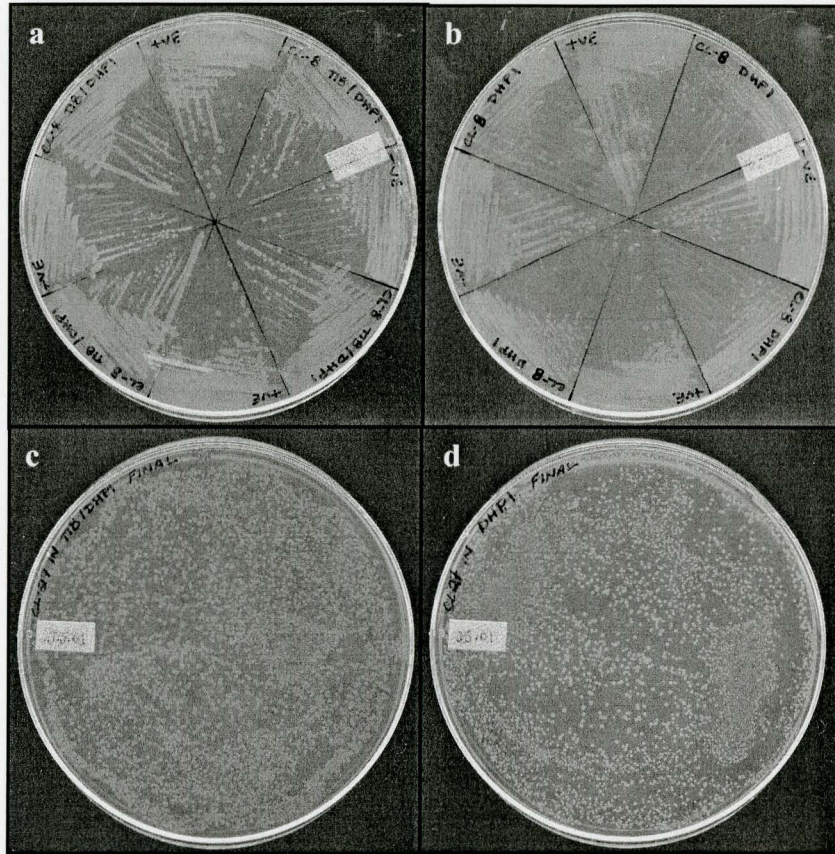
### **Candidates:**

Twelve candidates exhibiting the characteristics described above, were obtained from the screen and analyzed by sequencing (Table 1) using an oligonucleotide that sequenced into the library candidate fused to the T25 fragment in the pT25 plasmid (Eccleston *et al.*, accepted for publication in the “Journal of Bacteriology”, 2002). The candidates from library aliquots 4 and 23 corresponded to two different hypothetical proteins of unknown functions, highly rich in aspartic acid and glycine residues, within *S. coelicolor*. The candidates from library aliquots 7 and 27(#2) corresponded to two polypeptide fragments within *S. coelicolor*, which may not have interacted in frame or in the correct orientation with BldB. The candidate from library aliquot 22(#1) corresponded to a homologue of ChiR, a two-component response regulator involving ChiS and ChiC in *Streptomyces thermoviolaceus* (Kormanec *et al.*, 2000). The candidate from library aliquot 28 corresponded to the adenylate cyclase protein of *S. coelicolor*, and the candidate from library aliquot 14 corresponded to a 99 amino acid, asparagine-rich polypeptide of unknown function. While we do not rule these out as bona fide BldB-binding proteins, we suspect that these interactions may be artifactual.

**Table 1: A list of all the candidates for BldB-binding partners obtained from the two-hybrid screen.**

Library Aliquot Number	Identity	<i>S. coelicolor</i> Library Cosmid
4	Hypothetical Protein	SC7A1.09
7	X	SCE41.20c
8	UspA <i>homologue</i>	SCJ12.12c
14	99 amino acid Polypeptide	SC66T3.16
22 ( <i>candidate 1</i> )	ChiR <i>homologue</i>	SC26G5.21c
22 ( <i>candidate 2</i> )	SrmR <i>homologue</i>	SC2G2.24c
23	Hypothetical Protein	SCD10.01c
27 ( <i>candidate 1</i> )	SrmR <i>homologue</i>	SC2G2.24c
27 ( <i>candidate 2</i> )	Y	SC6D7.32
27 ( <i>candidate 3</i> )	SrmR <i>homologue</i>	SC2G2.24c
27 ( <i>candidate 4</i> )	SrmR <i>homologue</i>	SC2G2.24c
28	Adenylate Cyclase	SCK13.20

The two most compelling candidates, however, were those obtained from library aliquots 8 and 22(#2), named bbp1 and bbp2 respectively, for BldB-binding protein. Those corresponded to homologues of the Universal Stress Protein family of proteins, Usp, best studied in *E. coli*, and SrmR of *Streptomyces ambofaciens*, respectively. It is also interesting to note that the candidate from library aliquot 22(#2) was obtained three more times from library aliquots 27(#1), 27(#3) and 27(#4), independently. These two candidates can be seen in Fig. 5 streaked out in parallel with positive and negative controls for comparison of phenotypes. All of these successful candidates resulted in a pink phenotype in the presence of BldB and a white phenotype in the absence of BldB, indicating that BldB is responsible for the interaction and hence the pink phenotype observed. The significance of all of these interactions is discussed in a later section.



**Figure 5: Results from the two-hybrid system screen. Two successful candidates obtained through the two-hybrid system screen, from aliquots 8 (a & b) and 27 (c & d), corresponding to the Usp and SrmR homologues, respectively. Both were transformed in the absence of BldB, resulting in a white phenotype (b & d; respectively), and in the presence of BldB, resulting in a red, positive phenotype (a & c; respectively).**

Using a Blast program from NCBI, homology analysis was performed between the two candidates obtained through the screen, and Usp and SrmR, seen in Fig. 6 and 7, respectively. There was approximately 60% homology between Usp and its homologue from *S. coelicolor*, with two Usp ATP-binding domains in frame with the candidate obtained from the screen, and 40% homology between SrmR and its homologue from *S. coelicolor*.



