PROTEIN KINASES IN *STREPTOMYCES*: INVOLVEMENT IN GROWTH, GLYCOPEPTIDE PRODUCTION AND RESISTANCE.

By:

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The History of Medicine

- *2000 B.C. – Here, eat this root*
- *1000 A.D. – That root is heathen. Here, say this prayer.*
- *1850 A.D. – That prayer is superstition. Here, drink this potion.*
- *1920 A.D. – That potion is snake oil. Here, swallow this pill.*
- *1945 A.D. – That pill is ineffective. Here, take this penicillin.*
- *1955 A.D. – Oops....bugs mutated. Here, take this tetracycline.*
- *1960-1999 – 39 more "oops"...Here, take this more powerful antibiotic.*
- *2000 A.D. – The bugs have won! Here, eat this root.*

— Anonymous

Abstract

With the recent crisis in hospital-acquired infections, a renewed sense of urgency for studying bacterial species carrying antibiotic resistance determinants has developed. Much of the focus has been centered on the glycopeptide antibiotics including vancomycin, which represent the last main line of defense against many hospital-acquired infections. These clinically pivotal antibiotics are produced by filamentous bacteria in the order Actinomycetes including the genus *Streptomyces*. The investigations presented in this thesis were undertaken to address fundamental questions of glycopeptide production and resistance in the glycopeptide-producing *Streptomyces toyocaensis* NRRL 15009 and non-producing *Streptomyces coelicolor* A3(2). Specifically, questions with respect to the regulation of antibiotic production and resistance have been addressed in light of the relatively recent isolation of protein kinase genes with high similarity to those of eukaryotic origin. A link between glycopeptide production, resistance and protein phosphorylation in *S. toyocaensis* NRRL 15009 is presented as a basis for a subsequent degenerate PCR strategy to clone putative protein kinase genes from this organism.

Using the PCR strategy four putative protein kinase gene fragments were cloned and one of these, *sto*PK-1 was isolated in its entirety and characterized by biochemical and genetic methods. Analysis of StoPK-1 revealed a bona fide serine/threonine protein kinase with localization to membrane fractions. Disruption of the genomic *sto*PK-1 gene in *S. toyocaensis* NRRL 15009 brought about an increased sensitivity to oxidative stress

which could be reversed by supplying *sto*PK-1 *in trans,* but not with a catalytically dead mutant, suggesting a role for the active kinase in responding to oxidative stress in *S. toyocaensis* NRRL 15009.

Additionally, studies examining glycopeptide resistance were undertaken through genomic gene disruption and complementation on the previously isolated vancomycin resistance gene cluster (*van*HAX), supporting a role for this cluster in *Streptomyces* similar to its function in vancomycin resistant *Enterococcus* (VRE). Furthermore, analysis of *Streptomyces* DNA sequences revealed putative two component regulatory systems composed of histidine kinase and response regulator pairs associated with the *van*HAX genes in *S. coelicolor* A3(2) and the glycopeptide biosynthesis genes in *S. toyocaensis* NRRL 15009. These putative VanRS systems share homology to proteins shown to be critical for inducible resistance in VRE, and these observations encouraged an assessment of the response regulator (VanR) homologue in regulating resistance in *S. coelicolor* A3(2). The results of these studies support the conclusion that the regulation of vancomycin resistance in *Streptomyces* is similar to *Enterococcus* and provide further evidence that these soil-born bacteria may be the original source of the clinically important resistance genes.

Dedication

I would like to dedicate this work to my dad and mom, Nic and Anne Neu, whose commitment to my education made possible the work undertaken here. And to my wonderful wife Charmaine, whose love and support inspire me to achieve higher goals in all areas of life.

Acknowledgements

Firstly I wish to acknowledge the tireless supervision of Dr. Gerard Wright whose love of scientific inquiry is contagiously inspiring. My greatest respect and admiration goes to him for his patience and caring.

I also thank my wife Charmaine for her understanding of the "odd" hours that must be kept when investigating matters of scientific importance, and for her love and support through the past years.

A special thanks goes to Dr. Justin Nodwell for his advise and expertise with matters pertaining to *Streptomyces*.

I also thank my other supervisor committee members Dr. David Andrews and Dr. Astrid Petrich for their advise and direction over the work period.

Finally I thank the members of my laboratory for making the time there fun and exciting, with special mention to Gary Marshall for his ever encouraging help and advise in matters pertaining to *Streptomyces*.

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Chapter 1.

Introduction

1.1. Antibiotics and Antibiotic Resistance

Prior to the 19th century human knowledge of infectious disease was limited to the symptoms of each disease and the often terrible consequences of infection. People frequently believed evil spirits were to blame and the true causative agents went undiscovered. The effects of epidemics such as the plague were responsible for widespread hysteria and death (Fig. 1.1). In just under five years between 1347 and 1352, 25 million people or one third of the entire population of Europe had died from the Black Death. The lack of knowledge of the causative agent (in this case the bacterium *Yersinia pestis*) lead people to try a variety of remedies including burning incense, ringing church bells, firing cannons and purchasing all manner of charms and spells (Nohl, 1971). It wasn't until the 1800's when Louis Pasteur developed the "germ theory of disease", that the popular opinion of infectious diseases changed.

The use of small molecules to kill or inhibit the growth of disease causing organisms first came about in 19th century when the French chemist Louis Pasteur noticed that certain bacteria have the ability to kill bacteria that cause the cattle disease anthrax. Following this discovery, Paul Ehrlich began to synthesize chemical compounds that could attack infectious bacteria, and in 1909 his experiments led to the discovery of salvarsan, an arsenic-containing compound effective in treating syphilis. Later, in 1928 Alexander Fleming observed the effects of a substance he called "penicillin" produced by the fungi *Penicillium notatum,* which had prevented the growth of a neighbouring colony of bacteria (Fleming 1929). By 1938 work by Howard Florey and Ernst Chain at Oxford

University on developing methods for growing, extracting and purifying penicillin eventually proved its value as an antibiotic. For their work, Fleming,

Figure 1.1: The Plague as visualized by Arnold Böcklin, circa 1898. This painting was unfinished as the artist and his family twice had to flee cholera epidemics.

Florey and Chain were awarded the Nobel Prize for medicine in 1945. However by 1946, only three years after drug companies began mass-producing penicillin, one hospital had reported that 14% of isolated *Staphylococcus* strains were penicillin resistant and by the end of the decade, the rate had increased to 59% (Levy 1992).

The success of penicillin and emergence of resistance lead to the discovery of many other antibiotics. The aminoglycoside streptomycin was discovered in 1943 and was quickly followed by the isolation of tetracycline in 1948. The glycopeptide vancomycin was introduced in 1956. The importance of the advent of antibiotic therapy cannot be underestimated. Deaths by infectious bacterial diseases now are thought to be only one-twentieth of what they were in 1900, before the development of antibiotics. A time line for antibiotic discovery is shown in Figure 1.2. Of note is the lack of discovery of new antibiotic classes between 1962 and 2000 when linezolid was approved for clinical use. During this time little or no research was carried out on drug discovery, rather chemical modifications were made to existing antibiotics to improve their effectiveness.

The recent push for the development of novel antibiotics has largely come about because of the emergence of antibiotic resistant strains of pathogenic bacteria. The Centers for Disease Control and Prevention estimates that each year two million hospital-acquired (nosocomial) infections occur in the United States, resulting in approximately 90,000 deaths. The agency also reports that in 1992, 13,300 hospital patients died of bacterial infections that were resistant to various antibiotic treatments (CDC 2000). Thus the scope of antibiotic resistance in infectious diseases is a large and serious one.

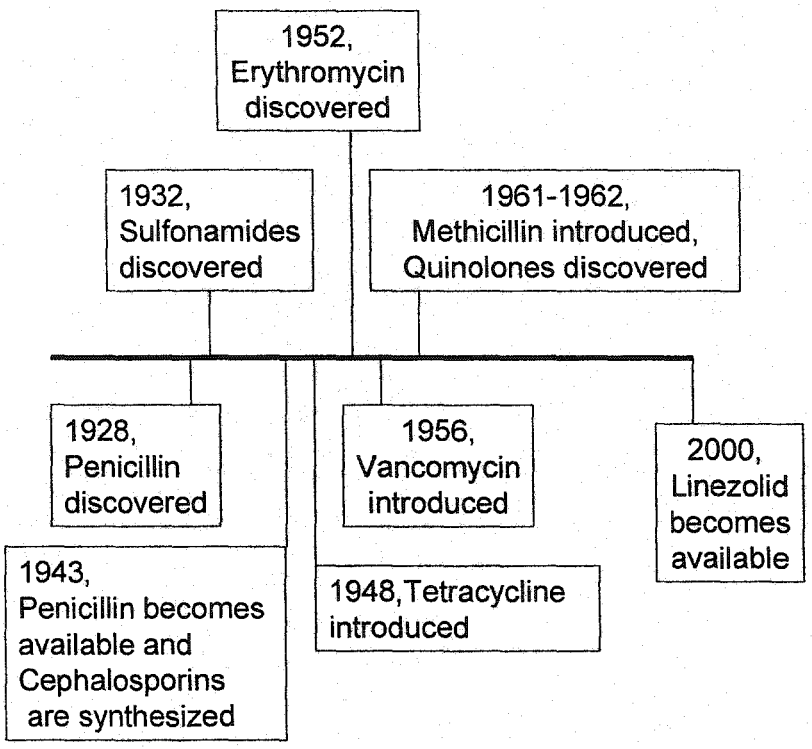


Figure 1.2: Time Line for the discovery and introduction of various classes of antibiotics.

Enterococcus and Staphylococcus aureus are two of the most common causes of nosocomial infections. Treatment of *S. aureus* infections with penicillin was originally indicated, but the development of resistance was quick (see above) and the use of methicillin in the early 1960's became common place. However, methicillin resistance also appeared shortly after, and today nosocomial methicillin-resistant *Staphylococcus aureus* (MRSA) infections have become common, accounting for 40% of isolated stains (Chambers 2001). Since the widespread emergence of MRSA in the 1980s in the United States, vancomycin has been the antimicrobial agent of choice for serious infections. The greatest fear now is the development of vancomycin resistance in MRSA, a situation that would create a truly dangerous "superbug". Indeed, the development of intermediate

resistance has already been documented (Perl 1999), and the acquisition of resistance may be inevitable. Table 1.1 shows the development of antibiotic resistance in *S. aureus* since the introduction of penicillin in 1941. Although resistance in *S. aureus* has taken 40 years to develop, the use of vancomycin in the treatment of *Staphylococcus* infections is a recent phenomenon (Kirst *et al* 1998). However, vancomycin has been used more extensively to treat infections caused by *Enterococcus* (see below and Chapter 5).

Table 1.1: Time required for prevalence rates of resistance in *S. aureus* to reach 25% in hospitals.

Drug	Year drug introduced	Years to report of resistance	Years until 25% rate in hospitals	Years until 25% rate in community
Penicillin	1941	1-2	6	15-20
Vancomycin	1956	40	?	?
Methicillin	1961	<1	25-30	40-50 (projected)

Modified from Chambers (2001).

In the 1960's, when enterococci became resistant to aminoglycosides, vancomycin became a popular drug used to treat infections caused by these enteric organisms. Vancomycin is now the only effective treatment for enterococcal infections (Moellering 1998). Vancomycin resistant *Enterococcus* (VRE) with high-level resistance was first isolated in France in 1986 (Leclercq *et al* 1988). In the early 1990's enterococci accounted for approximately 176,000 nosocomial infections annually in the United States and the percentage of nosocomial infections caused by VRE increased more than 20-fold (from 0.3% to 7.9%) between 1989 and 1993 (Emori and Gaynes 1993, Huycke *et al* 1998). More recent surveys have shown a continual increase, and the incidence of nosocomial VRE in intensive care patients was as high as 25 % as of 1999 (CDC 2000).

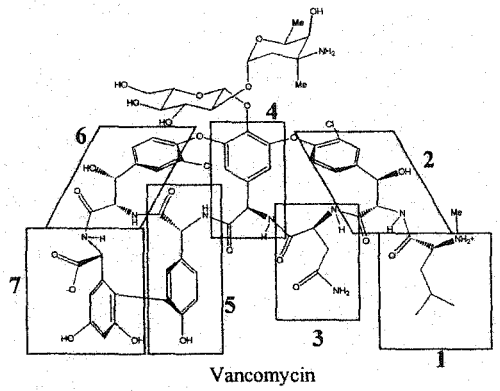
One worry is that the high incidence of VRE can act as a reservoir for resistance genes. This concern has merit because high-level vancomycin resistance can be experimentally transferred from *Enterococcus faecalis* to *S. aureus* in both *in vitro* and *in vivo* models (Noble *et al* 1992). Thus the emergence of a vancomycin-resistant *Staphylococcus aureus* (VRSA) "super bug" could inevitably result from the gene transfer of glycopeptide resistance determinants in a hospital setting.

1.2. Structure and Activity of Glycopeptide Antibiotics

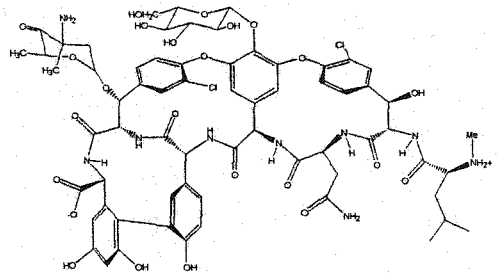
1.2.1. Glycopeptides Classification

Glycopeptide antibiotics are produced by filamentous bacteria of the order *Actinomycetes*. Over 100 different glycopeptides have been isolated and vancomycin is the prototypical compound. Almost all glycopeptides contain a heptapeptide core of seven amino acid residues with amino acids number 2 & 4, 4 & 6, and 5 & 7 covalently linked. (Fig. 1.3) The use of unusual amino acids is a common phenomenon, including β-hydroxytyrosine, 4-hydrophenylglycine and 3,5-dihydroxphenylglycine. Chlorine, sulphate, sugars and lipids can be substituents at various places. Classification is based on residues at positions 1 & 3 and characterized into three groups. Group I glycopeptides contain aliphatic residues and include vancomycin and balhimycin. Group II contain aromatic residues and include the glycopeptide avoparcin. Group III glycopeptides all contain covalently joined aromatic residues with A47934 and teicoplanin representative members (see Fig. 1.3).

