Analysis of *blm*: a gene of unclear function involved in the biosynthesis of clavulanic acid in *Streptomyces clavuligerus*

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Abstract

Streptomyces clavuligerus produces the medicinally important specialized metabolite clavulanic acid (CA), but detailed understanding of the roles played by many genes involved in CA biosynthesis is still lacking. These include the overlapping *blm (orf12*), *orf13* and *orf14* genes, disruptions of which cause defective CA production in *S. clavuligerus*. I investigated the potential polarity associated with disrupting *blm* in *S. clavuligerus*, along with the cellular localization of Blm. This was accomplished using complementation of prepared *blm* deletion mutants and western analysis of strains expressing epitope (FLAG) tagged copies of the protein. *blm*-like genes from other microorganisms containing sequenced CA gene clusters were examined for their ability to complement a *S. clavuligerus blm* in-frame mutant. Results demonstrate that the *blm* mutation is not associated with polar effects and that Blm is a cytoplasmic protein, suggesting that it could either play a regulatory or enzymatic role during CA production in *S. clavuligerus*.

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List of Abbreviations and Symbols

The following list includes the various abbreviations and symbols used throughout this thesis. Acronyms used superfluously in the life sciences are not included.

Abbreviation	Definition
β	Beta
Δ	Delta
µ	Micro
Apra	Apramycin
Amp	Ampicillin
BSA	Bovine serum albumin
CA	Clavulanic acid
CSR	Cluster situated regulators
DAD	Diode array detector
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
HPLC	High performance liquid chromatography
Hyg	Hygromycin
IPTG	Isopropyl-beta-D-thiogalactopyranoside
ISP-4	International *Streptomyces* Project Media #4
kDa	Kilodalton
LB	Lennox broth
MOPS	3-[N-morpholino]propanesulfonic acid
MYM	Maltose-Yeast Extract-Malt Extract
NAG	*N*-acetylglucosamine
NAM	*N*-acetylmuramic acid
NCBI	National Center for Biotechnology Information
OD	Optical density
Ori	Origin of replication
PAG	Polyacrylamide gel
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
SA	Starch asparagine media
SDS	Sodium dodecyl sulfate
TBE	Tris-borate-EDTA
TEMED	Tetramethylethylenediamine
Tm	PCR annealing temperature
TSBS	Trypicase soy broth, supplemented with starch
v/v	volume/volume
wt	Wild-type
w/v	weight/volume
w/w	weight/weight
YT	Yeast extract-tryptone medium

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Chapter 1: Introduction

1.1: The *Streptomyces*

The *Streptomyces* are ubiquitous Gram-positive, filamentous bacteria that dwell in diverse environments such as soil and marine sediments. Their diverse metabolic capabilities place the *Streptomyces* centrally in the environmental carbon and nutrient cycle as they are responsible for the degradation of insoluble organic material (such as lignocellulose and chitin) through the action of secreted hydrolytic enzymes (Chater et al. 2010). *Streptomyces* are unusual among prokaryotes in that they follow a complex multicellular life cycle, which begins as a single unigenomic spore that grows and germinates to produce branched substrate mycelia (Chater and Losick 1996). When nutritional stress occurs, a process of morphological differentiation is initiated by the substrate mycelia and aerial hyphae grow upward out of the vegetative mycelial mat. The aerial hyphae then begin to coil and septate, leading to the formation of mature unigenomic spores, which are resistant to many environmental factors, including desiccation and low nutrient availability (Ensign 1978). Once in a supportive environment, the spores germinate and the life cycle begins again.

Recently, a new mode of apical mycelial *Streptomyces* growth was discovered called 'exploratory growth' (Jones et al. 2017). This process involves the interaction between *S. venezulae* and fungi, whereby the latter deplete glucose in the growth medium. This change causes the *S. venezulae* colonies to transform into non-branching vegetative hyphae, which can cross biotic and abiotic surfaces. Additionally, it was found that this mode of growth can be communicated to other *Streptomyces* spp. through signalling

using Volatile Organic Compounds (VOCs) that affect the growth and exploratory behaviour of the other bacteria (Jones et al. 2017).

VOCs and other specialized metabolites (aka secondary metabolites) are produced during the process of physiological differentiation where the bacteria alter their physiology, which in most cases is coordinated with morphological differentiation (Chater 1993). Specialized metabolites are generally produced during times of slow or no growth, as they are nonessential for the growth and survival of the producing organism under laboratory conditions. Therefore, they are called specialized metabolites as opposed to primary metabolites (like amino acids, nucleotides, lipids and carbohydrates) that are essential for growth and survival. When placed in their natural environment, the production of specialized metabolites is important for Streptomycetes as it provides them with a competitive advantage against competing organisms (Van der Mei et al. 2017).

1.2: Specialized metabolites

Streptomyces produce a variety of structurally diverse specialized metabolites, mostly during stationary phase (idiophase). Cultures transition from primary growth phase (trophophase) to idiophase upon sensing nutritional stress (Barrios-González et al. 2003). Therefore, development and specialized metabolism are often coordinated and linked in *Streptomyces.* In addition, sometimes specialized metabolite production is regulated through a negative feedback loop, where the final metabolite inhibits an early step of its own production pathway (Wang et al. 2009). In general, metabolite production in *Streptomyces* is highly regulated by many complex mechanisms (discussed further in section 1.6) due to the coordination of growth and metabolite production.

Bacterial specialized metabolites are medically, economically and industrially important for human society. Some are used as pharmaceuticals and function as antibiotics, anti-tumour agents, immunosuppressants, etc. (Figure 1.1). Streptomycete specialized metabolites account for over two-thirds of naturally derived antibiotics in current use (Challis and Hopwood 2003). In addition, specialized metabolites produced by *Streptomyces* are used in agriculture as plant growth promoters, plant protection agents (fungicides), and environmentally friendly herbicides/antiparasitics (Barrios-González et al. 2003). Due to the widespread importance of these metabolites, a vast industry exists that is involved in metabolite production, strain maintenance and strain improvement.

Bacteria are grown using large batch fermenters to produce specialized metabolites on an industrial scale. Specialized metabolite production is controlled by regulating growth conditions such as media, aeration etc. (Ser et al. 2016). Another way of improving specialized metabolite production is by introducing targeted genetic modifications to the producer organism, which is referred to as strain improvement (Barrios-González et al. 2003). As a result, specialized metabolite production is a growing subject of scientific study because improving production may increase drug efficacy and availability as well as profit for industry.

1.3: β-lactam antibiotics and their mechanism of action

For over 50 years, β-lactam antibiotics have been one of the most important agents used in the treatment of bacterial infections (Schmidt 2002). Their limited toxicity towards humans and wide range of antibacterial activities make them some of our most successful medicines (Schmidt 2002). Currently, β-lactam antibiotics make up over 60%

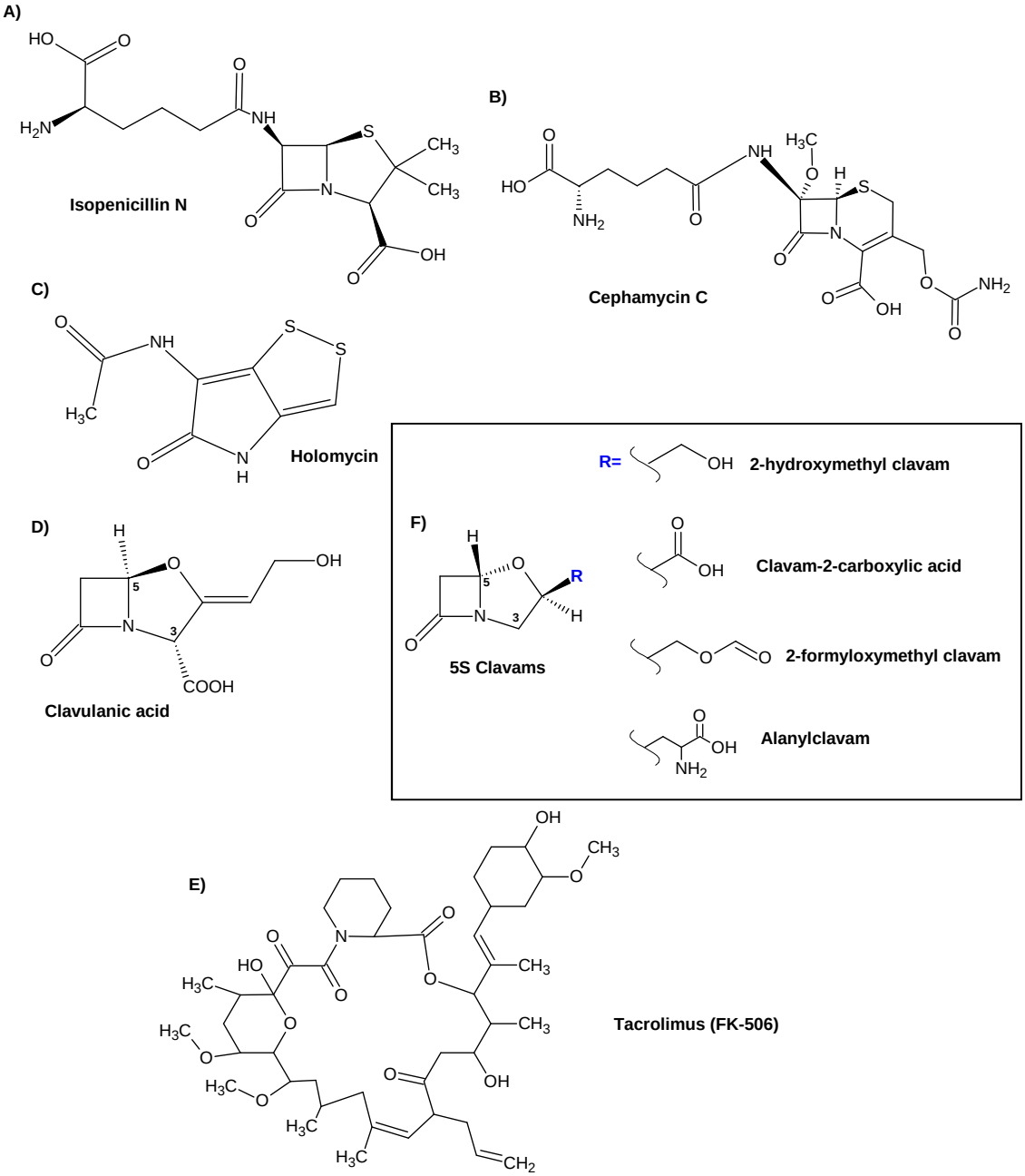
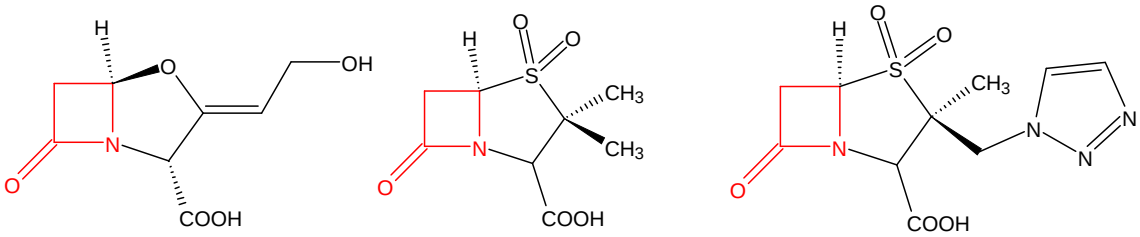


Figure 1.1: Secondary metabolites produced by *S. clavuligerus*. Shown are the β-lactam antibiotics **(A)** isopenicillin N and **(B)** cephamycin C and the pyrrothine antibiotic **(C)** holomycin. The β-lactamase inhibitor **(D)** clavulanic acid and the immunosuppressant **(E)** Tacrolimus (FK-506) are also shown. **(F)** depicts the core structure of the 5*S* clavams with respective side chains represented by R. The C3 and C5 atoms of the oxazolidine ring are highlighted and represent sites of important differences between clavulanic acid and the 5*S* clavams.

of the world antibiotic market (Van Boeckel et al. 2014). Antibiotics in this family all contain a four-member heterocyclic ring (Figure 1.2) and are further classified based on their chemical structure, solubility or spectrum of activity. Some members contain a second ring attached to the β-lactam ring and together the rings form a bicyclic core. The β-lactams are classified based on the nature of the second ring found in these molecules. For example, if they contain a five membered, sulfur-containing ring they are called the penams, and if there is a six-membered, sulfur-containing ring, they are called the cephams (Dalhoff et al. 2006). To this end, β-lactams can be divided into the monobactam, penam, cephem, clavam or carbapenem groups (Table 1.1) Many β-lactams are naturally produced but some of them are chemically modified after purification and these are called the semi-synthetics. Presently, the cephalosporins and broad spectrum, semi-synthetic penicillins account for the greatest usage of antibiotics worldwide (Van Boeckel et al. 2014).

The cell wall is the target of the β-lactams. Cell walls in bacteria are comprised of many components, one of which is peptidoglycan. Peptidoglycan is made up of glycan (NAG-NAM) chains that are crosslinked by peptide bridges. The crosslinking of peptides is essential for the rigidity and stability of the bacterial cell wall and is carried out by DD-transpeptidases, which are the targets of the β-lactams. Therefore, β-lactams target actively growing cells that are crosslinking their peptidoglycan. The inhibition of peptidoglycan synthesis leads to structural defects in the cell wall and ultimately cells lyse due internal pressure (Andersson et al. 2001).



Clavulanic acid **Sulbactam** **Tazobactam**

Figure 1.2: Chemical structures of currently used natural product (clavulanic acid) and synthetic (sulbactam and tazobactam) β-lactamase inhibitors containing the β-lactam core. Clavulanic acid is produced by the bacterium *S. clavuligerus*, while the other two are completely synthetic. The mode of action of all three inhibitors is the same with the β-lactam ring (in red) being the target for β-lactamase inhibition.

Table 1.1: Classes of β-lactam antibiotics with commercially relevant examples and the microorganisms that produce them

Class	Antibiotic example	Producer organism
Penam	Penicillin	*Penicillium chrysogenum*
Cephems (Cephalosporin/Cephamycin)	Cephamycin C	*Penicillium chrysogenum* *Streptomyces clavuligerus* *Streptomyces lipmanii* *Nocardia lactamdurans*
Clavam	Clavulanic acid	*Streptomyces clavuligerus* *Streptomyces jumonjinensis* *Streptomyces katsurahamanus*
Carbapenem	Thienamycin	*Streptomyces cattleya*
Monobactam	Aztreonam	*Chromobacterium violaceum*

1.4: Resistance, β-lactamases and inhibitors

Resistance to β-lactams has resulted from bacteria evolving to avoid the lethal action of these metabolites. There are three primary mechanisms that lead to resistance to β-lactam antibiotics. First and foremost is the production of β-lactamase enzymes, which hydrolyze the β-lactam ring and thereby inactivate the antibiotic (Bush 1989a). The second mechanism employs efflux systems that actively pump β-lactam antibiotics out of the cell and thereby prevent their accumulation at the cell wall target site. A third mechanism involves mutations in the genes of the DD-transpeptidases that lead to enzymes that no longer bind to the β-lactam and therefore are not inhibited by it. This is a very important mechanism which is responsible for methicillin resistance in *Staphylococcus aureus* (MRSA) due to target modification (Walsh 2000). Together, these primary mechanisms, and the ease in which they are transferred to other bacteria are responsible for the worldwide epidemic of antibiotic resistance.

β-lactams went into widespread clinical use in the early 1940s but by this time β-lactamases had already been identified in *Escherichia coli*, suggesting that they were going to be a problem (Abraham and Chain 1940). Since then, more than 850 different types of β-lactamase enzymes have been identified and reported. β-lactamases can be classified based on different systems, one of which uses the four molecular classes: A, B, C and D (Drawz and Bonomo 2010). These enzyme classes are based on amino acid sequence similarity and catalytic mechanism (Drawz and Bonomo 2010). β-lactamases in classes A, C and D use an active-site serine in the catalytic mechanism leading to the inactivation of the β-lactam antibiotic (process reviewed in Drawz and Bonomo 2010). In

contrast, the class B β-lactamases do not use an active-site serine and instead are metalloenzymes that use metal ions for catalysis (Drawz and Bonomo 2010). This makes the mechanism of inactivation very different between the two groups of β-lactamases. Recently, the Gram negative pathogen *Klebsiella pneumoniae* carrying a class B metallo-β-lactamase, called New-Delhi Metallo-β-lacatmase (NDM), has been reported to confer resistance to all classes of β-lactams (Chen et al. 2016).

To overcome β-lactam resistance, two main strategies have been developed (Andersson et al. 2001). The first is to alter the structure of the β-lactam antibiotic so that it is no longer recognized by the β-lactamase and therefore is not inactivated. The other strategy employs the use of β-lactamase inhibitors along with β-lactam antibiotics. In this strategy, the β-lactamase inhibitor inactivates the β-lactamase enzyme so that the β-lactam antibiotic can inhibit the DD- transpeptidase and cause cell lysis. There are two classes of β-lactamase inhibitors; one that binds to the enzyme reversibly such as cephalosporins, monobactams and carbapenems (Drawz and Bonomo 2010) and the other that binds to the enzyme irreversibly such as CA, Sulbactam and Tazobactam (Figure 1.2), a process known as suicide inhibition.

Sulbactum and Tazobactum are synthetic compounds developed by the pharmaceutical industry, whereas CA is a natural product produced by *Streptomyces clavuligerus* (English et al. 1978; Fisher et al. 1980). Overall, the structures of β-lactamase inhibitors mimic conventional β-lactam antibiotics. However, in the case of CA, an oxygen group is present instead of a sulfur group, and this produces an oxazolidine ring rather than a thiozolidine ring. As a result, CA binds irreversibly to the serine hydroxyl group of the β-lactamase, hence this is why it is called a suicide inhibitor

as the β-lactamase is inhibited permanently (Drawz and Bonomo 2010). On its own, CA is a very weak antibiotic (Brown 1986) but when given in combination with another β-lactam antibiotic (e.g. amoxicillin) it can treat resistant infections. Soon after CA's discovery, it became the first clinically used β-lactamase inhibitor, thereby extending the use of β-lactam antibiotics against otherwise resistant infections (Drawz and Bonomo 2010). In particular, CA actively inhibits most of the class A β-lactamases and the cloxacillin-hydrolysing enzymes of class D; however, CA is not active against cephalosporinases of class C and Zn^{2+} metallo- β-lactamases of class B (Bush 1989a, b).

1.5: Clavulanic acid and *S. clavuligerus*

Streptomyces clavuligerus is industrially used to produce CA for medicinal use. In addition to CA, *S. clavuligerus* also produces several other specialized metabolites (Figure 1.1) and according to genome mining, has the capability to produce numerous more (Medema et al. 2010). The initial discovery of *S. clavuligerus* was a result of its ability to produce cephamycin C (CEF), and only later on was it found to produce CA upon subsequent re-examination (Howarth et al. 1976).

In addition to *S. clavuligerus*, two other organisms, *Streptomyces jumonjinensis* (Vidal 1987) and *Streptomyces katsurahamanus* (Kitano et al. 1978), have been shown to produce CA. The genes involved in CA production are clustered together forming the CA gene cluster, which resides next to the CEF gene cluster on the chromosome, thus creating a super-cluster of genes involved in β-lactam antibiotic production (Ward and Hodgson 1993). Recent genome sequences have shown that *Streptomyces flavogriseus* and *Saccharomonospora viridis* have gene clusters resembling the CA gene cluster from

S. clavuligerus, but they have not been shown to produce CA under laboratory conditions (Alvarez-Alvarez et al. 2013). While *S. flavogriseus* is a *Streptomyces* species, *S. viridis* is a member of the *Pseudonocardiaceae* family, but both are classified as Actinomycetes.

In addition to CA, *S. clavuligerus*, also produces other clavam metabolites such as clavam-2-carvoxylate, 2-hydroxymethylclavam, 2-formyloxymethylclavam, and alanylclavam (Figure 1.1). The clavams are classified together due to the presence of a five membered oxazolidine ring fused to the four membered β-lactam ring (Figure 1.1). Within the clavams, CA is unique due to its 3*R*, 5*R* stereochemistry (Figure 1.1) and because of this stereochemistry it can inhibit β-lactamases (Baggaley et al. 1997), whereas all other 5*S* clavams have not been shown to exhibit β-lactamase inhibitory activity. Although the 5*S* clavams do not have β-lactamase inhibitory activity, they display some antibacterial/antifungal properties due to other mechanisms. In addition to the clavams, *S. clavuligerus* also produces the industrially important antibiotics CEF and penicillin N (Figure 1.1), which is an intermediate in the CEF biosynthetic pathway (Higgens and Kastner 1971; Nagarajan et al. 1971). Both of these antibiotics are clinically used to treat bacterial infections.

The *Streptomyces* chromosomes are linear and contain high GC (~72%) content DNA (Lin et al. 1993). Each chromosome can be divided into conserved core regions (containing genes essential for survival) flanked by variable arms (Hopwood 2006). The *S. clavuligerus* genome consists of a 6.8 Mb linear chromosome and four self-replicating, linear plasmids, including a 1.8 Mb mega-plasmid (pSCL4) (Medema et al. 2010; Song et al. 2010). This genome was mined for potential specialized metabolite gene clusters and a total of 48 were found; 25 on the mega plasmid and 23 on the chromosome. Some of

these include known antibiotic biosynthetic gene clusters (e.g. CA), while others either resemble known gene clusters or are completely novel (Medema et al. 2010). Therefore, *S. clavuligerus* has the genetic potential to produce many different specialized metabolites, but only a few are able to be produced under laboratory conditions (Medema et al. 2010). For now, *S. clavuligerus* is known to produce five medically important compounds: a β-lactamase inhibitor (CA), a pyrrothine antibiotic (holomycin), two β-lactam antibiotics (penicillin N and CEF) and an immunosuppressant (Tacrolimus) (Figure 1.1).

Nearly all studies on the biosynthesis of the clavams (which includes CA and the 5*S* clavams) have been conducted in *S. clavuligerus* (Jensen 2012). *S. clavuligerus* is unique among the clavam producing *Streptomyces* because it can produce both CA and 5*S* clavams, while all others produce either CA or the 5*S* clavams only (Jensen 2012).

The genes involved in CA and 5*S* clavam biosynthesis reside in three unlinked gene clusters in *S. clavuligerus.* The "primary" CA gene cluster is the one situated next to CEF gene cluster as mentioned above (Figure 1.3 A) (Tahlan et al. 2004 a, Ward and Hodgson 1993). The second gene cluster, the clavam gene cluster (Figure 1.3 B), is situated on the *S. clavuligerus* chromosome and is comprised of genes predominately involved in 5*S* clavam production (Tahlan et al. 2007). The third gene cluster, the paralogue gene cluster (Figure 1.3 C), is located on the pSCL4 mega-plasmid (Medema et al. 2010) and contains second copies of genes involved in both CA and 5*S* clavam biosynthesis (Tahlan et al. 2004a; Tahlan et al. 2004b). Therefore, the genetics of CA-5*S* clavam biosynthesis is somewhat complicated involving "cross talk" between chromosomally encoded and plasmid borne gene clusters in *S. clavuligerus*.

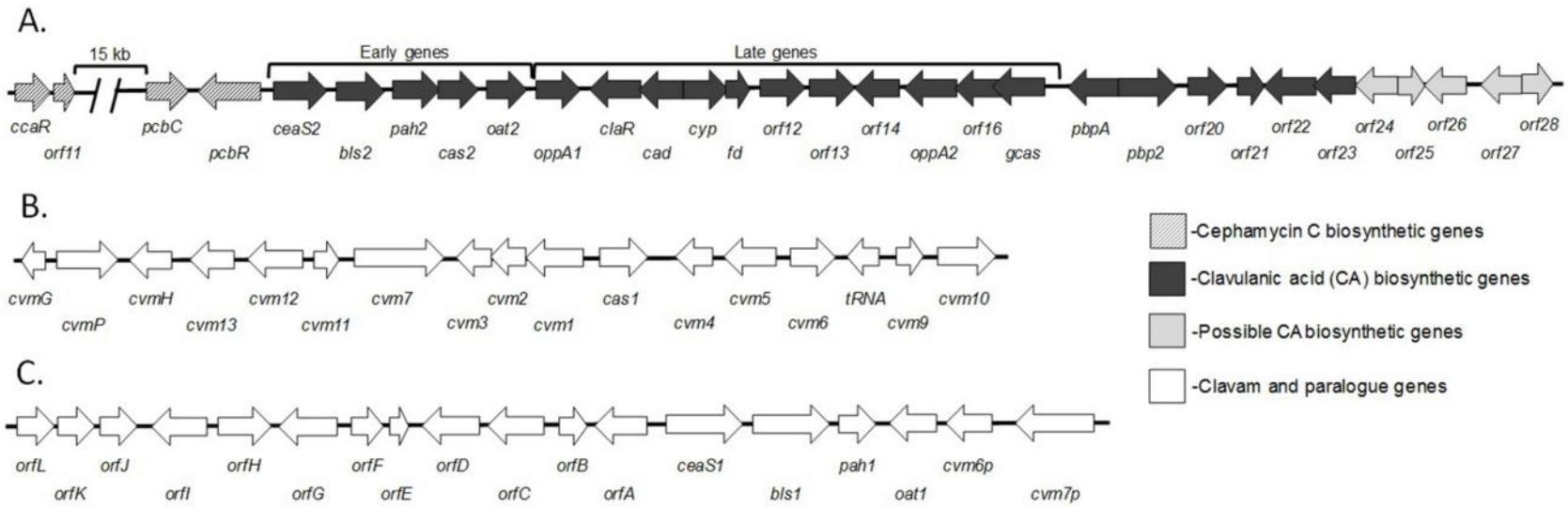


Figure 1.3: The three unlinked gene clusters responsible for clavulanic acid and 5*S* clavam biosynthesis in *S. clavuligerus*. Each arrow represents a gene and points in the direction in which it is transcribed. The name is located below each gene. The solid line backbone represents the *S. clavuligerus* chromosome. (**A)** The clavulanic acid gene cluster. A few genes from the cephamycin C gene cluster that are important for clavulanic acid biosynthesis are included. **(B)** The clavam gene cluster. **(C)** The paralogue gene cluster.

(Tahlan et al 2004a, Liras et al. 2008).

1.6: Clavulanic acid biosynthesis and its regulation

CA and the 5*S* clavams share a common biosynthetic pathway up to the intermediate clavaminic acid, after which the pathway diverges (Figure 1.4) (Ward and Hodgson 1993, Egan et al. 1997). The biosynthetic reactions shared by the two metabolite groups are referred to as the "early stages" of the CA-5*S* clavam pathway (Ward and Hodgson 1993).

The biosynthetic pathway leading to CA and the 5*S* clavam is very different than the one involved in the formation of the conventional penicillin and cephalosporin family of antibiotics. The precursors of the biosynthesis of penicillins and cephalosporins arise using a non-ribosomal peptide synthetase (Martin 1998); whereas an enzyme called carboxyethylarginine synthase (CEAS) catalyzes the initiation of the CA-5*S* clavam biosynthetic pathway using L-arginine and glyceraldehyde-3-phosphate (Figure 1.4).