<i>S.coel.</i> hit	-----MARTITVGLDGSPE\$R\$AAAEWA	22
<i>S.coel.</i> prot.	MSEDEWPYGAQLRDTLESEGP\$T\$REVRDMTRTITV\$GIDGSPESHAAAEWA	50
<i>M.tb.</i> prot	-----MSGRGEPTMKTII\$V\$GIDGSHAAITAAALWG	29
<i>M.tb.</i> Usp	-----MSKPRKQHG\$V\$V\$V\$GVDG\$S\$LESDAAACWG	27
<i>S.coel.</i> hit	ARECTLR\$R\$V\$P\$V\$R\$LL\$H\$V\$W\$Q\$P\$V\$E\$P\$M\$A\$Q\$A\$P\$L\$G\$A\$E\$T\$H\$Q\$H\$W\$T\$E\$R\$I\$P\$-R\$D\$T\$A\$E\$G	71
<i>S.coel.</i> prot.	AREAE\$R\$D\$L\$P\$V\$R\$LL\$H\$V\$W\$E\$P\$A\$P\$A\$V\$L\$A\$Q\$D\$S\$I\$L\$G\$A\$K\$T\$H\$Q\$H\$W\$T\$E\$R\$V\$P\$-Q\$K\$V\$G\$E\$G	99
<i>M.tb.</i> prot	VDEA\$I\$S\$R\$A\$V\$P\$L\$R\$L\$V\$S\$V\$I\$K\$--P\$T\$H\$P\$P\$D\$D\$Y\$D\$R\$D\$L\$A\$H\$A\$E\$R\$S\$I\$R\$E\$A\$Q\$S\$A\$V\$E\$A\$A	77
<i>M.tb.</i> Usp	ATDA\$A\$M\$R\$N\$I\$P\$L\$T\$V\$V\$H\$V\$V\$N\$--A\$D\$V\$A\$T\$W\$P\$P\$M\$P\$Y\$P\$E\$T\$W\$G\$V\$W\$O\$E\$D\$E\$G\$R\$Q\$I\$V\$A\$N\$A	75
<i>S.coel.</i> hit	L\$R\$L\$R\$H\$P\$G\$V\$-----E\$V\$T\$T\$E\$Q\$A\$T\$G\$N\$P\$A\$D\$A\$L\$L\$A\$G\$H\$L\$D\$A\$E\$L\$L\$V\$L\$G\$S\$R\$A\$L\$S\$G\$I\$T	115
<i>S.coel.</i> prot.	L\$R\$L\$R\$H\$P\$G\$V\$-----V\$V\$T\$S\$D\$Q\$R\$S\$G\$P\$P\$A\$D\$T\$L\$V\$R\$D\$A\$D\$G\$A\$E\$L\$L\$V\$L\$G\$S\$R\$A\$P\$S\$G\$I\$G	143
<i>M.tb.</i> prot	G\$K\$L\$V\$K\$-----I\$E\$T\$D\$I\$P\$R\$G\$P\$A\$G\$P\$V\$L\$V\$E\$A\$S\$R\$D\$A\$E\$M\$I\$C\$V\$G\$S\$V\$G\$I\$G\$R\$Y\$A	117
<i>M.tb.</i> Usp	V\$K\$L\$A\$K\$E\$A\$V\$G\$A\$D\$R\$K\$L\$S\$V\$K\$S\$E\$L\$V\$F\$S\$T\$E\$V\$P\$T\$M\$V\$E\$I\$S\$N\$E\$A\$E\$M\$V\$L\$G\$S\$S\$C\$R\$G\$A\$L\$A	125
<i>S.coel.</i> hit	G\$F\$L\$V\$G\$S\$V\$G\$Q\$S\$V\$I\$A\$R\$T\$E\$T\$P\$V\$I\$L\$V\$R\$A\$G\$E\$Q\$A\$A\$D\$E\$H\$L\$K\$D\$P\$T\$G\$I\$P\$S\$A\$A\$T\$G\$F\$R\$P\$V\$V	165
<i>S.coel.</i> prot.	G\$F\$L\$A\$G\$S\$V\$G\$Q\$S\$V\$I\$A\$H\$S\$E\$T\$P\$V\$V\$L\$V\$R\$A\$G\$E\$Q\$A\$A\$G\$E\$H\$V\$V\$D\$S\$V\$G\$V\$E\$P\$A\$A\$N\$R\$R\$R\$P\$V\$V	193
<i>M.tb.</i> prot	S\$S\$I\$L\$G\$S\$T\$A\$T\$E\$L\$A\$E\$K\$A\$H\$C\$P\$V\$A\$V\$M\$R\$S\$-----K\$V\$D\$Q\$P\$A\$S\$D\$I\$N\$W\$I\$V	154
<i>M.tb.</i> Usp	R\$G\$L\$L\$G\$S\$V\$S\$S\$S\$L\$V\$R\$R\$A\$G\$C\$P\$V\$A\$V\$I\$H\$S\$-----D\$D\$A\$V\$I\$E\$D\$P\$Q\$--H\$A\$P\$V\$L	163
<i>S.coel.</i> hit	V\$G\$L\$D\$I\$G\$S\$P\$D\$E\$A\$V\$L\$S\$F\$A\$F\$E\$E\$A\$R\$R\$R\$R\$A\$P\$L\$T\$A\$V\$R\$A\$W\$N\$L\$P\$S\$S\$Y\$T\$Y\$S\$I\$A\$A\$G\$F\$D\$P\$R	215
<i>S.coel.</i> prot.	V\$G\$L\$D\$I\$G\$S\$P\$D\$D\$G\$V\$L\$S\$F\$A\$F\$D\$E\$A\$Q\$R\$R\$G\$T\$A\$V\$H\$V\$V\$S\$G\$W\$R\$P\$P\$P\$Y\$P\$-S\$I\$T\$A\$G\$G\$V\$P\$D	243
<i>M.tb.</i> prot	V\$R\$M\$T\$D\$A\$P\$D\$N\$E\$A\$V\$L\$E\$Y\$A\$A\$R\$E\$A\$K\$L\$R\$Q\$A\$P\$I\$L\$A\$L\$G\$G\$R\$-----P	188
<i>M.tb.</i> Usp	V\$G\$L\$D\$G\$S\$P\$V\$S\$E\$L\$A\$T\$A\$V\$A\$F\$D\$E\$A\$S\$R\$R\$G\$V\$E\$L\$I\$A\$V\$H\$A\$W\$S\$----D\$V\$E\$V\$V\$E\$L\$P\$G\$L\$D\$F	209
<i>S.coel.</i> hit	E\$E\$L\$A\$R\$A\$Q\$A\$E\$A\$L\$G\$E\$A\$L\$L\$P\$W\$R\$E\$K\$Y\$P\$D\$V\$E\$V\$T\$E\$T\$C\$R\$L\$G\$S\$P\$A\$E\$H\$L\$I\$D\$A\$A\$R\$D\$A\$S\$I\$V	265
<i>S.coel.</i> prot.	Q\$G\$V\$A\$R\$R\$K\$A\$A\$D\$L\$T\$R\$L\$L\$P\$W\$R\$Q\$S\$Y\$P\$D\$V\$E\$V\$V\$E\$A\$S\$R\$P\$G\$S\$P\$A\$D\$L\$L\$A\$E\$A\$S\$H\$E\$A\$S\$I\$V	293
<i>M.tb.</i> prot	E\$E\$L\$R\$E\$I\$P\$D\$G\$E\$F\$E\$R\$R\$V\$Q\$D\$W\$H\$H\$R\$H\$P\$D\$V\$R\$V\$Y\$P\$I\$T\$H\$T\$G\$I\$A\$R\$F\$L\$A\$D\$H\$D\$E\$R\$V\$Q\$L\$A	228
<i>M.tb.</i> Usp	S\$A\$V\$Q\$Q\$E\$A\$E\$L\$S\$I\$A\$E\$R\$I\$A\$G\$W\$Q\$E\$R\$Y\$P\$D\$V\$P\$V\$S\$R\$V\$V\$C\$D\$R\$P\$A\$R\$K\$L\$V\$Q\$K\$S\$A\$S\$A\$Q\$L\$V	259
<i>S.coel.</i> hit	V\$V\$G\$R\$R\$I\$R\$R\$S\$P\$F\$G\$V\$H\$I\$G\$A\$V\$A\$H\$A\$V\$M\$H\$A\$T\$T\$P\$V\$A\$V\$V\$A\$H\$D	301
<i>S.coel.</i> prot.	V\$V\$G\$R\$R\$I\$R\$P\$S\$P\$L\$G\$V\$H\$I\$G\$A\$V\$A\$H\$A\$V\$L\$H\$R\$V\$S\$A\$P\$V\$A\$V\$V\$A\$H\$P	329
<i>M.tb.</i> prot	V\$I\$G\$G\$G\$-E\$A\$G\$Q\$L\$A\$R\$L\$V\$G\$P\$S\$G\$H\$P\$V\$F\$R\$H\$A\$E\$C\$S\$V\$L\$V\$V\$R\$R\$-	262
<i>M.tb.</i> Usp	V\$V\$G\$S\$H\$G\$R\$G\$G\$L\$T\$G\$M\$L\$L\$G\$S\$V\$S\$N\$A\$V\$L\$H\$A\$A\$R\$V\$P\$V\$I\$V\$A\$R\$Q\$S	295