GROUP I

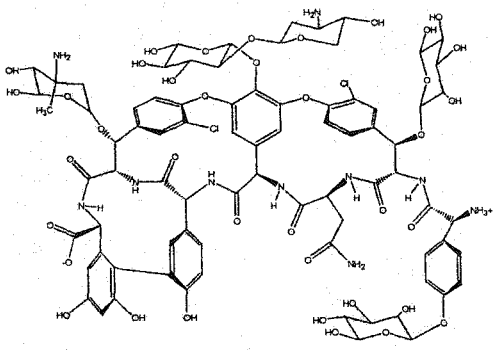


Vancomycin



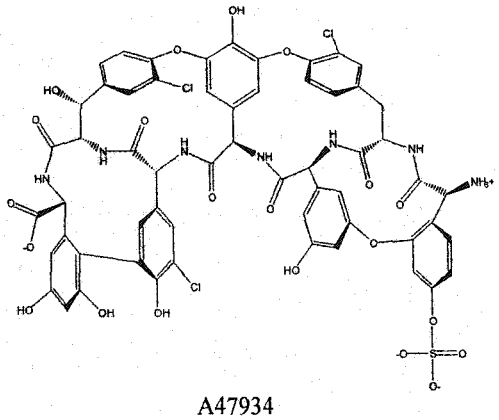
Balhimycin

GROUP II

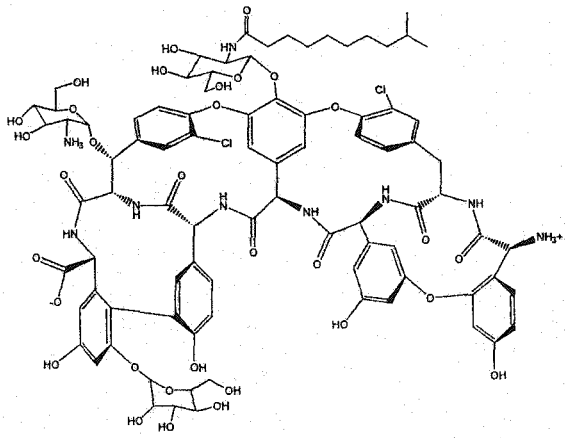


Avoparcin

GROUP III



A47934



Teicoplanin

Figure 1.3: Classification of glycopeptide antibiotics showing amino acid numbering (vancomycin). See text for classification details.

1.2.2. Dimerization

Glycopeptide dimerization may play an important role in antibacterial activity, and most glycopeptides can form dimers (Waltho and Williams 1989, Gerhard *et al* 1993) with the exception of teicoplanin (Mackay *et al* 1994A). Dimerization may be promoted by additions to the heptapeptide backbone including chlorines and sugars (Mackay *et al* 1994B). and can occur 'back to back' (Sheldrick *et al* 1995, Schafer *et al* 1996) or 'face to face' (Loll *et al* 1998). Face to face dimerization has been shown to be ligand-mediated but its role in antibiotic activity is not clear (Loll *et al* 1998). However, back to back dimerization is thought to promote antimicrobial action by bringing second molecule in close proximity to peptidoglycan ligand (Beauregard *et al* 1995). Covalently-linked monomers of vancomycin have been shown to increase affinity to cell wall ligands as well as increase antimicrobial action and may mimic the back to back dimers. (Sundram *et al* 1996).

Dimerization may increase antibacterial activity through several mechanisms. These include inducing conformational changes to increase ligand affinity (Mackay *et al* 1994A, Loll *et al* 1998, Beauregard *et al* 1995), positioning the glycopeptide charged groups so that the carboxy terminus groups in the growing peptidoglycan are attracted to the glycopeptide (Mackay *et al* 1994(A), Loll *et al* 1997), and enhancing ligand affinity by strengthening hydrogen bonds to the ligand through electronic polarization by hydrogen bonds across the dimer interface (Loll *et al* 1997).

1.2.3. Peptidoglycan Assembly

Like the penicillin group of antibiotics, the target of glycopeptide action is the synthesis of the cell wall peptidoglycan. Unlike the penicillins however, the glycopeptide group of antibiotics are inactive against gram negative organisms due to their inability to cross the outer membrane barrier. Therefore, access to the growing peptidoglycan layer only allows their use as antimicrobial agents in gram positive infections.

Peptidoglycan synthesis in gram positive bacteria has been well studied and an outline of the events in cell wall construction are shown in Figure 1.4 (for reviews see Bugg and Walsh 1992, Navarre and Schneewind 1999, Mazmanian *et al* 2001). Glycopeptides are thought to specifically target transpeptidation and to a lesser extent, transglycosylation events in the growing cell wall to cause cell lysis and death (Fig. 1.4E and D). Binding to the terminal D-alanine-D-alanine dipeptide (D-ala-D-ala) of the lipid-disaccharide-pentapeptide precursor of peptidoglycan in the growing cell wall is thought to be the principle site of activity (see Fig. 1.4). As shown in Figure 1.5A, this model is supported by studies showing that glycopeptides bind tightly to the terminal D-ala-D-ala dipeptide through five hydrogen bonds between the peptide backbone and the cell wall peptide (Williams and Kalmer 1977). It is believed that this tight interaction physically blocks transpeptidase access thereby blocking formation of peptide cross-bridges (Walsh 1999).

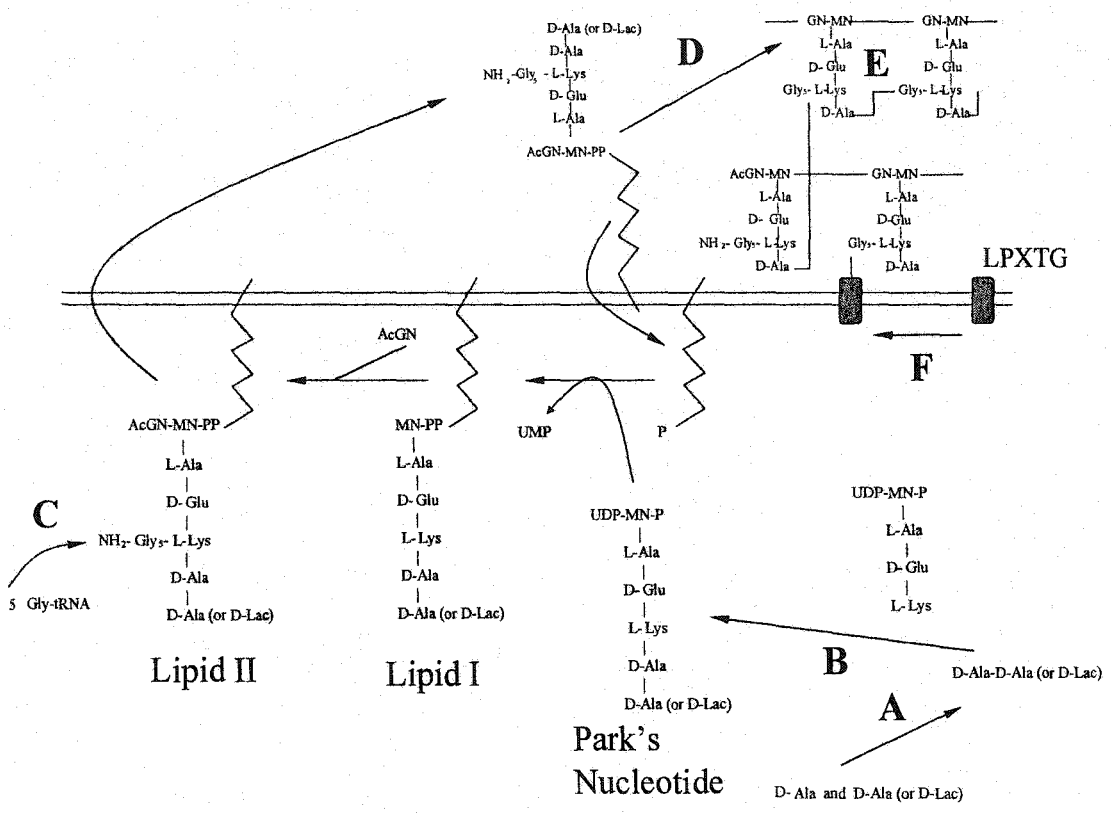


Figure 1.4: Assembly of peptidoglycan. The generation of dipeptide precursors occurs in the cytoplasm **(A)** through the actions of D-alanine-D-alanine ligases (eg. DdlA or VanA) and culminates with the production of Park's nucleotide peptidoglycan precursor by the actions of adding enzymes such as MurF **(B)**. This precursor is then transferred to a lipid carrier to generate lipid I which is modified with N-acetylglucosamine (AcGN) generating lipid II, and is decorated with glycine residues (usually 5) by FEM proteins **(C)**. The precursor is then translocated across the membrane and incorporated into the cell wall by transglycosylation **(D)** displacing the lipid carrier, and by transpeptidation **(E)**. In addition, linking of membrane proteins to the pentaglycine bridge can also occur through the cell anchor signal LPXTG **(F)**. MN is N-acetylmuramic acid and P is phosphate.

1.3. Glycopeptide Antibiotic Resistance

1.3.1. Resistance in *Enterococcus*

The emergence of vancomycin resistance in *Enterococcus* in the 1980's generated tremendous interest and has brought about a large amount of research to determine the resistance mechanism. Early in this research it became apparent that a target modification was responsible for resistance: the replacement of the D-ala-D-ala terminal dipeptide with a D-ala-D-lactate substrate. As shown in Figure 1.5B, this replacement leads to a loss of a single hydrogen bond and results in a 1000 fold affinity drop, preventing substrate binding and allowing transpeptidation to proceed (Walsh *et al* 1996). In addition, the loss of binding to the terminal dipeptide is sufficient to reverse the inhibition of transglycosylation normally seen with vancomycin. The inhibition of transglycosylation is thought to be mediated by the vancomycin carbohydrate residues, but the inhibition can be restored through the use of various carbohydrate substrates independent of the peptide backbone and therefore is apparently not dependent on terminal dipeptide binding (Ge *et al* 1999).

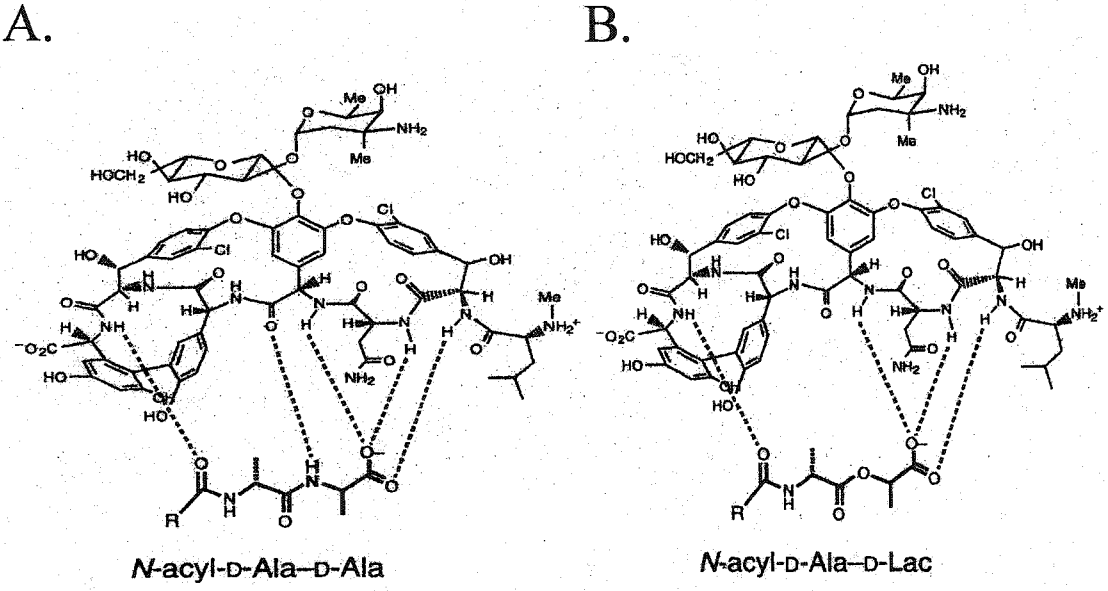


Figure 1.5: (A) Binding of vancomycin to D-alaine-D-alanine. Five hydrogen bonds are involved in producing a tight interaction. **(B)** Binding of vancomycin to D-alanine-D-lactate. Loss of a single hydrogen bond prevents strong interaction and results in loss of activity.

The determinants of vancomycin resistance in *Enterococcus* were first characterized by a series of experiments carried out by Patrice Courvalin and his infectious diseases group at the Pasteur Institute in France (Arthur and Courvalin 1993). This group determined that vancomycin resistance was dependent on five genes named *van*S, *van*R, *van*H, *van*A and *van*X (Fig. 1.6). The functions of these genes and their protein products have been determined and are summarized in Table 1.2. The *van*HAX genes are clustered in a co-ordinately regulated operon and encode enzymes that produce an altered peptidoglycan with a D-alanine-D-lactate terminal dipeptide, reducing glycopeptide affinity. Their regulation is inducible and controlled by the VanRS two component system (See section 1.3.3 below).

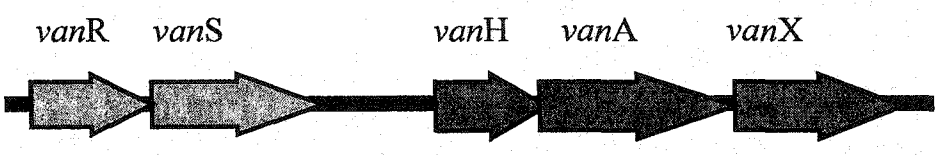


Figure 1.6: Organization of glycopeptide resistance cluster (VanA) in *Enterococcus*. See Table 1.2 for gene functions.

Table 1.2: Gene Products of the *van* gene cluster in Enterococcus.

Gene Product	Type	Function	References
VanR	Response regulator	Regulation of *van*HAX	Wright *et al* 1993.
VanS	Histidine kinase	Regulation of *van*HAX	Wright *et al* 1993.
VanH	D-lactate dehydrogenase	Generates D-lactate	Bugg *et al* 1991.
VanA	D-ala-D-lactate ligase	Generates D-ala-D-lactate dipeptide	Bugg *et al* 1991, Wright and Walsh 1993.
VanX	D-ala-D-ala dipeptidase	Cleaves D-ala-D-ala dipeptide	Wu *et al* 1995.

1.3.2. Resistance in *Streptomyces*

Gram positive glycopeptide producing bacteria such as the vancomycin producer *Amycolatopsis orientalis* C329.2 (originally named *Streptomyces orientalis*) and the A47934 producer *Streptomyces toyocaensis* NRRL 15009 require the ability to grow in the presence of the glycopeptides they produce and therefore are likely candidates for resistance genes. The discovery of the *van*HAX gene cluster in these filamentous bacteria has lead to the speculation that glycopeptide producing organisms may be the source of the resistance genes in enterococci (Marshall *et al* 1998). Whether this is true or not is a matter of some debate, however, it is very likely that the original resistance mechanism

emerged in these organisms. In addition to glycopeptide producing *Streptomycetes*, analysis of sequencing data from the prototypical organism *Streptomyces coelicolor* A3(2), which does not produce glycopeptides, also revealed the presence of the vancomycin resistance cluster. The organization of the *S. coelicolor* resistance genes are shown in Figure 1.7 together with the genes cloned by Marshall *et al* (1997) and J. Pootoolal (unpublished) from *S. toyocaensis* and *A. orientalis*. The presence of two novel glycopeptide resistance associated genes (*gra*1 and *gra*2) are shared by both *S. coelicolor* and *S. toyocaensis* and are of unknown function although the predicted protein Gra2 shares high homology to the FemA proteins from *Staphylococcus* which are involved in adding glycine to lipid II (see Fig. 1.4, Ton-That *et al* 1998). In addition, a two component system appears just upstream of the *van*HAX cluster in *S. coelicolor* and is clustered with the glycopeptide biosynthesis genes in *S. toyocaensis* (Figure 1.7, J. Pootoolal unpublished).