The early part of the CA-5*S* clavam biosynthetic pathway involves the carboxyethylarginine synthase (*ceaS2*), beta-lactam synthase (*bls*), clavaminic acid synthetase (*cas*) and proclavaminate amidinohydrolase (*pah*) gene products leading to the formation of clavaminic acid (Figure 1.4). The proteins encoded by these genes have known functions and are well characterized (entire process is reviewed in Jensen 2012, Hamed et al. 2013). The only other proteins in the CA pathway that have known functions are glycylclavaminic acid synthase (GCAS) and clavaldehyde dehydrogenase (CAD) (Figure 1.4). GCAS is the first enzyme specifically responsible for the CA arm of the pathway (Arulanantham et al. 2006) and CAD catalyzes the final step in CA

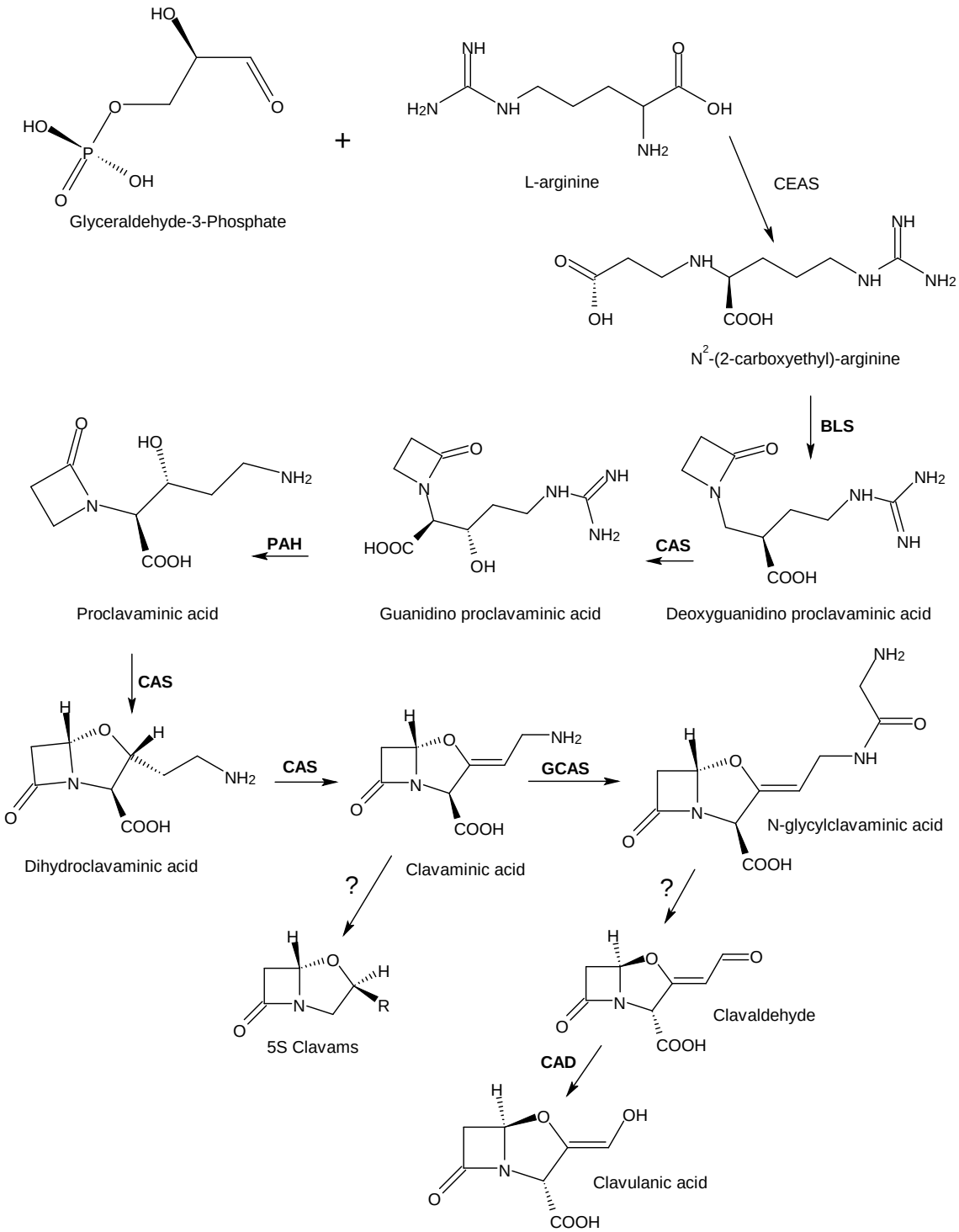


Figure 1.4: Schematic of the currently proposed biosynthetic pathway leading to clavulanic acid and 5*S* clavam production in *S. clavuligerus*. Names of intermediates are given under the corresponding structure and involved enzymes are in bold above corresponding arrows. Question marks represent unknown enzymes that catalyze multiple reactions leading to the respective metabolite.

biosynthesis (Perez-Redondo et al. 1998). There are numerous genes from the CA gene cluster that have not been assigned functions (Figure 1.3 and 1.4), but it has been shown that knocking each of them out leads to loss of CA production (Jensen et al. 2000, Jensen et al. 2004, Li et al. 2000, Mellado et al. 2002, Song et al. 2009). Further study is required to elucidate the function of all genes essential to the CA production pathway.

There are number of regulatory proteins and factors involved in regulating specialized metabolite production in *S. clavuligerus* (Jensen 2012). There are two regulatory genes with known functions from the CEF and CA super-cluster. CcaR (cephamycin and clavulanic acid regulator) is present in the CEF arm of the CEF and CA super-cluster (Figure 1.3 A) and encodes the cluster situated regulator (CSR). It regulates the production of both CEF and CA but not the 5*S* clavams (Perez-Llarena et al. 1997). In the case of the regulation of CA, CcaR regulates the transcription of *claR*, which is a pathway specific regulator required only for the biosynthesis of CA (Paradkar et al. 1998). CcaR also directly regulates the early genes (*ceaS2*, *bls2*, *pah2* and *cas2*) involved in the formation of CA and the 5*S* clavams as well as its own transcription by binding its promoter region (Santamarta et al. 2002). ClaR is encoded by a gene found within the CA gene cluster (Figure 1.3 A) and it regulates the transcription of a few genes (*oppA1*, *cad* and *cyp*) involved in the late stages of CA biosynthesis but not the genes involved in the biosynthesis of the 5*S* clavams (Paradkar et al. 1998).

Multiple systems of global genetic regulation control CA and 5*S* clavam biosynthesis. One such system, the BldA system is quite complex in *S. clavuligerus* and possesses several unique features. The *bldA* gene encodes a leucine tRNA that recognizes UUA codons (Guthrie and Chater 1990). TTA codons are rare because *S. clavuligerus*

has high GC content (72%) DNA, therefore not many T or A codons are present. TTA codons are found mostly in non-essential genes involved in differentiation and specialized metabolism. As a result, *bldA* mutants cannot undergo differentiation (resulting in "bald" colonies) or specialized metabolite production (reviewed in Hackl and Bechthold 2015). *bldA* mutants still produce normal levels of CEF and CA even though CcaR, the CSR of CEF and CA, carries a TTA codon (Perez-Llarena et al. 1997). All 5*S* clavams (except for alanylclavam) are overproduced in *bldA* mutants because *res1*, a gene encoding a 5*S* clavam regulator, carries a TTA codon that exhibits *bldA* dependence (Kwong et al. 2012). These two observations infer that some TTA codon-containing genes are dependent on *bldA* while others are not (Trepanier et al. 2002). *bldA* dependence was found to be based on the residue immediately following the TTA codon (Trepanier et al. 2002).

Although *ccaR* expression is not regulated by *bldA*, its transcription is dependent on another "bald" gene, *bldG* (Bignell et al. 2005). BldG is similar to anti-anti sigma factors from *Bacillus subtilis* (Bignell et al. 2000). The *bldG* homologue in *S. clavuligerus* is required for aerial hyphae formation and the production of CEF, CA and the 5*S* clavams. Through the regulation of *ccaR*, *bldG* controls the production of CEF and CA (Bignell et al. 2005). However, *bldG* must also control another regulator since *ccaR* does not regulate 5*S* clavam production.

Recently, a group of small diffusible extracellular regulators called the γ-butyrolactones (GBLs) have been under intensive study. They are produced by several *Streptomyces* species to regulate the onset of antibiotic production (Santamarta et al. 2005). In the cell, butyrolactone receptor proteins (BRPs) are bound to regulatory

sequences called ARE boxes and usually act as repressors (Onaka et al. 1995; Onaka and Horiniouchi 1997). ARE boxes are located upstream from genes that are responsible for the regulation of specialized metabolite production and differentiation (Kinoshita et al. 1999; Onaka and Horiniouchi 1997). When GBLs are released in the cell, they bind to BRPs causing the BRPs to disassociate from the ARE boxes, which relieves repression and triggers antibiotic production (Onaka et al. 1995; Onaka and Horiniouchi 1997). In *S. clavuligerus*, there is only one BRP that is encoded on the pSCL4 mega plasmid as *scaR* (Kim et al. 2004; Medema et al. 2010). ScaR has been tied to the regulation of CA and CEF biosynthesis as both metabolites are overproduced in *scaR* mutants (Santamarta et al. 2005). In addition, an ARE box was found 815 nt upstream of the *ccaR* transcription initiation site; further indicating a relationship between CcaR and ScaR (Santamarta et al. 2005). Most likely, ScaR functions as a negative repressor of *ccaR* gene expression, and this repression is relieved by the binding of a GBL to ScaR, though the identity of this GBL remains to be determined (Santamarta et al. 2005).

1.7: Putative genes involved specifically in the biosynthesis of clavulanic acid

How clavaminic acid is converted to clavaldehyde is still not well understood. Information on this part of the pathway is missing. Although several genes (*oppA1, cyp, fd, orf12, orf13, orf14, orf16, orf21, orf22* and *orf23*) have been implicated, there is no clear role for each (Jensen et al. 2000, Jensen et al. 2004, Li et al. 2000, Mellado et al. 2002, Song et al. 2009). Clavaldehyde has the same 3*R*, 5*R* stereochemistry of its oxazolidine ring as CA, whereas clavaminic acid has the same stereochemistry as the 5*S* clavams. Therefore, there has to be an enzymatic reaction that results in the

stereochemical ring inversion from clavaminic acid to clavaldehyde. Recently, it has been proposed that the protein encoded by *orf12* (Figure 1.3) could somehow be involved in the process of cleaving the oxazolidine ring so that ring inversion takes place (Jensen 2012; Valegard et al. 2013). Still, the mechanism of this inversion is not well understood.

1.7.1: *orf12*

As mentioned above, *orf12* has been a target of research interest due to its largely unknown function but possible involvement in ring inversion. It has been found that *S. clavuligerus orf12* mutants are completely unable to produce CA, thus rendering Orf12 essential for the production of CA (Li et al. 2000; Jensen et al. 2004). Orf12 shares the closest amino acid sequence homology to two hypothetical proteins; one found in *S. flavogriseus* (61% identity) and one in *S. viridis* (51% identity). It is more distantly related to uncharacterized serine hydrolases and LpqF lipoproteins in *Rhodococcus* and *Mycobacterium* species with ~35% identity across the majority of these sequences. Sequence similarity (32% identity) has been found between Orf12 (hereafter called Blm) and class A β-lactamases such as Spyr1 from *Mycobacterium gilvum*. However, a β-lactamase function for Blm is not consistent with its essential role in CA biosynthesis (Li et al. 2000). Blm's proposed involvement in ring opening or re-closing (for sterochemical ring inversion) is similar to the β-lactamase activity of binding and cleaving the β-lactam ring (Jensen 2012; Valegard et al. 2013). Nonetheless, this suggestion is weakened by the fact that Blm only retains one of the necessary β-lactamase catalytic motifs; the SDN motif (Mellado et al. 2002).

β-lactamases have four catalytic motifs necessary to their function. The first motif, common to all class A β-lactamases, is S-T-F-K, where S is the active serine responsible for the nucleophilic attack on the carbonyl group of the β-lactam ring (Herzberg and Moult 1987; Meroueh et al. 2005). Within this region, Blm differs from class A β-lactamases in the second (T→A) and third (F→Y) residues (Valegard et al. 2013). Nevertheless, during biochemical studies on Blm, the S residue in the first position was found to act as the nucleophilic catalyst to convert cephalosporin C to deacetylcephalosporin C (Valegard et al. 2013). The second catalytic motif is S-D-G, where the G residue is important for penicillinase activity and an N residue (in place of G) is important for cephalosporinase activity (Jacob et al. 1990). Analysis of the Blm amino acid sequence indicated that it contains the latter S-D-N motif (Li et al. 2000). The third motif, common to class A β-lactamases is E-P-E-L-N, which is replaced with D-P-E-R-R in Blm. The first E residue is always found in class A β-lactamases while it is substituted to a D residue in class C β-lactamases (Lobkovsky et al. 1993). It has been demonstrated that the E residue is important for catalysis and the substitution of E with D increases substrate affinity (Gibson et al. 1990). The final conserved class A β-lactamase motif is K-(T or S)-G (Herzberg and Moult 1991). The K residue is important for hydrogen bonding and the G residue is important for deacylation (Knox et al. 1993; Escobar et al. 1994). In class A β-lactamases, this motif is particularly important for proper positioning of the substrate during acyl-enzyme formation (Zhang et al. 2007) as it forms part of an external β pleated sheet near the substrate binding pocket (Matagne et al. 1990). Blm does not possess any of these residues. Instead, it has an F-K-A motif, which does not act similarly to the β-lactamase residues. Although Blm does not contain all of

the motifs required for β-lactamase activity, its motifs have the potential for cephalosporin esterase activity as Blm can hydrolyse the ester group of 3'-*O*-acetyl cephalosporins (Valegard et al. 2013). Regardless, this activity is not believed to be its essential function but rather low level activity due to substrate similarity (Valegard et al. 2013). Further investigation is required to determine the activity of this protein.

Recent biochemical and structural studies on Blm have revealed that it is a monomer consisting of two protein domains: a C-terminal penicillin-binding protein (PBP)/β-lactamase type fold with highest structural similarity to the class A β-lactamases, fused to an N-terminal domain with a fold similar to steroid isomerases and polyketide cyclases (Valegard et al. 2013). The C-terminal domain did not show β-lactamase or PBP activity, but did show the low-level esterase activity towards 3'-*O*- acetyl cephalosporins and a thioester substrate (Valegard et al. 2013). Based on this, a few functional possibilities of Blm in CA production have been proposed. One possibility is that Blm is involved in the epimerization step during the conversion of clavaminic acid to clavulanic acid (Jensen 2012, Valegard et al. 2013). This was proposed because the fold of the N-terminal domain is related to the structure of steroid isomerases, which upon Blm-CA binding could lead to epimerization (Valegard et al. 2013). However, no apparent active site or conserved catalytic residues analogous to steroid isomerases have been found in Blm.

Biochemical studies have shown that two molecules of CA bind to one molecule of Blm (Valegard et al. 2013). The first molecule of CA binds to the Blm active site, which is lined with residues from both the N and the C terminal domains. The other molecule binds between the two domains. The first CA molecule is orientated in such a

way that the carbonyl O of the β- lactam ring is positioned in the opposite direction of the nucleophilic serine (part of the SXXK motif). This likely is a reason Blm does not show any β-lactamase activity. The second molecule of CA does not form any direct hydrogen bonds to Blm. Instead, it binds in a hydrophobic cleft that may act as a "gatekeeper" to the active site. In summary, CA has been shown to bind Blm; however, the biological significance of that binding is not well understood. It is possible that there is only one active site and the second site binds to a CA precursor or ligand in its native host, *S. clavuligerus*. Further study is required to give a more complete description of the enzymatic mechanism that Blm uses in the biosynthesis of CA and what other proteins in the CA biosynthetic pathway it may be interacting with.

1.8: *blm, orf13* and *orf14*

DNA sequencing studies have revealed that the stop codon of *blm* overlaps with the start codon of the adjacent *orf13* gene by 4 bp (Figure 1.5 A; Tahlan unpublished). As a result, *blm* and *orf13* are transcriptionally coupled (Mellado et al. 2002) and may be translationally coupled. *orf13* is predicted to encode a protein that is similar to amino acid metabolite efflux pumps, suggesting a role in transporting clavulanic acid out of the cell (Mellado et al. 2002). In addition, the 3' ends of *orf13* and *orf14* (encoded on the opposite DNA strand from *orf13*) overlap by 50 nt (Figure 1.5 A; Tahlan unpublished), where *orf14* also has an unknown function in CA biosynthesis. This phenomenon is quite rare as genes encoded on opposite DNA strands do not overlap normally. In previous studies, when *blm*, *orf13* or *orf14* were disrupted, CA production was severely compromised (Li et al. 2000, Mellado et al. 2002, Jensen et al. 2004), whereas,

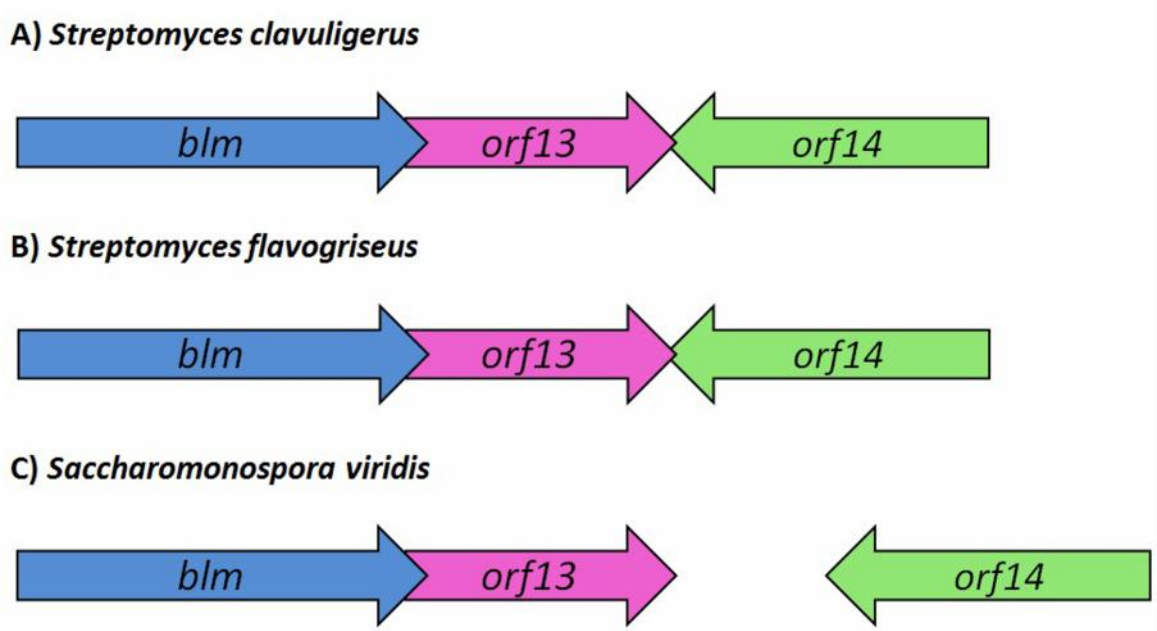


Figure 1.5: A comparison of *blm*, *orf13* and *orf14* from the clavulanic acid biosynthesis pathways of *S. clavuligerus*, *S. flavogriseus* and *S. viridis*. Arrows indicate transcriptional direction. The overlapping and spacing of genes is not to scale.

overexpression of *blm* and *orf14* showed a significant increase in CA production (Mellado et al. 2002).

Due to the transcriptional arrangement of *blm*, *orf13* and *orf14*, particular attention must be given to the genetic reading frame when creating knock-out mutants. All previous mutants of *blm* (Li et al. 2000, Jensen et al. 2004), *orf13* (Jensen et al. 2004) and *orf14* (Mellado et al. 2002, Jensen et al. 2004) were created by inserting an antibiotic resistance cassette at specific positions within each gene. These studies showed that the three genes are essential for CA production. However, it is possible that the *blm* insertional mutations also affected the transcription and translation of *orf13* and *orf14*, and thus the observed effect on CA production in the *blm* mutants may have been due to polar effects on expression of the downstream genes. Therefore, there is a need for examining the phenomenon of transcriptional and translational polarity in the *blm* mutant to confirm its role in CA production.

Genome sequencing studies have shown that orthologues of *blm*, *orf13*, and *orf14* are present in *S. flavogriseus* and *S. viridis* in arrangements slightly different than in *S. clavuligerus* (Figure 1.5). Each predicted protein has ~60% amino acid identity with its orthologue in *S. clavuligerus*, except for Blm from *S. viridis*, which has a 52% amino acid identity. Of particular note, both Orf13 and Blm from *S. clavuligerus* contain additional amino acid stretches that are not present in either of the predicted *S. flavogriseus* and *S. viridis* protein sequences. Transcriptional studies have shown that the late genes from the CA gene cluster (including *blm*) are barely expressed in *S. flavogriseus* (Alvarez-Alvarez et al. 2013). This lack of expression is likely the reason for the inability of *S. flavogriseus* to produce CA, but it is not known if the *blm* gene from *S. flavogriseus* has the same

function as its orthologue from *S. clavuligerus*.

1.9: Main objectives of this thesis

The complexity of CA biosynthesis in *S. clavuligerus* is one of the reasons why this pathway has not yet been completely elucidated. Several questions still remain unanswered, including the elusive mechanism behind the stereochemical inversion leading to the 5*R* configuration of CA and the function of the "silent" CA gene clusters of *S. flavogriseus* and *S. viridis*. The main goal of this thesis was to verify the role of Blm in CA biosynthesis, to determine its cellular location, and to determine if the genes from *S. flavogriseus* and *S. viridis* are functionally equivalent to their counterpart from *S. clavuligerus*. Two *blm* mutant strains of *S. clavuligerus* were prepared and tested in complementation studies using the *blm* gene. One mutant was created to test the possibility of downstream polar effects due to the overlap present between *blm* and *orf13*. The other mutant, an in-frame *blm* deletion mutant, was created to investigate the sole effects of Blm on CA production and whether CA production could be rescued by the *blm*-like genes from *S. viridis*, and *S. flavogriseus*. It is possible that Blm from *S. viridis* and *S. flavogriseus* is defective, or it may have a different function in the production of some other related compound. The goals of the cellular localization studies of Blm provide insight into its possible function since most β-lactamases are secreted proteins. This was accomplished by performing western analysis on cellular and secreted fractions using FLAG epitope tagged (Hopp et al. 1988) Blm as well as two control proteins with known cellular locations: CcaR (cytoplasmic) and Blip (secreted).

Chapter 2: Materials and Methods

2.1: General procedures and supplies

All solutions and media were prepared using HPLC-grade H_2O, and were sterilized before use by autoclaving or by filter sterilization using 2 µm filters (28145-481, VWR International, Canada). Unless stated otherwise, all reagents, antibiotics and dehydrated media were purchased from Sigma Aldrich Canada, Fisher Scientific Canada or VWR International. Aseptic techniques with sterile reagents and glassware were used while handling and manipulating bacterial cultures. All centrifugation steps throughout this study were performed using either an Eppendorf 5424 micro-centrifuge (022620401, Germany) or a Thermo Scientific Sorvall ST 16 R centrifuge (75004380, USA) using a TX-400 Swinging Bucket Rotor (75004380), unless otherwise indicated. Optical densities (ODs) and nucleic acid concentrations were measured using an Implen p300 NanoPhotometer (Implen GmbH, Germany). All polymerase chain reactions (PCR) were conducted using an Eppendorf VapoProtect Mastercycler Pro (950040015, Germany) and subsequent gel electrophoresis was performed using Thermo Scientific OWL easycast gel boxes and a VWR International 300V Power Source (100414605). All restriction enzymes used in this study were obtained from New England BioLabs (Canada). Gel purification steps were conducted using the EZ-10 Spin Column DNA Gel Extraction Kit (Bio Basic Canada Inc., Canada) and performed according to manufacturer's instructions except that 34 µl of 50°C H_2O was used to elute the DNA and the column was incubated at 37°C for 5 minutes before the final centrifugation step. All *E. coli* and *S. clavuligerus* strains were stored as 20% glycerol stocks at -80°C, and all DNA samples were stored at -20°C.

2.2: General *E. coli* procedures

2.2.1: Maintenance and stock preparation

E. coli liquid cultures were grown at 37°C with 200 rpm shaking for 16-18 hours in 3-5 ml of Lennox broth (LB) (BP1426500, Thermo Fisher Scientific, USA). Strains were maintained on LB agar. When required, media was supplemented with the appropriate antibiotics as listed in Table 2.1. All bacterial strains and plasmids used in this study are described in Table 2.2.

2.2.2: Cosmid and plasmid DNA isolation

For routine screening purposes, cosmid and plasmid DNA was isolated using a modified alkaline lysis method (Birnboim and Doly 1979). An overnight culture (1.5 ml) was transferred to a 1.5 ml microfuge tube and pelleted by centrifugation at 12,000 rpm for 30 seconds at room temperature. The supernatant was discarded and the cell pellet was re-suspended in 100 µl of ice cold Solution I (50 mM glucose, 25 mM Tris-Cl- pH 8.0, 10 mM ethylenediaminetetracetic acid (EDTA)-pH 8.0). Next, 200 µl of freshly prepared Solution II (0.2 N NaOH, 1% w/v SDS) was then added and the tube was gently inverted 5-7 times. A total of 150 µl of ice cold Solution III (3 M potassium acetate, 2 M glacial acetic acid, and topped up with H_2O, pH 4.6) was added to the tube before inverting gently 5-7 times. The tube was incubated on ice for 5 minutes before centrifugation at 10,000 rpm for 10 minutes at room temperature. The resulting supernatant was then transferred to a new 1.5 ml microfuge tube containing 450 µl of ice cold 95% ethanol. The contents were vortexed and incubated on ice for 5 minutes before centrifugation at 10,000 rpm for 10 minutes at room temperature. The supernatant was

Table 2.1: Stock and experimental concentrations of antibiotics used throughout this study

Antibiotic	**Stock Concentration (mg/ml)**	**Experimental Concentration (µg/ml)**	
		E. coli	*S. clavuligerus*
Ampicillin	100	100	N/A*
Apramycin	50	50	25
Chloramphenicol	34	25	N/A*
Hygromycin	50	50 (liquid broth) and 100 (solid media)	200
Kanamycin	50	50	50
Nalidixic Acid	50	40	40

*N/A: Not applicable -the antibiotic was not used with this organism.

Table 2.2: Cosmids, plasmids and bacterial strains used in this study

Name	Antibiotic concentration for selection (µg/ml)	Genotype and relevant features	Reference
Cosmids			
12B8	Ampicillin 100 Kanamycin 50	pWE15 cosmid clone containing *blm* and surrounding region from the *S. clavuligerus* chromosome	Jensen et al. 2004
Cloning vectors			
pGEMT®-Easy	Ampicillin 100	*E. coli* general cloning vector	Promega
pHM11a	Hygromycin*	Integrative *Streptomyces* expression vector	Motamedi et al. 1995
Plasmids for antibiotic resistance cassettes			
pIJ10700	Hygromycin 50	Hyg^R cassette in pBluescript II KS(+)	Gust et al. 2003
pIJ773	Apramycin 50	$Apra^R$ cassette in pBluescript II KS(+)	Gust et al. 2003
Gene disruption constructs			
12B8 *Δblm-AP*	Apramycin* Kanamycin 50	12B8 Δ*blm*::*apra-oriT* cassette (*blm* mutant cosmid)	This study
12B8 Δ*blm::scar*	Ampicillin 100 Kanamycin 50	12B8 Δ*blm*::in-frame scar	This study
12B8 Δ*blm*::*scar* ::Δ*amp*::*hyg*^{R}	Hygromycin* Kanamycin 50	12B8 Δ*blm*::in-frame scar Δ*amp*::*hyg-oriT*	This study

Complementation/localization constructs			
pH:*blm*^{SC}	Hygromycin*	pHM11a with *blm* from *S. clavuligerus*	This study
pH:*blm*^{SF}	Hygromycin*	pHM11a with *blm* from *S. flavogriseus*	This study
pH:*blm*^{SV}	Hygromycin*	pHM11a with *blm* from *S. viridis*	This study
pH:*ccaR*-FLAG	Hygromycin*	pHM11a with *ccaR* from *S. clavuligerus* with FLAG tag	This study
pH:*blm*-FLAG	Hygromycin*	pHM11a with *blm* from *S. clavuligerus* with FLAG tag	This study
pH:*blip*-FLAG	Hygromycin*	pHM11a with *blip* from *S. clavuligerus* with FLAG tag	This study
E. coli strains			
NEB5α	N/A	DH5α derivative cloning host	New England Biolabs
ET12567(pUZ8002)	Chloramphenicol 25 Kanamycin 25	DNA methylation deficient host used for conjugation with *S. clavuligerus.* pUZ8002 provides DNA transfer functions	MacNeil et al. 1992
BW25113 (pIJ790)	Chloramphenicol 25 Kanamycin 50	Host for Redirect PCR targeting system with pIJ790 for λ RED proteins	Gust et al. 2003
DH5α (BT340)	Ampicillin 100 Chloramphenicol 25	Produces FLP recombinase from BT340 plasmid	Cherepanov and Wackernage 1995
S. clavuligerus strains			
ATCC 27064	N/A	Wild-type (wt) strain	American Type Culture Collection
Δ*blm*::*apra*^{R}	Apramycin 25	Δ*blm::apra-oriT* mutant (cassette in opposite orientation to transcription)	This study

Δ*orf12::apra*	Apramycin 25	Δ*blm::apra*^{R} mutant (*apra*^{R} in opposite orientation to *blm*)	Jensen et al. 2004
Δ*blm*-INF	N/A	Δ*blm:: scar* in-frame mutant	This study
***S. flavogriseus* and *S. viridis* strains (respectively)**			
ATCC 33331	N/A	Wild-type (wt) strain	American Type Culture Collection
ATCC 15386	N/A	Wild-type (wt) strain	American Type Culture Collection
Klebsiella pneumoniae			
ATCC 15380	N/A	Indicator organism for bioassay experiments	Reading and Cole 1977

*Final antibiotic concentration for this item is dependent on the bacterial species- See Table 2.1 for further information.
N/A-either strain is antibiotic sensitive or antibiotics were not used in selection

discarded and the pellet was washed with 300 µl of ice cold 70% ethanol and centrifuged at 10,000 rpm for 5 minutes. Gentle aspiration was used to remove the supernatant before air drying under a laminar flow hood for 20 minutes. The clear, dried pellet was re-suspended in 50 µl of H_2O containing 20 µg/ml RNaseA (AC117, Omega Bio-Tek, USA) before being incubated at 37°C for 20 minutes and stored at -20°C for subsequent use.