**Figure 6: The sequence alignment of *bbp1* (*S. coelicolor* hit) from the two-hybrid system screen (aliquot 8) with other homologous Universal Stress Proteins.**

<i>S.coel.</i> hit	-----MSRDHAQSAETPFLELLARGASADAYEQPVLLARAEGG	38
<i>S.amb.</i> SrmR	-----	
<i>S.coel.</i> hit	PAERTDALOR--AKHLALRVRSELEGRRRREAELSALFFETAHD-----	79
<i>S.amb.</i> SrmR	----MRRLEER--FNRLALTAQSMIEYRRDREAELAALVDAACHE-----	37
<i>S.coel.</i> hit	LACLRLDAVIRRAIVQARASLLGTDVITYLSLND--PDRGDTYMRVTEGVS	127
<i>S.amb.</i> SrmR	FVAARRGKDLLESIAARRALLKLDVSYVGLHE--EDRPGTIVVLSADGNA	85
<i>S.coel.</i> hit	AARFQQLRLGMGEG-----LGGLVAQTARP--YVTDYFKDARFOH	166
<i>S.amb.</i> SrmR	VKVAESYRIPADGG-----LGAMVVRTCRAP--FWTPDYLGDNSFTH	124
<i>S.coel.</i> hit	TGTIDAGVRDEGLVALLGVPMLMGHHVIGVLFADRRARVFEREQIALLG	216
<i>S.amb.</i> SrmR	VEAVDDIVRAEGLRAVLAVPLCAGGEPMGVLYVADRQVRHLTPNEVTLLC	174
<i>S.coel.</i> hit	SFAALAAAAIDTANLLAETRSLAACLERANEILRDRSGVIERASDVHDLR	266
<i>S.amb.</i> SrmR	SLADLAAVAIERNRIVEELHDTIGQLRQDIGEARTALARTRRSADLQSHL	224
<i>S.coel.</i> hit	AELVLRGGGVHDVAAAVSQVLDG-----TVEFTEATAAPEYALEASRA	309
<i>S.amb.</i> SrmR	VTQVMDRRCADSLATAAEALGG-----GAGLCSPLGRPLAAYGTLRP	267
<i>S.coel.</i> hit	DC-----HVVRHKDDWIAAVAAGDEIFCALVLRGHPG-----LDPVDQ	347
<i>S.amb.</i> SrmR	VAPTELRAACRRAAETGRPTSVAIPGVWTVPLIPGGNAGFLLTDLGPDADH	317
<i>S.coel.</i> hit	LTLEERAAMVTSLLLLARRSAAEAEQVR--GELLDDLLDAR----DRDPR	391
<i>S.amb.</i> SrmR	TAVPLLPVARTLALHILRVQHDDSPKAQSHQEFFDDLIGAP-----RSPT	362
<i>S.coel.</i> hit	LIDDRARRLHADLSATHVLAARLDATETDADQEAARRRLWAAAASHLAA	441
<i>S.amb.</i> SrmR	LIRERALMFSLSFRRPHVVLVA-----GGPRGTSPRLDRSGADYAK	403
<i>S.coel.</i> hit	TRHGLAAARDGG----TVLLPLPLTSDSATDLARRIARHLGRAVHEAVTV	487
<i>S.amb.</i> SrmR	ELGGLCVSRDGA----VVLLPLPGDDE---VAVAQTAAPELTDRAGHPVTV	446
<i>S.coel.</i> hit	GASAPVRDLAARPEAVAAAYTEAGRCIDALRLLGRGGDGA AAEDFCFLGL	537
<i>S.amb.</i> SrmR	GVAGPASTVDG----IADAHREAAKCLETLRALGGDGGTACASDLGFLGM	492
<i>S.coel.</i> hit	LIAGDQDVPGFVDRITICQVWAYDERRGTELVRTLDAYFACGMSPARTKEA	587
<i>S.amb.</i> SrmR	LLAEBENDVPGYIRTTIGPVVDYDTHRFTDILVPTLIRVYLESGRSPTRAAET	442
<i>S.coel.</i> hit	LHVHINTVAQRLERVGRLLG-----DDWQSPDRALETQLAIRLHRLSAP	631
<i>S.amb.</i> SrmR	LIRVHPNTVSRRLERICVLLG-----EDWQSPERVIDIQLAIRLYQVRSAP	486