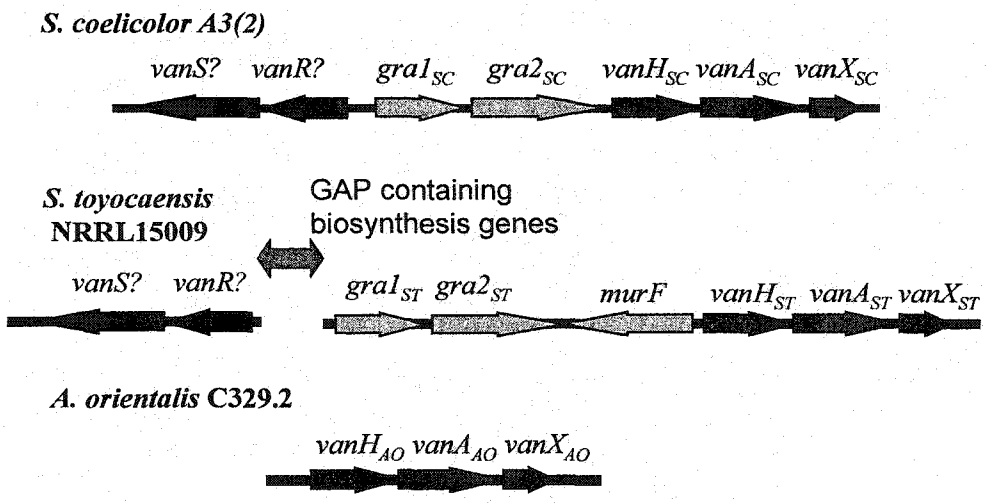


Figure 1.7: Organization of vancomycin resistance genes isolated from various Actinomycetes. vanS? and vanR? are a two component system similar to that found in enterococci. *gra*1 and *gra*2 are glycopeptide resistance associated genes.

1.3.3 Regulation of Glycopeptide Antibiotic Resistance

In enteroccoci, the regulation of vancomycin resistance is generally inducible in nature. However, at least six different phenotypes have been characterized depending on inducibility, resistance profiles and levels of resistance (for review Pootoolal *et al* 2002). In the majority of clinical isolates two phenotypes dominate: VanA and VanB. The VanA phenotype is characterized by high levels of inducible glycopeptide resistance including vancomycin and teicoplanin, and is associated with a plasmid born transposon Tn1546 (Arthur *et al* 1993). On the other hand, the VanB phenotype is associated with inducible resistance to vancomycin, but low levels of resistance to teicoplanin. This effect is thought to be due to an inability of the regulating system to detect the presence of teicoplanin (Baptista *et al* 1996).

Regulation of the vancomycin resistance gene cluster is controlled by the VanRS two component system and a schematic of induction events is shown in Figure 1.8. This system is very similar to other bacterial two component systems which sense an environmental stimulus and through the actions of a histidine kinase and response regulator pair, modulate target gene transcription (for recent reviews see Stock *et al* 2000, Foussard *et al* 2001, West and Stock 2001).

The exact mechanism(s) which trigger induction of the resistance genes is still a matter of controversy (for comprehensive review see Arthur and Quintiliani 2001). Several groups have found that VanA type resistance is inducible by glycopeptides and the transglycosylase inhibitor moenomycin, but not by drugs inhibiting reactions preceding (eg ramoplanin, D-cycloserine) or following (eg. penicillin, bacitracin)

transglycosylation (Handwerger and Kololathis 1990, Baptista *et al* 1996). These researchers have suggested that a build up of the pentapeptide precursors such as lipid II (see Fig. 1.4) may act as a ligand for activating the kinase $VanS_A$ (subscript indicates phenotype). This conclusion is also supported by results found by others where only late stage inhibitors of cell wall biosynthesis (vancomycin, penicillin, bacitracin) but not early stage inhibitors (D-cycloserine, fosfomycin) could induce resistance (Allen and Hobbs 1995). However, Ulijasz *et al* 1996 have suggested extending this list to include early stage precursors, since the induction of a *lacZ* reporter in *Bacillus subtilis* driven by the *van*H promoter region was found to occur in the presence of both early and late stage inhibitors.

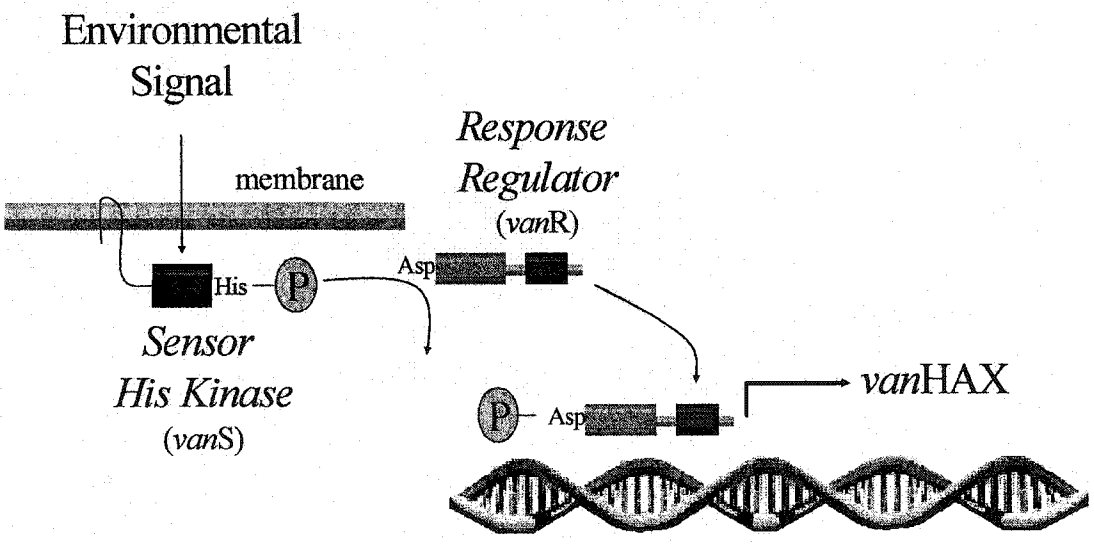


Figure 1.8: VanRS two component system. Two component systems sense an environmental stimulus and a sensor histidine kinase auto-phosphorylates on histidine (encoded by *van*S). This phosphate is then passed to an aspartate residue of a response regulator (encoded by *van*R) which modulates the expression of resistance enzymes (encoded by *van*HAX).

An apparent contradiction with the VanA induction model comes from results with the teicoplanin-sensitive VanB phenotype. Baptista *et al* (1999) have reasoned that $VanS_A$ and $VanS_B$ (subscript indicating phenotypes) are activated by different mechanisms and that activation of $VanS_B$ may involve direct interaction with glycopeptide. This is suggested by differences in the N-terminal sensing domain (they are not related) (Baptista *et al* 1996) and the observation that vancomycin is the only inducer for VanB strains. However, deletion of *van*S_B also allows induction of resistance by teicoplanin and moenomycin, which are not normally inducers (Baptista *et al* 1999). Apparently other heterologous kinase(s) can activate vancomycin resistance gene transcription via cross-talk with the VanR response regulator. Removal of VanS (which also has phosphatase activity) seems to prevent VanR dephosphorylation and inactivation, at least in VanB strains. In addition, the putative heterologous kinase(s) in VanB and the $VanS_A$ kinase from VanA-type enterococci are stimulated by the same antibiotics (including vancomycin and moenomycin) supporting the contention that the host kinase and $VanS_A$ may respond to the same signals (Arthur *et al* 1999, Batista *et al* 1999). However, it should be noted that induction can also occur in *van*S_A null mutants (Arthur *et al* 1992, Arthur *et al* 1999) and the responses seen in VanA strains (through a heterologous kinase) may therefore be similar to VanB.

Unlike *Enterococcus*, studies on the nature of glycopeptide resistance in *Streptomyces* are limited to the isolation of genes by our laboratory with predicted protein sequence and organizational homology to enterococcal gene products (Figure 1.7, Marshall *et al* 1998, J. Pootoolal unpublished). Given the observed homology and the

apparent requirement on the expression of these genes for the presence of glycopeptides (Marshall and Wright 1997), it seems reasonable that the regulation of resistance in *Streptomyces* may be similar to that of *Enterococcus*. However, the presence of two novel genes *gra*1 and *gra*2 (Fig. 1.7), which are not seen in *Enterococcus* but are present in the glycopeptide producer *S. toyocaensis* and the non-producing *S. coelicolor*, suggest that important regulatory differences may exist.

1.4. Glycopeptide Antibiotic Biosynthesis

1.4.1. Non-ribosomal Peptide Synthases

Filamentous bacteria such as *Streptomyces* produce many linear and cyclic peptide natural products. Although diverse in structure, non-ribosomally synthesized peptides have a common mode of biosynthesis. The peptides for glycopeptide biosynthesis are assembled on very large protein templates called peptide synthetases that exhibit a modular organization.

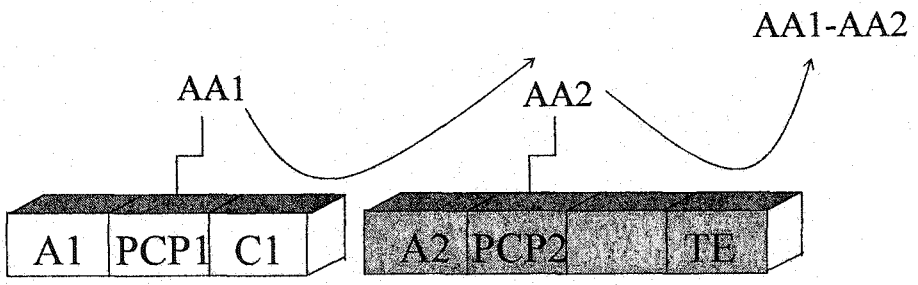


Figure 1.9: "Assembly line" of peptides in modules of a non-ribosomal peptide synthase. Adenlyate activation of the amino acid (AA1 and AA2) is carried out by the A-domain and the amino acid is transferred to the peptide carrier protein (PCP) by a thioester arm. Transpeptidation is carried out by the C domain and product is released by the actions of a thioesterase domain (TE).

These multifunctional "megasynthases" employ a thio-template mechanism similar to that utilized by polyketide synthases. (for reviews see Kleinkauf and Dohren 1990, Marahiel 1997, Cane and Walsh 1999). Each peptide synthase is composed of modules

dedicated to the processing of individual amino acids in an assembly line-like organization. Figure 1.9 outlines the functional domains of a typical peptide synthase.

Modules contain domains with sites for binding each amino acid, and enzyme activities for amino acid adenylate formation (A-domain), thioester formation of the adenylated amino acid to a phosphopentetheinyl arm of the peptide carrier protein (PCP-domain), transpeptidation between PCP-linked aminoacids (C domain) and a thioesterase domain for substrate release (TE domain). Other domains can also be present to perform functions such as epimerization and cyclization (Stachelhaus and Marahiel 1995, Stachelhaus *et al* 1996, Marahiel 1997). The linear sequence of DNA modules encoding the functional domains generally corresponds to the linear sequence of amino acids in the peptide generated.

1.4.2. Regulation of Glycopeptide Antibiotic Production

Secondary metabolites like the glycopeptides are produced during specific times in the life cycle and appear dependent on differentiation and cell density. Typically, the onset of production occurs late in the growth phase or early in the stationary phase. Streptomycetes are complex, filamentous bacteria that can exhibit differing morphology depending on the exact composition of the media for growth. It is not surprising therefore that secondary metabolism and drug production are highly dependent on the available nutrients. Specific nutrients such as phosphate, oxygen and calcium have all been shown to play an important role in vancomycin production (Mertz and Doolin 1973, Dunstan *et al* 2000). Furthermore, cell density has been liked to the onset of secondary metabolism in non-glycopeptide producers through a quorum sensing (cell density-dependent) process. In several *Streptomyces*, the signalling molecules in these systems are small γ-

buterolactones and are akin to hormones in eukaryotic systems (for reviews see Martin and Liras 1989, Horinouchi and Beppu 1992, Kleerebezem *et al* 1997). The first of these was isolated in 1967 and was termed A-factor. It has been shown to be essential for aerial mycelium formation and streptomycin production in *Streptomyces griseus* (Ueda *et* al 1996), and in regulating secondary metabolism and morphogenesis in *S. coelicolor* (Onaka *et al* 1998). A-factor is thought to interact with specific receptors, which are generally repressors of DNA transcription, by inhibiting their DNA binding and freeing the DNA target for transcription (Horinouchi and Beppu 1992).

In addition to A-factor quorum sensing, other signal transduction pathways have been implicated in differentiation and secondary metabolism in *Streptomycetes*. These include transcriptional activators and eukaryotic-type protein kinases (ePKs), the latter have only been recently identified in bacteria (reviewed in Kennelly and Potts 1996, Leonard *et al* 1998). Evidence for the involvement of ePKs in secondary metabolism has been for the most part indirect (Hong *et al* 1993), however, the ePK AfsK isolated from *S. coelicolor* A3(2) has been shown to regulate polyketide biosynthesis to some extent (Matsumoto *et al* 1994).

Although the study of small signalling molecules and ePKs has been limited to non-glycopeptide producing organisms, the temporal regulation and specific growth requirements of glycopeptide producing organisms such as the one used in our laboratory (*S. toyocaensis* NRRL 15009, Marshall and Wright 1996) suggests that the use of specific signal transduction pathways in this organism may be similar to other *Streptomyces*.

1.5. Project Goals and Strategies

In order to gain a better understanding of the regulation of growth, glycopeptide antibiotic production and resistance in glycopeptide producing and non-producing *Streptomyces*, a series of studies were carried out involving two well characterized Streptomycetes: the glycopeptide antibiotic producer *S. toyocaensis* NRRL15009 and the prototypical Streptomycete *S. coelicolor* A3(2). Both organisms have established transformation procedures, cloning vectors and characterized genomic integration sites, making them extremely attractive to study. In addition, access to *S. coelicolor* A3(2) DNA sequence is available on an ordered set of sequenced cosmids, and this genomic sequencing data is readily available through the Sanger Centre web site (http://www.sanger.ac.uk/Projects/S_coelicolor/). The entire project presented in this thesis can be neatly divided into five main goals as listed below:

1.5.1. Characterizaton of conditions for A47934 antibiotic production.

Although a *Streptomyces* antibiotic media (SAM) has been established for the production of the glycopeptide A47934 in *S. toyocaensis* NRRL 15009 (Marshall and Wright 1996), little is known about the effects of individual components. As outlined in Chapter 2, examination of individual media components, including the effects of various carbon and nitrogen sources in the presence of changing phosphate levels was carried out in an attempt to gain insights into the regulation of secondary metabolism in this organism.