2.2.3: Preparation and transformation of competent cells

2.2.3.1: Chemically competent cells

A single colony of the appropriate *E. coli* strain was used to inoculate 5 ml of LB broth (containing the appropriate antibiotics if necessary) and was cultured at 37°C (or 30°C in the case of DH5α/BT340). The next day, 500 µl of the overnight culture was used to inoculate 50 ml of LB broth, which was incubated at the appropriate temperature with shaking until $OD_{600} \sim 0.4$. The culture was then transferred to a 50 ml falcon tube and pelleted by centrifugation at 1,000 x *g* for 10 minutes at 4 °C. The supernatant was discarded and 5 ml of ice cold transformation and storage buffer [LB broth (pH 6.1), 10% PEG v/v, 5% DMSO w/v, 20 mM Mg^{2+} (10 mM $MgCl_2$ + 10 mM $MgSO_4$)] was used to re-suspend the pellet. The cell suspension (100 µl) was rapidly aliquoted into pre-chilled 1.5 ml microfuge tubes and each tube was flash frozen in a liquid nitrogen bath before being stored at -80°C for subsequent use. Before DNA transformation, competent cells were thawed on ice for 30 minutes. The DNA to be transformed (10 µl ligation reactions or 5 ng pure plasmid/cosmid DNA) was added to 50 µl of thawed competent cells and incubated on ice for 30 minutes. Following incubation, the mixture was heat-shocked at 42°C for 30 seconds before being placed immediately on ice for 3-5 minutes. The cells

were then transferred to a 1.5 ml microfuge tube containing 950 µl of SOC (2% w/v tryptone, 0.5% w/v yeast extract, 0.05% w/v NaCl, 0.0186% w/v KCl in dH_2O with 10 mM $MgCl_2$ and 20 mM of glucose added after autoclaving) and incubated at 37°C for 1 hour with shaking. After incubation, the cells were pelleted via centrifugation at 10,000 rpm for 2 minutes and 900 µl of the resulting supernatant was removed and discarded. The pellet was re-suspended in the remaining 100 µl of supernatant and the entire volume was plated onto a single LB plate containing the appropriate antibiotics. Transformation plates were incubated at the appropriate temperature overnight and successful transformants were identified based on the growth of bacterial colonies on the following day. In the case of transforming ligations involving the vector pGEMT-Easy, (A1360, Promega, Canada) blue/white screening was employed and white colonies were selected for further analysis.

2.2.3.2: Electrocompetent cells

A single colony of *E. coli* BW25113/pIJ790 was used to inoculate 10 ml of LB broth containing chloramphenicol before the culture was incubated overnight at 30°C with shaking. Afterwards, 100 µl of overnight culture was sub-cultured into 10 ml of Super Optimal Broth (SOB) (2% w/v tryptone, 0.5% w/v yeast extract, 0.05% w/v NaCl, 0.0186% w/v KCl in dH_2O) containing 20 mM $MgSO_4$ and chloramphenicol (25 µg/ml). This culture was incubated at 30°C with shaking until an OD_{600} of 0.4 was reached. Cells were pelleted by centrifugation at 4000 rpm for 5 minutes at 4°C. The supernatant was discarded and the pellet was re-suspended in 10 ml of ice cold, sterile 10% glycerol. The pellet was washed two more times with 5 ml ice cold 10% glycerol in this manner and

finally the supernatant was discarded and the pellet was re-suspended in the remaining ~100 µl of 10% glycerol. 50 µl of the electrocompetent cell suspension was added to an ice cold 0.2 cm electroporation cuvette and mixed with 100 ng of cosmid DNA. Electroporation was carried out using a BioRad GenePulser Xcell™ (165-2662, Canada) set to: 200 Ω, 25 µF and 2.5 kV. The time constant of successful transformations was usually in the range of 5.0-5.2 ms. Immediately after electroporation, 1 ml of ice cold LB was added to the cells, which were then gently transferred to a fresh microfuge tube and incubated at 30°C for 1 hour with shaking. Afterwards, the cells were pelleted by centrifugation at 4000 rpm for 5 minutes at and 900 µl of supernatant was drawn off and discarded. The cells were re-suspended in the remaining ~100 µl of supernatant before being spread onto two LB agar plates supplemented with the appropriate antibiotics. These plates were incubated overnight at 28°C and resulting antibiotic resistant colonies were used to inoculate 5 ml LB broth supplemented with ampicillin (100 µg/ml), kanamycin (50 µg/ml) and chloramphenicol (25 µg/ml). These cultures were incubated overnight at 30°C with 200 rpm shaking and glycerol stocks were prepared.

2.3: General *Streptomyces* procedures

2.3.1: Maintenance and stock preparation

All cultures of *S. clavuligerus* were grown at 28°C, with liquid cultures being incubated with agitation at 200 rpm in 125 ml flasks with springs (unless indicated otherwise). Strains of *S. clavuligerus* were maintained on either international ISP-4 medium (M359, HiMedia, India) or on MYM plates (0.4% w/v maltose, 0.4% w/v yeast extract, 1% w/v malt extract and 1.8% w/v agar) and liquid cultures were grown in TSB

(236950, BD Biosciences, USA) supplemented with 2% w/v starch (TSBS) (RM3029, HiMedia, India). *S. flavogriseus* was cultured and maintained in TSB using the same incubation conditions mentioned above. *S. viridis* was grown in liquid cultures of Nutrient Broth (2340000, BD Difco, USA) at 45°C with agitation at 200 rpm in 125 ml flask (with no spring). Glycerol stocks were prepared by scraping growth (spores or mycelia) from agar plates into a 1.5 ml microfuge tube containing 500 µl of 20% sterile glycerol, before being stored at -80°C. When required, antibiotics were included as listed in Table 2.1. All exconjugants were allowed to recover on AS-1 medium (0.01% w/v yeast extract, 0.02% w/v L-alanine, 0.02% L-arginine, 0.05% w/v L-asparagine, 0.5% soluble starch, 0.25% w/v NaCl, 1.0% w/v Na_2SO_4, 2.0% w/v agar, pH to 7.5 and 1% v/v sterile 1 M $MgCl_2$ added post- autoclaving). For bioassays and HPLC analysis, strains were grown in SA broth [1% w/v soluble starch; 0.2% w/v L-asparagine; 2.1% w/v MOPS buffer; 0.06% w/v $MgSO_4$; 0.44% w/v K_2HPO_4 in H_2O; pH 6.8 and 1 ml of sterile trace elements solution (0.10% w/v of each $FeSO_4$, $MnCl_2$, $ZnSO_4$ and 0.13% w/v $CaCl_2$) per L of media (added after autoclaving].

2.3.2: Chromosomal DNA preparation

Chromosomal DNA was extracted from all *S. clavuligerus*, *S. flavogriseus* and *S. viridis* cultures using the QIAamp DNA Mini Kit (QIAGEN Inc., Canada) using a modified protocol. From 25 ml of TSBS cultures, 1.5 ml was drawn and added to a microfuge tube before the mycelia were harvested by centrifugation. Subsequently, 200 µl of both ATL and AL buffer were added. Approximately 600 µl of this cell suspension was added to a 2 ml sterile screw cap tube containing sterile, acid-washed beads (BAWZ

400-250-35, OPS Diagnostics, USA). The samples were homogenized using a SpeedMill PLUS Bead Homogenizer (845-00008-2, Analytik Jena AG, Germany) under the pre-programmed "Bacteria" protocol (2 x 3 minute on and 3 minute off cycles). Afterwards, samples were centrifuged at 10,000 rpm for 1 minute, and the supernatants were transferred to fresh 1.5 ml microfuge tubes containing 200 µl of 95% ethanol. After vortexing briefly, the tubes were centrifuged again and the supernatants were transferred to spin columns and centrifuged at 8,000 rpm for 1 minute. Filtrates were discarded and 500 µl of AW2 Buffer was added to each column before centrifugation at 14,000 rpm for 3 minutes. The filtrates were again discarded and tubes were centrifuged as above for an additional minute to remove residual buffer and ethanol. Columns were placed into clean, sterile 1.5 ml microfuge tubes and 100 µl of dH_2O was added to each column. Columns were incubated at room temperature for 5 minutes before the DNA was eluted via centrifugation at 8000 rpm for 1 minute and was stored at -20°C for subsequent use.

2.3.3: Chemical denaturation of *S. flavogriseus* chromosomal DNA for use in PCR

Genomic DNA (9 µl) prepared via the above method was mixed with 2 µl of 1 M NaOH before incubating at 37°C for 10 minutes. Afterwards, 2 µl of 1 M HCl was added to the genomic DNA solution, which was then mixed immediately and placed on ice. After the solution had cooled, 1 µg of genomic DNA from the sample was used for PCRs amplification (refer to section 2.4.1.2 for PCR instructions).

2.3.4: Standardization of cell growth for bioassays and HPLC

Cultures of different *S. clavuligerus* strains were started by dispensing a 1% v/v mycelial inoculum into 25 ml of TSBS containing antibiotics as necessary and then incubating for 36 hours. Mycelia were then harvested by centrifugation at 3,500 rpm for 7 minutes before being washed twice with 10.3% w/v sucrose. The pellet was re-suspended in 2 ml of 20% glycerol and the OD_{595} of the suspensions were measured, which were determined following a protocol modified from Brana et al. (1985). Mycelial suspension (500 µl) was mixed with 500 µl of 0.62 M HCl before being sonicated in a water bath (Ultrasonic cleaner, ME 4.6, Mettler Electronics corp., USA) at room temperature for 30 seconds. Samples were then diluted 1/100 with 0.25 M HCl and the OD_{595} was recorded for each sample. According to the protocol, one unit of OD_{595} is equivalent to 0.59 mg/ml dry cell weight when the final readings are below 0.6. Final standardized stocks were made by diluting all samples with 20% glycerol to an OD_{595} of 25. These diluted suspensions, were aliquoted into a microfuge tubes and stored as mycelial stocks at -80°C.

2.3.5: Liquid cultures for bioassays and HPLC analysis

Cultures of each *S. clavuligerus* strain were started using a 1% v/v inoculum from the standardized mycelial stocks (see section 2.3.4) in 10 ml of TSBS plus antibiotics (as necessary) and incubated for 48 hours with shaking in 50 ml flasks without springs. SA medium (25 ml) was inoculated with 200 µl of the above starter cultures in 125 ml flasks without springs, and the flasks were incubated for 120 hours with shaking. Culture supernatants were sampled aseptically every 24 hours starting at 48 hours, with 1 ml

being removed and transferred to clean sterile microfuge tubes. Samples were stored at -80°C for subsequent use in bioassays and HPLC analysis.

2.3.6: Liquid cultures for protein extraction

TSBS medium (10 ml) supplemented with hygromycin (200 μg/ml) was inoculated with *S. clavuligerus* strains harbouring the FLAG tag expression plasmid for *blm*, *blip* or *ccaR* (Table 2.2), and were incubated for 24 hours at 28°C with shaking. Three ml from each starter culture was sub-cultured into 100 ml SA broth in 500 ml flasks without springs. These cultures were incubated at 28°C with shaking for 48 hours before being used immediately for protein extraction or were frozen at -80°C for further analysis (as described later).

2.3.7: Mycelial conjugations

Cultures of either *S. clavuligerus* Δ*blm::apra*^{R} or Δ*blm*-INF were started in 50 ml of 2% TSBS containing appropriate antibiotics and were incubated for 36-40 hours at 28°C with shaking. Subsequently, the cultures were centrifuged at 3,500 rpm for 7 minutes and washed twice in 25 ml of 2x YT medium (1.6% w/v tryptone, 1% w/v yeast extract, 0.5% w/v NaCl, in H_2O, pH 7.0) before being re-suspended in 5 ml of 2x YT media. The mycelial suspension was then homogenized using a Duall tissue grinder with glass pestle (KT885450-0023, VWR International, Canada). A 50 ml culture of the donor *E. coli* strain ET12567/pUZ8002 containing the plasmid/cosmid to be mobilized was grown in LB supplemented with the appropriate antibiotics (Table 2.2) to an OD_{600} of

0.3-0.5. Cells were centrifuged at 3,500 rpm and washed twice with LB before being re-suspended in 1 ml of LB. The homogenized mycelial were centrifuged as above and re-suspended in 1 ml of 2x YT media. Mycelial suspension (500 µl) was transferred to a sterile 1.5 ml microfuge tube and heat-shocked at 40°C for 5 minutes with 300 rpm shaking in an Eppendorf thermomixer (22331, Germany). Immediately after heat-shock, 500 µl of the *E. coli* suspension was added to the mycelial suspension and mixed by vortexing. The entire 1 ml suspension was spread evenly onto AS-1 agar plates and after 24 hours of incubation at 28°C, the plates were overlaid with nalidixic acid (40 µg/ml, final concentration) and an antibiotic for plasmid/cosmid transfer selection, before being incubated for a further 1-2 weeks. When adequate growth was obtained, colonies were picked and homogenized with a sterile pipette tip in a 1.5 ml microfuge tube containing 20 µl of sterile H_2O. This suspension was plated onto ISP-4 media containing nalidixic acid (40 µg/ml) and the previously used selection antibiotic before being incubated at 28°C for 2 weeks. After incubation, growth was collected and scraped into 500 µl of 20% glycerol. These stocks were vortexed and stored at -80°C for subsequent use.

2.3.8: Spore conjugations

Spore conjugations were only performed using the wt *S. clavuligerus* strain. A culture of the donor *E. coli* ET12567/pUZ8002 containing the plasmid/cosmid to be transferred (Table 2.2) was grown and processed as described above. *S. clavuligerus* wt spores (100 µl) were washed in 1 ml of 2x YT broth followed by suspension in a 50°C water bath for 10 minutes. Spores were allowed to cool before mixing 500 µl of washed *E. coli* cells and 500 µl of heat shocked spores in a microfuge tube. The contents were

pelleted by centrifugation at 10,000 rpm for 1 minute. The supernatant was discarded and the pellet was gently re-suspended in residual broth. The entire suspension was spread onto a freshly made AS-1 agar plates and incubated at 28°C for 24 hours. The following recovery and selection process was the same as described in the above mycelial conjugation protocol (see section 2.3.7).

2.3.9: Determination of dry cell weights

After 96 hours of incubation during fermentation studies using SA medium, 1 ml of each culture (section 2.3.5) was removed and transferred to microfuge tubes. Mycelial pellets were obtained by centrifugation at 10,000 rpm and the majority of the supernatant was drawn off with special consideration to not disturb or remove any of the pellets. The pellets were re-suspended in the remaining supernatant and each one was transferred to one, pre-weighed, small aluminum weigh boat (12577-062, VWR International, Canada). H_2O (100 µl) was added to each microfuge tube to resuspend any remaining mycelia and to ensure that all growth was removed and transferred to each weigh boat. Afterwards, the pellets in the weigh boats were dried for 72 hours at 100°C and were then left to cool to room temperature before weights were recorded. Dry cell weights were determined by subtracting the weight of the weigh boat from that of the weigh boat and dried pellet.

2.4: General DNA procedures

2.4.1: PCR and agarose gel-electrophoresis conditions

The primers listed in Table 2.3 were used for performing the various PCR reactions and DNA sequencing analysis conducted during the described study.

Table 2.3: Oligonucleotide primers used in this study with their primer pair, Tm and purpose

Primer Name	Sequence	Restriction enzyme site	Used with	Tm (°C)	Purpose
blm-RD-F	5'-ATG ATG AAG AAA GCT GAT TCC GTC CCG ACC CCG GCT GAG ATT CCG GGG ATC CGT CGA CC-3'	None	blm-RD-R	Multi	Introduction of *apra-oriT* cassette in place of *blm* in genetic reading frame
blm-RD-R	5'-TCA TCG CCG GGC GGC TTC TCC GGC GCT CGC GCG GTC GTC TGT AGG CTG GAG CTG CTT C-3'	None	blm-RD-F	Multi	
blm-CONF-F	5'- CCG ACA AGG AGC GAT GAT GA-3'	None	blm-CONF-R	57 (Taq) 68 (Phus)	Confirm DNA in position of *blm*, could be *blm*, *apra-oriT* cassette or scar
blm-CONF-R	5'- GGA TCA CAC CGA AGC CCA GT-3'	None	blm-CONF-F	57 (Taq) 68 (Phus)	
pIJ773-R	5'-TCG CTA TAA TGA CCC CGA AG-3'	None	blm-CONF-R	51 (Taq)	Shows direction of *apra-oriT* cassette in *S. clavuligerus* Δ*blm::apra*^{R} mutants
pWE15-amp-RD-F	5'-TGA TAA ATG CTT CAA TAA TAT TGA AAA AGG AAG AGT ATG ATT CCG GGG ATC CGT CGA CC-3'	None	pWE15-amp-RD-R	Multi	Introduction of *hyg-oriT* cassette into *amp*^{R} in 12B8 cosmid (Redirect)
pWE15-amp-RD-R	5'-AAT CTA AAG TAT ATA TGA GTA AAC TTG GTC TGA CAG TTA TGT AGG CTG GAG CTG CTT C-3'	None	pWE15-amp-RD-F	Multi	
pIJ799-F	5'-CCCTGATAATGCTTCAATAATATTGAAAA-3'	None	pIJ799-R	49 (Taq)	Confirms DNA in position of *amp*^{R}, could be *amp*^{R} or *hyg-oriT* cassette, in 12B8 cosmid
pIJ799-R	5'-AAT CAA TCT AAA GTA TAT ATG AGT AAA CTT GGT C-3'	None	pIJ799-F	49 (Taq)	
HygR-F	5'-CGC ATA GAC GTC GGT GAA GT-3'	None	HygR-R	56 (Taq)	Confirms presence or absence of *hyg*^{R}
HygR-R	5'-TAC CTG GTG ATG AGC CGG AT-3'	None	HygR-F	56 (Taq)	Confirms presence or absence of *hyg*^{R}
blip-FLAG-F	5'-ATA CAT ATG AGG ACA GTG GGG ATC G-3'	*NdeI*	blip-FLAG-R	3-step	Amplification of *blip* from *S. clavuligerus* with FLAG tag attached
blip-FLAG-R	5'-ATG GAT CCT CAC TTG TCG TCG TCG TCC TTG TAG TCT ACA AGG TCC CAC TGC CGC TTG-3'	*BamHI*	blip-FLAG-F	3-step	
ccaR-FLAG-F	5'-TAC ATA TGA ACA CCT GGA ATG ATG TGA C-3'	*NdeI*	ccaR-FLAG-R	3-step	Amplification of *ccaR* from *S. clavuligerus* with FLAG tag attached
ccaR-FLAG-R	5'-ATG GAT CCT CAC TTG TCG TCG TCG TCC TTG TAG TCG GCC GGG GTA CCG ACC-3'	*BamHI*	ccaR-FLAG-F	3-step	
blm-FLAG-F	5'- TAC ATA TGA TGA AGA AAG CTG ATT CCG TC-3'	*NdeI*	blm-FLAG-R	3-step	Amplification of *blm* from *S. clavuligerus* with FLAG tag attached
blm-FLAG-R	5'- ATG GAT CCT CAC TTG TCG TCG TCG TCC TTG TAG TCT CGC CGG GCG GCT TC-3'	*BamHI*	blm-FLAG-F	3-step	

blm-SC-F	5’-ATA CAT ATG ATG AAG AAA GCT GAT TCC G-3’	*NdeI*	blm-SC-R	67 (Phus)	Amplification of *blm* from *S. clavuligerus*
blm-SC-R	5’-ATA GGA TCCTCA TCG CCG GGC GGC TTC-3’	*BamHI*	blm-SC-F	67 (Phus)	
blm-SF-F	5’-TAC ATA TGA TCG ATC TAC CCG GTT CCG-3’	*NdeI*	blm-SF-R	72 (Phus)	Amplification of *blm* from *S. flavogriseus*
blm-SF-R	5’-ATA AGC TTC ACCGGG AGG TGC CGG-3’	*HindIII*	blm-SF-F	72 (Phus)	
blm-SV-F	5’-TAC ATA TGT TGA CCA CTA CCG AGA C-3’	*NdeI*	blm-SV-R	66 (Phus)	Amplification of *blm* from *S. viridis*
blm-SV-R	5’-ATA AGC TTC ACG GCA GCA ACG AGT G-3’	*HindIII*	blm-SV-F	66 (Phus)	

Multi- Redirect PCR method outlined by Gust et al. 2003 was used. See section 2.5.1; (Taq)- Tm used in conjugation with Taq DNA polymerase; (Phus)- Tm used in conjunction with Phusion DNA polymerase; 3-step- Phusion 3-step PCR method was used. See section 2.4.1.1

2.4.1.1: PCR to confirm the deletion of the *blm* gene in cosmid and genomic DNA

Several DNA oligonucleotide primer pairs (Table 2.3) were used to confirm the replacement of the *blm* gene with either the apramycin cassette or the 81 bp scar DNA sequence. These PCR reactions were carried using either purified cosmid DNA or *S. clavuligerus* genomic DNA as template, depending on the stage of the Redirect PCR targeting process (Gust et al. 2003). Either 1 µg of *S. clavuligerus* genomic DNA or 200 ng of cosmid DNA was used as template along with Fisher BioReagents® *Taq* DNA polymerase (FB-6000-15, Fisher Scientific, Canada,) with Buffer A, 3% v/v DMSO and 1 M betaine, as per manufacturer's instructions. The thermocycling conditions were as follows: initial denaturation at 95°C for 3 minutes with 35 cycles of denaturation at 95°C for 30 seconds, variable annealing at temperatures for 30 seconds and extension at 72°C for 2 minutes, followed by a final extension step at 72°C for 5 minutes. Annealing temperatures (X°C) are variable and DNA oligonucleotide primer dependent as indicated in Table 2.3.

2.4.1.2: PCR of *blm* from *S. clavuligerus, S. viridis* and *S. flavogriseus*

The *blm* gene from each species was amplified using oligonucleotide primers described in Table 2.3 along with 1 µg of purified genomic DNA as template from each organism (*S. flavogriseus* genomic DNA was chemically denatured prior to use in PCR; see section 2.3.3) and Phusion High-Fidelity DNA Polymerase (F-5302, Fisher Scientific, Canada) with high GC buffer, 3% v/v DMSO and 1 M betaine, as per manufacturer's instructions. The following thermocycling conditions were used: initial denaturation at 98°C for 1 minute and then 30 cycles of denaturation at 98°C for 15 seconds, annealing at

variable temperatures for 15 seconds and extension at 72°C for 1.5 minutes, followed by a final extension at 72°C for 5 minutes. Annealing temperature (X°C) is variable and depends on the oligonucleotide primer pair used as described in Table 2.3.

2.4.1.3: PCR and FLAG tagging of *blm, ccaR* and *blip* for protein localization studies

All three *S. clavuligerus* genes were amplified using oligonucleotide primers described in Table 2.3 along with 1 µg of *S. clavuligerus* genomic DNA as the template, Phusion High-Fidelity DNA Polymerase with High GC buffer, 3% v/v DMSO and 1 M betaine, as per manufacturer's instructions. The following three step thermocycling conditions were used for the reactions: initial denaturation at 98°C for 30 seconds; then 10 cycles of denaturation at 98°C for 10 seconds, annealing at 60°C for 30 seconds and extension at 72°C for 1 minute; followed by 10 cycles of denaturation at 98°C for 10 seconds, annealing at 65°C for 30 seconds and extension at 72°C for 1 minute; and finally 15 cycles of denaturation at 98°C for 10 seconds, annealing at 72°C for 30 seconds and extension at 72°C for 30 seconds. A final extension at 72°C for 3 minutes was conducted at the end.

2.4.1.4: Agarose gel-electrophoresis

All reactions were loaded on to either a 0.8% w/v (for fragments >1 kb) or 1.5% w/v (for fragments ≤1 kb) agarose gels using 6x loading dye (30% v/v glycerol, 0.25% w/v bromophenol blue, 0.25% xylene cyanol). The GeneRuler™ 1 kb ladder (SM0321, Thermo Scientific, USA) or the GeneRuler™ 100 bp Plus ladder (SM0321, Thermo Scientific, USA) were used as molecular weight markers for the analysis. Electrophoresis

was performed in 1x TBE buffer (89 mM Tris base, 89 mM Boric acid, 2 mM EDTA, pH 8) at a constant voltage between 95-120 V, depending on the gel size. Before imaging, gels were stained in 0.5 µg/ml ethidium bromide solution (L07482, Alfa Aesar, USA) for 30 minutes and then de-stained in H_2O for 15 minutes. Images of the gels were then obtained using a ChemiImager™ Ready imager (DE500, Canberra Packard, Canada).

2.4.2: Cloning with pGEM®-T Easy and pHM11a

2.4.2.1: pGEM®-T Easy

All genes later expressed in *S. clavuligerus* (*blm*^{SC}, *blm*-like genes and FLAG-tagged genes) were originally cloned into the pGEM®-T Easy vector (A1360, Promega, USA). Ligation of PCR products with the pGEM®-T Easy vector was carried out according to the manufacturer's instructions. Polyadenylation of PCR products was achieved through the use of Fisher BioReagents® *Taq* DNA polymerase (recombinant, FB-6000-15, Fisher Scientific Canada) in 10 µl reactions with the following components: 1x Taq Buffer A, 0.2 mM dNTPs and up to 8.7 µl of PCR product. The reactions were incubated at 72°C for 10 minutes before immediately being added to ligation reactions containing the pGEM®-T Easy vector. The reactions were left at 4°C overnight before transformation into *E. coli* NEB5α (as discussed in section 2.2.3.1). After blue/white screening, plasmid DNA from each strain was isolated using the modified alkaline lysis method. Restriction enzyme digestion using *Eco*RI and subsequent gel electrophoresis was performed to screen plasmids for the presence of positive clones.

2.4.2.2: pHM11a

Each positive PGEM®-T Easy clone (1 µg) was digested to excise the previously cloned insert from above (*blm*^{SC}, *blm*-like genes and flag-tagged genes) with restriction enzymes corresponding to the sites engineered into the respective primers (Table 2.3). *SspI* was also included in each digestion to cut the pGEM®-T Easy backbone in order to promote ligation of DNA fragments containing genes of interest into pHM11a (Motamedi et al. 1995) instead. After overnight digestion, the DNA was added to 0.5 ng of pHM11a previously digested with the same enzymes in addition to FastAP thermosensitive alkaline phosphatase (EF0654, Thermo Fisher Scientific Inc., Canada). Before combining the two samples, all enzymes were heat inactivated by incubating at 80°C for 20 minutes. The combined DNA was co-precipitated by adding 1/10 volume of 3 M sodium acetate and 2 volumes of ice cold absolute ethanol and contents were vortexed. The samples were incubated overnight at -20°C, after which they were centrifuged at 14,000 rpm at 4°C for 15 minutes in an Eppendorf 5417R centrifuge with an F45-30-11 rotor. The supernatant was discarded and the pellet was dried by aspiration. Subsequently, 300 µl of ice cold 70% ethanol was added and the tubes were centrifuged again (as above) for 5 minutes. The supernatant was discarded and the pellet was dried again in laminar flow hood with the caps open until they became colourless. The DNA pellets were re-suspended in 35 µl of sterile H_2O for use in ligations.

Ligations with the pHM11a vector were carried out in 10 µl reactions using 1 µl of T4 DNA Ligase (M0202S, New England Biolabs, USA), 1x NEB ligation buffer and 8 µl of ethanol-precipitated DNA from above. All ligation reactions were incubated overnight

at 18-20°C before being transformed into *E. coli* NEB5α (as discussed in section 2.2.3.1). The DNA sequence of each insert was determined using a combination of primers (Table 2.3) at The Centre for Applied Genomics (TCAG, University of Toronto, Canada) to confirm the absence of mutations, before the plasmids were introduced into *S. clavuligerus Δblm-INF, Δblm::apra*^{R} and wt strains via mycelial or spore conjugation.

2.5: The creation of *S. clavuligerus* Δ*blm* mutants

2.5.1: PCR targeting and preparation of the 12B8 *Δblm-AP* cosmid

Redirect PCR primers were designed to amplify the apramycin cassette from the plasmid pIJ773 while adding DNA sequences from the upstream and downstream region of *blm* to each end of the PCR product. The apramycin resistance cassette was released from pIJ773 as a result of an *Eco*RI and *HindIII* digest. The cassette was then gel purified as described above in section 2.1. The purified apramycin cassette was used as the template DNA in a PCR reaction using the blm-RD primers (Table 2.3) along with Phusion High-Fidelity DNA Polymerase with high GC Buffer, 3% v/v DMSO and 1 M betaine, as per manufacturer's instructions. The following two step thermocycling conditions were used: initial denaturation at 98°C for 1 minute; then 10 cycles of denaturation at 98°C for 5 seconds, annealing at 50°C for 15 seconds and extension at 72°C for 2 minutes; followed by 15 cycles of denaturation 98°C 5 seconds, annealing at 55°C for 15 seconds and extension at 72°C for 2 minutes; and finally a final extension at 72°C for 5 minutes. The resulting PCR product contained the apramycin cassette flanked by sequences complementary to DNA directly upstream and downstream of *blm* from the

S. clavuligerus chromosome. The size of the PCR product was confirmed by gel electrophoresis followed by gel purification.