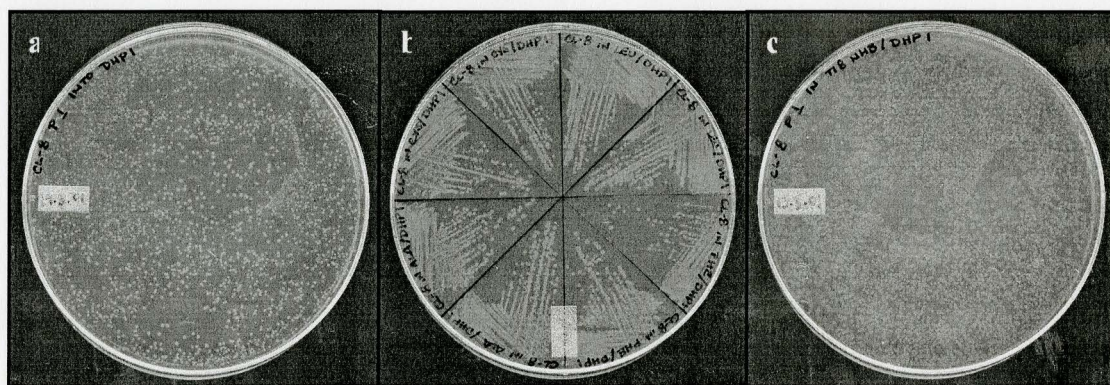
Figure 7: The sequence alignment of *bbp2* (*S. coelicolor* hit) from the two-hybrid system screen (aliquot 27) with SrmR of *S. ambofaciens*.

### **Effects of BldB Mutations on Binding:**

Two of the point mutants in *bldB* documented by Pope *et al.*, 1998, altered the same tyrosine residue at position 21, and suggested its significance for *bldB* function. Tyrosine residues are known to mediate protein-protein interactions through enabling hydrogen bonding, which was suggested to help in the multimerization of BldB or the interaction of BldB with other proteins (Pope *et al.*, 1998). The tyrosine 21 residue of BldB was mutated to 4 other residues (Eccleston *et al.*, accepted for publication in the “Journal of Bacteriology”, 2002), modifying its hydroxyl group that is suggested to be the main mediator of these interactions. These mutations replaced the tyrosine 21 residue with an alanine, cysteine, leucine and phenylalanine, in the context of the T18 plasmid to be tested against all the successful candidates obtained from the two-hybrid system screen. If this tyrosine residue was necessary for protein interactions with BldB, mutations in this residue should disrupt these interactions resulting in a white phenotype on MacConkey plates supplemented with maltose, similar to that obtained in the absence of BldB.

Each *bldB* mutant’s ability to support the apparent interaction with each candidate was tested in DHP-1. Each candidate was transformed into each of the T18-Mut/DHP-1 competent cells, plated on MacConkey medium supplemented with 1% maltose, 100µg/mL ampicillin, and 30µg/mL chloramphenicol, and incubated for 48 hours at 30°C. As a positive control, each candidate was transformed into the T18-BldB/DHP-1 competent cells, in parallel. As a negative control each candidate was transformed into DHP-1 competent cells alone.

The BldB tyrosine 21 mutants did not affect the interactions with the candidates obtained from the two-hybrid screen. All candidates produced a pink phenotype in the presence of all four tyrosine 21 mutations, a result similar to that obtained in the presence of wild type BldB. Figure 8 represents the results obtained when testing the tyrosine mutants against the Usp homologue; data for other candidates is not shown.



**Figure 8: Results from the tyrosine 21 mutants. One successful candidate from the two-hybrid system screen, *bbp1*, transformed in the absence of BldB (a), in the presence of BldB (c), and in the BldB Tyr21 mutants (b), where each quarter of the plate represents a different mutation in the Tyr21 residue (clockwise from the top midpoint: Leu, Phe, Ala, and Cys), all resulting in a red phenotype.**

## Discussion:

All of the results obtained from the two-hybrid system screen of the *S. coelicolor* genomic library represent possible BldB binding partners, with some representing more likely candidates than others. Such results as those candidates from library aliquots 7 and 27(#2) (X and Y respectively) represent polypeptide fragments which may not have interacted in frame, or not in the correct orientation with BldB, and hence represent

artifacts. Other candidates corresponding to other hypothetical proteins represent possible binding.