1.5.2. Role of protein phosphorylation in A47934 production and resistance.

As mentioned above, several studies have implicated the role of kinases in the regulation of differentiation and secondary metabolism in various *Streptomyces* species

(Hong *et al* 1993, Matsumoto *et al* 1994). Therefore, examination of *S. toyocaensis* NRRL 15009 was carried out to establish the types and amounts of protein phosphorylation, and a role for protein phosphorylation in glycopeptide production. This was achieved through careful examination of phosphoproteins over the growth phase (producing and non-producing time points) and included phosphoprotein localization studies, and phosphoamino acid and Western blotting analysis. In addition, examination of differentiation and drug production was undertaken in the presence of several protein kinase inhibitors. These studies are summarized in Chapter 2.

1.5.3. Cloning and characterization of protein kinases in *S. toyocaensis*.

In order to identify tyrosine, serine and threonine protein kinases in *S. toyocaensis* NRRL 15009, a degenerate PCR strategy based on conserved sequences was utilized. This type of strategy has been employed successfully by others to clone novel kinase genes (Wilkie and Simon 1991, Zhang 1993, Urabe and Ogawara 1995). Initial estimates of the numbers of kinases present in *S. toyocaensis* were set at under 20 based on the sequenced genome of *Mycobacterium tuberculosis* (which has at least 11, Cole *et al* 1998) and on the phosphoprotein work undertaken in Chapter 2. The goal of this work was to clone and characterize any putative kinases and establish their functions with sight on their possible involvement in glycopeptide production or resistance. This involved the characterization of a single gene product StoPK-1 (*Streptomyces toyocaensis* protein kinase 1) through genetic analysis, protein expression studies, and genomic disruption and complementation. In addition, the search for an *afs*K orthologue in *S. toyocaensis* was carried out to establish its presence in this glycopeptide producer. The results of these studies are presented in Chapter 3 and Chapter 4.

1.5.4. Necessity of the *van*HAX cluster in *S. toyocaensis* NRRL 15009.

Although the organizational and predicted protein sequence homology between the *van*HAX genes in *Enterococcus* and those recently cloned from *Streptomyces* strongly suggest that these are bona fide resistance genes, this conclusion must be firmly established. This goal was investigated by insertional disruption of the genomic copy of the *van*A gene ($vanA_{ST}$) in *S. toyocaensis* and subsequent complementation. This work is presented in Chapter 5.

1.5.5. Role of the vanHAX-associated two component system in *S. coelicolor* A3(2).

The presence of a two component system just upstream of the *van*HAX cluster in *S. coelicolor* A3(2) and a highly homologous pair (~80% at the protein level) clustered with the glycopeptide biosynthesis genes in *S. toyocaensis* NRRL 15009 (Figure 1.7) suggests the possibility that these genes act in a similar fashion to the VanRS system in *Enterococcus*. Since VRE *van*S insertional and null mutants (but not *van*R mutants) retain vancomycin resistance *in vivo*, insertional inactivation and complementation of the response regulator in *S. coelicolor* A3(2) was carried out to establish the involvement of this system in resistance. These experiments are outlined in Chapter 5.

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Chapter 2.

Regulation of A47934 Glycopeptide Antibiotic Production and Resistance in *Streptomyces toyocaensis* NRRL 15009

2.1. Background

2.1.1. Secondary Metabolism in *Streptomyces toyocaensis:* Temporal and Nutrient requirements

Streptomycetes are Gram positive bacteria which show a complex life cycle of growth development through the formation of mycelia followed by sporulation, and are important producers of secondary metabolites, many of which find use as medicinal agents. Important secondary metabolites of medicinal value such as glycopeptide antibiotics are produced during specific times in the life cycle, typically late in the growth phase or early stationary phase (Mertz and Doolin 1973, Neu and Wright 2001). This is also the trend for other antibiotics such as actinorhodin and undecylprodigiosin production in *S. coelicolor* (Kang *et al* 1998), neomycin and tylosin production in *S. fradiae* (Majumdar and Majumdar 1967, Vu-Trong *et al* 1981) and streptomycin production in *S. griseus* (Horinouchi and Beppu 1993).

The temporal regulation of secondary metabolism in *Streptomyces* suggests the existence of controlling regulatory networks. Such systems respond to environmental and internal stimuli through signal transduction pathways. Environmental stimuli can include specific compounds such as phosphate, oxygen and calcium, nutrients which have been shown to play important roles in vancomycin production (Mertz and Doolin 1973, Dunstan *et al* 2000) and tylosin production (Vu-Trong 1981, Elizarov and Danilenko 2001). Many growth media that support antibiotic production, including the media developed previously in our laboratory for A47934 production contain complex

components (Marshall and Wright 1996), and this can make analysis of the effects of individual components on drug production difficult. Therefore, in order to better characterize A47934 production in *S. toyocaensis*, several defined media were examined for their ability to support antibiotic production. In addition, analysis of temporal and nutrient requirements were undertaken, including media component testing and carbon/nitrogen source utilization.

2.1.2. Antibiotic Drug Production and Phosphorylation

Cellular signal transduction is carried out by several mechanisms including the transfer of phosphate groups from ATP to various amino acid residues through the actions of protein kinases. Until relatively recently, most controlling regulatory networks in bacteria have been thought to be simple "two component" systems. In these systems, a protein kinase undergoes autocatalytic transfer of phosphate to a histidine and then delivers the phosphate to an aspartate residue of a partner response regulator protein (Parkinson and Kofoid 1992). Recently, however, evidence of other signal transduction pathways in prokaryotic cells has emerged. These include eukaryotic-like phosphorylation at serine and threonine (Munuz-Dorado *et al* 1991, Stowe *et al* 1989, Zhang *et al* 1993) and tyrosine residues (Dadssi and Cozzone 1990, Atkinson *et al* 1992, Frasch and Dwortin 1996, Smith *et al* 1997, McCartney *et al* 1997). In most of these cases, the organisms involved display complex life cycles such as fruiting body formation (Myxococci) or sporulation (Actinomycetes).

Phosphorylation at Ser/Thr and Tyr residues on various proteins has been demonstrated in several Streptomycetes (Stowe *et al* 1989, Waters *et al* 1994) and a

number of eukaryotic-like Ser/Thr kinases have been isolated and cloned (Matsumoto *et al* 1994, Urabe and Ogawara 1995). Studies conducted on one of these, AfsK, has lead to the conclusion that this protein is involved in the regulation of the biosynthesis of polyketide antibiotic actinorhodin in *Streptomyces coelicolor* (Matsumoto *et al* 1994, also see Chapter 3). Furthermore, studies on *S. griseus* growth in the presence of various compounds known to inhibit eukaryotic protein kinases, have revealed an inhibition of aerial mycelium formation and pigmentation, consistent with a role for protein kinases in secondary metabolite regulation (Hong *et al* 1993).

One pathway of regulation of streptomycin production in *S. griseus* has been shown to be controlled by a butanolide autoregulator termed A-factor (Horinouchi and Beppu 1992). A-factor was originally discovered in culture broth as an agent that could induce streptomycin production in a mutant strain of *S. griseus* (Horinouchi and Beppu 1992). Other A-factor-like regulatory factors have been isolated from *S. virginiae,* which control the production of virginiamycin (staphylomycin) (Yamada *et al* 1987). A-factor has also been found to be widely produced by Actinomycetes (Hara and Beppu 1982) and may be involved in regulating secondary metabolism in *S. coelicolor* (See Chapter 3).

None of the above studies examined the role of protein phosphorylation or autoregulators in glycopeptide producing *Streptomyces,* and it remains unclear what role these may play in glycopeptide production or resistance. Glycopeptide resistance has recently gained prominence with the emergence of vancomycin resistant strains of *Enterococcus* (VRE) and S*taphylococcus*, and recent work in our laboratory has shown that the genes conferring resistance in *S. toyocaensis* and *S. coelicolor* to glycopeptides

are very similar to those found in VRE (Marshall *et al* 1997, Chapter 5). This resistance system relies on switching cell wall biosynthesis from incorporation of D-alanine-D-alanine to D-alanine-D-lactate for crosslinking peptidoglycan and is regulated by a two component system (Wright *et al* 1993). In *S. toyocaensis*, the expression of these genes probably occurs after drug production commences (CG Marshall, unpublished observation). Similarly, these genes are expressed in VRE in the presence of glycopeptides (Arther *et al* 1997). In this chapter, the effects of various protein kinase inhibitors was examined on this inducible vancomycin resistance in *S. toyocaensis*. Comparisons with inhibitor effects on A47934 production were also carried out and the results suggest that different regulatory pathways are involved in glycopeptide drug production and resistance in this organism.

2.1.3. Antibiotic Drug Production and Gene Expression

Secondary metabolism and drug production require the controlled expression of different gene products, including various regulatory and biosynthesis proteins. The temporal regulation of these genes makes studying their differential expression an attractive strategy for the isolation of new biosynthesis-related genes. In eukaryotes various techniques have been employed to isolate differentially expressed genes including (1) subtractive hybridization strategies (Lee *et al* 1991, Utt *et al* 1995, Diatchenko *et al* 1996, Zeng *et al* 1994, Yang and Sytkowski 1996, Takahashi *et al* 1999), and (2) differential display PCR (DD-PCR) (Liang and Pardee 1992, Schroeder *et al* 1998, Chin *et al* 2000). The latter technique (DD-PCR) involves random PCR of two separate RNA populations and the cloning of differing amplification products (Fig.

2.1A). Subtractive hybridization techniques involve subtraction of expressed mRNAs common to two different samples leaving unique mRNAs which are cloned and screened (Fig 2.1B).

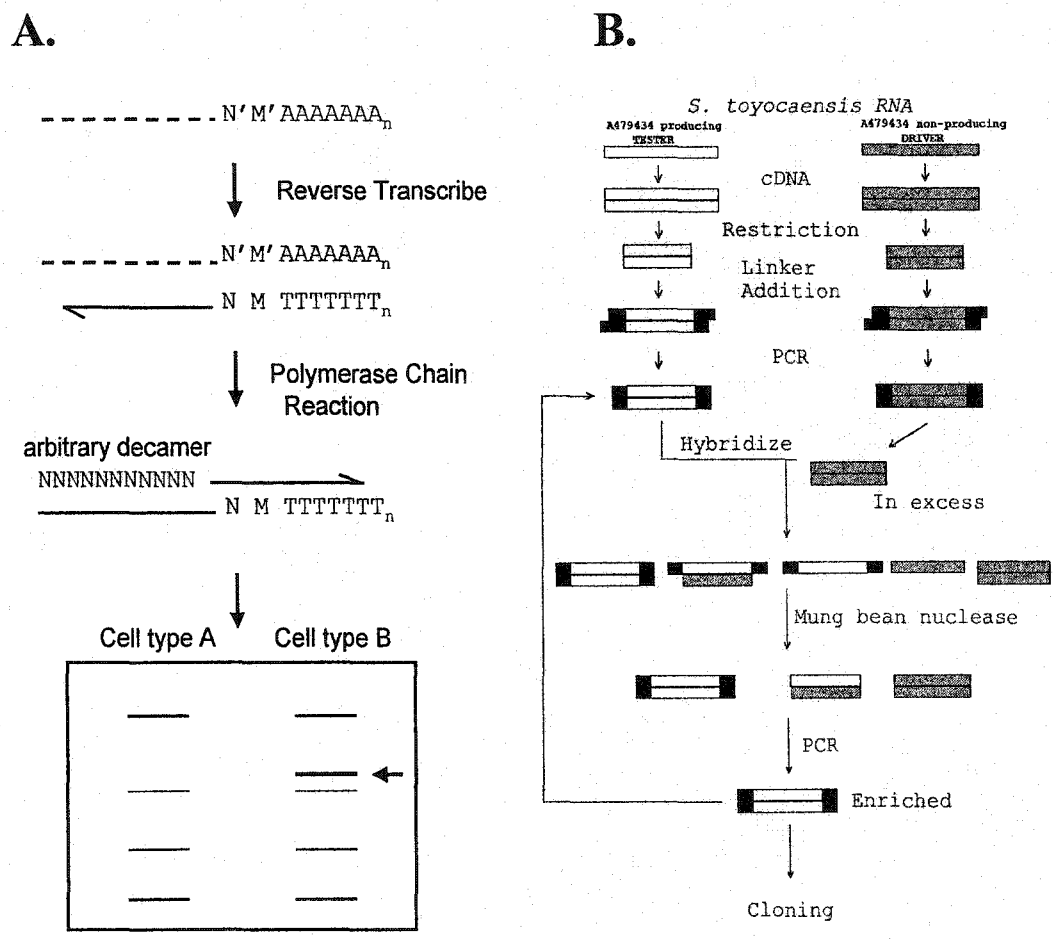

Figure 2.1: Strategies for isolating differentially expressed genes. **(A)** Differential display PCR uses 3' end fragments generated from reverse transcribed polyA mRNA from two separate samples. Reproducible differences in amplification products (arrow) are cloned and sequenced. **(B)** Subtractive hybridization. Schematic shows one possible strategy (Linker Capture) for isolating gene fragments expressed in small scale A47934-producing *S. toyocaensis* cultures. PCR with primers specific for linkers enriches differentially expressed Tester DNA after subtraction of Driver DNA and single stranded digestion with Mung bean nuclease. Cloning of the enriched fragments allows later hybridization screening of the library for specific gene products.

Both of these techniques contain elements that are attractive but also have potential pitfalls for application to the isolation of genes differentially expressed between *S. toyocaensis* A47934-producing and non-producing cultures. Although the simplicity and speed of DD-PCR is attractive, the success of this technique is highly dependent on the PCR step (Liang and Pardee 1992, Liang *et al* 1993). False positives can be a problem and differences between non-specific amplification and true expression levels must be differentiated by reproducing results a number of times (Liang *et al* 1993). Also, since prokaryotic cells contain no polyA messenger RNA (mRNA), the use of four sets of degenerate oligo(dT) primers successful for amplifying the majority of eukaryotic mRNAs could not be employed and major modifications and optimizations would probably be necessary .

On the other hand, subtractive hybridization can allow the cloning of the majority of differentially expressed mRNAs and is less affected by relative abundance (Lee *et al* 1991, Fig. 2.1B). Although this technique is more complex when compared to DD-PCR, it can also allow hybridization screening of the products for specific gene types (eg. Kinases) before sequencing would commence. In addition, Utt *et* al (1995) have successfully applied subtractive hybridization to characterizing expression differences between related bacterial strains. For these reasons, preliminary studies were carried out to assess the feasibility of constructing a subtractive library for analysis of expression differences between A47934-producing and non-producing *S. toyocaensis* cultures.

2.2. Materials and Methods

2.2.1. Organism and Culture Conditions.

The glycopeptide-producing organism *Streptomyces toyocaensis* NRRL 15009 was used throughout this study. This organism produces the teicoplanin-like antibiotic A47934 and cultures were maintained as spore suspensions in 20% glycerol at -70°C as described previously (Marshall and Wright 1996). In all studies, 200 μL of spores were first inoculated into a vegetative medium to produce a seed culture for subsequent studies. This vegetative medium consisted of 25 mL of 5% glucose, 2% potato starch, 1.5% soybean grits, 1% yeast extract, 0.2% corn steep liquor, pH 6.5 in a baffled 125 mL Erlenmeyer flask grown 48 hrs at 30°C and 225 rpm. Subsequent antibiotic producing cultures were grown in baffled 50 mL or 250 mL flasks in 15 or 50-75 mL respectively of *Streptomyces* antibiotic medium (SAM) consisting of 1.5% soytone, 1.5% glucose, 0.5% NaCl, 0.1% $CaC0_3$, 2.5% glycerol, pH 7.0. A tryptone containing media (TCM) with identical composition to SAM except for the replacement of soytone with an equal concentration of tryptone was also tested in some studies. Cells were grown for up to 120 hrs and harvested or 0.5-1.0 mL samples were taken at appropriate times.