SOB broth (50 ml) containing the appropriate antibiotics was inoculated with 1 ml of overnight *E. coli* BW25113 containing pIJ790 and cosmid 12B8 (12B8 was previously electroporated into this strain via the method outline in section 2.2.3.2). Five hundred microliters of 1 M L-arabinose stock solution (10 mM final) was added to induce the *red* genes of pIJ790 before the culture was incubated at 30°C with 200 rpm shaking until the OD_{600} approached ~0.3 (~ 3 hours). An additional 500 µl of 1 M L-arabinose solution (10 mM final) was then added and the culture was incubated for another 30-40 minutes. Cultures were transferred into sterile 50 ml falcon tubes and cells were recovered by centrifugation at 4000 rpm for 5 minutes at 4°C. The supernatant was discarded and the pellet was re-suspended in 10 ml of ice cold 10% glycerol before centrifuging as above. The process was repeated after which the supernatant was discarded and the pellet was re-suspended in the ~100 µl residual 10% glycerol. Fifty microliters of the cell suspension was mixed with ~500-800 ng of purified apramycin PCR product (from above) before electroporation was carried out in an ice cold 0.2 cm electroporation cuvette using a BioRad GenePulser set to: 200 Ω,25 µF and 2,5 kV. Ice cold LB (1 ml) was immediately added and cells were incubated at 37°C with 200 rpm shaking for 1 hour. Afterwards, the cells were pelleted by centrifugation at 4000 rpm for 5 minutes at 4°C and 900 µl of the resulting supernatant was discarded. The cells were re-suspended in the remaining ~100 µl of supernatant and the cell mixture was spread onto two low sodium LB (1% w/v tryptone, 0.5% w/v yeast extract, 0.25% w/v sodium chloride) agar plates supplemented with kanamycin (50 µg/ml) and apramycin (100 µg/ml). These cultures were incubated

for 16-24 hours at 37°C to promote the loss of pIJ790. Antibiotic resistant colonies were used to inoculate 5 ml of LB broth supplemented with kanamycin (50 µg/ml) and apramycin (100 µg/ml). These cultures were incubated overnight at 37°C with 200 rpm shaking and glycerol stocks were made the following day.

The resulting 12B8 Δ*blm-AP* cosmid was verified via PCR amplification of the *blm* region and was transformed into *E. coli* ET12567/pUZ8002 and plated onto LB agar supplemented with apramycin (50 µg/ml), chloramphenicol (25 µg/ml) and kanamycin (25 µg/ml). Isolated colonies were picked and cultured in 5 ml of LB broth supplemented with antibiotics (as above) before being incubated overnight at 37°C with 200 rpm shaking for preparing glycerol stocks.

2.5.2: Preparation of the *S. clavuligerus* a*blm::apra*^{R} mutant

The engineered cosmid from above (12B8 Δ*blm-AP*), was conjugated from *E. coli* ET12567/pUZ8002 into *S. clavuligerus* wt via spore conjugation (section 2.3.8). After the initial 30 hours incubation, the conjugation plates were overlaid with nalidixic acid (40 µg/ml) and apramycin (25 µg/ml) before being incubated for a further 2 weeks. Thereafter, colonies were picked and homogenized with a sterile pipette tip in a 1.5 ml microfuge tube containing 20 µl of sterile H_2O. This suspension was plated onto ISP-4 medium containing nalidixic acid and apramycin before being incubated at 28°C for 2 weeks. After incubation, growth was collected in a microfuge tube and 500 µl of 20% glycerol was added. These stocks were vortexed and placed at -80°C for subsequent use.

To verify the *blm* replacement, glycerol stocks of suspected *S. clavuligerus* a*blm::apra*^{R} colonies were thawed on ice and serially diluted to 10^{-6} using sterile H_2O. Each dilution (100 µl) was spread onto MYM agar supplemented with nalidixic acid and apramycin and incubated at 28°C. After 1 week, isolated colonies from each strain were crushed with a pipette tip in 30 µl sterile H_2O. Twenty microliters of this suspension was plated onto ISP-4 media containing apramycin and the remaining 10 µl was plated onto MYM agar containing kanamycin. These plates were left to incubate for 2 weeks before colony growth on each plate was compared. Growth from plates containing mutant strains sensitive to kanamycin and resistant to apramycin were scraped and placed in a 1.5 ml tube containing 500 µl of 20% glycerol before being stored at -80°C for subsequent use. The *S. clavuligerus* a*blm::apra*^{R} mutants were genetically confirmed via PCR of the *blm* region (section 2.4.1.1) using genomic DNA (section 2.3.2) as the template DNA.

2.5.3: FLP-mediated excision of the apramycin cassette from *12B8 Δblm-AP*

The 12B8 Δ*blm-AP* cosmid was transformed into chemically competent *E. coli* DH5α/BT340 cells as outlined above (section 2.2.3). All steps including incubation were done at a temperature of 30°C or lower to maintain FLP synthesis and plasmid replication. The transformation mixture was plated onto LB agar supplemented with the appropriate antibiotics (Table 2.2) before being incubated at 30°C for 48 hours. Subsequent colonies were streaked onto LB agar plates without antibiotics and these plates were incubated at 42°C overnight to promote FLP recombinase expression and loss of the BT340 plasmid. The following day, the antibiotic resistance profile of 10 colonies

from the growth plates was tested against ampicillin and apramycin. This was conducted by dividing a single colony into 3 portions and spotting each third of a colony onto the following media: a LB agar plate, LB agar containing ampicillin and LB containing apramycin. Afterwards, these plates were incubated at 37°C overnight. Colonies that were ampicillin resistant and apramycin sensitive were verified by PCR analysis (see section 2.4.1.1) before glycerol stocks were made and stored at -80°C. These colonies contained the engineered cosmid 12B8 Δ*blm::scar.*

2.5.4: Replacement of the ampicillin resistance gene with the hyg^{R} cassette

The hygromycin resistance cassette was released from pIJ10700 as a result of a *SmaI* and *HindIII* digestion before being gel purified using the EZ-10 Spin Column DNA Gel Extraction Kit (section 2.1). The purified hygromycin cassette was used as template DNA in a PCR reaction using the pWE15-amp-RD primers (Table 2.3), Fisher BioReagents® *Taq* DNA polymerase with Buffer A, 3% v/v DMSO and 1 M betaine, as per manufacturer's instructions. The following two step thermocycling conditions were used for the reactions: initial denaturation at 95°C for 3 minutes; then 15 cycles of denaturation 95°C for 30 seconds, annealing at 50°C for 30 seconds, and extension 72°C for 2 minutes; followed by 30 cycles of denaturation 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension 72°C for 2 minutes; and finally a final extension at 72°C for 5 minutes. The resulting PCR product contained the hygromycin gene flanked by sequences complementary to the beginning and end of the ampicillin resistance gene located on the 12B8 Δ*blm::scar* cosmid (pWE15 backbone, Wahl et al. 1987). The PCR product size was confirmed with gel electrophoresis before gel purification. PCR

targeting was carried out as above (section 2.5.1) except for the following changes. The SOB broth contained ampicillin (100 µg/ml), kanamycin (50 µg/ml) and chloramphenicol (25 µg/ml) with 1 ml of overnight BW25113/pIJ790/*12B8* Δ*blm::scar* culture (created via electroporation of *12B8* Δ*blm::scar* via the method outlined above in section 2.2.3.2). Before electroporation, 50 µl of cells were mixed with ~500-800 ng of purified hygromycin PCR product (from above). Afterwards the cells were spread onto two low sodium LB agar plates supplemented with kanamycin and hygromycin. Inoculated plates were incubated for 24 hours at 37°C to promote the loss of pIJ790. The resulting antibiotic resistant colonies were used to inoculate 5 ml low sodium LB supplemented with kanamycin and hygromycin. These cultures were incubated overnight at 37°C with 200 rpm shaking and glycerol stocks were made. The resulting 12B8 Δ*blm::scar Δamp::hyg-oriT* cosmid was verified via PCR amplification of both the scar sequence and a 400 bp portion of the hygromycin gene. As well, the antibiotic resistance profile was determined for individual colonies of each strain by streaking half of a colony onto LB agar containing ampicillin and streaking the remaining half of the colony onto LB agar containing hygromycin. After a 24 hour incubation period, colonies that were ampicillin sensitive and hygromycin resistant were used to start new liquid cultures in order to make glycerol stocks. Upon cosmid confirmation, 12B8*::scar Δamp::hyg-oriT* was transformed into *E. coli* ET12567/pUZ8002 and plated onto two low sodium LB agar plates supplemented with the appropriate antibiotics (Table 2.2). Isolated colonies were selected and cultured in low sodium LB broth supplemented with the same antibiotics before being incubated overnight at 37°C with 200 rpm shaking. Glycerol stocks were made and stored.

2.5.5: Preparation of the *S. clavuligerus blm-INF* mutant

The cosmid engineered above (*12B8::scar Δamp::hyg-oriT*), was conjugated from *E. coli* ET12567/pUZ8002 into the *S. clavuligerus Δblm::apra*^{R} mutants (section 2.5.2) via mycelial conjugation (section 2.3.7). After the initial 24 hour incubation, the conjugation plates were overlaid with nalidixic acid and kanamycin before being incubated for a further 1.5 weeks. Thereafter, colonies were picked and homogenized with a sterile pipette tip in a 1.5 ml microfuge tube containing 20 µl of sterile H_2O. This suspension was plated onto ISP-4 media containing nalidixic acid and incubated at 28°C. After 2 weeks, growth was collected glycerol stocks were made and stored for further analysis.

To verify gene replacement, glycerol stocks of suspected *Δblm-INF* colonies were thawed on ice and serially diluted to 10^{-6} dilution using sterile H_2O. Each dilution (100 µl) was spread onto ISP-4 medium and incubated at 28°C for 2 weeks. Once large, isolated colonies appeared, a replica plating tool (25395-380, VWR International, Canada) and a sterile velveteen square was used to replica plate each ISP-4 dilution onto the following MYM agar plates: one without antibiotics, one containing kanamycin, and containing apramycin. These plates were left to incubate for 2 weeks before colony growth on each plate was compared. Colonies sensitive to both kanamycin and apramycin were picked and homogenized with a sterile pipette tip in a 1.5 ml tube containing 100 µl of 20% glycerol. Of this suspension, 50 µl was plated onto ISP-4 media and incubated at 28°C for 2 weeks. After incubation, growth was collected and glycerol stocks were made and stored for further analysis. The *Δblm-INF* mutants were

genetically confirmed via PCR of the *blm* region (section 2.4.1.1) using genomic DNA (section 2.3.2) from each as the template DNA.

2.6: Determination of antibiotic production

2.6.1: Bioassays for the detection of clavulanic acid

Bioassays were used to detect CA produced in culture supernatants (Mosher et al. 1999). TSB agar (TSBA; 200 ml) containing 1 ml of an overnight culture of *Klebsiella pneumoniae* (grown in TSB) and penicillin G (6 µg/ml) was poured into an ethanol-sterilized 22 x 22 cm plastic bioassay tray. As a control, another sterile tray was set up the same way, but without the addition of penicillin G to the medium. Sterile filter paper discs (10 mm, 1001-185, Whatman, UK) were aseptically placed on top of the agar in each tray and 10 µl of *S. clavuligerus* culture supernatant was spotted onto the filter paper discs. The trays were incubated overnight at 37°C right side up and the zones of inhibition were measured and recorded. Bioassay plates were imaged using a UVP Gel Doc-IT^{TS2} 310 Imager under white light.

2.6.2: HPLC analysis for the detection of clavulanic acid

HPLC analysis of *S. clavuligerus* culture supernatants was performed to detect and quantify CA production by different strains as described before (Foulstone and Reading 1982, Paradkar and Jensen 1995) with some modifications. An Agilent Technologies 1260 Infinity Quaternary LC system with a 100 x 8 mm Bondclone^{TM} 10µm C18 148 Å LC column (00D-2117-L0, Phenomenex Inc., USA) was used in the analysis. Supernatants in microfuge tubes were centrifuged at 12,000 rpm for 5 minutes before 100 µl was taken and derivatized with imidazole (10284730, Fisher Scientific

Canada) (25 µl of 20.6% w/v imidazole in water solution, pH 6.8 using concentrated HCl) for 15 minutes in the dark at room temperature. Control samples were prepared in the same way with 100 µl of supernatant mixed with 25 µl HPLC-grade H_2O. All samples were centrifuged at 12,000 rpm for another 5 minutes to remove particulate matter before 100 µl of each sample was transferred to flat bottom glass inserts (5181-3377, Aglient Technologies Canada Inc.). Each insert was placed inside a screw-cap glass vial (5181-3377, Aglient Technologies Canada Inc.) and loaded into the LC system. Samples (25 µl) were injected onto the column at a flow rate of 2 ml/min, and sample components were separated using a run time of 15 minutes with an isocratic mobile phase of sodium phosphate buffer (1 M Na_2HPO_4, 6% v/v CH_3OH, pH to 3.2 with glacial acetic acid). Absorbance at wavelengths corresponding to 311 nm, 317 nm and 320nm was monitored using the diode array detector and the Agilent ChemStation for LC 3-D Systems (G2170BA, revision: B.04.03, Agilent Technologies Canada Inc.) software was used to analyze the resulting data.

2.7: Western analysis of flag tagged proteins

2.7.1: Cytoplasmic protein extraction

One hundred milliliters of *S. clavuligerus* cultures were transferred to 2 x 50 ml tubes and centrifuged at 3,500 rpm for 7 minutes. The supernatant and the mycelial pellet were separated and frozen at -80°C if not used immediately. The pellet volume was made up to 5 ml with lysis buffer (150 mM HEPES and 150 mM NaCl, pH 7.5) and vortexed vigorously. Afterwards, 3 ml of each mycelial suspension was transferred to 3 ml screw cap cryovials (0566965, Fisher Scientific, Canada) and sonicated on ice using a QSonica

sonicator (Q125-110, VWR International, Canada) with a 5/64 inch probe (FB4423, Fisher Scientific, Canada). The sonication program consisted of 12 cycles of 15 seconds on and 15 seconds off for a total of 6 minutes. Sonicated mycelia were aliquoted into 3 x 1 ml microfuge tubes and centrifuged at 10,000 rpm for 10 minutes before the supernatants were carefully removed from the pellet and placed in fresh microfuge tubes. Protein quantification was performed using the OmniPur Bradford Method Protein Assay (2740-OP, EMD Millipore, Canada). Previously, a standard curve using this kit was prepared according to manufacturer's instructions through the use of Bovine Serum Albumin (BSA). All protein supernatants were stored at -80°C when not in use.

2.7.2: Ammonium sulfate precipitation of proteins from culture supernatant

Approximately eighty-seven millilitres of culture supernatant (separated from the above mycelial pellet in the first step; section 2.7.1) were transferred to 2 x 50 ml tubes and centrifuged in a Thermo Scientific Sorvall RC 6+ centrifuge at 27,000 *xg* for 15 minutes. The supernatant was carefully poured into a vacuum filtration apparatus (87006-062, VWR International, Canada) and filtered through a 0.2 µm filter to remove any residual mycelia. Filtered supernatant was transferred into a 500 ml flask containing a stir bar. Over a period of 1 hour, 44.9 g of ammonium sulfate was added gradually with constant stirring at 4°C to give 80% saturation. The protein that precipitated from solution was collected by high speed centrifugation as described above. The supernatant was discarded and the protein pellet was left to air dry in the inverted tubes. After 10 minutes, the pellet was re-suspended in 500 µl of 1 M phosphate buffer (sodium

phosphate, pH 7.0) and protein quantification was performed as mentioned above (section 2.7.1). Protein samples were stored at -80°C for subsequent analysis.

2.7.3: SDS-PAGE and western blot analysis

To visualize proteins, 20 or 50 µg of each protein sample was placed in a microfuge tube along with 1x protein loading dye (EC-887, National Diagnostics, USA), 2.5% v/v of DTT and sample buffer (either lysis buffer or phosphate buffer depending on sample type) to make up the final volume to 20 µl. Samples were boiled at 100°C for 5 minutes before immediately being put on ice. A total of ~20 µl of each sample was loaded onto an SDS 12% PAG (resolving gel: 12% w/v acrylamide/bis-acrylamide, 50% w/v 1.5 M Tris-HCl- pH 8.8, 0.01% w/v each of SDS and ammonium persulfate (APS) and 0.1% v/v TEMED; stacking gel: 3.2% w/v acrylamide/bis-acrylamide, 25% w/v 0.5 M Tris-HCl-pH 6.8, 0.01% w/v each of SDS and APS and 0.1% v/v TEMED). Both the Thermo Scientific PageRuler Plus Prestained Protein Ladder (26619) and the PiNK Plus pre-stained protein ladder (PM005-0250) (5 µl each) were loaded and used as protein size markers. Electrophoresis was performed at room temperature and 150 V for 75 minutes using a BioRad Mini-PROTEAN® Tetra System (165-8001, BioRad, Canada) with 1x Tris-glycine electrophoresis buffer (50 mM Tris-HCl pH 8.3, 380 mM glycine and 0.1% w/v SDS). Gels were soaked in transfer buffer (19.2 mM Tris-HCl pH 8, 192 mM glycine, 0.015% w/v SDS, 20% v/v methanol) for 10 minutes before the proteins were transferred from the gel to a Millipore Immobilon®-P PVDF (polyvinylidene difluoride) Membrane (IPFL00010, Canada) using the BioRad Trans-Blot® Cell (170-3930, Canada) according to manufacturer's instructions. The transfer was performed at 100 V with 380

mA for 1 hour at 4 °C. The membrane was washed 3 x 5 minutes with HPLC-grade H_2O and 1 x 5 minutes with TBS-T buffer (50 mM Tris-HCl pH 7.6, 150 mM NaCl, and 0.5% v/v Tween-20). The membrane was blocked overnight at 4°C in blocking buffer (TBS-T with 10% w/v non-fat milk: Instant Skim Milk, Carnation). Anti-FLAG antibodies (PA1 984B, Thermo Scientific Pierce, USA) were diluted to 1:500 in blocking buffer and were incubated on the membrane for 1 hour with gentle rocking. TBS-T was used to perform several washes of the membrane (2 quick washes, 2 x 5 minute washes and 1 x 15 minute wash) before the secondary antibody (diluted to 1:400, SA1200, Thermo Scientific Pierce, USA) was added and incubated on the membrane for 1 hour with gentle rocking. The membrane was washed again (as above) before being developed with ECL Western Blot Substrate (W1001, Promega, USA) and imaged with a GE ImageQuant LAS 4000 Digital Imaging System (28-9558-10, GE Healthcare). Resulting images were analyzed using the ImageQuant TL software (GE Healthcare).

2.7.4: Determining protein weights using a standard curve

A standard curve (Supplementary Figure 12) was created for each membrane to accurately determine the weights of each protein in comparison to the protein weight ladders. The program ImageJ (Schneider et al. 2012) was used to analyze each membrane. First, a measurement was set in ImageJ by drawing a short line across a photo of a 30 cm ruler taken with the digital imaging system set to the same settings used to develop the membranes. Thus, the line drawn was of known length and was used to set the ImageJ scale. Next, the distance travelled (in millimeters) by each band of both ladders as well as the loading dye (dye front) was determined and recorded in Microsoft

Excel. Subsequently, as laid out in Bio-Rad's guide to Molecular Weight Determination by SDS-PAGE (Tech Note 3133), the R_f value of each band was calculated using the following equation: R_f = migration distance of the protein/migration - distance of the dye front. Once this was completed, a graph plotting the R_f values versus the log of the known molecular weight of each band (log MW) was created. Through the addition of a trend line to the data, a straight line equation (y=mx + b) was obtained for each data set (Supplementary Figure 12). Since the accuracy of the calculated molecular weight depends on the linearity of the relationship, the two largest proteins of each ladder were removed from the graph. This moved the r^2 value closer to 1, which is an increase in linearity. Using these two equations, the molecular weights of the unknown proteins were determined by entering the R_f values of each unknown protein into the respective line equation and solving for "y".

The expected weights of each protein were determined using the published nucleotide sequence of each gene (*blm*: AY258009; *ccaR*: AH006362; *blip*: M34538) and translating them to their amino acid sequences. The molecular weight (1 kDa) of the FLAG tag was added to the expected weight and this value (theoretical) with the experimentally determined value was used to calculate percent error (% error) in the equation % error = $\left|\frac{(experimental-theoretical)}{(theorectical)}\right| \times 100.$

Chapter 3: Results

3.1: Preparation of the Δ*blm* mutants

In order to create a *blm* in-frame mutant using the Redirect method, *blm* on the *S. clavuligerus* chromosome must be replaced with an 81 bp scar sequence in the correct reading frame. PCR primers (blm-RD-F and –R; Table 2.3) were designed so that the 5' ends were homologous to the beginning and the end region of the *blm* gene. The 3′ ends of these primers were homologous to the apramycin resistance cassette from pIJ773. The resistance cassette in pIJ773 consists of the apramycin resistance gene (*aac(3)IV*) and an origin of transfer (*oriT*; RK2), both of which are flanked by two FRT (FLP recombinase target) sites. The FRT sites were used to excise the cassette from the cosmid later on while preparing an in-frame deletion mutant. This is accomplished using an *E. coli* strain that expresses the *Saccharomyces cerevisiae* FLP recombinase enzyme. The apramycin resistance cassette is used in the first step in preparing a mutant using the Redirect protocol. PCR primers were used to amplify the cassette, and the resulting PCR product was electroporated into an *E. coli* strain harbouring the *blm*-containing 12B8 cosmid (Table 2.2 and Figure 3.1A) and expressing the λ RED proteins. The *blm* gene on Cosmid 12B8 was replaced by the apramycin resistance cassette via homologous recombination mediated by the λ RED proteins (Figure 3.1 B). The 12B8 cosmid containing the apramycin cassette was verified by PCR (Figure 3.1; see next section 3.1.1), after which it was conjugated into wt *S. clavuligerus*. Through homologous recombination between the mutant cosmid and the chromosome, *blm* on the wt *S. clavuligerus* chromosome was replaced with the apramycin cassette.

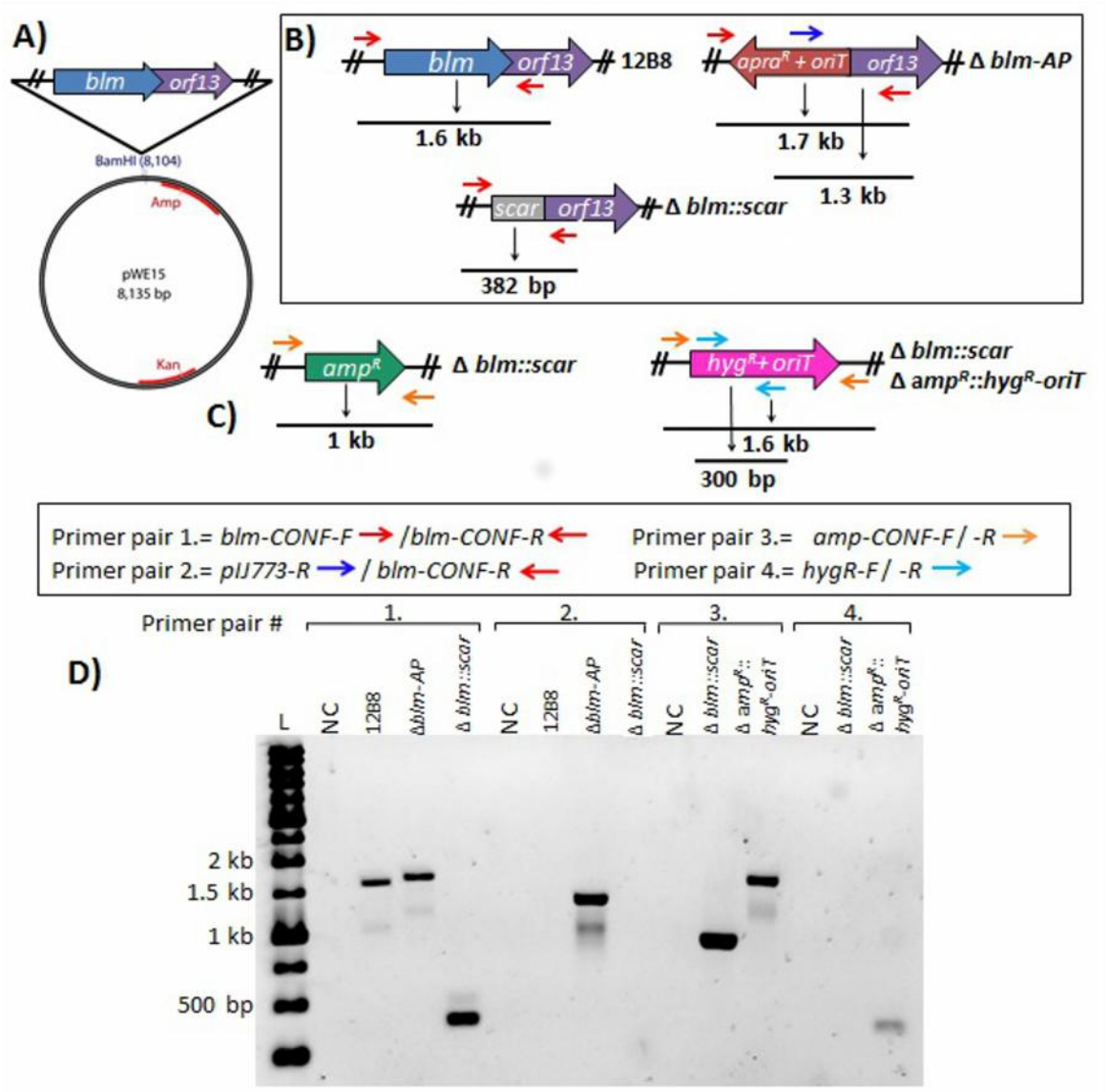


Figure 3.1: Preparation and analysis of the *Δblm-AP*, *Δblm::scar* and *Δblm::scar* Δ*amp*^{R}*:: hyg*^{R}*+oriT* cosmids. **(A)** A schematic representation of the pWE15-based cosmid, 12B8 (Tahlan et al. 2004 b). **(B)** Insertions preformed on the 12B8 cosmid. Primer pairs inside (blue and red arrows) and outside (red arrows) of *blm* are shown. Expected fragment size is dependent on size of insertion and is shown below each primer pair. Note that the entire *blm* gene is not deleted as depicted in the figure (refer to Figure 3.2). **(C)** Analysis of the replacement of *amp*^{R} from 12B8 with *hyg*^{R}*+oriT*. Primer pair 3 (orange arrows) anneals outside of *amp*^{R}. Primer pair 4 (light blue arrows) amplifies inside *hyg*^{R}. The expected fragment size is dependent on size of insertion and is shown below each primer pair. **(D)** Gel electrophoresis results of PCR products from reactions using the primer pairs and cosmid templates described above in (B and C). In each case, (L) is the 1 kb ladder and (NC) is the negative control. Using primer pair 1 (outside primers in red), PCR resulted in the following expected product sizes: (12B8) 1.6 kb, (*Δblm-AP*) 1.7 kb, and (*Δblm::scar*) ~400 bp. Using primer pair 2 (two reverse primers, red and blue), PCR resulted in the following expected product sizes: (12B8) no product, (*Δblm-AP*) 1.3 kb and (*Δblm::scar*) no product. Using primer pair 3 (outside primers in orange), PCR resulted in the following expected product sizes: (*Δblm::scar*) 1 kb and (Δ*amp*^{R}*:: hyg*^{R}*+oriT*) 1.6 kb. Using primer pair 4 (inside primers in light blue), PCR resulted in the following expected product sizes: (*Δblm::scar*) no product and (Δ*amp*^{R}*:: hyg*^{R}*+oriT*) 300 bp.

This produced the *S. clavuligerus blm* mutant (Δ*blm*::*apra*^{R}) with the apramycin cassette inserted in the reverse orientation in place of *blm* (Figure 3.1 B). However, 39 bp of the 5′ and 3′ ends of *blm* remained. This mutant was created to examine the potential of polarity on the expression of *orf13* in the Δ*blm*::*apra*^{R} mutant due to the genetic overlap present between *blm* and *orf13*. Since the apramycin cassette was inserted in the direction opposing natural transcription, this mutant had the highest potential to cause polar effects.

Another *blm* mutant was created from Δ*blm*::*apra*^{R} in order to obtain a *blm* in-frame mutant that had the least potential to cause polar effects. In order to produce the Δ*blm*-INF mutant, the 12B8 Δ*blm-AP* cosmid was transformed into the *E. coli* DH5α strain containing the BT340 plasmid. The BT340 plasmid contains the *flp* gene, encoding the FLP recombinase enzyme. FLP recombinase catalyzed the recombination between the FRT sites surrounding the apramycin cassette and caused the excision of the DNA between them. This left 39 bp of the beginning and the end of the *blm* gene as well as an 81 bp scar sequence in place of the original *blm* gene while maintaining the gene's reading frame (Figure 3.2). This reaction resulted in the production of the 12B8 Δ*blm::scar* cosmid. In order to conjugate the 12B8 Δ*blm::scar* cosmid into *S. clavuligerus*, an *oriT* was required. Since the apramycin cassette was removed, the linked *oriT* was also lost in 12B8 Δ*blm::scar*; therefore an *oriT* needed to be re-introduced into the cosmid.