BldB has an overall negative charge of approximately  $-15$  ( $pI = 4.1$ ). Some of the candidates obtained from the two-hybrid system screen as possible binding partners may represent meaningful interactions with BldB, yet some may represent a charge interaction rather than a functional interaction. The two most likely candidates were therefore those corresponding to the homologues of Usp and SrmR. These proteins have been well studied in bacteria such as *E. coli* and *S. ambifaciens*, respectively, yet little is known about their homologues in *S. coelicolor*.

UspA is an *E. coli* protein belonging to the large family of universal stress proteins found in all bacteria. UspA in *E. coli*, 15.8kDa, is synthesized during growth inhibition caused by starvation for carbon, nitrogen, sulphates and phosphates or by a variety of toxic reagents (Freestone *et al.*, 1997). Its expression is shut off during nutritional upshifts and is shown to increase during periods when growth ceases, therefore suggesting its production to be related to growth phase rather than growth rate (Diez *et al.*, 1997). The function of UspA was also thought to be related to prokaryotic defense against aging as it causes decreased growth rate under minimal conditions. Mutants of *uspA* were found to have the impaired ability to survive complete and prolonged growth inhibition caused by starvation and stress and therefore UspA may have a general protective function related to growth arrested state (Freestone *et al.*, 1997). The possibility then, that BldB might interact with a UspA homologue could be consistent

with a role for BldB in starvation or other stress responses. This will suggest that starvation is an important trigger for aerial mycelium formation in *S. coelicolor*.

SrmR is a *S. ambofaciens* regulatory protein found to regulate the repression of the *srmG* gene, which is involved in the synthesis of the macrolide antibiotic spiramycin. By regulating the accumulation of transcripts from *srmG*, *srmR* acts as a putative regulatory gene for spiramycin biosynthesis (Geistlich *et al.*, 1992). Mutations in *srmR* prevent the regulation of transcripts from *srmG*, thereby preventing spiramycin production. Therefore, the product of *srmR* is thought to act as a transcriptional activator of the *srmG* promoter (Geistlich *et al.*, 1992). SrmR is also thought to represent a prototype for a new class of regulatory proteins involved in the early stages of antibiotic production. The binding of BldB then to an SrmR homologue presents a possibility consistent with the significance of BldB for antibiotic production as shown earlier by complementation.

The BldB tyrosine 21 residue appeared to have no effect on the interactions observed through the two-hybrid screen. Previous literature had suggested the involvement of this tyrosine 21 residue in BldB interactions. Results obtained from this screen prove otherwise, and suggest this tyrosine residue to be involved in other intercellular interactions possibly involving BldB. These however, will not be detected through a genetic screen, as the changes they may infer may be more biochemical instead of phenotypic and will therefore require more detailed biochemical assays.

## **PART B: AFFINITY CHROMATOGRAPHY**

### **Introduction:**

To strengthen the results of the two-hybrid screen, I carried out some biochemical experiments in an effort to pull out BldB partners by an independent technique. BldB protein had been previously purified in the Nodwell Lab (Eccleston *et al.*, accepted for publication in the “Journal of Bacteriology”, 2002) as an N-terminal his-tagged protein. I repeated the purification technique with an extra incubation step with *S. coelicolor* cell extract prior to elution. This allowed BldB present on the column to interact with its binding partners present in the extract, and hence co-elute.

### **Materials & Methods:**

The pBB801 plasmid containing the his-tagged BldB was used to express his-tagged BldB in *E. coli* BL21 DE3 (Eccleston *et al.*, accepted for publication in the “Journal of Bacteriology”, 2002). The QIAGEN Nickel Resin was purchased from QIAGEN (Mississauga ON). Tris HCl was obtained from Roche Diagnostics (Laval QC). Sodium Chloride was obtained from Bioshop (Burlington ON). Imidazole, ampicillin, lysozyme, protease inhibitor cocktail, DNase, and RNase were all purchased from Sigma (Oakville ON). Glycerol was obtained from BDH (Toronto ON).

**BldB Purification:**

The strain containing the pBB801 plasmid was streaked out on a Luria-Bertani (LB) agar plate supplemented with ampicillin and incubated overnight at 37<sup>0</sup>C. One isolated colony was used to inoculate 50mL of LB broth supplemented with ampicillin and incubated with shaking overnight at 37<sup>0</sup>C. Two 4L flasks, each containing 1L of LB broth supplemented with ampicillin, were inoculated with 10mL of the previously grown 50mL culture and incubated with shaking until OD<sub>600</sub> reached a value of 0.6. Expression of *bldB* was induced with 1mM IPTG for 3 hours, incubated with shaking at 37<sup>0</sup>C. The cells were harvested by spinning at 6000rpm for 15 minutes at 4<sup>0</sup>C, and resuspended in a total of 25mL of freshly prepared lysis buffer. The cells were disrupted by sonication at 40% for 3 cycles of 30 seconds alternating with 3 incubations on ice. Lysed cells were incubated with 2 units of DNase and 10μg/μL of RNase on ice for 30 minutes. Cells were then harvested by spinning at 12000rpm for 30 minutes at 4<sup>0</sup>C. The supernatant was incubated with 8ml of QIAGEN Nickel Resin while rotating for one hour at 4<sup>0</sup>C. This mixture was poured into a 30mL column, set at 4<sup>0</sup>C, allowed to drain by gravity flow, and washed three time with 30mL of wash buffer (50mM Tris HCl pH 7.5, 500mM NaCl and 20mM imidazole). BldB was eluted with 10mL of elution buffer (50mM Tris HCl pH 7.5, 500mM NaCl and 500mM imidazole), and analyzed on a 12% SDS-PAGE gel for purity. Half of this sample was dialyzed into 1L of wash buffer and stored at 4<sup>0</sup>C. This was quantitatively analyzed using the Bradford Protein Assay.