Media components from SAM were analyzed for their contributions to A47934 production. For instance, glucose was replaced on solid media with various carbon sources such as galactose, lactose, sucrose, potato starch, mannitol, sorbitol, acetate and citrate. Plates were incubated at 30°C for 3-4 days and drug production and sporulation examined. In addition, the major nitrogen source from SAM (soytone) was replaced in 15

mL liquid cultures with different sources including tryptone, $NaNO_3$, and casaminoacids. Phosphate levels (supplied as 0.1 g/L K_2HPO_4) were also varied in the nitrogen replacement experiments.

Defined media tested included a modified liquid I-media (75 mL cultures) (Mertz and Doolin 1973) containing 0.75% (w/v) $NaNO_3$, 0.34% $(NH_4)SO_4$, 2.5 g/L KCl, 10 mg/L $MnCl_2$, 1.04 g/L $MgSO_4 \cdot 7H_2O$, 30 mg/L $Fe(NH_4)_2(SO_4)_2$, 30 mg/L $ZnCl_2$, 50 mg/L $CaCO_3$, 3 mg/L cupric acetate, 10 mg/L niacin, 66 mg/L L-citruline, 985 mg/L glutamic acid, 1% D-glucose and 2.5 mL/L glycerol (pH 6.8 with NaOH), and liquid Czapek Dox media (15 mL cultures) containing 3% D-glucose, 0.3% $NaNO_3$, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.5 g/L KCl and 10 mg/L $FeSO_4 \cdot 7H_2O$ (pH to 7.3 with HCl). To examine the effects of phophate both media were used with two different inorganic phosphate concentrations (from K_2HPO_4): a low level (0.1 g/L) and a high level (1 g/L).

2.2.2. A47934 and Other Antibiotic Bioassays.

A47934 adheres to *S. toyocaensis* NRRL 15009, therefore the antibiotic was extracted from the cell mass with 1 M 3-(cyclohexylamino)-1-propanesulfonic acid, pH 10 (50 µL/100 mg wet weight of cells) (Zmijewski Jr. *et al* 1987). Mycelia were then removed by centrifugation and the pH of the supernatant was neutralized with an equal volume of 1M KH_2PO_4 pH 6.0. A fraction of the extract (10-20 µL) was applied onto 7 mm sterilized filter disks and placed on a fresh lawn of 100 µL overnight *Bacillus subtilis* culture on Luria-Bertani (LB) agar. Plates were incubated overnight at 37 °C and zones of inhibition measured.

An agar plug method was used to test for production of A47934 on solid media. Agar plugs (~8 mm) from plates of interest were removed and transferred to fresh LB agar plates by fitting into an identical hole cut in these plates. Plates containing plugs were overlaid with a thin layer of LB agar and incubated overnight at room temperature after which a fresh lawn of 100 µL overnight *Bacillus subtilis* spread on top. Antibiotic production was considered positive if plugs produced zones of inhibition.

2.2.3. γ[^{32}P]-ATP Labeling of *S. toyocaensis* NRRL 15009 cell extracts.

S. toyocaensis NRRL 15009 cultures (50 mL) were harvested after various growth periods by low speed centrifugation, and washed 3 times in 20 mL of 50 mM Tris/HCl, 1 mM EDTA (pH 8.0). Washed cells were resuspended in 5 mL 2X kinase buffer consisting of 100 mM Tris pH 8.0, 100 mM NaCl, 2 mM EDTA, 2 mM dithiothreitol, 5% (v/v) glycerol and 1 mM freshly prepared phenylmethanesulfonyl fluoride, which was added immediately prior to lysis. Cells were lysed by three passages through a French Pressure cell at 20,000 psi and crude cell-free extract was collected by centrifugation (10,000 x g for 20 min). The supernatant was recovered and further fractionated into membrane (pellet) and cytoplasmic fractions by ultracentrifugation (1 hr at 100,000 x g). The membrane containing pellet was subsequently washed three times in 2X kinase buffer and suspended in the same buffer.

Protein concentrations were determined by the method of Bradford (Bradford 1976) and the fractions diluted with 2X kinase buffer to achieve a final protein concentration of 3-5 mg/mL. γ[^{32}P]-ATP labeling was carried out in the presence of 15-

40 μg protein with 15 Ci γ[^{32}P]-ATP, 10 mM $MgCl_2$, 2 mM $MnCl_2$, with and without 10 μM okadaic acid and 1 mM sodium orthovanadate in a total volume of 10-40 μL and incubated at room temperature for 10 min. The reaction was stopped using an equal volume of 2X SDS-PAGE loading buffer (4% (v/v) sodium dodecylsulphate (SDS), 2 mM EDTA, 500 mM dithiothreitol, 0.01% (w/v) bromophenol blue, 10% (v/v) glycerol, 100 mM Tris-HCl pH 8.8), and the samples subsequently separated on a 9-18% polyacrylamide gradient or 15% polyacrylamide gel containing 0.1 % SDS. Gels were either exposed directly to Kodak X-OMAT film for 2-3 hrs for the detection of phosphorylated proteins or electroblotted onto polyvinylidene difluoride (PVDF) membranes and then exposed to X-ray film. Transfer of proteins to PVDF membranes was accomplished at 70V for 50 min. in a transfer buffer consisting of 25 mM Tris, 15% methanol and 192 mM glycine. Parallel gels were soaked in 20%:10% acetic acid:ethanol to identify proteins phosphorylated on histidine, which undergo non-enzymatic dephosphorylation under these conditions.

2.2.4. $H_3{}^{32}PO_4$ Whole Cell Labeling.

S. toyocaensis NRRL 15009 cultures (15 mL) were grown for 24 and 48 hrs at which time 1 mL and 0.5 mL aliquots were removed for analysis of protein phosphorylation and antibiotic production respectively. Samples were prepared for protein phosphorylation studies by first collecting the cells from a 1 mL culture sample followed by resuspension in 1 mL Czapek Dox (lacking phosphate) medium (see section 2.2.1 for recipe). The cells were then collected by centrifugation, suspended in 0.2 mL

Czapek Dox (lacking phosphate) media and 25 μL (250 μCi, 300 Ci/mmol) of $H_3{}^{32}PO_4$ (NEN Laboratories), and incubated for 2 hrs at 30°C. Cells were then washed three times in 50 mM Tris-HCl pH 7.4, containing 1 mM β-mercaptoethanol, and suspended in 200 μL of a 10 mg/mL lysozyme solution containing 10 μM okadaic acid and 1 mM sodium orthovanadate, 1 mg/mL each of deoxyribonuclease I and ribonuclease A, and 5 mg of 0.5 mm diameter glass beads. The mixture was incubated 10-20 min at 30°C with vigorous shaking every 2 min. An equal amount of 2X SDS-PAGE loading buffer (section 2.2.3) was then added and the sample boiled for 5 min, centrifuged briefly to precipitate insoluble material, and 10-20 μL of the supernatant applied to a 9-18% polyacrylamide gradient or 15% polyacrylamide gel containing 0.1 % SDS. Gels were exposed to X-ray film directly or electroblotted onto PVDF membranes (section 2.2.3) and then exposed to X-ray film for 4-16 hrs.

2.2.5. Phosphoamino acid analysis.

2.2.5.1. PhosphoSer, phosphoThr and phosphoTyr.

[^{32}P]-Labeled proteins from PDVF blots from the $H_3{}^{32}PO_4$ and γ[^{32}P]-ATP labeling experiments were excised and placed in 500 μL 6M HCl, heated to 110°C for 60 min, dried under vacuum, and the residue resuspended in 10 μL distilled water. A 5 μL aliquot was spotted onto glass-backed cellulose thin layer plates along with 1 μL of 1 mM phosphoamino acid standards. Two-dimensional electrophoresis was then performed at a constant voltage of 1500 V for 20 min (first phase) in formic acid/glacial acetic acid pH =1.9 (2.5%:8% respectively) followed by 1500V for 20 min (second phase) in 5%

glacial acetic acid + 0.5% pyridine + 0.5 mM EDTA, pH = 3.5. Dried plates were then sprayed lightly with ninhidrin solution to visualize amino acid standards and then exposed to X-ray film overnight to visualize [^{32}P]-labelled amino acids (Ausubel *et al* 1994).

2.2.5.2. PhosphoHis.

Phosphoproteins that were sensitive to acid hydrolysis were further analyzed for phosphoHis incorporation. Radiolabeled proteins were excised from PVDF blots as described above and treated with 100 μL of 3M KOH at 60°C for 5 hrs. The solution was cooled and 10 μL $HClO_4$ added followed by brief centrifugation. A sample of the supernatant (10 μL) was spotted onto a silica gel TLC plate and the amino acids separated by two dimensional TLC as previously described (Smith *et al* 1978). An authentic sample of [^{32}P]-phosphoHis was prepared by [^{32}P]-ATP labeling purified MBP-VanS, the cytoplasmic His kinase domain of the two component sensor kinase VanS (Wright *et al* 1993).

2.2.6. Detection of Protein-Tyr Phosphorylation by Western Analysis.

Approximately 30 μg of total cell protein obtained by lysis of cells through a French pressure cell as described above, were separated on an SDS 10-15% polyacrylamide gel. Proteins were electroblotted at 9 V overnight onto nylon membranes and probed with antiphosphotyrosine monoclonal antibodies 4G10 (Upstate Biotechnology, Lake Placid, NY) or PY20 (Transduction Laboratories, Lexington, KY) as per manufacturer's recommendations. The blots were then treated with a goat anti-mouse IgG-HRP

conjugate for 20 min at room temperature and developed for visualization using chemiluminescence blotting substrate (Boehringer Manheim). Parallel control blots were also performed in the presence of 2 mM phosphoTyr, phosphoSer and phosphoThr (mixed with secondary antibody). Additionally, some blots were preincubated for 3 hr in water:acetic acid:ethanol (7:2:1) to remove histidine phosphates before incubation with antiphosphoTyr antibodies.

2.2.7. Subtraction Library Design and Construction

2.2.7.1. General Considerations

Today, many variations on the subtraction library strategy have been developed. These include enzymatic degrading subtraction (Zeng *et al* 1994), linker capture (Yang and Sytkowski 1996), suppression subtraction (Clontech kit), and biotinylated subtraction (Invitrogen kit). One of the simplest PCR based techniques which could be applied to small scale *S. toyocaensis* expression is the linker capture method (Fig. 2.1B). However, as with any of the above techniques, the lack of poly adenylated mRNA in bacterial cells including *Streptomyces* creates a dilemma because mRNA only makes up about 4% of the total cellular RNA (Brock *et al* 1984). Therefore, a subtraction strategy utilizing total RNA without any attempt remove the predominating ribosomal (rRNA) and transfer RNA (tRNA) species could preferentially isolate these in a differential screen unless their relative amounts are almost exactly the same.

Another potential pitfall in creating a differential library from *Streptomyces* is the relatively high GC content of the genomic DNA (typically about 70%). mRNA isolated

would therefore have secondary structure of increased stability due to the high GC content, which could create significant problems for reverse transcription reactions which are performed at relatively low temperatures (37-42°C). In order to circumvent this problem and address the purification of mRNA from *Streptomyces*, a scheme was devised to eliminate rRNA and tRNA as well as increase the likelihood of successful reverse transcription of the majority of mRNAs isolated. This scheme is outlined in Figure 2.2 below.

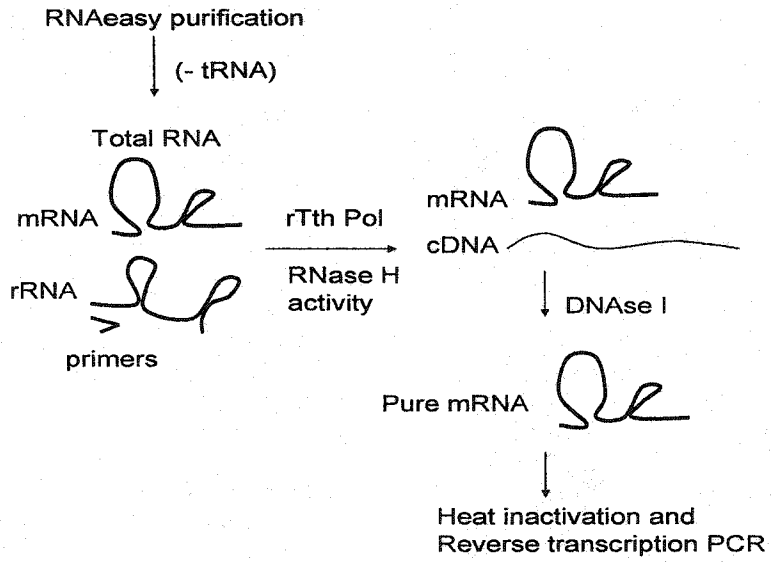

Figure 2.2: Proposed strategy for the isolation and reverse transcription of mRNA from *Streptomyces*. Removal of rRNA relies on the RNase H activity of rTth polymerase that destroys template RNA as reverse transcription goes forward.

This strategy is based on the reverse transcriptase and RNAse H activities of the rTth polymerase in the presence of manganese (Myers and Gelfand 1991, Auer *et al* 1996). Initially, primers designed to complement sections of the 16S and 23S rRNAs of *Streptomyces* are used to reverse transcribe these RNAs, and the copy DNA (cDNA)

generated is subsequently eliminated by DNAse I (Fig 2.2). In addition, small tRNAs are eliminated by the use of column purification (RNAeasy kit, Qiagen) which have size cutoffs of approximately 100-200 nucleotides.

2.2.7.2. Preliminary Studies

In order to test the validity of the proposed strategies for constructing *S. toyocaensis* differential libraries, several preliminary feasibility studies were performed. These studies specifically focused on the isolation of stable total RNA from 15 mL liquid SAM cultures from A47934-producing and non-producing time points. Harvested mycelia were washed twice in 0.4% DEPC treated distilled water. Different lysis techniques were employed including sonication (2 times for 30s at #35 setting, Fischer model 300), 1 mg/mL lysozyme treatment in phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 7H_2O$, and 1.4 mM KH_2PO_4, pH 7.3) with 10% sucrose followed by sonication as above, and French pressure cell lysis (3 passages at 20,000 psi). In all cases cell debris was removed by centrifugation (10,000 x g for 20 min). Total RNA was isolated from a sample of supernatant (50 or 100 μL) using RNAeasy purification kit (Qiagen) or by Trizol extract (Gibco BRL) as per manufacturers' instructions. Additional purification steps were also performed after each extraction including incubation for 1 hr. at 55 °C with 100 μg/mL proteinase K (Sigma) followed by up to 3 precipitations with $1/10^{th}$ volume 5 M sodium acetate (pH 5.0) and 4 volumes ice cold absolute ethanol.