Following the same Redirect process as above, PCR primers (pWE15-amp-RD-F and –R; Table 2.3) were designed to amplify the hygromycin (*hyg*) resistance cassette

Figure 3.2: Diagram of the in-frame mutation of *blm* present in the 12B8 Δ*blm::scar* cosmid and the *S. clavuligerus* Δ*blm*-INF mutant. The first 39 bp of *blm* including the start codon (STRT), and the first FRT (FLP recognition target) site comprise the Redirect primer blm-RD-F (Table 2.3), used to create the in-frame mutation. Both FRT sites (20 bp and 19 bp respectively) and 42 bp of FLP core recombination site comprise the 81 bp "scar" sequence. The last 39 bp of *blm* including the stop codon (STP), and the last FRT site comprise the Redirect primer blm-RD-R. The start of *orf13* overlaps with *blm* by 4 bp and as such they are likely co-transcribed.

from pIJ10700 to replace the ampicillin resistance gene (*amp*^{R}) located on 12B8 Δ*blm::scar* (Figure 3.1 A). The ampicillin resistance gene is part of the pWE15 cosmid backbone (Figure 3.1 A). Like the apramycin cassette described above, the hygromycin cassette contains an *oriT* and two FRT sites. The knock-in of this cassette effectively changed the resistance marker on 12B8 Δ*blm::scar* as well as provided an origin of transfer for conjugation into *S. clavuligerus* to occur. This newly created cosmid is called 12B8 Δ*blm*::*scar*::Δ*amp*::*hyg*^{R}.

Once the knock-in of the hygromycin cassette was confirmed, 12B8 Δ*blm*::*scar* ::Δ*amp*::*hyg*^{R} was conjugated into the *S. clavuligerus* Δ*blm*::*apra*^{R} mutant described above. Through homologous recombination of the genetic regions before and after *blm* that are present on both the 12B8 Δ*blm*::*scar*::Δ*amp*::*hyg*^{R} cosmid as well as the Δ*blm*::*apra*^{R} mutant chromosome, the *apra-oriT* cassette was replaced with the scar sequence (Figure 3.2). The desired Δ*blm*-INF mutants are identified by the concomitant loss of all antibiotic resistance and genetic screening across the native *blm* location.

3.1.1: Verification of all cosmid DNA used to create the *blm* in-frame mutants

Before transferring the cosmids from *E. coli* ET1256 to *S. clavuligerus*, each cosmid was verified using PCR. All cosmid DNA was verified using the same set of PCR primers (blm-CONF-F and –R; Table 2.3) as PCR product sizes differed depending on the cosmid template used. As expected, a 1.6 kb product was observed when the native 12B8 cosmid DNA was used and a 1.7 kb product was observed when the 12B8 *Δblm-AP* cosmid DNA was used (Figure 3.1 D primer pair 1). The PCR product size change from 1.6 kb to 1.7 kb represents the apramycin cassette in the place of *blm* (Figure

3.1 D primer pair 1) making the PCR product larger. Furthermore, two reverse primers were used in PCR to validate the reverse orientation of the apramycin cassette in relation to the direction of *blm* transcription (Figure 3.1 B). When the apramycin cassette is in the reverse orientation to transcription, one primer (pIJ773-R) anneals inside the apramycin cassette in the forward direction. The second primer (blm-CONF-R) anneals in the reverse direction outside of the cassette and is the same one used in the other cosmid confirmation reactions. If the apramycin cassette was in the same orientation as transcription, these two primers would not produce a PCR product because they would both anneal in the reverse direction. The backwards orientation of the cassette is validated due to the production of a 1.3 kb product upon PCR and gel electrophoresis (Figure 3.1 D primer pair 2). The final cosmid DNA, 12B8 *Δblm::scar*, produced the expected ~400 bp product upon PCR and gel electrophoresis (Figure 3.1 D primer pair 1). This ~400 bp product included the 81 bp scar sequence as well as the flanking DNA (Figure 3.2). In addition to the replacement of *blm* on the 12B8 *Δblm::scar* cosmid, changes to the ampicillin resistance gene also occurred and therefore required confirmation.

Before 12B8 *Δblm::scar* was conjugated into wt *S. clavuligerus*, the insertion of the hygromycin cassette (containing the *oriT*) into the ampicillin resistance gene on 12B8 *Δblm::scar* was confirmed using PCR. There is a large size difference between the ampicillin resistance gene (1 kb) and the hygromycin cassette (1.6 kb). Hence, a set of primers (amp-CONF-F/-R) were created to amplify across the ampicillin resistance gene so the change in PCR product size could be verified (Figure 3.1 C). Upon PCR and gel electrophoresis, the size difference noted above was verified (Figure 3.1 D primer pair 3).

As an added confirmation step, a small 300 bp section of the hygromycin resistance gene was amplified via PCR (Figure 3.1 D primer pair 4). Both of these PCR results confirmed that the hygromycin cassette including an *oriT* was inserted into the ampicillin resistance gene in 12B8 *Δblm::scar,* which created the 12B8 Δ*blm*::*scar* ::Δ*amp*::*hyg*^{R} cosmid. In conclusion, all PCR product sizes appeared as expected after gel electrophoresis.

3.1.2: Verification of strains prior to clavulanic acid production analysis

3.1.2.1: Screening for antibiotic resistant phenotypes in the *S. clavuligerus* mutants

The antibiotic resistance profiles of both mutants were screened to select individual strains to test and genetically verify. The *S. clavuligerus* Δ*blm::apra*^{R} mutant was used to create the Δ*blm*-INF mutant, therefore exconjugates were screened for mutants containing apramycin resistance (stemming from the apramycin cassette). Genomic DNA from confirmed strains was then used in PCR reactions to verify the replacement of *blm* with the apramycin cassette (see next section 3.1.2.2).

The *S. clavuligerus* Δ*blm::apra*^{R} mutant was used to create the *S. clavuligerus* Δ*blm*-INF mutant. Once this mutant was created, the Δ*blm*-INF mutant went through two sets of antibiotic resistance screening. The first round occurred after the conjugation of the 12B8 Δ*blm*::*scar*::Δ*amp*::*hyg*^{R} cosmid into the *S. clavuligerus* Δ*blm::apra*^{R} mutant. These exconjugates were screened for kanamycin resistant strains that contained the entire scar cosmid integrated by a single crossover. The kanamycin resistance stems from the kanamycin resistance gene (Kan^{R}), which is present on the pWE15 cosmid backbone (Figure 3.1 A). These kanamycin resistant strains were isolated on antibiotic free

medium before being screened for the loss of both kanamycin and apramycin resistance. The antibiotic sensitive clones were the desired *S. clavuligerus* Δ*blm*-INF mutants. Genomic DNA from these confirmed strains was then used in PCR reactions to verify the replacement of *blm* with the scar sequence (see next section 3.1.2.2).

3.1.2.2: PCR verification of the *S. clavuligerus blm* mutants

Using the same primer pair (blm-CONF-F and -R) and the same reaction conditions as above, all mutant *S. clavuligerus* strains were confirmed through PCR using genomic DNA as a template (Figure 3.3 A). Since the cosmids above (section 3.1.1) were used to create the *S. clavuligerus* mutants, the sizes of the confirmation products are the same. As a control, wt *S. clavuligerus* genomic DNA was used as template, which produced the expected 1.6 kb product (Figure 3.3 B primer pair 1). When genomic DNA from the *Δblm::apra*^{R} mutant was used, a 1.7 kb product was observed (Figure 3.3 B primer pair 1). The reverse orientation of the apramycin cassette was also confirmed using genomic DNA (Figure 3.3 B primer pair 2). The *Δblm::apra*^{R} mutant was the only reaction expected to produce a product (Figure 3.3 B primer pair 2). This confirms the reverse orientation of the cassette in the genomic DNA of the *Δblm::apra*^{R} mutant. When genomic DNA of the Δ*blm*-INF mutant was used, the expected ~400 bp product was observed (Figure 3.3 B primer pair 1). To confirm that the Δ*blm*-INF mutant was genetically correct and in-frame, the ~400 bp PCR product was cloned into pGEMT-Easy and sent for sequencing. According to the results (Supplementary Figure 1), the 81 bp scar sequence including both *FRT* sites and 39 bp of the beginning and end portions of *blm* (Figure 3.2) were present as expected.

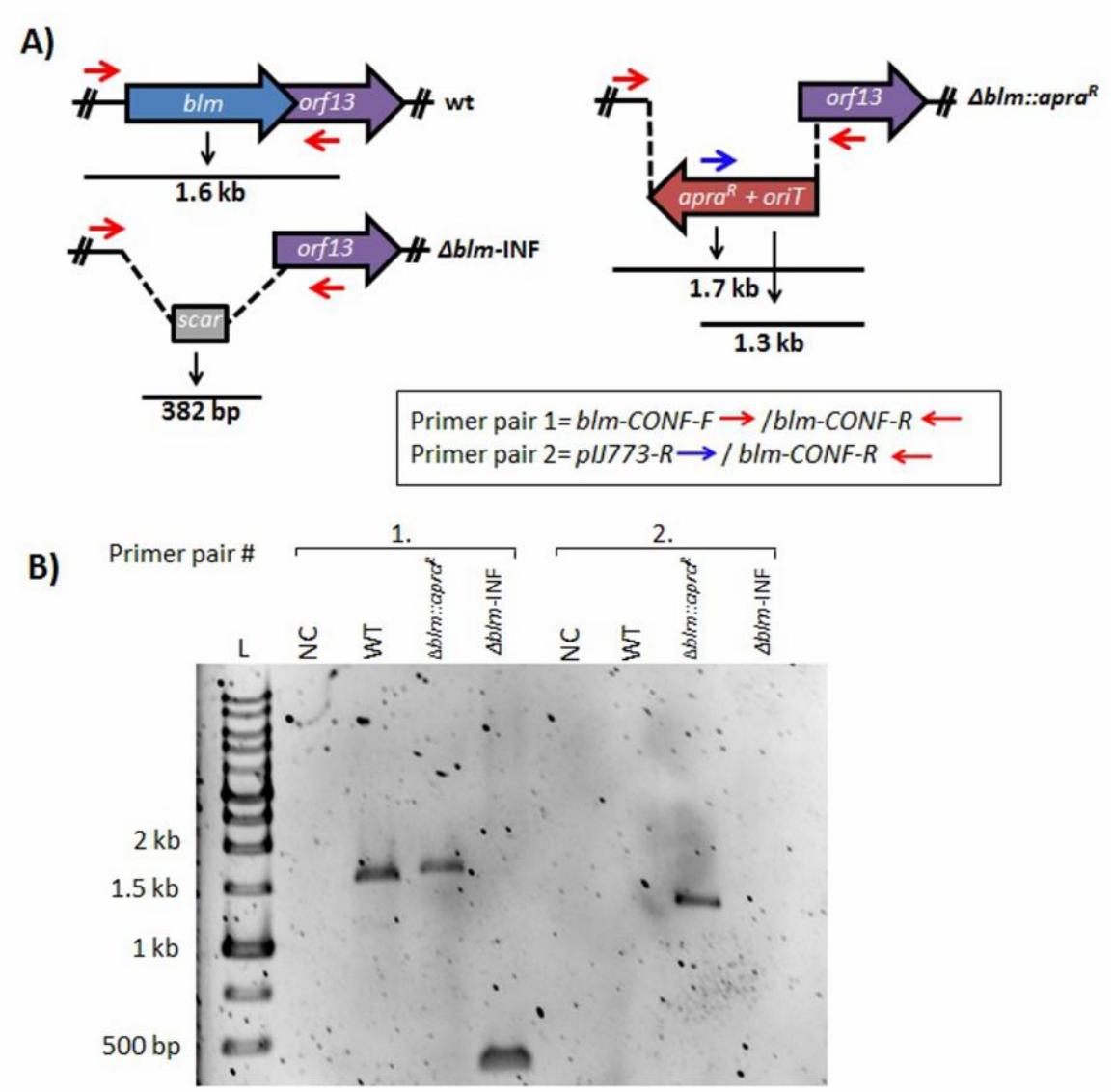


Figure 3.3: Preparation and analysis of the *S. clavuligerus Δblm::apra*^{R} and *Δblm*-INF mutants. **(A)** The insertions performed on wt *S. clavuligerus*. Primers used for mutant verification which anneal to regions surrounding *blm* (red arrows) or within the apra cassette (blue arrow) are shown. Expected fragment size is dependent on size of insertion and is shown below each primer pair. Note that the entire *blm* gene is not deleted as depicted in the figure (refer to Figure 3.2). **(B)** Gel electrophoresis results of PCR products from reactions using the primer pairs and genomic templates described above in (A). In each case, (L) is the 1 kb ladder and (NC) is the negative control. Using primer pair 1 (outside primers in red), PCR resulted in the following expected product sizes: (WT) 1.6 kb, (*Δblm::apra*^{R}) 1.7 kb and (*Δblm*-INF*)* ~400 kb. Using primer pair 2 (two reverse primers, red and blue), PCR resulted in the following expected product sizes: (WT) no product, (*Δblm::apra*^{R}) 1.3 kb and (*Δblm*-INF*)* no product.

Negative control reactions using sterile H_2O instead of template DNA were completed using both primer pair 1 and primer pair 2. Both negative control reactions did not produce PCR products so contaminating DNA was not present in any of the reactions.

3.2: Complementation of *blm* mutants to assess for polar effects

Previous studies have demonstrated that when either *blm* or *orf13* are mutated, CA production drastically decreases (Li et al. 2000; Jensen et al. 2004). Accordingly, both the *Δblm::apra*^{R} mutant and Δ*blm*-INF should not produce the metabolite whereas the wt strain should. Complementation studies were carried out to verify these phenotypes as well as to determine if the mutation of the *blm* gene has a polar effect on *orf13*. CA production was first verified via bioassay against *K. pneumoniae* before the same supernatants were analyzed via HPLC. Each strain was grown in SA medium for up to 96 hours, and samples were collected as described in section 2.6.1. Zones of inhibition were observed around discs infused with supernatants from wt *S. clavuligerus* and wt + pHM11a cultures (Table 3.1, Figure 3.4). Since the plasmid pHM11a is used as the expression vector for all complementations, the empty vector was used as a control to show that did not have a significant effect on the strain's ability to produce CA. Supernatants from the *Δblm::apra*^{R}, Δ*blm*-INF and *orf12::apra* (Jensen et al. 2004) mutants did not produce zones of inhibition (Table 3.1, Figure 3.4). All three of these results are to be expected because in each strain *blm* is disrupted. Additionally, when pHM11a is introduced into each strain, CA production was not restored; therefore, the expression vector has no effect on their phenotype, which was to be expected (Table 3.1, Figure 3.4). CA production is restored in the three non-producing mutants when *blm* was

Table 3.1: Diameter (in mm) of zones of inhibition of *Klebsiella pneumoniae* by clavulanic acid containing culture supernatants from two culture replicates of various mutant strains of *Streptomyces clavuligerus*

Time-point		48 hours		96 hours	
Strain	**Culture Replicate**	**1**	**2**	**1**	**2**
Wt		25	23	28	27
wt+pHM11a		13	19	21	25
wt+pH*:blm*^{SC}		25	20	29	25
Δ*blm::apra*^{R}		0	0	0	0
Δ*blm::apra*^{R}+pHM11a		0	0	0	0
Δ*blm::apra*^{R}+ pH*:blm*^{SC}		26	20	29	24
orf12::apra		0	0	0	0
***orf12::apra*+pHM11a**		0	0	0	0
***orf12::apra*+ pH*:blm*^{SC}**		21	19	25	22
Δ*blm*-INF		0	0	0	0
Δ*blm*-INF+pHM11a		0	0	0	0
Δ*blm*-INF+ pH*:blm*^{SC}		20	20	22	20
Δ*blm*-INF+ pH*:blm-flag*		21	15	22	18
Δ*blm*-INF+ pH*:blm*^{SF}		0	0	0	0
Δ*blm*-INF+ pH*:blm*^{SV}		0	0	0	0

"0"= zone of clearing not observed, *orf12::apra = S. clavuligerus blm* insertional mutant strain (Jensen et al. 2004)

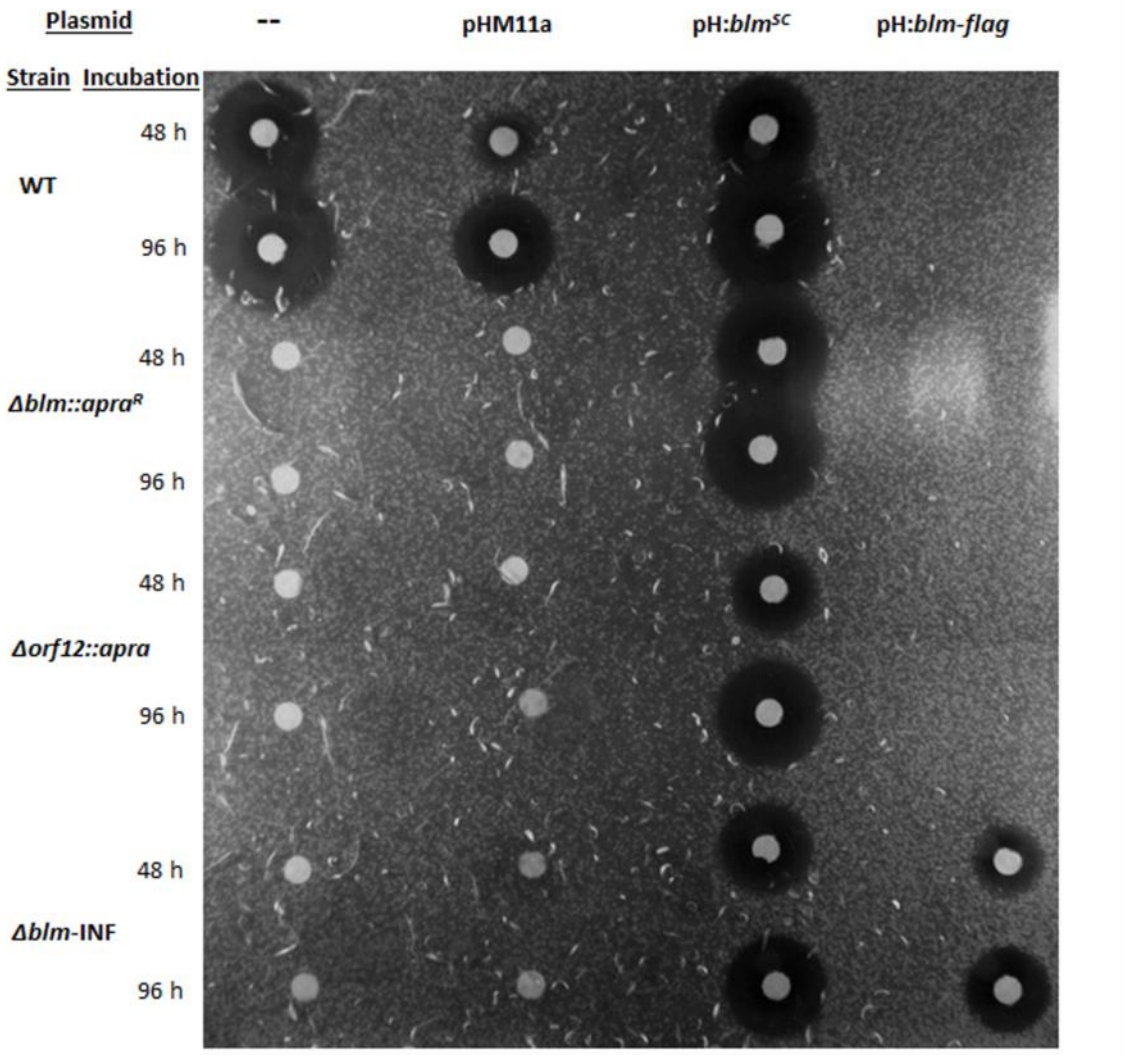


Figure 3.4: Bioassay results for the detection of CA in liquid culture supernatants from various *S. clavuligerus* strains. Strains include wt, *Δblm::apra*^{R}, *Δorf12::apra*^{R} (Jensen et al. 2004) *and Δblm*-INF containing either pHM11a, pHM11::*blm*^{SC}, pHM11a::*blm-flag* or no plasmid (-). Each culture was grown for 48 and 96 hours in SA medium. Image shows results for one of the two replicates completed for each culture and incubation period.

re-introduced into the mutants (Table 3.1, Figure 3.4).

Since the zones of inhibition were larger at the 96 hour time point, these supernatants were chosen to be analyzed by HPLC to confirm the presence of CA in the sample. Supernatants from two replicates of each strain were prepared as described in section 2.6.2 and CA production was determined by observing peak areas absorbing at 311 nm. Five CA standards with the following concentrations of 0.01, 0.13, 0.25, 0.38 and 0.5 µg/µl were used as positive controls and to create a standard curve (Supplementary Figure 2). The standard curve was used to determine the CA concentration within each supernatant. Derivatized supernatants from the following strains showed a detectable peak absorbing at 311 nm at ~ 10 minutes: wt, wt+pHM11a, wt+pH:*blm*^{SC}, Δ*blm::apra*^{R} +pH:*blm*^{SC}, *blm::apra*^{R}+pH:*blm*^{SC}, Δ*blm::*INF+pH:*blm*^{SC}, and Δ*blm::*INF+pH:*blm*-FLAG (discussed later in section 3.4.3). Derivatized supernatants from all mutant strains containing a knockout of *blm* (Δ*blm::apra*^{R}, *orf12::apra,* and Δ*blm::*INF) did not produce a signal absorbing at 311 nm. All strains containing the empty expression vector pHM11a displayed the same phenotype (producing/non-producing) as their parent strain, which do not contain the vector. However, the wt strain that contained pHM11a not produce the same level of CA as the wt type strain on its own. The vector, pHM11a, had an effect on the production of CA that lead to the decrease in CA production. Nonetheless, the results of the HPLC analysis concur with the results of the bioassays done above.

The CA production levels in the various strains were normalized using dry cell weight (Supplementary Table 1) to account for the differences in growth between the strains. Concentrations of CA calculated using the CA standard curve (Supplementary

Figure 2) were divided by the dry cell weights of each replicate. This resulted in a concentration of CA produced by each mg of cells in a culture, allowing the CA concentrations to be compared across all strains (Figure 3.5).

3.3: Alignment of Blm from *S. clavuligerus, S. viridis* and *S. flavogriseus*

The amino acid sequences of Blm from *S. clavuligerus, S. viridis* and *S. flavogriseus* have previously been aligned (Valegard et al. 2013). As mentioned previously, the Blm-like protein from *S. flavogriseus* has ~60 % amino acid identity and the Blm-like protein from *S. viridis* has 52 % amino acid identity with their orthologue in *S. clavuligerus*. Nonetheless, further focus on the hypothesized functional domains and CA binding locations may provide insight into whether or not the Blm-like proteins from the other two species will complement the *S. clavuligerus* Δ*blm*-INF mutant. Valegard et al. (2013) highlighted several key residues of Blm that are likely to compose its active site, CA binding pockets and catalytic motifs. Of particular interest are the three motifs that are characteristic of serine β-lactamase active-sites: SXXK, SDN and KTG. These domains contain the characteristic catalytic tetrad of Ser1/Ser2 (Tyr)/Lys1 (His)/Lys2, which has been found in *S. clavuligerus* Blm as Ser173/Ser234/Lys176/Lys375 (Figure 3.6, dark blue and red boxes). In the *S. viridis* and *S. flavogriseus* Blm homologues, these motifs (Figure 3.6, red boxes) are almost identical to the *S. clavuligerus* Blm motifs. Most importantly, the catalytic tetrad residues are conserved (Figure 3.6, dark blue and red boxes). According to Valegard et al. (2013), the most important catalytic residue of the tetrad is Ser173, since mutation of this residue resulted in a 100-fold reduction in

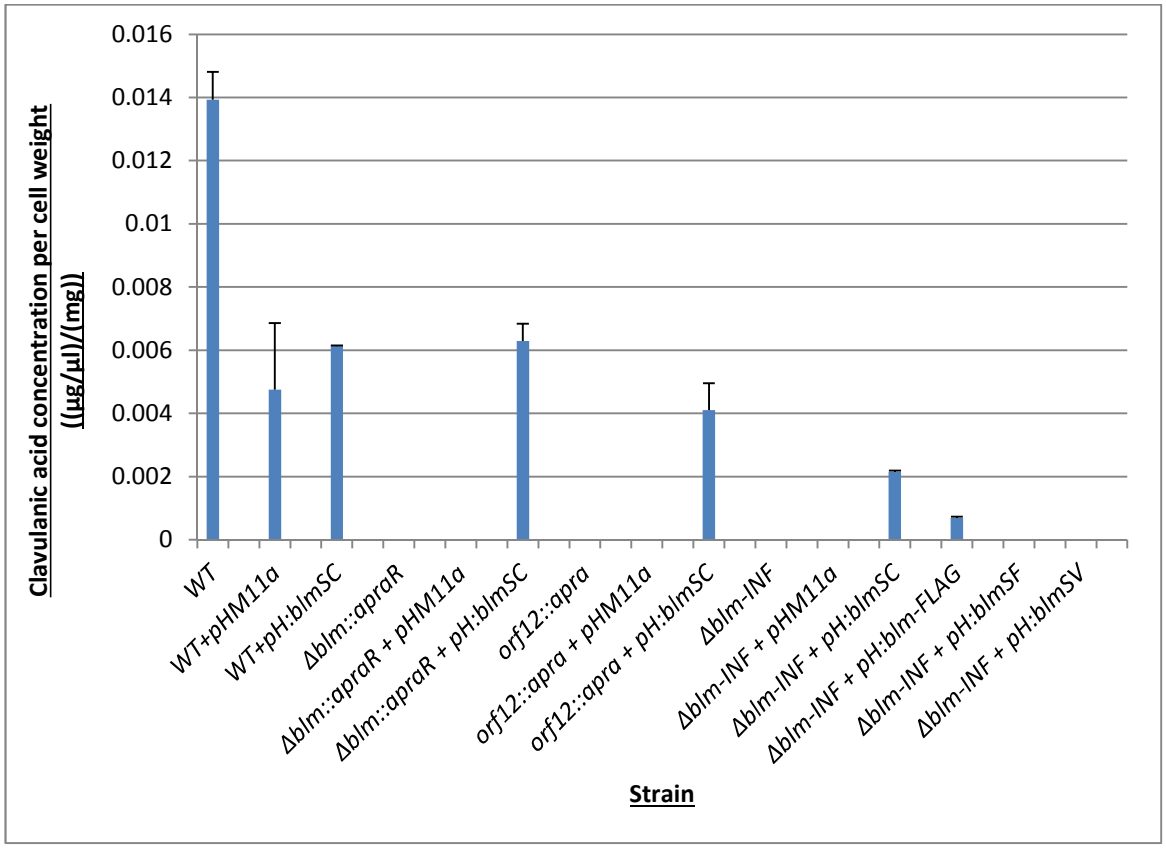


Figure 3.5: Clavulanic acid production by each strain in this study after 96 hours of incubation. Bar graph of the average concentration of CA produced by each strain used in this study. Concentrations (µg/µl) were calculated by comparison of HPLC determined values against a standard curve of known CA concentrations (Supplementary Figure 2). These values were then standardized using the dry cell weight (mg) determined for each culture. Concentrations and dry cell weights were determined in duplicate (n=2) for each strain. Strains with no bars on the chart did not produce detectable levels of CA. This experiment included *orf12::apra* (Jensen et al. 2004). Error bars indicate the standard error of the mean.

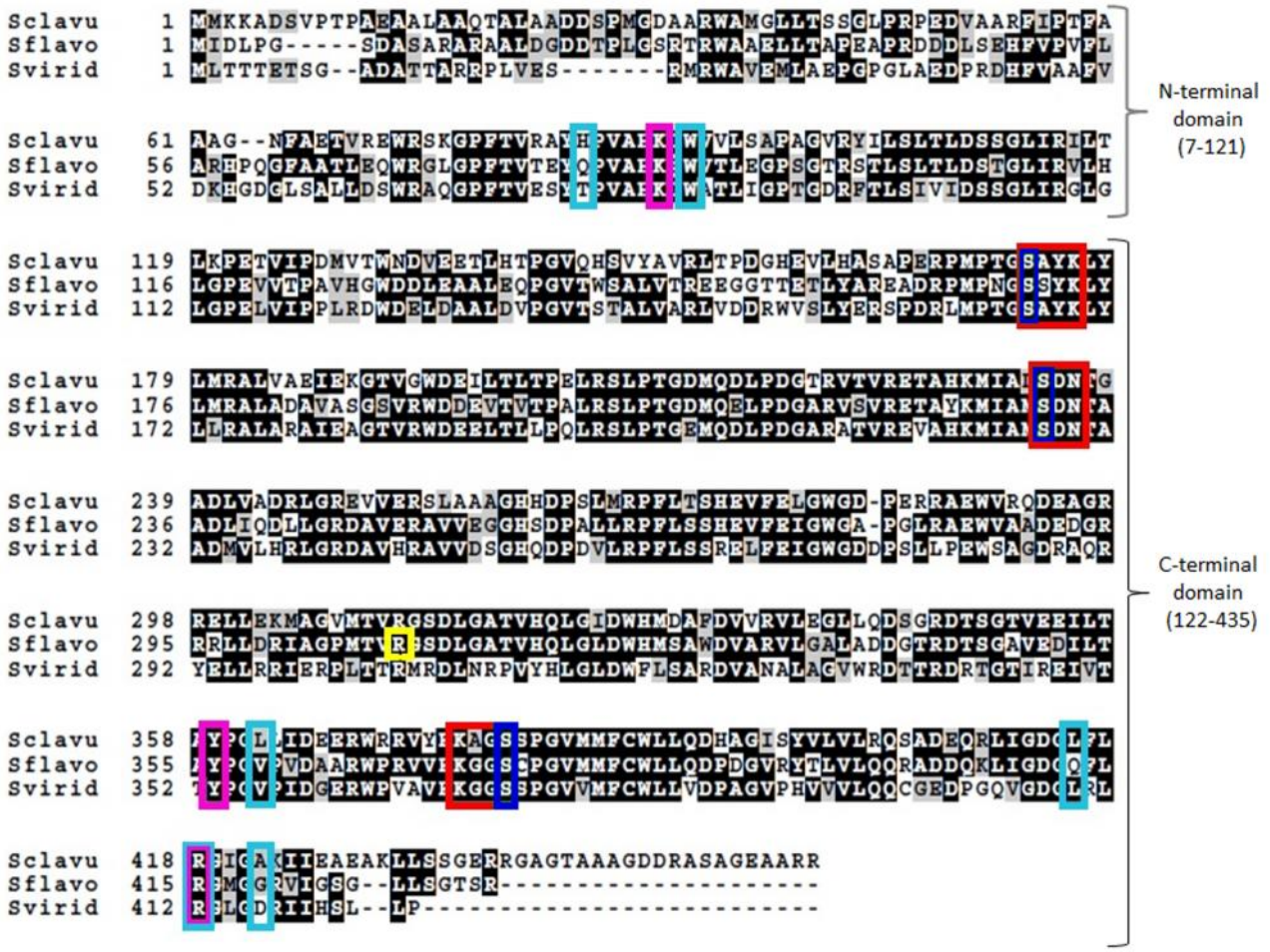


Figure 3.6: Protein alignment of Blm from *S. clavuligerus*, *S. flavogriseus*, and *S. viridis* with important residues highlighted. Three motifs that are characteristic of serine β-lactamase active-sites are enclosed in red boxes. Conserved residues important for catalytic activity in serine β-lactamases are indicated by dark blue boxes. Pink boxes highlight residues that comprise the Blm active site of *S. clavuligerus*. The residues that make up the hydrophobic cleft where a proposed second molecule of CA can bind (Valegard et al. 2013) are located in light blue boxes. The yellow box surrounds the Arg 308 residue of *S. flavogriseus*. This is a proposed silent mutation in the blm^{SF} gene used in this study. Labels on the side separate the N and C terminal domains. Alignment created using the BOXSHADE software (Hofmann & Baron, Institute Pasteur, France).