### **Preparation of *Streptomyces coelicolor* Whole Cell Extracts:**

Sterile porous cellophane discs were placed onto 10 freshly poured R2YE plates. A single colony of wild type M145 was plated on each disc and incubated for 48 hours at 30°C. The resulting cells were scraped off and washed twice with 10mL of 0.85% saline. The cells were harvested by spinning at 6000rpm for 10 minutes. After discarding the supernatant, the cells were resuspended in lysis buffer at a concentration of 1mg/mL per gram of cells collected, and protease inhibitor cocktail was added to a final concentration of 1x. The extract was incubated at room temperature for a minimum of one hour, after which it was incubated on ice for all consequent steps of this protocol. The cells were disrupted by sonication for 3 cycles of 30 seconds alternating with 3 incubations on ice. Lysed cells were incubated with 1µL of DNase-free RNase at a final concentration of 0.1µg/mL and harvested by spinning at 3000rpm for 20 minutes at 4°C. After discarding the supernatant, glycerol was added to a final concentration of 30% (v/v) and the extract was distributed into microfuge tubes and stored at -20°C.

### **Affinity Chromatography:**

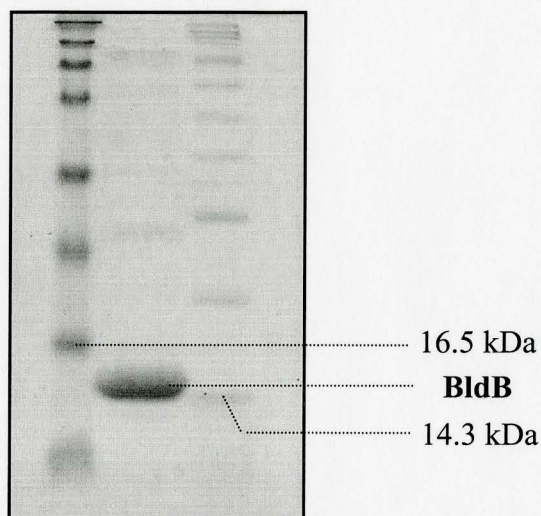
Increasing amounts of BldB (1, 2, 4, 8, 16, 20, 50 and 100µg) were incubated with 1mg of *S. coelicolor* cell extract while rotating for one hour at 4°C. Into each tube, 5µL of QIAGEN Nickel Resin were added and the mixtures were further incubated while rotating for one hour at 4°C. As a control, one tube contained cell extract and resin, lacking BldB, and another contained BldB and resin lacking cell extract. Following both incubations, the resin was harvested by spinning at 14000rpm for 5 minutes. The

supernatant was discarded and the pellet was washed twice with 50 $\mu$ L of wash buffer (50mM Tris HCl pH 7.5, 500mM NaCl and 5mM imidazole), each time harvesting the resin by spinning at 14000rpm for 5 minutes. The resin was resuspended in 10 $\mu$ L of elution buffer (50mM Tris HCl pH 7.5, 500mM NaCl and 500mM imidazole), and the elutions were collected by spinning at 14000rpm for 5 minutes. These were loaded onto a 12% SDS-PAGE gel. The remaining resin sampled were boiled in 10 $\mu$ L of SDS loading buffer and loaded on the same gel for comparison. The gel was stained using a silver staining method containing no gluteraldehyde and stored in distilled water.

## **Results:**

The purified BldB protein is shown in Fig. 9. There are some contaminants still visible on the SDS-PAGE gel, but compared to the amount of BldB present in the purified sample, these contaminants were disregarded for the purpose of the affinity chromatography experiment.

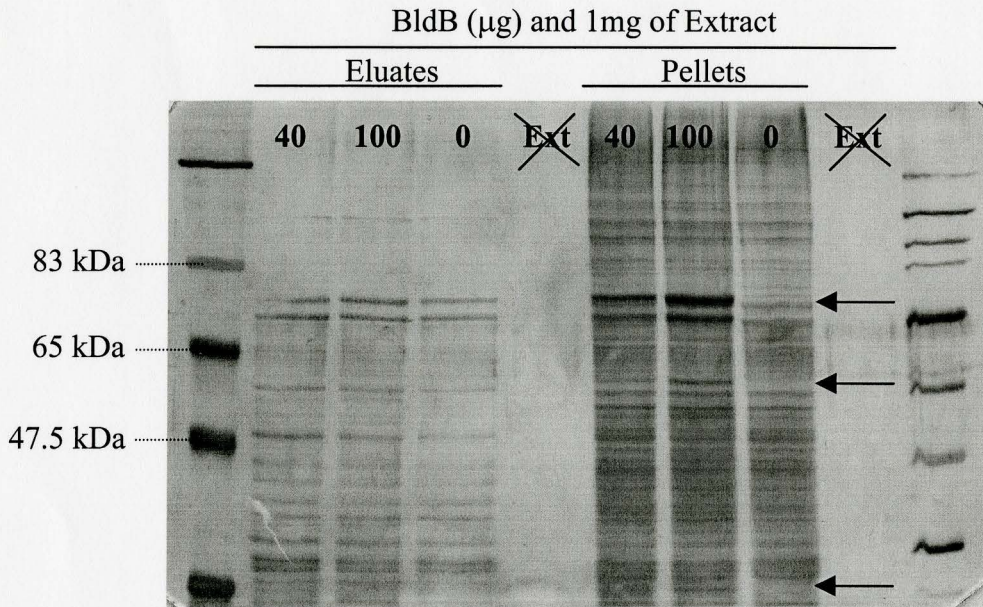
The data from affinity chromatography are not shown. The objective was to observe a band of increasing intensity corresponding to increasing amounts of BldB. The lanes containing the elutions showed no difference in the protein bands obtained throughout the different BldB concentrations used, as well as the two controls. A binding partner specific to BldB should only interact in the presence of BldB, and hence should be absent from the control in which BldB is not present. Such a band was not seen in any of the elutions. One band, however, was observed in the pellet lane containing the highest amount of BldB used, at a size of approximately 68kDa.