Purified RNA was quantified by ultraviolet absorbance at 260 nm and checked for purity using A260/A280. RNA was visualized using 1% denaturing formaldehyde agarose gel electrophoresis (Ausubel *et al* 1994). Samples (11μL, 2-8 μg) were mixed with 39 μL RNA loading buffer containing 5 μL 10X MOPS running buffer (0.4 M MOPS pH 7.0, 0.1 M sodium acetate and 0.01 M EDTA, with 0.125% bromophenol blue), 9 μL 12.3 M formaldehyde, and 25 μL formamide and incubated at 55 °C for 15 min. prior to loading on 1 % agarose gels containing 2.2 M formaldehyde in 1X MOPS running buffer (40 mM MOPS pH 7.0, 10 mM sodium acetate and 1 mM EDTA). Gels were electrophoresed at 5V/cm for 3 - 5 hrs or until bromphenol blue dye front was approximately two-thirds the length of the gel. RNA integrity was visualized with ethidium bromide (0.5 μg/mL in 0.5 M ammonium acetate) after removal of formaldehyde by two 20 min. washes in excess 0.5 M ammonium acetate.

Some samples were treated with 10U of RNAse free DNAse I (after addition of 1 mM $MgCl_2$, Boehringer) or 5U of RNAse A (Sigma) and incubated at 37 °C for 10-60 min. In addition, total RNA was also isolated using Trizol after single passage through a French pressure cell (20,000 psi) from *E. coli* grown overnight in LB, and *Saccharomyces cerevisiae* grown overnight in YEME media containing 0.3% yeast extract, 0.5% peptone, 1% glucose, 3.4% sucrose, and 5 mM $MgCl_2$-$6H_2O$.

Preliminary reverse transcription (RT) experiments were carried out on *S. toyocaensis* total RNA with r*Tth* polymerase in 5 mM manganese acetate (GeneAmp EZ r*Tth* RNA PCR Kit, Perkin Elmer) to examine purified *S. toyocaensis* RNA stability. A 16S PCR cocktail was used and included 0.3-0.9 μg total RNA, 0.5 μM of a 16S primer

(AB9518, 5'-TGATCCAGCCGCACCTTCC) which was based on the *Amycalotopsis orientalis* 16S sequence (Genbank accession AJ400711) and on universally conserved prokaryotic rRNA positions (Hopfl *et al* 1989), in 50 μL total volume with 25 mM manganese acetate, 2U r*Tth* polymerase and 300 μM dNTPs in EZ buffer (Perkin Elmer).

RT-PCR of *S. toyocaensis* and *E. coli* total RNA were also carried out using the r*Tth* kit. Amplification of specific fragments (*ddl*M from *S. toyocaensis* and *ddl*B from *E. coli*) were achieved with the degenerate primers (0.5 μM each) AB7074 (5'GGIGAGGACGGI(T/A)(C/G)I(C/A)TICAGGG) and AB7075 (5'GTGAAICC(C/G) GGIA(T/G)IGTGTT) for *ddl*M (*van*A_{st}), and the specific primers (5'GAGATATACATATGAGTGATAAAATCGCGGT) and (5'TGACATAAGCTT AGTCCGCCAGTTCCAGAATT) for *ddl*B. All primers were synthesized at the central facility of the Institute for Molecular Biology and Biotechnology, McMaster University.

2.3 Results

2.3.1 Temporal and Nutrient A47934 Production Requirements

S. toyocaensis NRRL 15009 displays exponential growth in liquid SAM medium during the first 36-40 hrs, after which it enters stationary phase and begins antibiotic production (Figure 2.3). Culture pH gradually increases during growth in this medium. In other media tested, a drop in pH occurs in late exponential phase and no antibiotic is produced (see Table 2.2). It should be noted here that there is no evidence that *S. toyocaensis* NRRL 15009 produces any other antibiotics besides A47934, and in the following experiments where zones of *B. subtilis* inhibition are measured it is assumed that these measurements are directly representative of A47934 production.

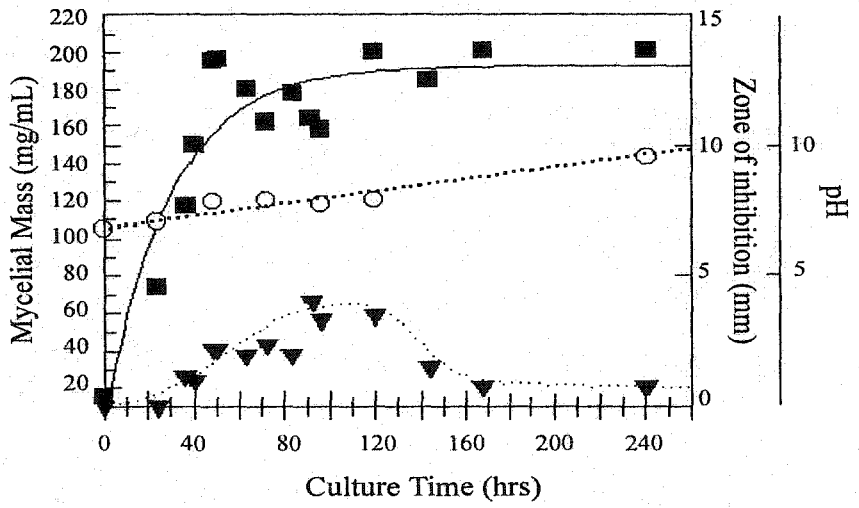

Figure 2.3: Growth of *S. toyocaensis* NRRL 15009 and production of A47934. The appearance of A47934 (▼) after ~36 hours occurs during late log phase of growth (■). In SAM growth coincides with a rise in culture pH (O).

Analysis of the ability of *S. toyocaensis* to utilize different carbon sources on SAM plates revealed that only potato starch could replace glucose for effective antibiotic production (Table 2.1). In contrast, several carbohydrates could stimulate sporulation, including galactose, lactose and mannitol, with potato starch being the most effective in this media.

Table 2.1: Effects on A47934 production and sporulation of replacing SAM carbon source (glucose) with various carbohydrates on solid SAM media.

Carbon Source (%)	Growth	Sporulation	A47934 Production
Glucose (1 %)	+	-/+	+
Galactose (1%)	+	+	-
Lactose (1 %)	+	+	-
Sucrose (2%)	+	-	-
Potato Starch (1%)	+	++	+
Mannitol (1.5%)	+	+	-
Sorbitol (1%)	+	-	-
Acetate (1%)	+	-	-
Honey (5%)	-	Not Applicable	Not Applicable
Agar Control	+	-	-

Initial studies on the effects of phosphate (supplied as K_2HPO_4) on A47934 production by *S. toyocaensis* in SAM, SAM without soytone (AM), SAM which had soytone replaced with tryptone (TCM) and a defined media (I-media) indicated that increasing phosphate could block antibiotic production, and the presence of soytone in SAM was not absolutely necessary for antibiotic production but was necessary for good growth (Table 2.2). However, when soytone was replaced with tryptone in the SAM, antibiotic production was not seen and culture pH tended to drop as with high phosphate media. The defined I-media was unable to sustain good growth or antibiotic production.

Table 2.2: **Effects of varying phosphate on A47934 drug production in *Streptomyces toyocaensis*. 50 mL cultures were grown in baffled 250 mL Erlenmeyer flasks at 30°C and 250 rpm for indicated times.**

Media	Weight (mg/mL) 96 Hrs	pH	A47934 Production[a]	Wet Cell Weight (mg/mL) 120 Hrs	pH	A47934 Production[a]
SAM	150	8.0	+++	145	7.8	+++
AM	30	6.3	++	45	6.5	++
I	11	6.2	-	12	6.2	-
TCM	180	4.2	-	ND	ND	ND
I+low Pi	11	4.5	-	-	-	-
SAM +high Pi	160	4.5	-	175	4.2	-
AM +low Pi	33	4.5	+/-	ND	ND	ND
AM +high Pi	6	4.6	-	ND	ND	ND
I+high Pi	8	4.9	-	ND	ND	ND

[a]- relative zones of clearance as judged by *B. subtilis* disk assay. ND – not determined.

Further analysis of phosphate effects utilizing other nitrogen sources including nitrate, casaminoacids and increasing yeast extract concentration revealed that higher levels of yeast extract could effectively replace soytone in SAM for relative A47934 production (AM + 5X yeast extract, Table 2.3). Growth in this media also appeared to be good although not as effective as SAM. In contrast, nitrate (supplied as $NaNO_3$) provided limited growth and drug production, and although casamino acids provided good growth with and without added phosphate, A47934 production was not seen (Table 2.3).

Overall, the best growth encountered occurred in SAM and TCM, but no drug production was observed in TCM (Table 2.2). Culture pH in this media dropped significantly and this drop could be mimicked by addition of high levels of phosphate to

SAM (Table 2.2). Low level phosphate addition did not appear to have this effect (Table 2.3), except in the case of AM (Table 2.2). However, increasing yeast extract concentrations appeared to reverse the trend (compare AM +low Pi, Table 2.2 with AM+ 5X yeast extract + phosphate, Table 2.3). Also evident is an apparent correlation between final culture pH and A47934 production with good production only evident in pH >7 culture conditions (Table 2.2 and 2.3), with only AM plus casaminoacids not following this trend (Table 2.3).

Table 2.3: Effects of nitrogen source and phosphate on A47934 production. 20 mL cultures were grown in baffled 50 mL Erlenmeyer flasks at 30°C and 250 rpm for 72 hrs. Additional nutrients as indicated included phosphate supplied as K_2HPO_4 (0.1 mg/mL), 5X yeast extract (5 g/L), casaminoacids (5 g/L) and nitrate as $NaNO_3$ (3 g/L).

Culture Media	Growth (mg/mL)	Zone of clearance in *B. subtilis* disk assay (net mm)	pH
SAM	180	4	8.0
AM + 5x yeast extract	128	4	7.8
AM + 5x yeast extract + phosphate	101	2	7.4
AM + casaminoacids	93	0	7.4
AM + casaminoacids + phosphate	105	0	7.6
AM + NO_3 + phosphate	49	1	8.0
Czapek Dox + phosphate	55	3	8.2

2.3.2 Protein phosphorylation in *S. toyocaensis* NRRL 15009.

Examination of $\gamma[^{32}P]$-ATP-dependent protein phosphorylation in whole cell extracts of cultures harvested both prior to (24 hr) and during (48 hr) A47934 production,

consistently revealed several discrete phosphoproteins (e.g. Fig. 2.4A, Table 2.4). The most abundant phosphoprotein was p36. (The abundance of p36 may not be evident in Fig. 2.6A, this is due to the separation of soluble and membrane associated components of p36). Several other phosphoproteins were identified, and most were Ser and/or Thr phosphorylated although one, p52, was Ser and Tyr phosphorylated (Table 2.4, Fig. 2.4B).

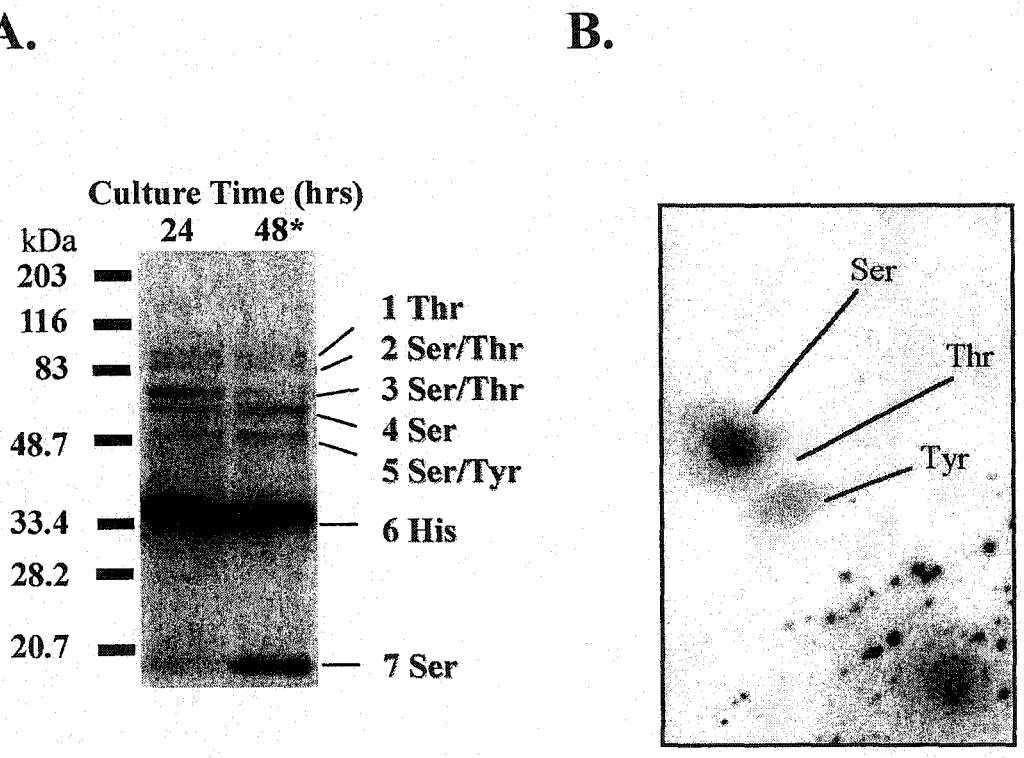

Figure 2.4: Protein phosphorylation in *Streptomyces toyocaensis*. (A) Phosphoproteins labeled from cell free extracts using $\gamma[^{32}P]$-ATP. (*) indicates A47934 producing culture. Phosphoaminoacid analysis revealed a variety of labeled amino acids (arrows). (B) phosphoaminoacid analysis of p52 showing phosphoserine and tyrosine.

Additional fractionation of the cell extract into membrane and soluble fractions revealed differential phosphorylation patterns (Fig. 2.6A). The abundant p36 band was found to be a mixture of a cytosolic protein, p36a, which was phosphorylated exclusively on His (Fig. 2.5), and a membrane associated protein, p36b, phosphorylated on Thr (Table 2.4). Three new phosphoproteins (p32, p34, and p40) were detected in membrane fractions of antibiotic producing cultures only, as revealed by $\gamma[^{32}P]$-ATP labeling (Fig. 2.6A). The p32 protein was Thr phosphorylated, while p34 and p40 were His phosphorylated (Table 2.4).

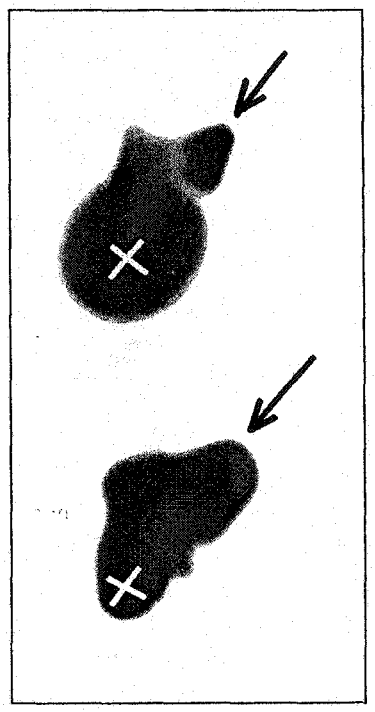

Figure 2.5: Two-dimensional TLC of p36 reveals phosphorylation on histidine residues. Basic phosphoamino acid analysis reveals labeling on p36 (bottom) similar to that of VanS (top). Arrows indicate phosphoHis residue and × marks origin.