S. clavuligerus Blm esterase activity, and this residue is conserved in the *S. viridis and S. flavogriseus* Blm homologues (Figure 3.6).

Valegard et al. (2013) also suggest that one Blm molecule binds two CA molecules. One CA molecule hydrogen binds to the active site, which is composed of the Tyr359, Lys89 and Arg418 residues (Figure 3.6, pink boxes). The other CA molecule binds in a hydrophobic cleft made up by His84, Trp91, Leu362, Leu415, Arg418, and Ala422 (Figure 3.6, light blue boxes). All three residues that make up the Blm active site are conserved in *S. viridis* and S. *flavogriseus,* but only two (Trp91 and Arg418) residues from the second site are conserved in these species.

3.3.1: Complementing the Δ*blm*-INF mutant with *blm*-like genes from *S. viridis* and *S. flavogriseus*

When the CA-like gene clusters were found in *S. viridis* and *S. flavogriseus,* they were determined to be silent clusters as studies were unable to detect any CA production by the organisms (Alverez-Alverez et al. 2013). The study determined that the lack of CA production in both organisms was likely due to either low or complete lack of expression of several genes essential for CA production, including *blm*. To determine if the Blm-like proteins from *S. viridis* and *S. flavogriseus* are similar in function to Blm from *S. clavuligerus,* they were cloned into the expression vector pHM11a. The resulting plasmids were conjugated into the Δ*blm*-INF mutant and the final mutant's ability to produce CA was tested.

The *blm* gene from *S. clavuligerus* and the *blm*-like genes from *S. viridis* and *S. flavogriseus* were amplified via PCR using the primers and program described above

(section 2.4.1.2) before being ligated into pGEMT-Easy. Once cloned into pGEMT-Easy, the *blm*-like genes were sequenced before the genes were excised from pGEMT-Easy and cloned into the expression plasmid pHM11a (section 2.4.2.2). The *blm*-like genes were sequenced again in pHM11A before these final plasmids were introduced into the Δ*blm*-INF mutant.

The final sequencing results (Supplementary Figures 3, 4, 5) were aligned with the published sequence (*S. clavuligerus*: AY258009, *S. viridis*: CP001683, *S. flavogriseus*: CP002475) of each gene. It was determined that *blm* from *S. clavuligerus* and *S. viridis* did not contain any mutations, whereas *blm* from *S. flavogriseus* contained a single point mutation (C→T) at nucleotide position 924 (Supplementary Figure 5). When translated, the mutated codon results in the same amino acid (Figure 3.6, yellow box), therefore the mutation may be considered silent.

3.3.2: CA production analysis of Δ*blm*-INF strains containing the *blm*-like genes from *S. viridis* and *S. flavogriseus*

Bioassays and HPLC analysis (section 2.6) were performed on supernatants from these mutant cultures to observe whether CA production is rescued by these foreign *blm*-like genes. CA production in *S. viridis* and *S. flavogriseus* was previously tested in the defined SA medium and the results were negative (Alverez-Alverez et al. 2013). CA production was also tested in the *S. clavuligerus* Δ*blm*-INF mutant (section 3.2) with negative results due to the deletion of the *blm* gene. CA production of the Δ*blm*-INF mutant containing the *blm*-like genes was first verified via bioassay against *K. pneumoniae* before the same supernatants were analyzed via HPLC. Duplicate cultures

of each strain were grown in SA medium for up to 96 hours and samples were collected as described in section 2.6.1. Zones of inhibition were not observed around discs infused with supernatants from either Δ*blm*-INF + pH:*blm*^{SF} or Δ*blm*-INF + pH:*blm*^{SV} (Table 3.1). The bioassay and HPLC analyses for these strains were conducted simultaneously with the bioassay and HPLC analyses completed in section 3.2. Accordingly, the control strains *S. clavuligerus* wt, wt+pHM11a, Δ*blm*-INF and Δ*blm*-INF+pH:*blm*^{SC} were tested at the same time. Derivatized supernatants from Δ*blm*-INF + pH:*blm*^{SF}, Δ*blm*-INF + pH:*blm*^{SV} and Δ*blm*-INF did not produce a signal absorbing at 311 nm by the 10 minute mark, while wt, wt+pHM11a and Δ*blm*-INF+pH:*blm*^{SC} did (Figure 3.5). The results of the HPLC analysis concur with the results of the bioassays completed above.

3.3.3: Examination of codon usage between *S. clavuligerus, S. flavogriseus* and *S. viridis*

To examine both Blm-like proteins further, each amino acid sequence from *S. flavogriseus* and *S. viridis* as well as Blm from *S. clavuligerus* was scanned against the known codon usage bias of *S. clavuligerus* (Nakamura et al. 2000) using the Graphical Codon Usage Analyser (Fuhrmann et al. 2004). This program returned a graphical representation (Supplementary Figure 6, 7, 8) depicting the relative adaptiveness of each codon used in the Blm-like proteins to the codon usage bias of *S. clavuligerus*. The Blm-like protein from *S. viridis* exhibited the most difference in codon usage (12.81%). The difference occurred in the codons used to code for four different amino acids. The codons used by *S. viridis* are all still used in *S. clavuligerus* but at varying rates for each (Supplementary Figure 6). The Blm-like protein from *S. flavogriseus* exhibited the least

difference in codon usage (5.09%) when compared to the *S. clavuligerus* codon usage (Supplementary Figure 7). The difference occurred in the codons used to code the amino acids arginine and proline; however, the codons used by *S. flavogriseus* are still frequently used in the *S. clavuligerus* genome at high rates (Supplementary Figure 7). To maintain consistency, Blm from *S. clavuligerus* was screened against its own codon bias and a difference of 6.61% was found (Supplementary Figure 8). The difference was in the codons used for the two amino acids alanine and proline. Of special note, the same codon was used for proline (CCC) in Blm from *S. clavuligerus,* and the Blm-like protein in *S. flavogriseus* but this codon (CCC) is not the most frequently used one for proline in the *S. clavuligerus* genome (Supplementary Figure 7 and 8). As a result, Blm from *S. clavuligerus* and Blm-like protein from *S. flavogriseus* only differ in codon usage by one codon.

3.4: Examination of the cellular localization of Blm

In order to determine the cellular location of Blm in *S. clavuligerus*, a short polypeptide sequence, known as the FLAG tag (Hopp et al. 1988), was added to the C-terminal end of each protein. The FLAG tag was engineered into the reverse primers (Table 2.3) used to amplify *blm* as well as two control genes, *blip* and *ccaR*. Once translated, the FLAG-tagged proteins were transported to their active sites either inside or outside the cell. Their cellular localization was determined by collecting different fractions of cell culture and performing western analysis with anti-FLAG antibodies. Since all FLAG-tagged genes were transferred into wt *S. clavuligerus* in pHM11a, their transcription was regulated by the constitutive *ermE** promoter (*ermE*p*).

Before being conjugated into wt *S. clavuligerus,* all three genes were sequenced in pHM11a to ensure each was free of mutation and contained the C-terminal FLAG sequence. Sequencing results (Supplementary Figures 9, 10, 11) were entered into the bioinformatic analysis program Geneious, and aligned with the published sequence of each gene. It was determined that *blm, ccaR,* and *blip* did not contain any mutations and each contained a C-terminal FLAG tag.

3.4.1: Analysis of CA production from *S. clavuligerus* Δ*blm*-INF +pHM11a::*blm*-FLAG

The FLAG tagged version of *blm* was introduced into the *S. clavuligerus* Δ*blm*-INF mutant to ensure that this version of *blm* is biologically active enough to restore CA production in the Δ*blm*-INF mutant. By doing this, we can confirm that Blm is located in its natural sub-cellular location because it is either biologically active or not. Bioassays against *K. pneumoniae* and HPLC analysis of culture supernatants were completed to ensure that the *S. clavuligerus* Δ*blm*-INF +pH:*blm*-FLAG strain was able to produce CA. Bioassays were completed with duplicate cultures of each strain (as described above in section 2.6.1) and supernatants from the 48 hour and 96 hour time point were tested. It was found that at both the 48 and 96 hour time points, the Δ*blm*-INF +pH:*blm*-FLAG strain was capable of producing CA (Table 3.1, Figure 3.4). Zones of inhibition were observed surrounding disks that were infused with culture supernatants from this strain.

HPLC analysis was completed on 96 hour supernatant of Δ*blm*-INF +pH:*blm*-FLAG due to increased CA production at this time point as shown by the bioassay results (Table 3.1, Figure 3.4). Analysis was conducted on duplicate culture replicates and a

peak representing CA absorbed at 311nm by ~10 minutes in both replicates (Supplementary Table 2). The area under the CA peak was compared to the CA standards graph (mentioned in section 3.2) and CA concentration was determined for each replicate. The dry cell weight of 1 ml of each Δ*blm*-INF +pH:*blm*-FLAG culture replicate was determined as described in section 2.3.9. The CA concentrations of each replicate were determined and put into the graph of the CA concentrations of all mutants (Figure 3.5) so that all could easily be compared.

3.4.2: Determination of a time point for protein extraction

According to the literature, CA production is well established after 48 hours incubation in SA medium and peak production occurs around 96 hours of growth (Paradkar et al. 1996). However, there was no significant size difference between the zones of inhibition at the 48 and the 96 hour time points during bioassay analysis. As a result, the 48 hour time point was chosen for protein isolation to determine the sub-cellular location of Blm during active CA production. The 96 hour time point was not selected due to the increased likelihood of cell lysis at the later time point, which would release intercellular FLAG-tagged proteins into the supernatant and produce conflicting results.

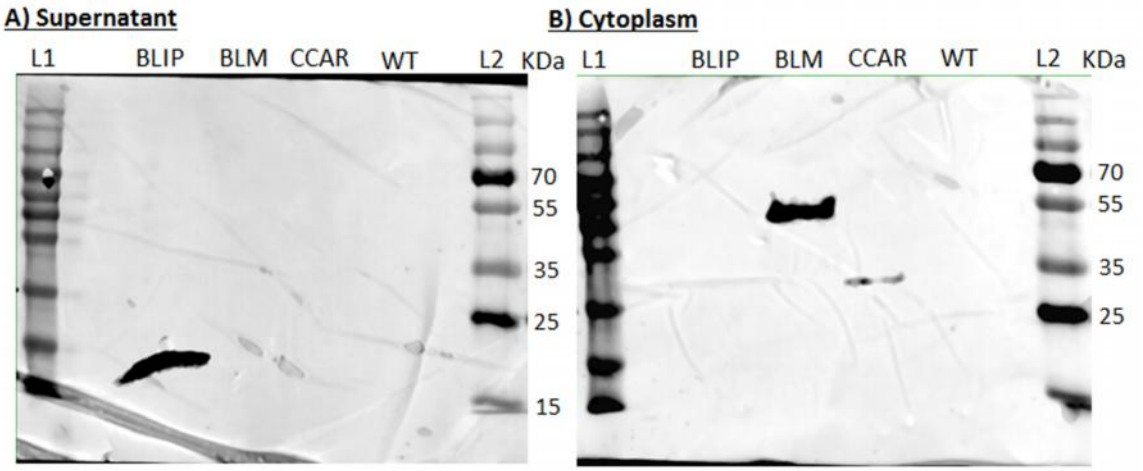
3.4.3: Localization of Blm, CcaR and Blip in *S. clavuligerus* through the use of western blotting

Protein was extracted from 48 hour cultures of wt+pHM11a, wt+pH:*blm*-FLAG, wt+ pH:*ccaR*-FLAG and wt+pH:*blip*-FLAG using two different methods. The first

method extracted cytoplasmic proteins by subjecting mycelial pellets to sonication, resulting in crude cellular lysates. The second method extracted secreted proteins from supernatant of the same cultures above, through a salting out process using ammonium sulfate. One culture of each strain was used for both methods, as one method extracts cytoplasmic protein and the other extracts secreted proteins.

Western analysis using FLAG-specific polyclonal antibodies was carried out to investigate the sub-cellular localization of Blm in comparison to the known control proteins CcaR and Blip. Salted out supernatant protein (50 µg) and cytoplasmic protein (20 µg) from wt+pHM11a, wt+pH:*blm*-FLAG, wt+ pH:*ccaR*-FLAG and wt+pH:*blip*-FLAG cultures were separated on 12% SDS-PAGE and transferred to separate PVDF membranes. After overnight membrane blocking, the membranes were incubated with anti-FLAG antibody and then incubated with secondary antibodies. The membranes were developed with chemiluminescence reagents and imaged with a digital imaging system. A standard curve (Supplementary Figure 12) was created for each membrane to be able to accurately determine the weights of each protein in comparison to the molecular weight ladders.

In the western blot analysis of cytoplasmic proteins, two signals were detected in the lanes containing cytoplasmic proteins from the wt+pH:*ccaR*-FLAG and wt+pH:*blm*-FLAG cultures (Figure 3.7 B). Using the standard curve generated (Supplementary Figure 12) and ImageJ software, the molecular weight of the protein from the wt+pH:*ccaR*-FLAG culture was determined to be 34 kDa and the protein from the wt+ pH:*blm*-FLAG culture was determined to be 54 kDa. The size of CcaR is 5 kDa larger



C) Protein size determination

Protein	Experimental (kDa)	Expected (kDa)	+FLAG tag (~1 kDa)	Percent error (%)
Blm	54	50	51	8%
Blip	18	17.5	18.5	3%
CcaR	34	29	30	13%

Figure 3.7: Analysis of western blots depicting the sub-cellular location of FLAG-tagged Blip, CcaR and Blm in *S. clavuligerus*. **A)** Western blot using anti-FLAG antibodies against proteins (50 µg) salted out from the supernatants of wt+pH:*blip*-FLAG (Blip), wt+pH:*blm*-FLAG (Blm), wt+pH:*ccaR*-FLAG (CcaR) and wt+pHM11a (WT). (L1), 5 µl of PiNK Plus pre-stained protein ladder; (Blip), a band at the expected size is present; (Blm) no banding present ; (CcaR) no banding present; (WT) no banding present; (L2), 5 µl of Thermo Scientific PageRuler Plus Prestained Protein Ladder. Sizes of marker bands in L2 are noted in kDa along the right. **B)** Western blot using anti-FLAG antibodies against proteins (20 µg) fractionated from the cell lysate of wt+pH:*blip*-FLAG (Blip), wt+pH:*blm*-FLAG (Blm), wt+pH:*ccaR*-FLAG (CcaR) and wt+pHM11a (WT). (L1), 5 µl of PiNK Plus pre-stained protein ladder; (Blip), no banding present; (Blm), a band at the expected size is present; (CcaR), a band of the expected size is present; (WT), no banding present; (L2), 5 µl of Thermo Scientific PageRuler Plus Prestained Protein Ladder. Sizes of marker bands in L2 are noted in kDa along the right. **C)** A table depicting the experimental and expected molecular weights for Blm, CcaR and Blip. The percent error between the experimental and expected weights is calculated while taking into account the molecular weight of the FLAG tag. Experimental weights were calculated using the standard curve generated (Supplementary Figure 12) and the expected weights were determined based on the published nucleotide sequence for each gene and translating them to their amino acid sequence (see section 2.7.4). The molecular weight (1 kDa) of the FLAG tag was added to the expected weight and this value (theoretical) along with the experimentally determined value was used to calculate percent error.

than the published size 29 kDa. The size of Blm is in agreement with the published size range of 50- 55 kDa that was purified from *E. coli* (Valegard et al. 2013).

In the western blot analysis of supernatant proteins, only one signal was detected in the lane containing supernatant proteins from the wt+pH:*blip*-FLAG culture (Figure 3.7 A). Using the standard curve generated (Supplementary Figure 12) and ImageJ software, the molecular weight of the protein from the wt+pH:*blip*-FLAG culture was determined to be 18 kDa. This size is in agreement with the published size range of Blip, 16.9-18 kDa (Doran et al. 1990). Signals were not observed in other lanes in this analysis. This was to be expected as the two other FLAG-tagged proteins were previously found in the cytoplasmic protein analysis. In addition, cell lysis was not a factor in the harvested cultures as additional signals in the Blm and CcaR lanes were not detected.

Chapter 4: Discussion

This study explored the polarity of *blm* mutants to determine if mutation of this gene leads to polar effects on the expression of downstream genes. This was done indirectly by the preparation of two mutants and subsequent complementation studies using native *blm* and the *blm*-like genes from *S. flavogriseus* and *S. viridis*. Additionally, this study also aimed to look at the cellular localization of the Blm protein in *S. clavuligerus*, as this information would provide insights into its biological function in CA production. The following sections discuss the major findings of this study as well as highlight areas in need of further investigation.

4.1: The creation and analysis of the *S. clavuligerus* Δ*blm* mutants

When the insertional mutants RFL 5-56 (Li et al. 2000) and *orf12::apra* (Jensen et al. 2004) were first analyzed, it was found that the production of CA was completely blocked in these mutants. More recently, it has come to light that the genetic sequences of *orf13*, *blm* and *orf14* overlap each other (Figure 1.5) and *orf13/blm* are co-transcribed while *orf14* is transcribed in the opposing direction (Mellado et al. 2002). As a result, the original thought was that the *blm/orf13* transcript may share the same ribosome binding site (RBS) and may be co-translated as well. Due to the overlapping nature, the insertional mutants created in previous studies may display polar effects on downstream genes as the mutant proposed in the Jensen study (2004) was not complemented. Therefore, the current study created a mutant (Δ*blm*::*apra*^{R}) by replacing *blm* with an apramycin cassette in reverse orientation. This was an attempt to produce polar effects and determine whether transcriptional and translational coupling is occurring between

blm and *orf13*. An in-frame *blm* mutant was later prepared using the Δ*blm*::*apra*^{R} mutant for complementation studies with *blm*-like genes from *S. viridis* and *S. flavogriseus*.

Both the Δ*blm*::*apra*^{R} and the Δ*blm*-INF mutant were created using the Redirect method (Gust et al. 2003). The replacement of *blm* with the apramycin cassette in the current study removes the native *blm* gene from the chromosome. In addition, the apramycin cassette is placed in the reverse orientation to the natural direction of transcription of *blm* and *orf13*. Having the cassette orientated in the direction opposing natural transcription could cause clashes between RNA and DNA polymerases leading to replication fork stalling or aborted chromosomal DNA replication (Rocha 2002). This would be particularly troublesome as bacteria exhibit gene orientation bias where highly expressed genes are present on the leading strand of replication in order to co-orient polymerases, thus reducing stalling and collisions (Rocha 2002). The stalling or abortion of transcription in the area of *blm* would have profound genetic effects on this mutant due to the co-transcription and possible co-translation of *blm* and *orf13.* In addition, there is the possibility of antisense mRNA binding to the natural promoter driven mRNA transcript and inhibiting translation. Due to the opposite orientation of the apramycin cassette, antisense mRNA transcript production is possible (Camblong et al. 2009; Lasa et al. 2012). Since the Δ*blm*::*apra*^{R} mutant retained a portion of the 5′ region of *blm* (start codon and 39 bp of the beginning of *blm* as in Figure 3.2), both the promoter driven mRNA and the antisense mRNA transcripts contain the same region. As a result, complementary base pairing is possible, that leads to the inhibition of translation through physical obstruction of the translation machinery (Brophy and Voight 2016) or

degradation by RNases. These complications would be described as causes of downstream polarity.

The mutation of *blm* in Δ*blm*-INF and Δ*blm::apra*^{R} lead to the complete cessation of CA production (Figure 3.4 and 3.5; Table 3.1). However, the existence of genetic polarity in the Δ*blm::apra*^{R} mutant can only be determined from complementation studies using *blm* on its own. Since both *blm* and *orf13* are crucial for the production of CA (Li et al. 2000; Jensen et al. 2003), the mutation of *blm* whether it displays polar effects or not would produce the same results: complete cessation of CA production.

4.2: Complementation of the of the *S. clavuligerus* Δ*blm* mutants

Complementation strains were created by introducing a native copy of the *S. clavuligerus blm* gene into all of the mutant strains as well as wt *S. clavuligerus*. The *Streptomyces* integrative expression vector pHM11a (Motamedi et al. 1995) was used to shuttle a native copy of *blm* into each mutant strain. In pHM11a, *blm* is under the control of the strong constitutive *ermE*p* promoter; therefore, it is constitutively expressed.

Complementation studies showed that both mutants could be successfully complemented by *blm*^{sc} (Figure 3.4 and 3.5; Table 3.1). Since pHM11a had an effect on CA production (Figure 3.4 and 3.5; Table 3.1), the level of complementation for each strain can only be compared to the wt+pHM11a control strain. Based on this comparison, both Δ*blm* mutants complemented completely. It is not an uncommon phenomenon for large expression vectors to have an effect on specialized metabolite production in *Streptomyces* (Tahlan personal communication). The vector, pHM11a, is a very large (9.7 kb) plasmid that is energetically expensive for the cell to maintain. As well, this

plasmid integrates into the chromosome at the mini-circle *att* site (Motamedi et al. 1995), which is located away from the CA genetic cluster and the native *blm* position. Both of these conditions could be causing the decrease in CA production observed in strains containing the pHM11a vector.

Due to the compete complementation of both Δ*blm* mutants, there is no transcriptional or translational polarity in either mutant and there are no other factors (besides *blm*) affecting their function. Accordingly, it can be concluded that the positioning of the apramycin cassette in the Δ*blm::apra*^{R} mutant does not affect expression of *orf13.*

Upon further examination of the *orf13* upstream region, a potential Shine-Delgarno (SD) sequence (GGAGAAG) was located 13 nt upstream from *orf13*'s start codon. This sequence shows similarity to the consensus *Streptomyces* SD sequence (GGAGG) as well as a few others that are less common (Strohl 1992). This potential RBS explains why the Δ*blm::apra*^{R} mutant does not display transcriptional or translational polarity. The RBS is conserved in both Δ*blm* mutants as 39 nt of both the beginning and the end regions of *blm* remained in each mutant.

4.3: The *blm*-like genes from *S. flavogriseus* and *S. viridis* do not complement the Δ*blm*-INF mutant

In previous studies, two putative CA producers were found: *S. viridis* and *S. flavogriseus* (Jensen 2012). Both organisms contain the entire CA gene cluster as well as orthologues of *nocE, blip* and *pcbR* that are found in *S. clavuligerus*. Efforts to try to either activate production or heterologously express the CA cluster in these organisms

have been unsuccessful (Jensen 2012) and CA production requirements have yet to be determined (Alvarez-Alvarez et al. 2013).

As well as having high nucleotide identity (*blm*^{SC} shares 71% and 69% nucleotide identity with *blm*^{SF} and *blm*^{SV} respectively), *blm* in these organisms maintains its overlapping nature (Figure 1.5) with the surrounding *orf13* and *orf14* orthologs. Previous studies have shown that *blm* is severely under expressed in *S. flavogriseu*s and therefore is considered silent (Alvarez-Alvarez et al. 2013). With *blm* being essential to the production of CA in *S. clavuligerus*, its silent nature in *S. flavogriseus* could be the reason this organism is unable to produce CA. As a result, this study aimed to determine how similar the Blm-like protein from either of these species is to Blm from *S. clavuligerus.* Bioinformatics analysis and experimentation were conducted to determine whether the similarity was sufficient enough to restore CA production in the *S. clavuligerus* Δ*blm*-INF mutant created in this study.

The alignment of the amino acid sequences of Blm from *S. clavuligerus, S. viridis* and *S. flavogriseus,* revealed the conservation of several key residues proposed by Valegard et al. (2013). The three motifs that are characteristic of serine β-lactamase active-sites, SXXK, SDN and KTG (Figure 3.6, dark blue and red boxes), are almost exclusively conserved in *S. viridis* and *S. flavogriseus* when compared to the same sites in *S. clavuligerus.* More importantly, the catalytic tetrad outline by Valegard et al. (2013) including the most essential residue of the tetrad (Ser173) is completely conserved. As well, all three residues that make up the Blm active site (Figure 3.6, pink boxes) are conserved in *S. viridis* and S. *flavogriseus,* but only two residues (Figure 3.6, light blue boxes) from the second CA binding site are conserved.

According to the bioinformatics analysis above, it seems logical that the *blm*-like genes from the other two species would complement the *S. clavuligerus* Δ*blm*-INF mutant and restore its CA production. Nevertheless, neither the *blm*-like gene from *S. viridis* nor *S. flavogriseus* restored CA production in the *S. clavuligerus* Δ*blm*-INF mutant. Both bioassay and HPLC analysis results revealed no detectable CA production by either mutant (Table 3.1, Figure 3.5). Almost all residues highlighted by Valegard et al. (2013) are conserved amongst the three species except in regards to the second CA binding site where only two of the six total residues are conserved. Valegard et al. (2013) proposed that the two conserved residues may act as gatekeepers for the active site. It is possible that the binding of the second CA molecule may be required to activate or release the first CA molecule from the active site. If the second CA binding site in *S. flavogriseus* and *S. viridis* does not contain the correct residues to bind a second molecule of CA, it is possible that Blm could be bound to one molecule of CA and remain permanently inactive in the Δ*blm*-INF mutant. It is also possible that the proposed CA gene cluster in *S. flavogriseus* and *S. viridis* does not synthesize CA but something related to or similar to CA. If this were the case, the second CA binding site in Blm from *S. flavogriseus* and *S. viridis* may bind more specifically to a CA related molecule whereas the active site contains more general catalytic residues to perform the same function on the CA related molecule. Additionally, even though most of the catalytic motifs are conserved in the three Blm proteins, overall there are sequence differences between them that could cause the Blm-like proteins from *S. flavogriseus* and *S. viridis* to be different enough that they cannot complement the *S. clavuligerus* Δ*blm*-INF mutant. In this case, it is possible that the *S. flavogriseus* and *S. viridis* CA gene clusters are evolutionary precursors to the *S.*

clavuligerus CA gene cluster or they are descendants of the *S. clavuligerus* CA gene cluster that have now become inactive.

Streptomyces exhibit a strong preference over which codon to use for each amino acid (Gustafsson et al. 2004). This is known as codon usage bias and occurs in all bacteria but is most extreme in high GC bacterial genomes. Codon usage bias is the single most important factor in prokaryotic gene expression because preferred codons correlate with the availability and abundance of the related tRNAs within the cell (Chaney and Clark 2015). This could pose potential problems during heterologous gene expression as could be the case in this study. Accordingly, the codon usage of each Blm-like protein studied (including Blm from *S. clavuligerus*) was compared against the documented codon usage present in *S. clavuligerus*. The relative adaptiveness of each Blm-like protein was determined through the use of the Graphical Codon Usage Analyser (Fuhrmann et al. 2004) and the mean difference in codon usage was reported. The mean difference was very low and similar (6.61% and 5.09% respectively) between Blm from *S. clavuligerus* and the similar protein in *S. flavogriseus* (Supplementary Figure 7 and 8). This is to be expected as both organisms are from the same genus and therefore have similar codon usage. The largest mean difference in codon usage came from the Blm-like protein in *S. viridis*, which is to be expected as *S. viridis* is more distantly related. In spite of its ancestry, the mean difference in codon usage between *S. clavuligerus* and *S. viridis* Blm is relatively low at only 12.81% (Supplementary Figure 6). In short, it is not likely that the codon usage bias exhibited by *S. clavuligerus* is a factor in the dormancy of the Blm-like proteins in the Δ*blm*-INF mutant.