**Figure 9: BldB Purification.** A Coomassie Blue stain of a 12% SDS-PAGE gel showing BldB obtained at 12 kDa.

In order to verify this band and reproduce it, another affinity chromatography was performed with some minor changes to further resolve this band. Since the initial increasing amounts of BldB previously used showed no significant results, they were omitted and replaced by two others; 40 $\mu$ g and 100 $\mu$ g. The same controls were used as in the initial affinity chromatography experiment and the protocol was repeated as previously stated, with the samples loaded onto a 10% SDS-PAGE gel to enhance resolution (Fig. 10). The band was reproduced with a clearer resolution, appearing again in the pellet samples and running at approximately 68kDa. Interestingly, two additional bands appeared in the same samples, one running at approximately 55kDa and one at 35kDa. The only band that appeared intense enough for identification was the one corresponding to approximately 68kDa. This band was analyzed in the Greenblatt Lab

(University of Toronto, Toronto ON) by trypsin digestion and mass spectrometry and screened against a database for *S. coelicolor* proteins.



**Figure 10:** Affinity Chromatography performed in the presence of BldB and extract, at two different BldB concentrations, in the absence of BldB, and in the absence of extract. Three bands can be seen in the presence of BldB and extract at 68kDa, 50kDa and 35kDa.

## Discussion:

The data obtained from the mass spectrometry analysis of the 68kDa band was inconclusive. The fragments resulting from mass spectrometry matched *S. coelicolor* proteins that did not agree with the size obtained for the 68kDa band, instead the best match was to a 55kDa protein not present in the strain we used. These results suggest the impurity of the band and possible contamination of the protein of interest with other proteins present in the sample.

These results do not eliminate the possibility of this 68kDa band to represent a protein that may be a BldB-binding partner. The data obtained from the affinity chromatography experiment is preliminary and the protocol used may be refined to produce bands pure enough to analyze using mass spectrometry. The fact that this particular band analyzed runs at 68kDa, which is the size of the SrmR homologue in *S. coelicolor*, is promising, as are the other two bands obtained in this trial of the experiment. It is difficult to analyze the interactions taking place between these candidates and BldB present in the sample until these partners are identified and binding assays are performed.

## **※CHAPTER 3: FUTURE DIRECTIONS**

### **BldB-Binding Partners**

The role of the BldB-binding partners, confirmed through affinity chromatography as well as the two-hybrid system screen, must be established for their respective interactions. Knockouts of these binding partners will be constructed and complementation experiments will be performed for observing the changes resulting upon the removal and reintroduction of these genes in *S. coelicolor*. If these partners are involved in *S. coelicolor* morphogenesis, as previously observed with *bldB* complementation, the role of BldB in aerial mycelium formation and antibiotic production will be further confirmed. Details of these interactions may then be investigated using *in vitro* binding assays to elaborate on the biochemical pathways involved in these interactions.

### **Localization of *bldB* Expression**

The technique of using the Green Fluorescent Protein (GFP) in identifying where and when gene expression is activated has been previously proven to be successful in providing useful data in many studies, including one performed in the Nodwell Lab (O'Connor *et al.*, accepted for publication in “Molecular Microbiology”, 2002). Constructing a GFP transcriptional fusion to the *bldB* promoter would allow identifying

when and where *bldB* gene expression is initiated and localized. The *bldB* promoter was amplified and cloned upstream of the GFP in the plasmid pIJ8630. This newly formed plasmid was transformed into wild type M145 and tested for fluorescence. This project is currently underway where fluorescence is being tested at six different time points during the *S. coelicolor* life cycle; 5-day, 4-day, 3-day, 2-day, 1-day and 12-hour time points. The signals observed thus far have been weak and, therefore, do not present data of ideal fluorescence. However, this represents preliminary data, which can be enhanced to view better fluorescence in future projects. It is hypothesized that *bldB* will be one of the genes activated earlier in the *S. coelicolor* life cycle if it represents a significant target in morphogenesis.

## **Future Perspectives**

For future analysis of BldB functional interactions involved in its role in morphogenesis, knockouts of the possible binding partners will be constructed as previously discussed. The tyrosine 21 residue remains an issue to be further investigated. In regards to the two-hybrid system, this residue did not affect the interactions observed with the candidates resulting from this screen. However, this does not eliminate the possibility of this tyrosine residue to be involved in the function of BldB. Tyrosine residues in proteins have been known to represent sites of phosphorylation, which may be the case with BldB, depending on the mechanism of its interaction. Proteins belonging to the family of Universal Stress Proteins, such as UspA, have been shown to autophosphorylate their target proteins (Freestone *et al.*, 1997). Since one of the

candidates, *bbp1*, obtained from the two-hybrid system screen represented a Usp homologue in *S. coelicolor*, BldB phosphorylation represents an interesting possibility to be explored.

The specific mechanism of interaction remains unclear for any of the candidates representing possible binding partners. Monitoring *bldB* transcript levels using S1 nuclease mapping may prove useful in elaborating on the relationship between BldB and its binding partners. SrmR is a transcriptional regulator of *srmG*, which is directly involved with antibiotic production in *S. ambofaciens*. The SrmR homologue in *S. coelicolor* resulting from the two-hybrid screen, *bbp2*, may act in a similar manner by regulating *bldB* transcripts, where BldB was proven to be significant for antibiotic production by the complementation experiment.



## ✳REFERENCES

- Bibb, M. (1996). The regulation of antibiotic production in *Streptomyces coelicolor* A3(2). *Microbiology* 142:1335-1344.
- Champness, W. (1988). New loci required for *Streptomyces coelicolor* morphological and physiological differentiation. *Journal of Bacteriology* 170:1168-1174.
- Chater, K. F. (1972). A morphological and genetic mapping study of white colony mutants of *Streptomyces coelicolor*. *Journal of Genetic Microbiology* 72:9-28.
- Chater, K. F. (1989). Multilevel regulation of *Streptomyces* differentiation. *Trends of Genetics* 5:372-376.
- Chater, K. F. (1998). Taking a genetic scalpel to the *Streptomyces* colony. *Microbiology*, 144:1465-1478.
- Chater, K. & Merrick, M. J. (1976). Approaches to a study of differentiation in *Streptomyces coelicolor*. Academic Press, London, England.
- Diez, A. A., Farewell, A., Nannmark, U. & Nystrom, T. (1997). A mutation in the *ftsK* gene of *Escherichia coli* affects cell-cell separation, stationary-phase survival, stress adaptation, and expression of the gene encoding the stress protein UspA. *Journal of Bacteriology* 179(18):5878-5883.
- Eccleston, M., Ahmed Ali, R., Seyler, R., Westpheling, J. & Nodwell, J. (2002). Structural and genetic analysis of the BldB protein of *Streptomyces coelicolor*. *Journal of Bacteriology* (in press).
- Freestone, P., Nystrom, T., Trinei, M. & Norris, V. (1997). The universal stress protein, UspA, of *Escherichia coli* is phosphorylated in response to stasis. *Journal of Molecular Biology* 274:318-324.
- Geistlich, M., Losick, R., Turner, J. R. & Rao, N. (1992). Characterization of a novel regulatory gene governing the expression of a polyketide synthase gene in *Streptomyces ambofaciens*. *Molecular Microbiology* 6(14):2019-2029.