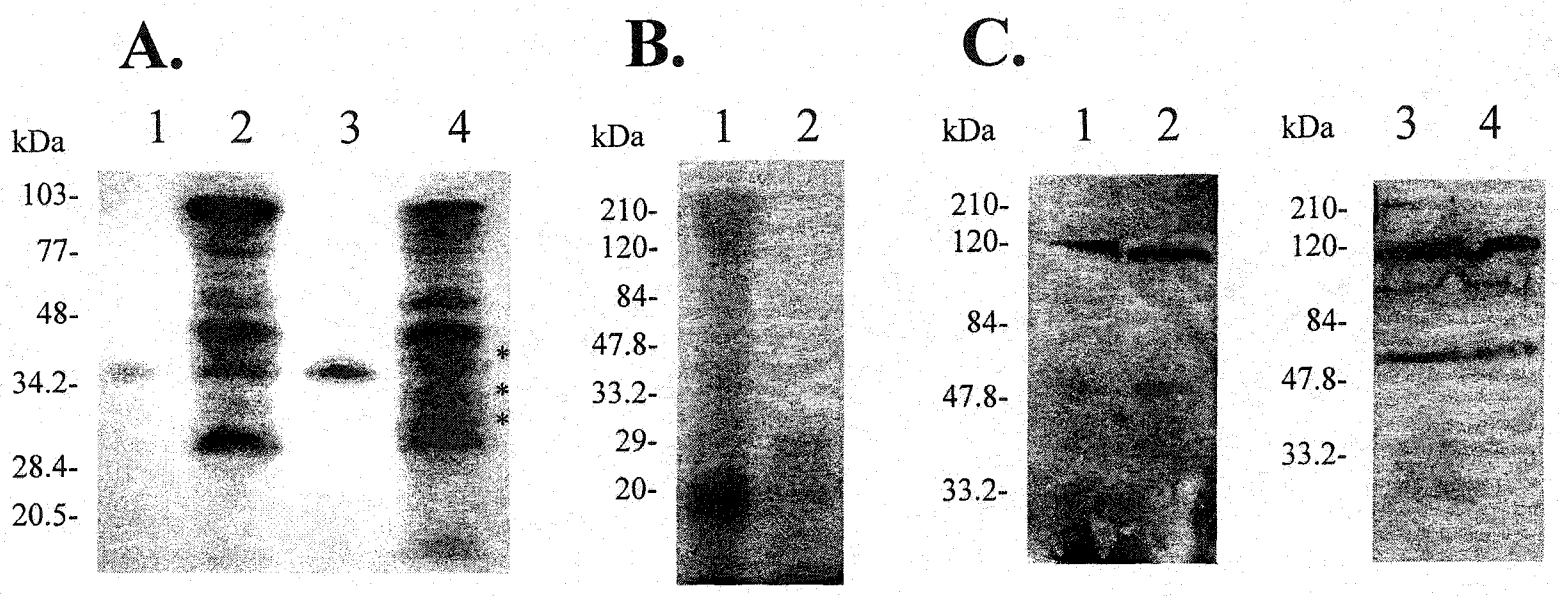

Figure 2.6: Protein phosphorylation in *S. toyocaensis* NRRL 15009. **(A)** Lanes 1 & 2: Soluble and membrane fractions from non-drug producing culture (24 hrs). Lanes 3 & 4: soluble and membrane fractions from drug-producing culture (48 hrs) showing three new phosphoproteins (*). **(B)** ^{32}Phosphate-labelled cells harvested from SAM at 24 (lane 1) and 48 hrs (lane 2) show differential phosphorylation with the appearance of a 28-29 kDa protein in A47934-producing cultures (lane 2). **(C)** Western blots of crude cell-free extracts from *S. toyocaensis* grown in liquid SAM for 24 (lanes 1 and 3) and 48 hrs (lanes 2 and 4). Two phosphoproteins are visible (lanes 1 and 2), and do not disappear in the presence of 2 mM phosphoserine and phosphothreonine (lanes 3 and 4).

Table 2.4: Analysis of phosphoproteins in *S. toyocaensis.*

Protein	Fraction	*in vitro* $\gamma[^{32}P]$-ATP or in vivo labeling: Phosphoamino acid	Anti-PhosphoTyr Ab
p8	memb	Ser	
p28*	nd	Ser/Thr	
p30	memb	His[a]	
p32*	memb	Thr	
p34*	memb	His[b]	
p36a	cyto	His[b]	
p36b	memb	Thr	
p40*	memb	His[a]	
p44	memb	Thr	
p50	memb	Thr	
p52	nd	Ser/Tyr	+
p65	memb	His[a]	
p70	nd	Ser/Thr	
p75	memb	His[a]	
p80	memb	Thr	
p83	nd	Ser/Thr	
p85	memb	Thr	
p89	nd	Thr	
p90	memb	Thr	
p120	nd	Tyr	+

nd - not determined. * - expressed only in A47934-producing cultures.
a – His phosphorylation based on acid sensitivity. b - His phosphorylation demonstrated by base hydrolysis followed by phosphoamino acid analysis.

Additionally, *in vivo* phosphoprotein labeling experiments using growth medium supplemented with $[^{32}P]$-phosphoric acid revealed both additional phosphoproteins and significant differences in proteins labeled after 24 and 48 hrs of growth (Fig. 2.6B). Since most of the radiolabel is localized in the nucleic acids in this kind of experiment, only a few phosphoproteins were detected compared to $\gamma[^{32}P]$-ATP labeling in cell free extracts and thus Figs. 2.6A and 2.6B are not directly comparable. The appearance of a new Ser/Thr phosphorylated 28 kDa protein occurred after 48 hrs, a time when antibiotic

production was underway. This protein was not detected in $\gamma[^{32}P]$-ATP labeling experiments, perhaps due to prior phosphate loading of this target or due to the actions of phosphatases.

Protein Tyr-phosphorylation was also assessed by Western analysis using two different anti-phosphoTyr monoclonal antibodies, PY20 and 4G10 (Fig. 2.6C). *S. toyocaensis* NRRL 15009 cultures grown to both 24 and 48 hr showed the presence of at least two distinct immuno-reactive proteins at 52 and 120 kDa. Addition of phosphoSer or phosphoThr (Fig. 2.6C), or preincubation of the blots in acidic solution to chemically eliminate phosphoHis, did not attenuate the signal, however, addition of phosphoTyr did, consistent with *bona fide* Tyr phosphorylation. The first band correlates with p52, which was identified in the $\gamma[^{32}P]$-ATP *in vitro* labeling experiments to be both Ser and Tyr phosphorylated (Fig. 2.4). However, p120 was not detected in these radiolabeling studies. Faint bands migrating with molecular masses in the vicinity of 120 kDa can be observed in some of these experiments, however, the very low abundance of these proteins precluded further characterization by phosphoamino acid analysis.

These experiments established not only that several proteins in the cell were His phosphorylated as expected in a prokaryotic organism, but also that many additional proteins were Ser, Thr and Tyr phosphorylated. Furthermore, the phosphoprotein pattern changed during liquid culture growth in a fashion parallel with A47934 production and growth phase.

2.3.3 Effect of protein kinase inhibitors on A47934 production and sporulation in *S. toyocaensis* NRRL 15009.

In order to establish whether protein phosphorylation plays a role in the production of A47934, several known inhibitors of eukaryotic Ser/Thr and Tyr protein kinases were added to growing liquid cultures of *S. toyocaensis* NRRL 15009. The compounds tested included the carbazole staurosporine, the flavonoids quercetin and genistein, and the isoquinoline sulfonamide H-9, the structures of which are shown in Figure 2.7. These compounds are all reversible inhibitors of protein kinases that show moderate discrimination between classes of enzymes; however, this is generally only in degree of inhibition. Thus staurosporine is an inhibitor of protein kinases A, C and G, as well as others, quercetin and genistein are know inhibitors of protein Tyr kinases (Akiyama *et al* 1987) and genistein has been shown to be a His-kinase inhibitor (Huang *et al* 1992), and H-9 is an inhibitor of protein kinases A, C and G (Hidaka and Koybashi 1992).

None of the compounds tested had any effect on the upward trend in culture pH, which is correlated with A47934 production. An apparent lag in growth was seen in early stages of culture growth when treated with H-9 or genistein, but did not effect overall growth (Fig. 2.8). However, H-9, genistein and quercetin had inhibitory effects on antibiotic production, although H-9 was only partially effective (Fig. 2.8A). Both quercetin (Fig. 2.8B) and genistein (Fig. 2.8C) completely blocked antibiotic production. Staurosporine (100 nM) had no effect on A47934 production (not shown).

Additional studies on solid media revealed that protein kinase inhibitors quercetin and genistein also blocked sporulation and formation of aerial mycelia (Fig. 2.9). In contrast, the isoquinoline sulfonamide H-9, showed no effect on sporulation (not shown).

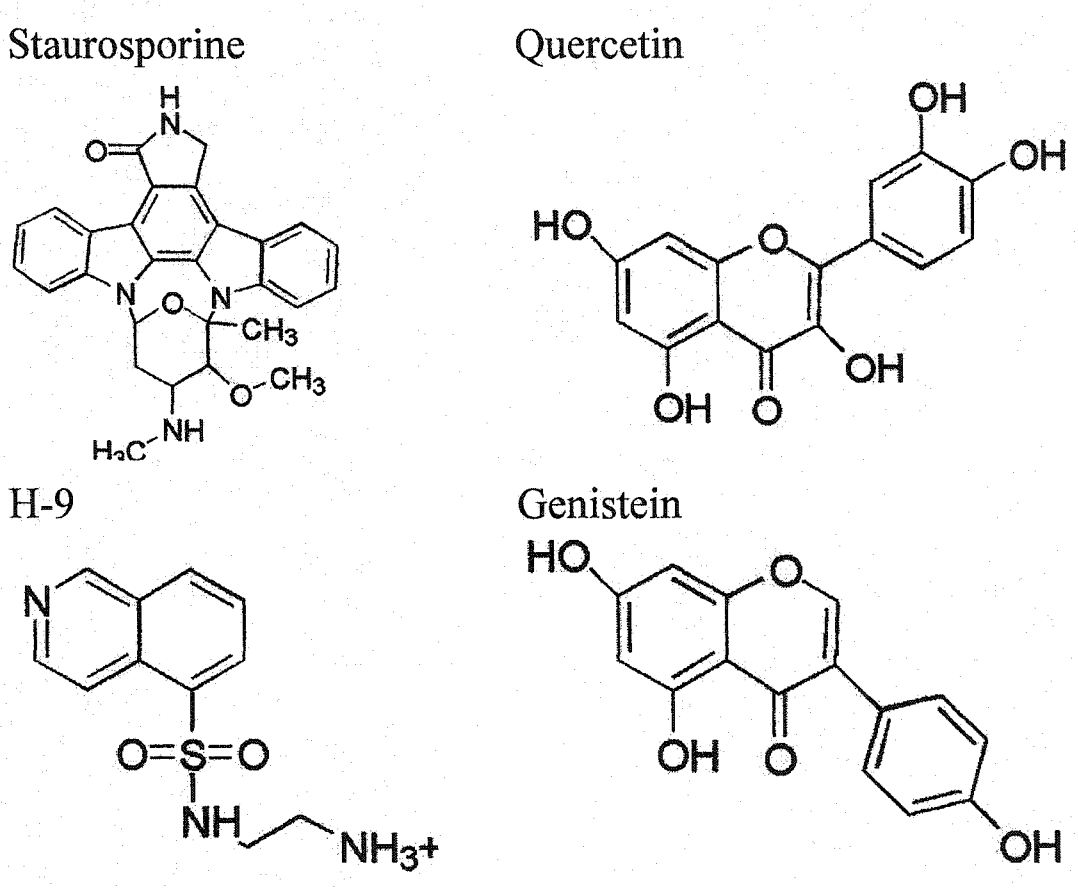

Figure 2.7: Structures of protein kinase inhibitors used in this study.

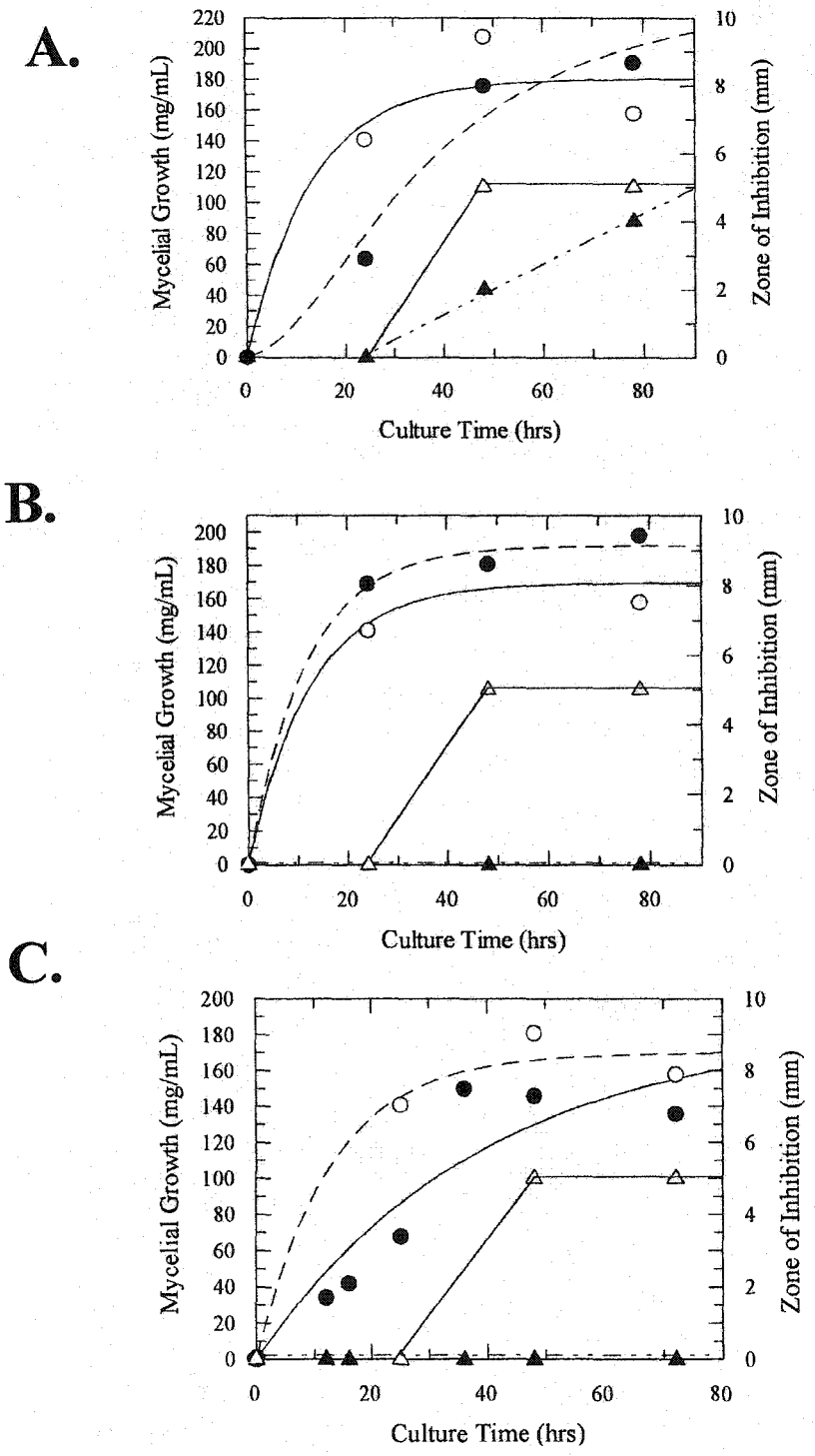

Figure 2.8: Effects of protein kinase inhibitors on the production of A47934 by *S. toyocaensis* NRRL 15009. Bacteria were grown in liquid SAM in the presence of 1 mM H-9 **(A)**, 1 mM quercetin **(B)**, or 1 mM genistein **(C)**. Key: (O) Control growth; (●) Growth in the presence of inhibitor; (Δ) *B. subtilis* disk assay of A47934 production from control culture. (▲) *B. subtilis* disk assay of A47934 production from *S. toyocaensis* culture in the presence of inhibitor.