In this study, a point mutation was found in the *blm*-like gene from *S. flavogriseus*. It occurs in the third position of the nucleotide codon triplet (Supplementary Figure 5) and produces a silent mutation. As a result, the amino acid (Arg 308) translated from that codon remains the same (Figure 3.6, yellow box). In addition, Arg 308 has not yet been highlighted as a residue important for catalytic function and is not in close proximity to already highlighted residues. Regardless, there is a large difference in the codon usage frequency between the original triplet (47.2 per thousand) and the mutated triplet (5.5 per thousand) (Nakamura et al. 2000). This difference may pose a problem for the translation of the codon to its amino acid.

4.4: Blm is located in the cytoplasm of the cell

It is widely agreed that Blm must have an important enzymatic function inside the cell as it is integral to the production of CA (Li et al. 2000; Jensen et al. 2004; de la Fuente et al. 2002). Blm is considered to be part of the "late steps" of CA production and some predict that it may be responsible for the epimerization step during the conversion of clavaminic acid to CA (Jensen et al. 2012; Valegard et al. 2013). On the other hand, if Blm plays any sort of role like its namesake, a β-lactamase, it would be secreted and be active outside of the cell (Drawz and Bonomo 2010). Therefore, determining the cellular location of Blm can help focus the functional possibilities because enzymes found within cell have different functions than ones found outside of the cell.

Through the use of FLAG tags, anti-FLAG antibodies and western analysis, the cellular location of the active Blm protein was determined. Blm was found to be localized to the cytoplasm of the cell and not excreted extracellularly, ruling out the

possibility of an extracellular function. Blm does not have a predicted secretion signal; however, this study aimed to completely rule out an extracellular function since this was the first time Blm was investigated in its native host. Protein secretion in Streptomycetes is not completely understood and there are alternative mechanisms of secretion that do not use a signal peptide, which have yet to be elucidated in Streptomycetes (i.e. the ESX-1 or type VII secretion system) (Mellado et al. 2011; Anne et al. 2014). As a result, determining the cellular location of Blm using western analysis ruled out the possibility of Blm being secreted by any mechanism.

In addition, CcaR, a cytoplasmic protein, was not detected in the supernatant fraction and Blip, a secreted protein, was not detected in the cytoplasmic fraction of the analysis, therefore there was no cross contamination of samples and cell lysis was not a factor while harvesting the cultures.

Previously hypothesized functions of being a catalytic enzyme acting as either an esterase on substrates similar to *3'-O-* acetyl cephalosporins or an isomerase enabling the epimerization step of clavaminic acid to CA (Valegard et al. 2013) remain possible as catalytic enzymes like these might be localized to the cytoplasm. It is also possible for Blm to be an intracellular transport protein responsible for binding CA and bringing it to the membrane where Blm would interact with Orf13, a proposed efflux pump, and CA would be transported out of the cell. The co-transcription of *blm* and *orf13* allows both proteins to be temporally active, therefore this proposed function of Blm gives further weight to why the *blm/orf13* overlap is present and maintained in homologous CA gene clusters. Blm also has the potential to be a CA regulator that controls the expression of downstream genes involved in the "late" steps of CA production. Although Blm does not

appear to possess any DNA binding domains, it is possible that Blm binds to and activates another protein responsible for CA production. This other mystery protein could be a protein previously identified as essential to CA production, such as *orf13*, *orf14*, *orf15*, *orf16*, or *orf17* (Jensen et al. 2004) and without its activation, CA is not produced.

It has been suggested that the post-translation modification of Blm is possible, such as Valegard et al. (2013) when they refer to the separation of the N and C termini through proteolysis. The separation of the termini is not congruent with the findings in this study. According to our western analysis, only a single band representing the full weight (~50 kDa) of the protein appears on the membrane in the Blm lane in the cytoplasmic fraction (Figure 3.7 B). If the termini were separated, there would be either only one smaller band (< 50 kDa) or two (one full weight 50 kDa) and one smaller (<50 kDa) bands would appear. Since the FLAG tag is present on the C-terminus, this would be the only terminus that would be detected during western analysis besides the full length protein. Nevertheless, only one full weight band is present after western analysis, therefore there is no evidence of post-translational proteolysis of Blm under the conditions of these assays.

4.5: Conclusions

This work elucidated some general attributes of *blm* and the Blm protein. Firstly, all of the *blm* mutants analyzed to date do not display polar genetic effects, as they all can be complemented to restore CA production. Secondly, the Blm-like proteins from the non-CA producing organisms *S. flavogriseus* and *S. viridis* cannot complement the *S. clavuligerus* Δ*blm*-INF mutant. Lastly, Blm was determined to be a cytoplasmic protein

that lacks post translational modification via proteolysis. Despite these accomplishments, there are still more questions to be answered concerning the function of this protein and how it fits into the late steps of CA production. In particular, this study bares the question of exactly where in the cytoplasm Blm is active. More specific cellular fractions can be generated and tested to narrow down the exact cytoplasmic location. It may be possible for Blm to be present in more than one fraction. For example, if Blm functions as an intracellular CA transport protein, it would be found in not only the cytoplasmic fraction but also the membrane fraction if it was interacting with membrane proteins to excrete CA out of the cell. Another question that is not known is if there are partner proteins interacting with Blm and when are they active? Answering both of these questions would narrow the functional possibilities of Blm. Continued examination of the late genes in CA biosynthesis will provide better understanding of how the conversion of clavaminic acid to CA takes place. More specifically, how the epimerization step takes place and what proteins are involved. If Blm plays a role in epimerization, we can engineer Blm to produce novel molecules that have 5*R* stereochemistry from 5*S* molecules. These novel molecules may hold the potential for antibiotic activity and aid in the fight against antibiotic resistance.

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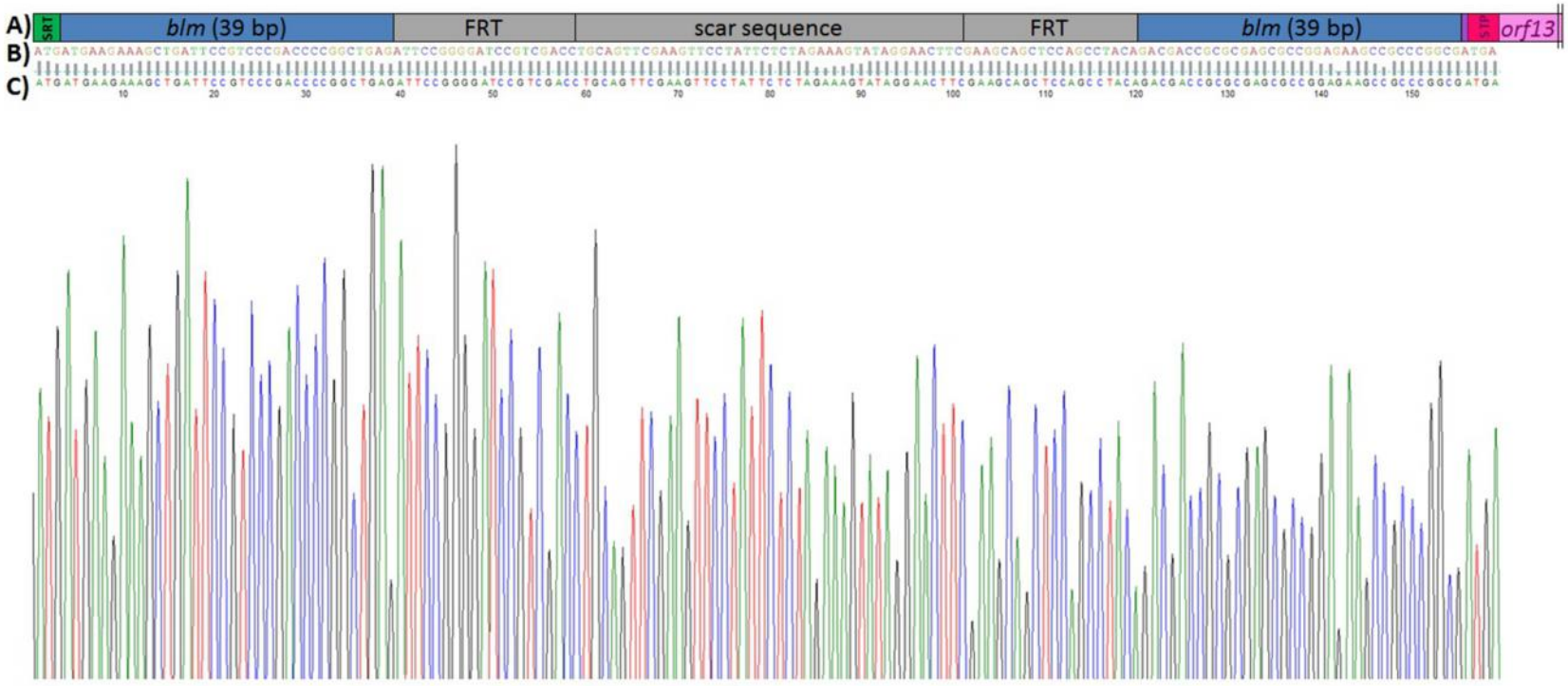
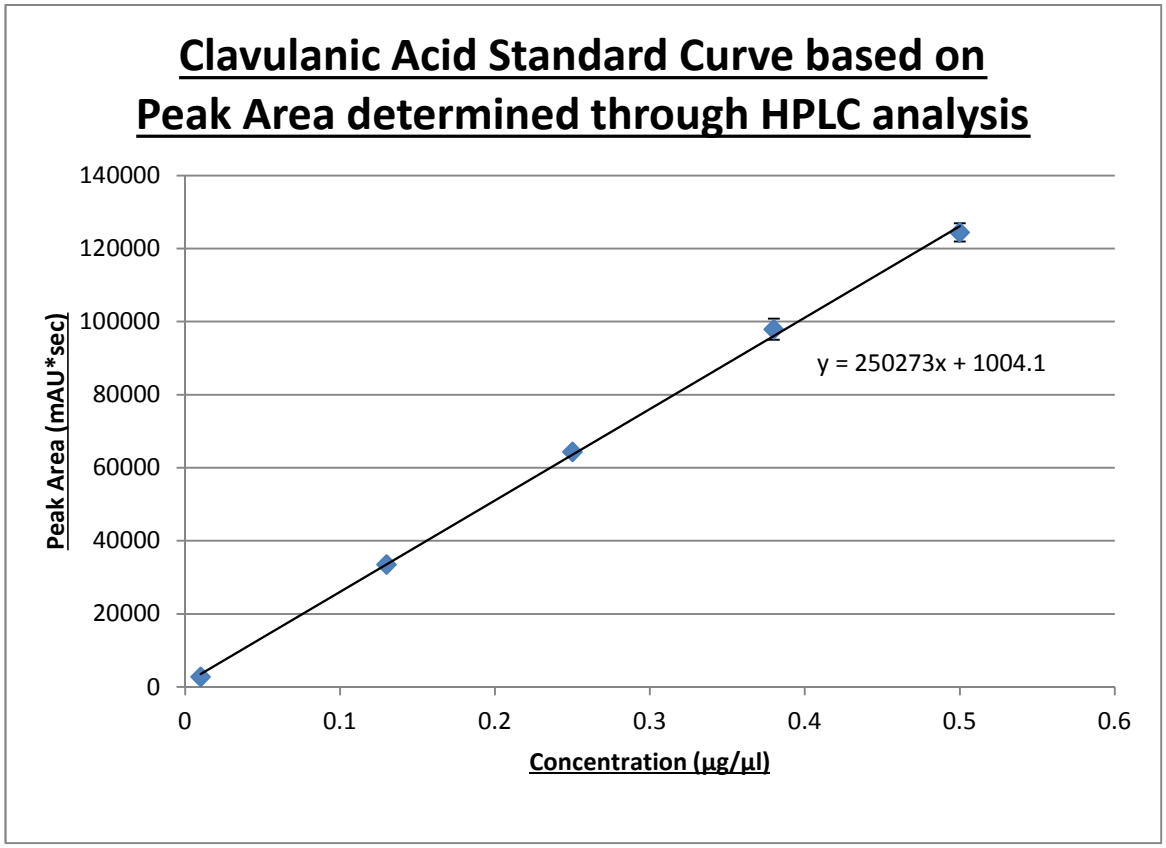
Appendix A- Supplementary data and sequences



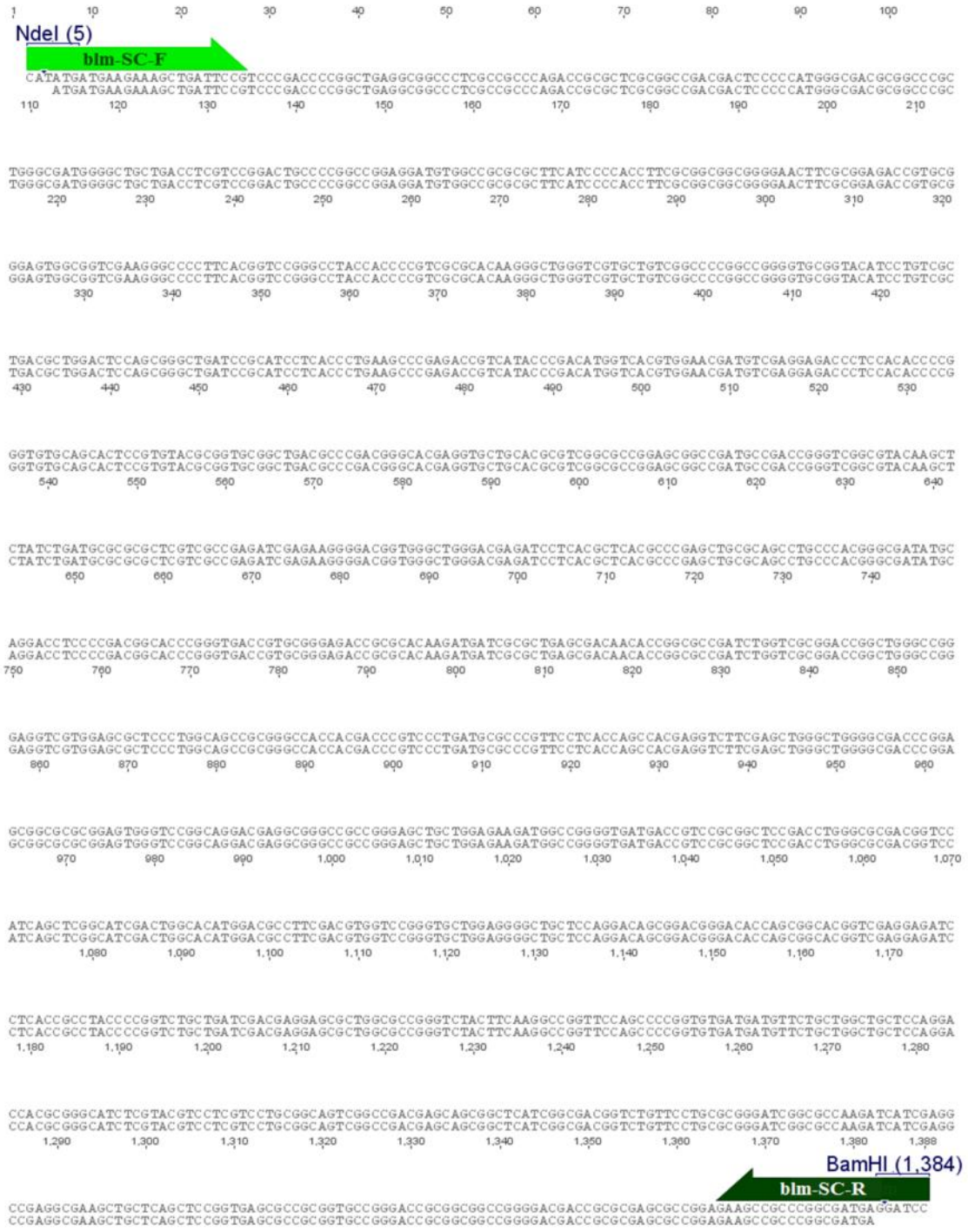
Supplementary Figure 1: The sequencing results of the scar region in the Δ*blm*-INF mutant. **A)** Diagram of the in-frame mutation of *blm* present in the *S. clavuligerus* Δ*blm*-INF mutant. The FRT sites and the scar sequence make up the complete 81 bp scar sequence. **B)** The expected nucleotide sequence for the "scar". **C)** The nucleotide sequence of the scar region as a result of Sanger sequencing and the resulting chromatogram.



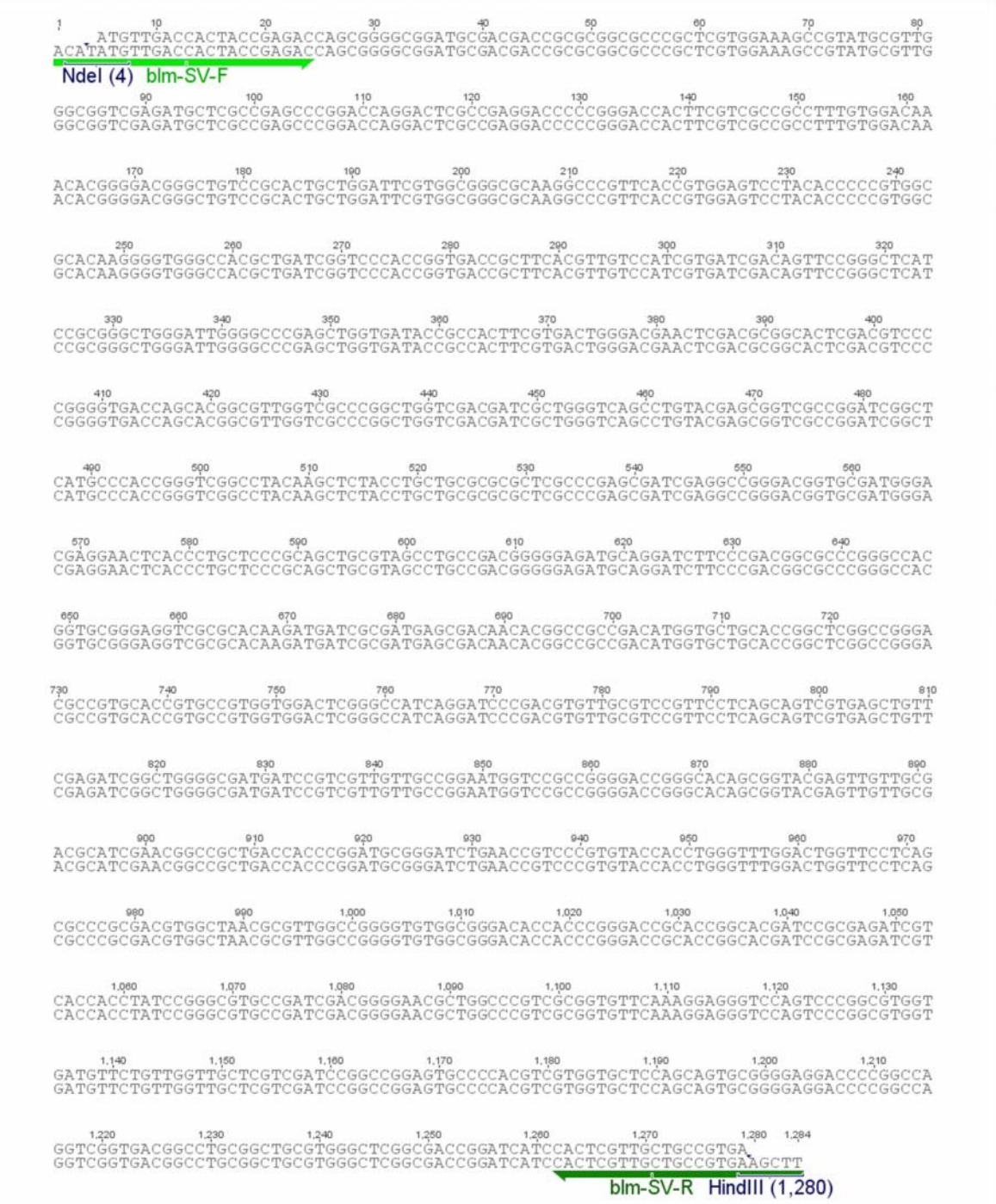
Supplementary Figure 2: Standard curve of the peak areas produced by clavulanic acid standards of known concentration. The concentrations of clavulanic acid used are: 0.01 µg/µl, 0.13 µg/µl, 0.25 µg/µl, 0.38 µg/µl, and 0.5 µg/µl. Each standard was prepared in duplicate (n=2) as described in section 2.6.2. The peak areas of each were determined using the Agilent ChemStation software. From the linear equation, experimental concentrations were determined for each sample based on the peak area produced. Error bars indicate standard error.

Supplementary Table 1: The dry cell weight (mg) of each replicate used to determine the clavulanic acid production of each strain used in this study.

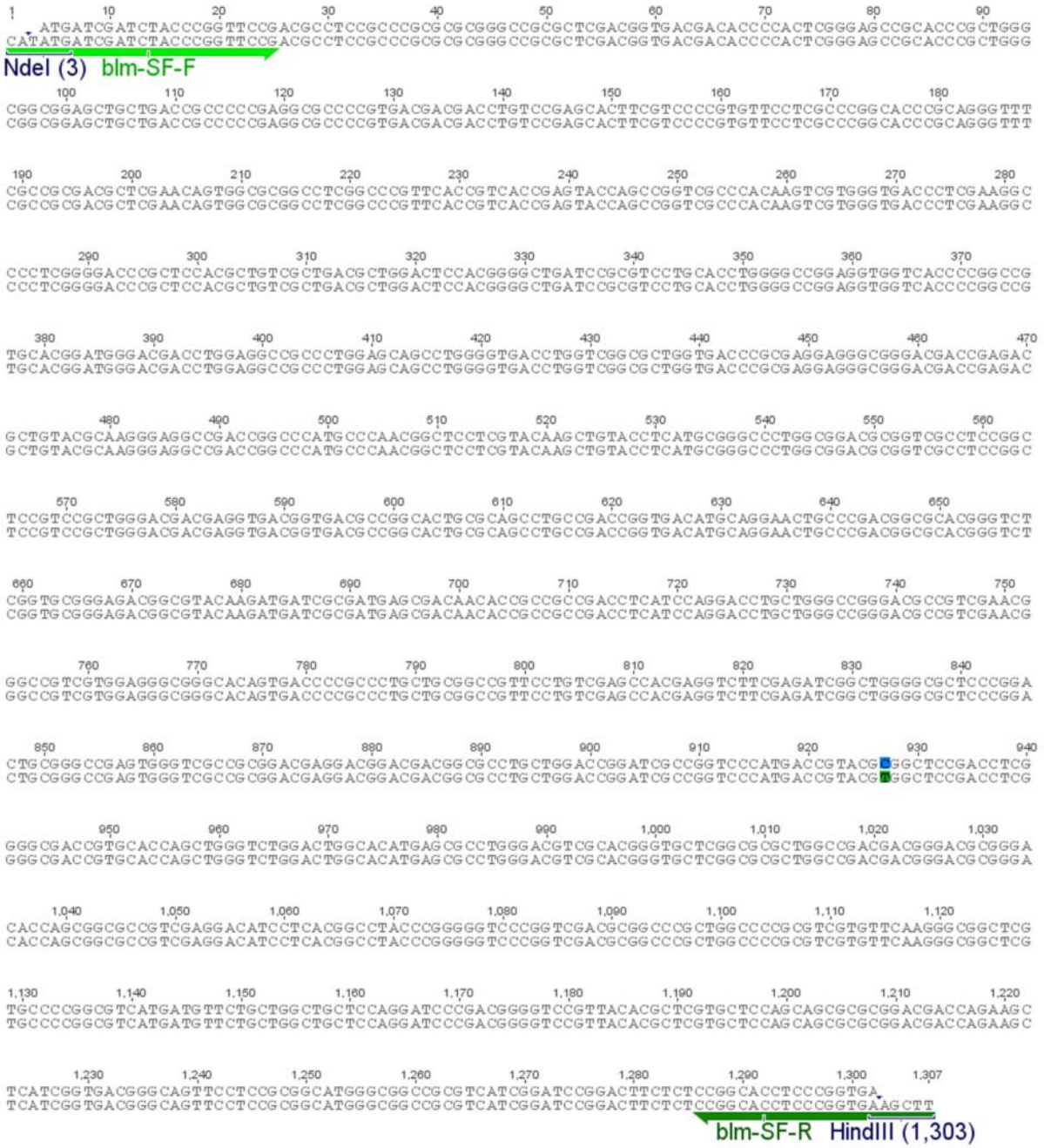
Strain	**Mass (mg)**
wt #1	0.6900
wt #2	0.9540
wt +pH #1	0.7710
wt + pH #2	1.1190
wt + pH:*blm*^{SC} #1	1.9700
wt + pH:*blm*^{SC} #2	1.2680
Δ*blm::apra*^{R} #1	0.8860
Δ*blm::apra*^{R} #2	0.9220
Δ*blm::apra*^{R} + pHM11a #1	1.3080
Δ*blm::apra*^{R} + pHM11a #2	1.0940
Δ*blm::apra*^{R} + pH:*blm*^{SC} #1	1.7440
Δ*blm::apra*^{R} + pH:*blm*^{SC} #2	1.2770
Δ*orf12::apra* #1	1.3880
Δ*orf12::apra* #2	1.4240
Δ*orf12::apra* + pHM11a #1	0.4190
Δ*orf12::apra* + pHM11a #2	0.4290
Δ*orf12::apra* + pH:*blm*^{SC} #1	0.8600
Δ*orf12::apra* + pH:*blm*^{SC} #2	1.0530
Δ*blm*-INF # 1	1.6970
Δ*blm*-INF # 2	1.1280
Δ*blm*-INF + pHM11a #1	1.5720
Δ*blm*-INF + pHM11a #2	1.3980
Δ*blm*-INF + pH:*blm*^{SC} #1	1.2780
Δ*blm*-INF + pH:*blm*^{SC} #2	1.2700
Δ*blm*-INF + pH:*blm*-FLAG #1	1.5510
Δ*blm*-INF + pH:*blm*-FLAG #2	1.2760
Δ*blm*-INF + pH:*blm*^{SF} #1	1.7540
Δ*blm*-INF + pH:*blm*^{SF} #2	0.9770
Δ*blm*-INF + pH:*blm*^{SV} #1	1.7600
Δ*blm*-INF + pH:*blm*^{SV} #2	1.7780



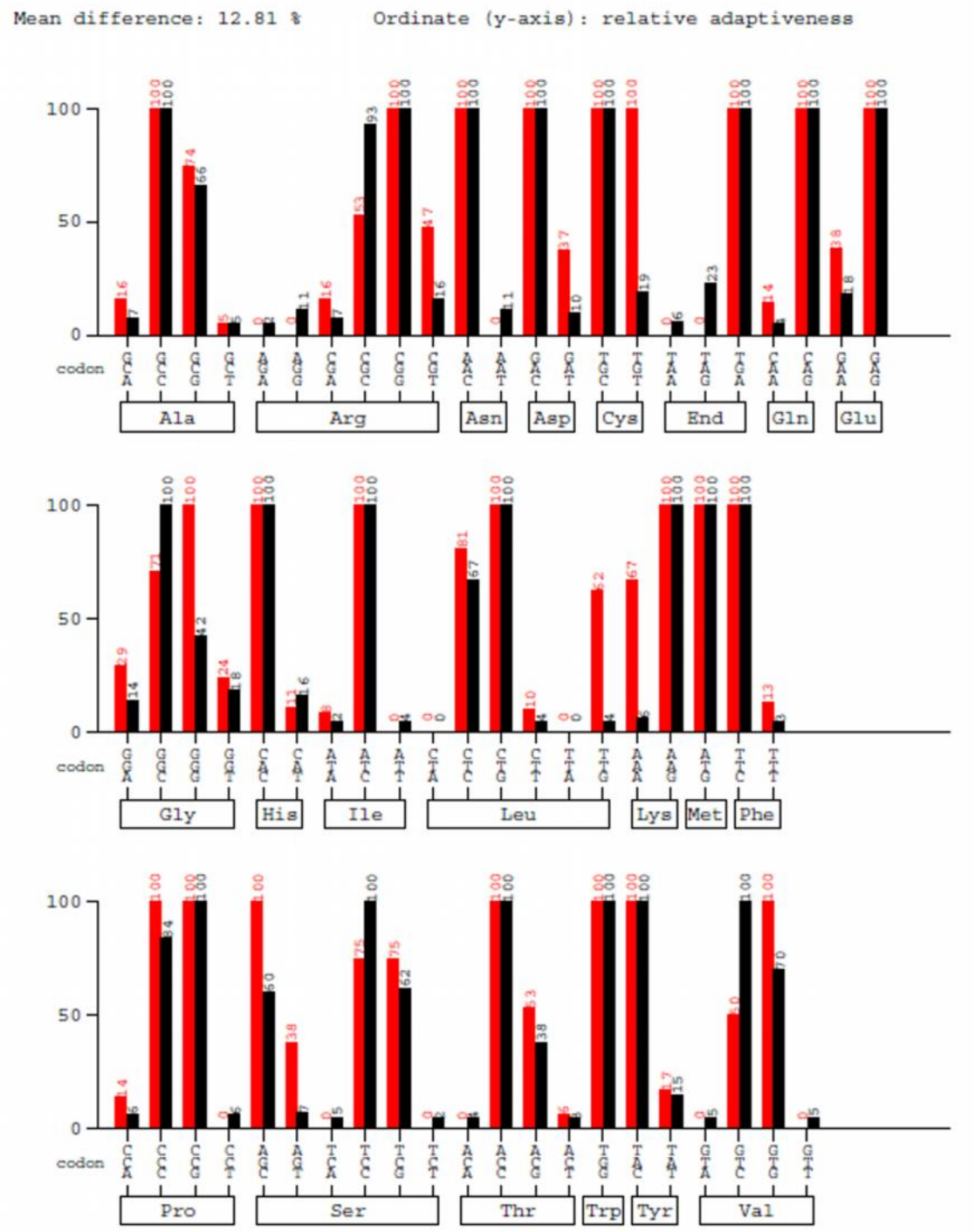
Supplementary Figure 3: A comparison of sequencing results (top line) of *blm* from *S. clavuligerus* against the published sequence (bottom line, AY258009). The forward and reverse primers (in green), used to amplify *blm* from *S. clavuligerus* genomic DNA, are included as well as the restriction enzyme sites that were used in cloning.