- Hobbs, G., Obanye, A. I. C., Petty, J., Mason, J. C., Barratt, E., Gardner, D. C. G., Flett, F., Smith, C. P., Borda, P. & Oliver, S. G. (1992). An integrated approach to studying regulation of production of the antibiotic methylenomycin by *Streptomyces coelicolor* A(3)2. *Journal of Bacteriology* 174:1487-1494.
- Hood, D. W., Heistra, R., Swoboda, U. K. & Hodgson, D. A. (1992). Molecular genetic analysis of proline and tryptophan biosynthesis in *Streptomyces coelicolor* A(3)2: interaction between primary and secondary metabolism – a review. *Gene* 115:5-12.
- Hopwood, D. A., Wildermuth, H. & Palmer, H. M. (1970). Mutants of *Streptomyces coelicolor* defective in sporulation. *Journal of Genetic Microbiology* 61:397-408.
- Karimova, G., Pidoux, J., Ullmann, A. & Ladant, D. (1998). A bacterial two-hybrid system based on a reconstituted signal transduction pathway. *Proceedings of the National Academy of Sciences* 95:5752-5756.
- Kieser, T., Bibb, M., Buttner, M. J., Chater, K. F., & Hopwood, D. A. (2000). *Practical Streptomyces genetics*. The John Innes Foundation, Norwich, England.
- Kormanec, J., Sevcikova, B. & Homerova, D. (2000). Cloning of a two-component regulatory system probably involved in the regulation of chitinase in *Streptomyces coelicolor* A3(2). *Folia Microbiology (Praha)* 45(5):397-406.
- Merrick, M. J. (1976). A morphological and genetic mapping study of bald colony mutants of *Streptomyces coelicolor*. *Journal of Genetic Microbiology* 96:299-315.
- Nodwell, J. R., McGovern K. & Losick, R. (1996). An oligopeptide permease responsible for the import of an extracellular signal governing aerial mycelium formation in *Streptomyces coelicolor*. *Molecular Microbiology* 22:881-893.
- Nodwell, J. R. & Losick, R. (1998). Purification of an extracellular signaling molecule involved in production of aerial mycelium by *Streptomyces coelicolor*. *Journal of Bacteriology* 180:1334-1337.
- Nodwell, J. R., Yang, M., Kuo, D. & Losick, R. (1999). Extracellular complementation and the identification of additional genes involved in aerial mycelium formation in *Streptomyces coelicolor*. *Genetics* 151:569-584.
- O'Connor, T. J., Kanellis, P. & Nodwell, J. R. (2002). The *ramC* gene is required for morphogenesis in *Streptomyces coelicolor* and expressed in a cell type specific manner under the direct control of RamR. *Molecular Microbiology* (in press).

- Pope, M. K., Green, B. D. & Westpheling, J. (1996). The *bld* mutants of *Streptomyces coelicolor* are defective in the regulation of carbon utilization, morphogenesis and cell-cell signaling. *Molecular Microbiology* 19:747-756.
- Pope, M. K., Green, B. & Westpheling, J. (1998). The *bldB* gene encodes a small protein required for morphogenesis, antibiotic production, and catabolite control in *Streptomyces coelicolor*. *Journal of Bacteriology* 180(6): 1556-1562.
- Potuckova, L., Kelemen, G. H., Findlay, K. C., Lonetto, M. A., Buttner, M. J. & Kormanec, J. (1995). A new RNA polymerase sigma factor,  $\sigma^F$ , is required for the late stages of morphological differentiation in *Streptomyces* spp. *Molecular Microbiology* 17:37-48.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular cloning – a laboratory manual* (2<sup>nd</sup> ed.). Cold Spring Harbor Laboratory Press, New York, USA.
- Strauch, M., Perego, M., Burbulys, D. & Hoch, J. (1989). The transition state transcription regulator ArbB of *Bacillus subtilis* is autoregulated during vegetative growth. *Molecular Microbiology* 3:1203-1210.
- Strauch, M., Spiegelmann, G., Perego, M., Johnson, W., Burbulys, D. & Hoch, J. (1989). The transition state transcription regulator ArbB of *Bacillus subtilis* is a DNA binding protein. *EMBO Journal* 8:1615-1621.
- Tan, H., Yang, H., Tian, Y., Wu, W., Whatling, C. A., Chamberlin, L. C., Buttner, M. J., Nodwell, J. & Chater, K. F. (1998). The *Streptomyces coelicolor* sporulation-specific  $\sigma^{\text{WhiG}}$  form of RNA polymerase transcribes a gene encoding a ProX-like protein that is dispensable for sporulation. *Gene* 212(1):137-146.
- Willey, J., Santamaria, R., Guijarro, J., Geislich, M. & Losick, R. (1991). Extracellular complementation of a developmental mutation implicates a small sporulation protein in aerial mycelium formation by *Streptomyces coelicolor*. *Cell* 65:641-650.
- Willey, J., Schwedock, J. & Losick, R. (1993). Multiple extracellular signals govern the production of a morphogenetic protein involved in aerial mycelium formation by *Streptomyces coelicolor*. *Genes Development* 7:895-903.