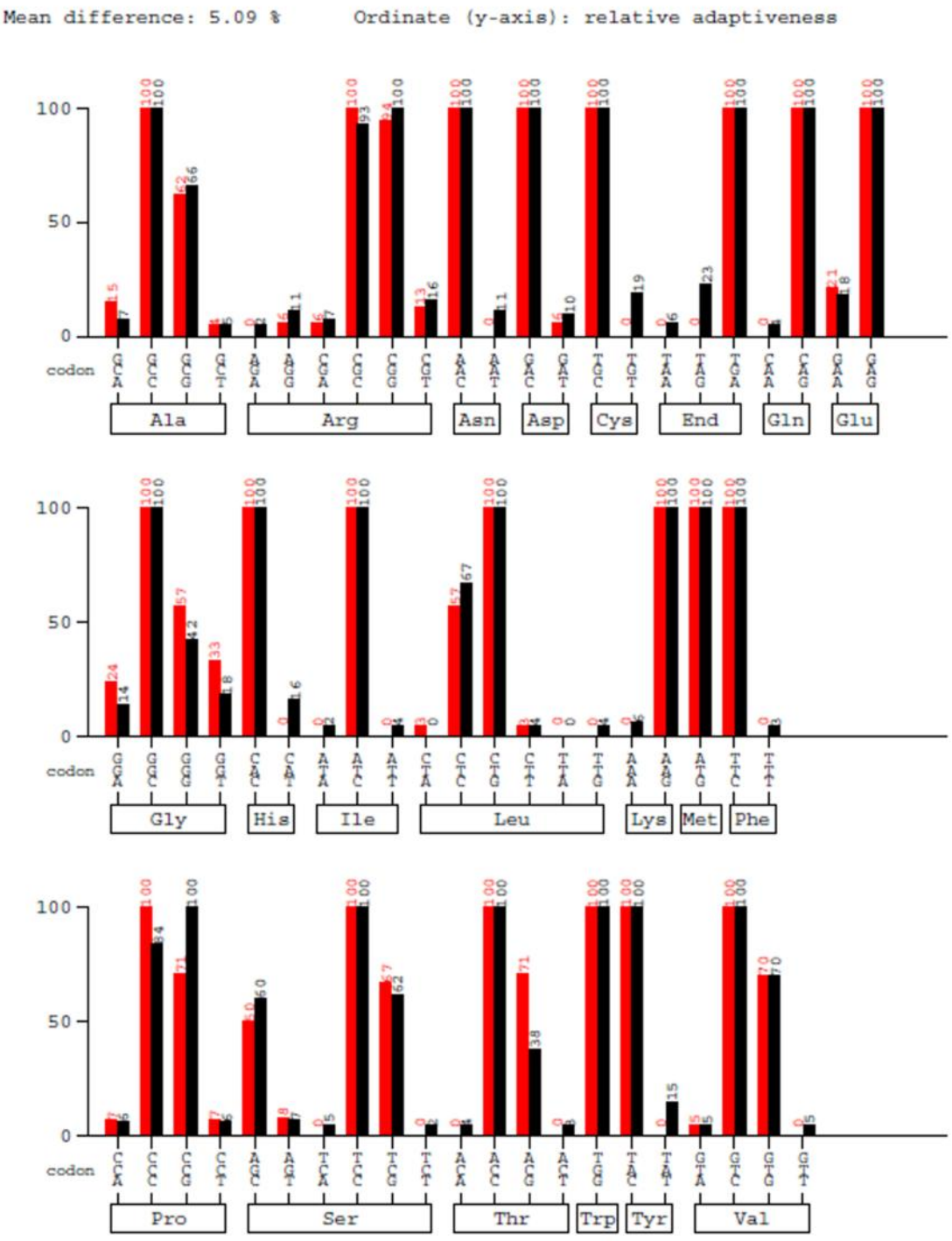
Supplementary Figure 4: A comparison of sequencing results (top line) of the *blm*-like gene from *S. viridis* against the published sequence (bottom line, CP001683). The forward and reverse primers (in green) used to amplify this gene from *S. viridis* genomic DNA, are included as well as the restriction enzyme sites used in cloning.



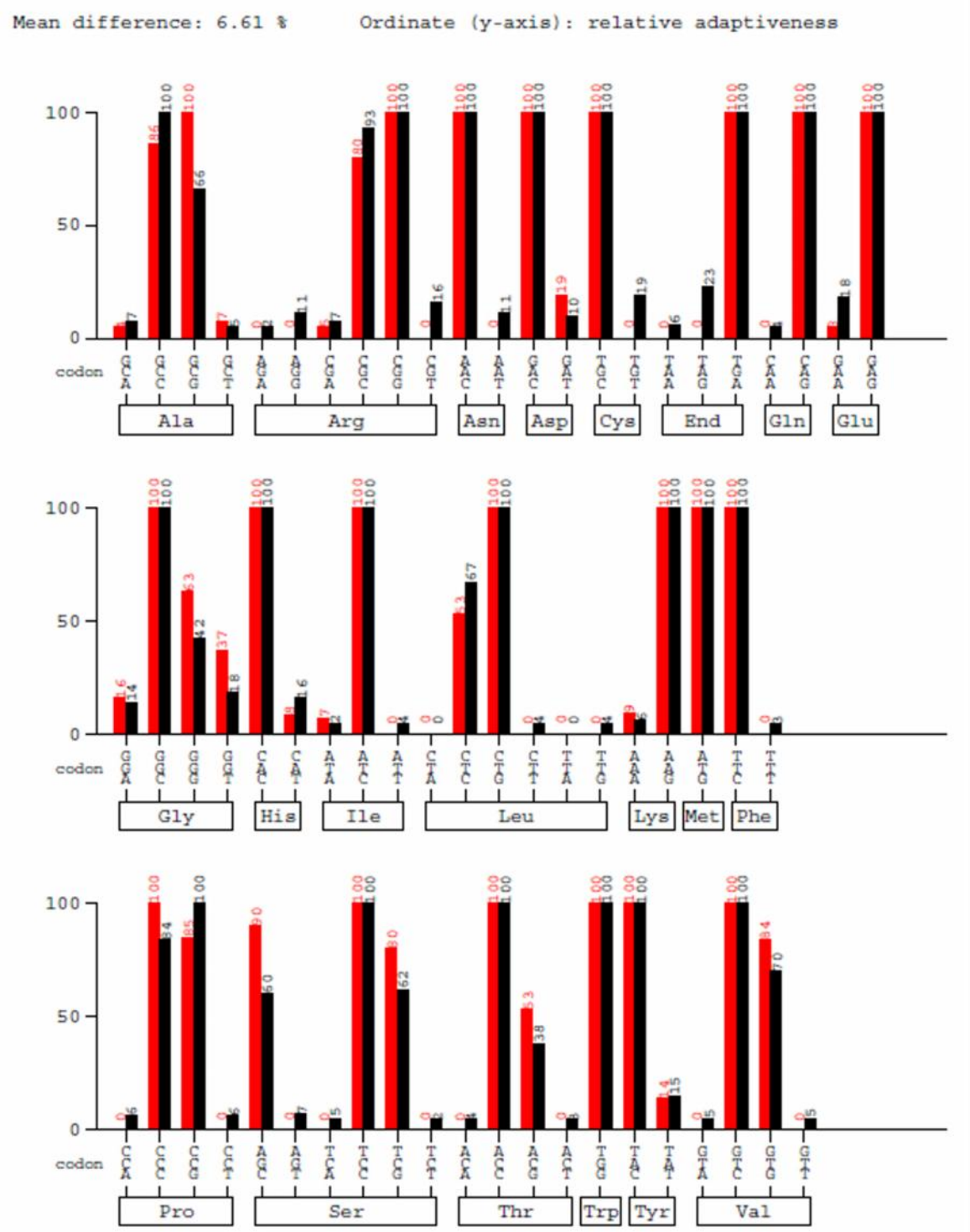
Supplementary Figure 5: A comparison of sequencing results (top line) of the *blm*-like gene from *S. flavogriseus* against the published sequence (bottom line, CP002475). The forward and reverse primers (in green) used to amplify this gene from *S. flavogriseus* genomic DNA, are included as well as the restriction enzyme sites used in cloning. A point mutation is highlighted at nucleotide position 927.



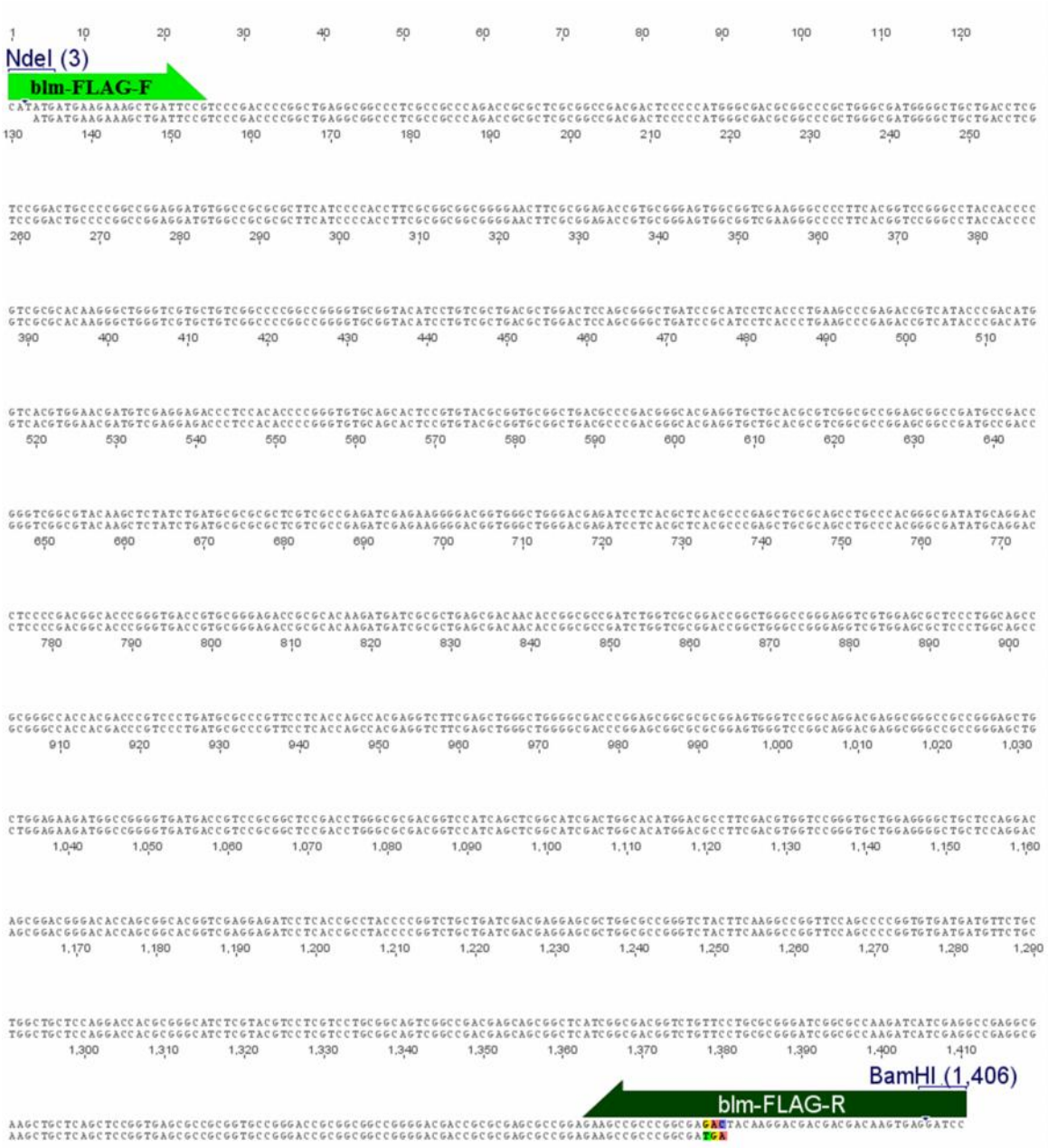
Supplementary Figure 6: A graphical representation of the relative adaptiveness of the amino acid codons used in the *blm*-like gene of *S. viridis* (red) to the codon usage profile of *S. clavuligerus* (black). A scale of relative adaptiveness is along the y-axis. 100 represents the most commonly used codon for each amino acid. Each amino acid is present on the x-axis along with its respective codon triplets. Each bar represents how often that codon is used for that particular amino acid in each species. Graph was produced using the Graphical Codon Usage Analyser (Fuhrmann et al. 2004).



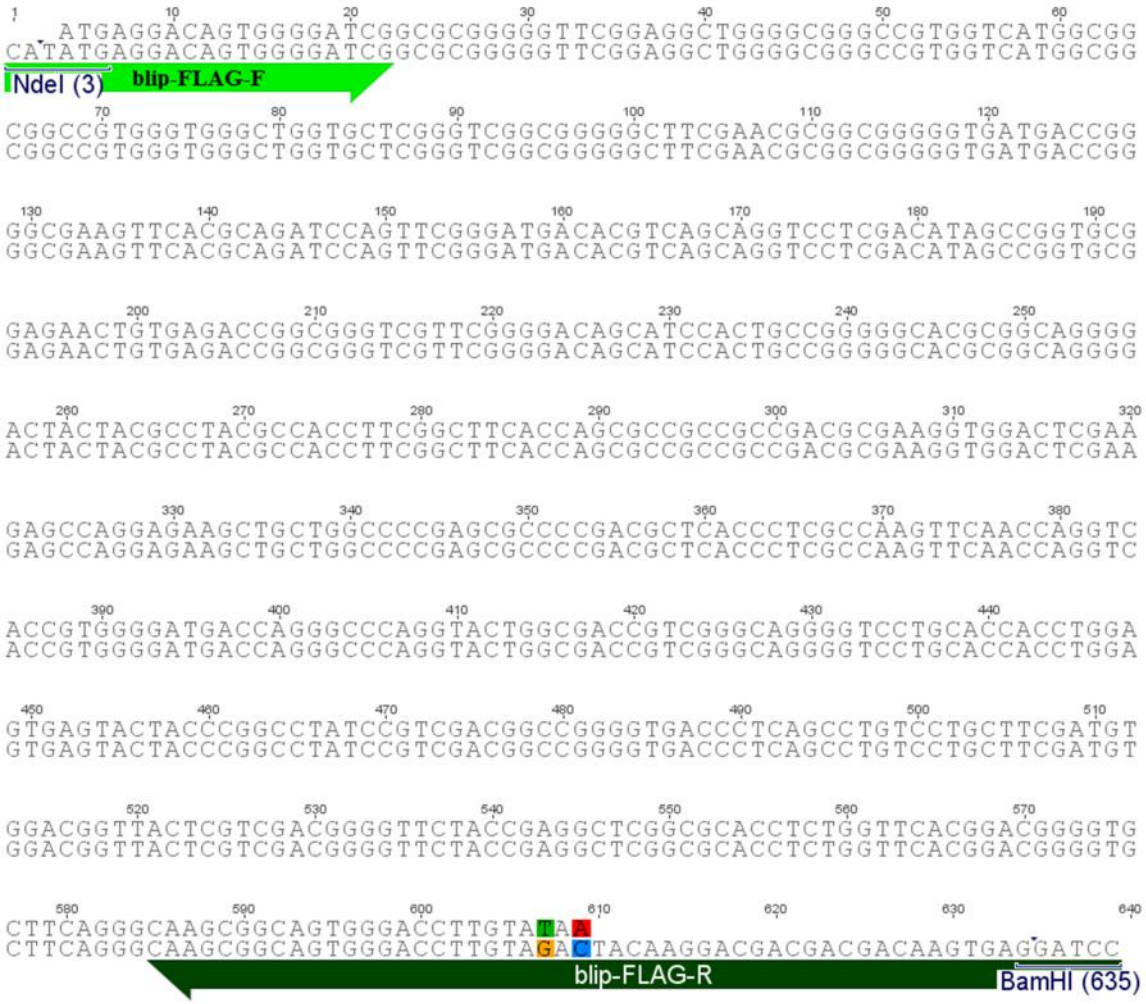
Supplementary Figure 7: A graphical representation of the relative adaptiveness of the amino acid codons used in the *blm*-like gene of *S. flavogriseus* (red) to the codon usage profile of *S. clavuligerus* (black). A scale of relative adaptiveness is along the y-axis. 100 represents the most commonly used codon for each amino acid. Each amino acid is present on the x-axis along with its respective codons triplets. Each bar represents how often that codon is used for that particular amino acid in each species. Graph was produced using the Graphical Codon Usage Analyser (Fuhrmann et al. 2004).



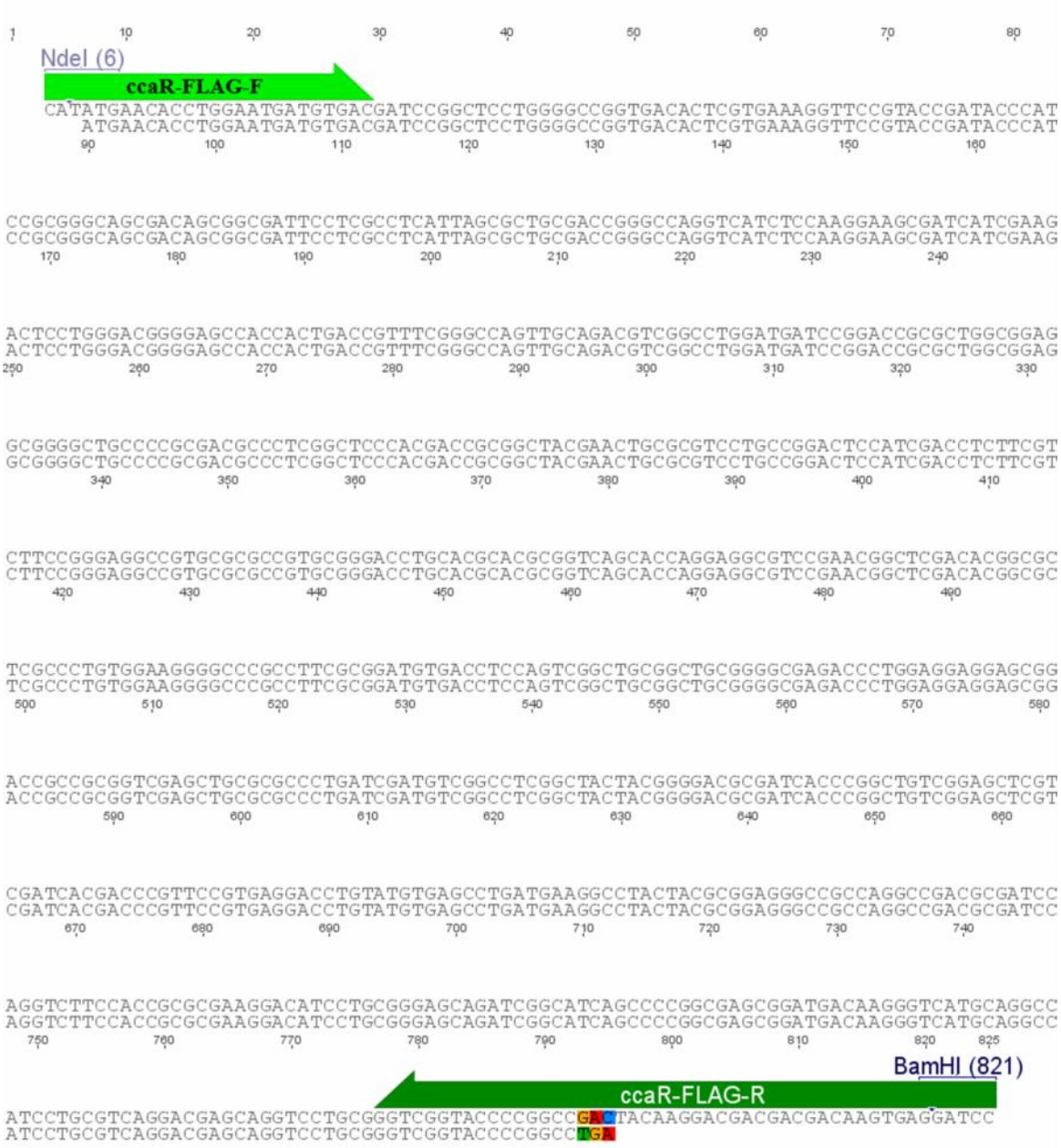
Supplementary Figure 8: A graphical representation of the relative adaptiveness of the amino acid codons used in *blm* of *S. clavuligerus* (red) to the codon usage profile of *S. clavuligerus* (black). A scale of relative adaptiveness is along the y-axis. 100 represents the most commonly used codon for each amino acid. Each amino acid is present on the x-axis along with its respective codons triplets. Each bar represents how often that codon is used for that particular amino acid in each species. Graph was produced using the Graphical Codon Usage Analyser (Fuhrmann et al. 2004).



Supplementary Figure 9: A comparison of sequencing results (top line) of *blm* from *S. clavuligerus* against the published sequence (bottom line, AY258009). The forward and reverse primers (in green) used to amplify this gene from *S. clavuligerus* genomic DNA, are included as well as the restriction enzyme sites used in cloning. The stop codon is removed at the end of the FLAG-tagged version, which is noted by the highlighted nucleotides at the end of the gene.



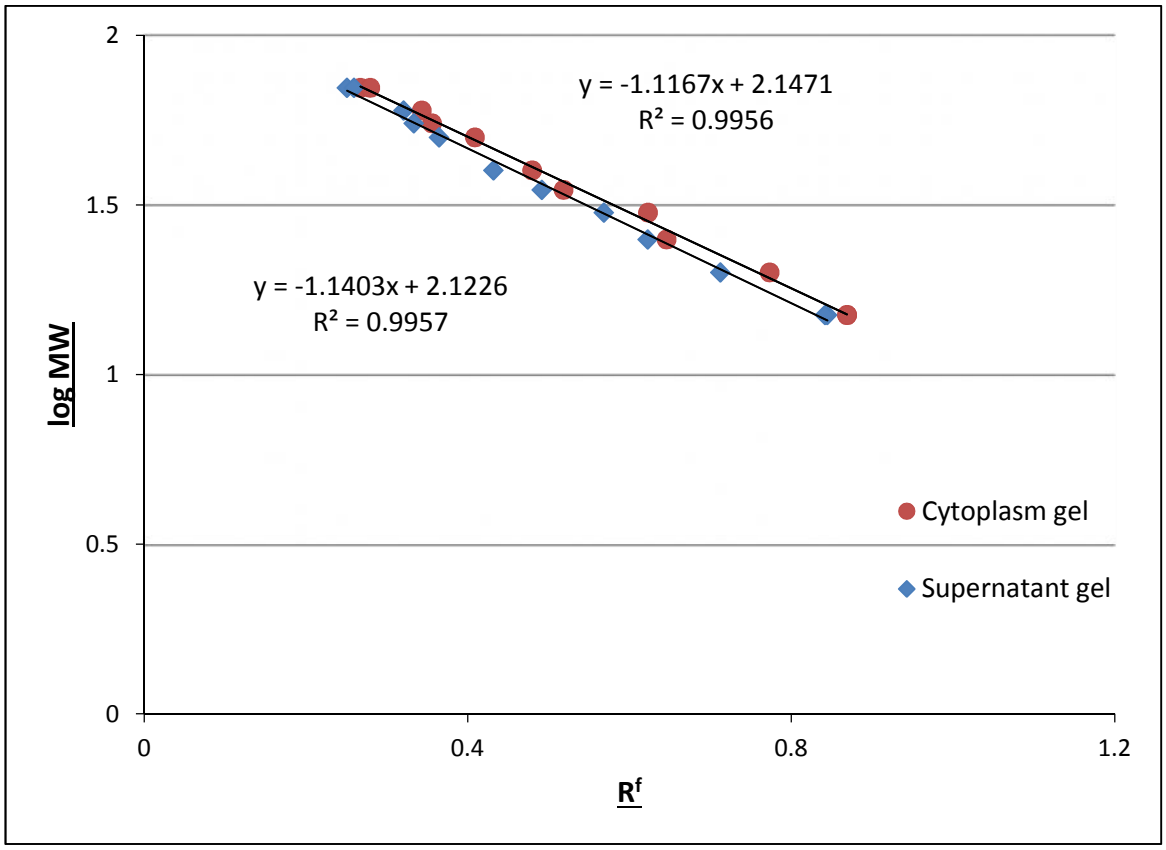
Supplementary Figure 10: A comparison of sequencing results (bottom line) of *blip* from *S. clavuligerus* against the published sequence (top line, M34538). The forward and reverse primers (in green) used to amplify this gene from *S. clavuligerus* genomic DNA, are included as well as the restriction enzyme sites used in cloning. The stop codon is removed at the end of the FLAG-tagged version, which is noted by the highlighted nucleotides at the end of the gene.



Supplementary Figure 11: A comparison of sequencing results (top line) of *ccaR* from *S. clavuligerus* against the published sequence (bottom line, AH006362). The forward and reverse primers (in green) used to amplify *ccaR* from *S. clavuligerus* genomic DNA, are included as well as the restriction enzyme sites used in cloning. The stop codon is removed at the end of the FLAG-tagged version, which is noted by the highlighted nucleotides at the end of the gene.

Supplementary Table 2: Peak time (mins) and peak area (mAU*sec) of clavulanic acid peaks generated through HPLC analysis of derivatized culture supernatants of *S. clavuligerus* strains used in this study.

Strain	**Peak Time (mins)**	**Peak Area (mAU*sec)**
WT #1	10.000	2.61333×10^4
WT #2	10.043	3.27849×10^4
WT +pH #1	10.008	7295.56592
WT + pH #2	10.046	1.83665×10^4
WT + pH:*blm*^{SC} #1	9.984	1.94081×10^4
WT + pH:*blm*^{SC} #2	10.039	2.03931×10^4
Δ*blm::apra*^{R} #1	-	-
Δ*blm::apra*^{R} #2	-	-
Δ*blm::apra*^{R} + pHM11a #1	-	-
Δ*blm::apra*^{R} + pHM11a #2	-	-
Δ*blm::apra*^{R} + pH:*blm*^{SC} #1	9.974	3.01581×10^4
Δ*blm::apra*^{R} + pH:*blm*^{SC} #2	10.084	1.99031×10^4
Δ*orf12::apra* #1	-	-
Δ*orf12::apra* #2	-	-
Δ*orf12::apra* + pHM11a #1	-	-
Δ*orf12::apra* + pHM11a #2	-	-
Δ*orf12::apra* + pH:*blm*^{SC} #1	9.970	1.11345×10^4
Δ*orf12::apra* + pH:*blm*^{SC} #2	10.062	1.02468×10^4
Δ*blm*-INF # 1	-	-
Δ*blm*-INF # 2	-	-
Δ*blm*-INF + pHM11a #1	-	-
Δ*blm*-INF + pHM11a #2	-	-
Δ*blm*-INF + pH:*blm*^{SC} #1	10.073	7816.24170
Δ*blm*-INF + pH:*blm*^{SC} #2	10.018	7939.66113
Δ*blm*-INF + pH:*blm-flag* #1	10.075	3813.06714
Δ*blm*-INF + pH:*blm-flag* #2	10.022	31439.1064
Δ*blm*-INF + pH:*blm*^{SF} #1	-	-
Δ*blm*-INF + pH:*blm*^{SF} #2	-	-
Δ*blm*-INF + pH:*blm*^{SV} #1	-	-
Δ*blm*-INF + pH:*blm*^{SV} #2	-	-



Supplementary Figure 12: Standard curve of the distance (R_f) each protein marker of known molecular weight (log MW) migrated on each western blot in this study. A line is generated for each blot (Figure 3.7) using the distance the protein markers ran (R_f) in comparison to their known molecular weights (log MW) to produce the linear equations above. From the linear equations, the experimentally determined weights of the FLAG-tagged proteins were determined. R_f = migration distance of the protein/migration - distance of the dye front. log MW is the natural log of the known molecular weight of each band in each protein ladder. R^2 values determine the linearity of the relationship; as validity increases, the closer the r^2 value is to 1. The molecular weights of each FLAG-tagged protein determined using this method are displayed in Figure 3.7.