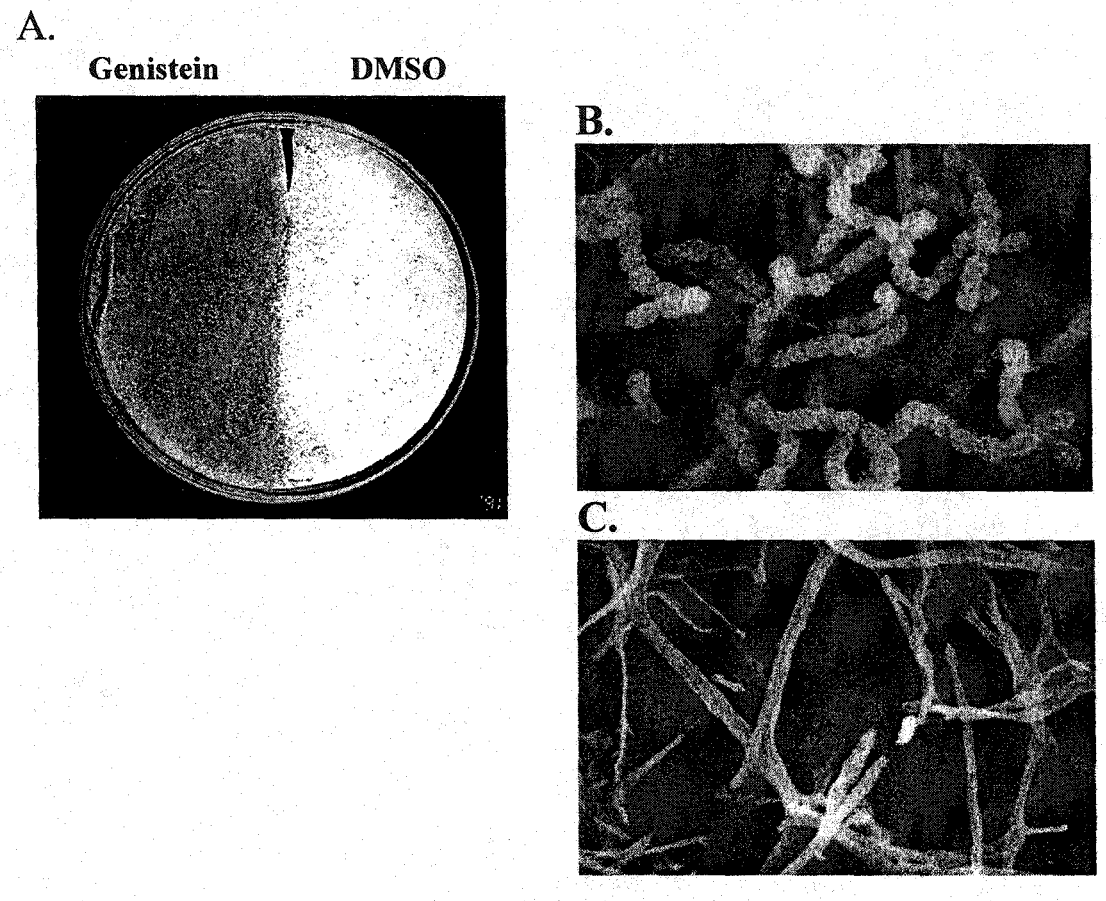

Figure 2.9: Effects of quercetin and genistein on sporulation in *S. toyocaensis* NRRL 15009. Scanning electron micrographs (5000x) of *S. toyocaensis* NRRL 15009 cultures grown 5 days on Bennett's agar. (A) Bennett's agar plate showing lack of sporulation (left side) in the presence of genistein (2 mM). (B) 1% DMSO control culture showing spore formation. (C) 2 mM quercetin showing lack of sporulation.

2.3.4. Protein Kinase Inhibitors and the Induction of Glycopeptide Resistance in *S. toyocaensis* NRRL 15009.

S. toyocaensis NRRL 15009 is resistant to A47934 as expected but unexpectedly is sensitive to the glycopeptide antibiotic vancomycin. Since the two N-terminal amino acids differ between A47934 and vancomycin, (3,5-dihydroxyphenylglycine, *O*-sulpho-4-hydroxyphenylglycine, and Asn, N-methyl-Leu respectively, see Fig. 1.3), resistance to the Group III glycopeptides ristocetin and teicoplanin (5 μg/mL), which share closer similarity with A47934, was investigated (Fig. 1.3). Results of these experiments revealed that *S. toyocaensis* NRRL 15009 was sensitive to these antibiotics as well (discussed in Chapter 5).

In an effort to determine whether the mechanism of resistance was specific to A47934 (eg. a modifying enzyme) or if A47934 was inducing a more general resistance mechanism (e.g. synthesis of D-alanine-D-lactate terminating peptidoglycan (Marshall *et al* 1998, Walsh et al 1996), several growth experiments were performed in liquid culture. Inoculation of cultures containing A47934 with cells that are not producing A47934 slightly delays the onset of growth and also induces resistance to vancomycin (Fig. 2.10A), an observation which is reminiscent of the induction of glycopeptide resistance in *Enterococus* (Nicas *et al* 1989). On the other hand, if A47934 was included in the seed culture at 5 μg/mL, the delay in growth was eliminated but resistance to vancomycin was not conferred (Fig. 2.10B). Vancomycin resistance could be induced by seeding cultures with A47934 producing cells grown in SAM medium (Fig. 2.10C). However, if the A47934 producing seed culture was first passaged for 24 hrs through TSB medium

(which does not support A47934 production), then vancomycin resistance was lost (not shown).

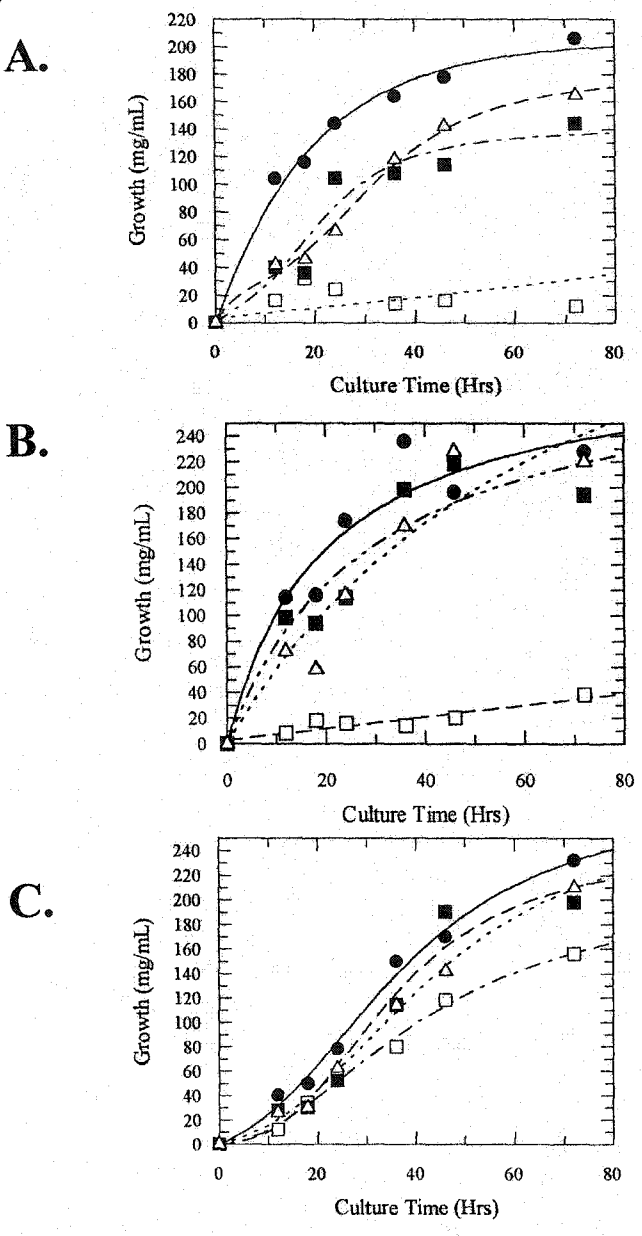

Figure 2.10: Induction of vancomycin resistance in *S. toyocaensis* NRRL 15009: **(A)** Control mycelial growth in liquid SAM seeded from a vegetative culture. **(B)** Mycelial growth in liquid SAM seeded from a vegetative culture grown in the presence of 5 μg/mL A47934. **(C)** Mycelial growth in SAM seeded from a 48 hr, A47934-producing SAM culture. **Key**: (●) control growth; (□) growth in the presence of 5 μg/mL vancomycin; (Δ) growth in the presence of 5 μg/mL A47934; (■) growth in the presence of 5 μg/mL A47934 and 5 μg/mL vancomycin.

Addition of the protein kinase inhibitor quercetin to cultures containing both A47934 and vancomycin had no effect on cell growth (Fig. 2.11A). On the other hand, the presence of the protein kinase inhibitors H-9 and genistein retarded growth in the presence of vancomycin (Fig. 2.11B and 2.11C), suggesting that inhibition of protein kinase(s) interfered with induction of vancomycin resistance.

We also assayed several other compounds including the protein phosphatase inhibitor sodium orthovanadate (1 mM), and bacterial cell wall and cell membrane-directed antibiotics D,L-cycloserine (20 μg/mL), penicillin G (50 μg/mL), bacitracin (50 μg/mL) and polymyxin B (5 μg/mL) for the ability to substitute for A47934 in the promotion of resistance to vancomycin. None of these compounds induced any resistance to vancomycin or adversely effected culture growth on their own (data not shown).

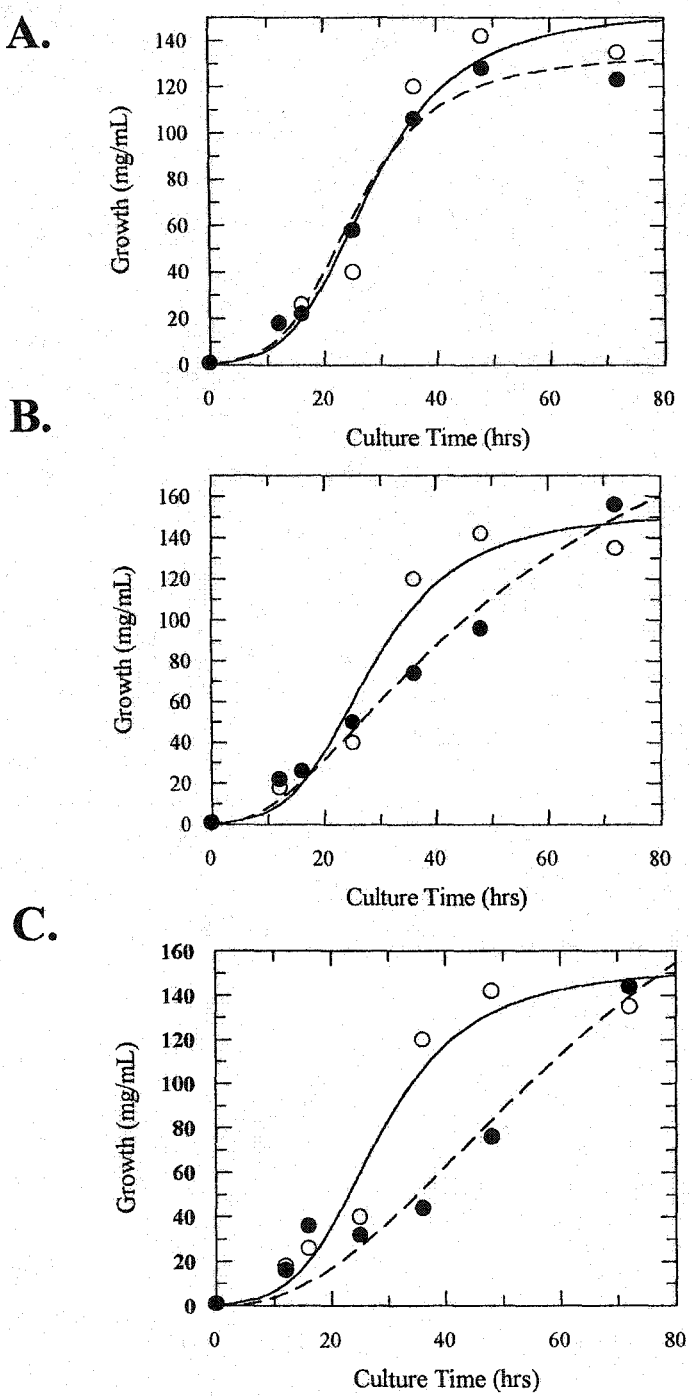

Figure 2.11: Inhibition of induced vancomycin resistance in *S. toyocaensis* NRRL 15009 by protein kinase inhibitors. Control curves (O) show growth in the presence of 5 μg/mL A47934 and 5 μg/mL vancomycin. The addition of various inhibitors reveals in some cases an inhibition of vancomycin resistance: **(A)** 1 mM quercetin (●). **(B)** 1 mM H-9 (●). **(C)** 1mM genistein (●). Note: for both H-9 and genistein a similar inhibition was also observed in the presence of 5 μg/mL A47934 only.

2.3.5 Subtraction Library

2.3.5.1. RNA Purification and Stability

Several different strategies were used in an effort to obtain pure full length total RNA from SAM cultured *S. toyocaensis*. Initial studies revealed that lysis through a French pressure cell gave the best and most consistent results as judged by the presence of 16S and 23S rRNA bands on denaturing gels (Fig. 2.12), therefore all subsequent RNA preparations were carried out using this method.

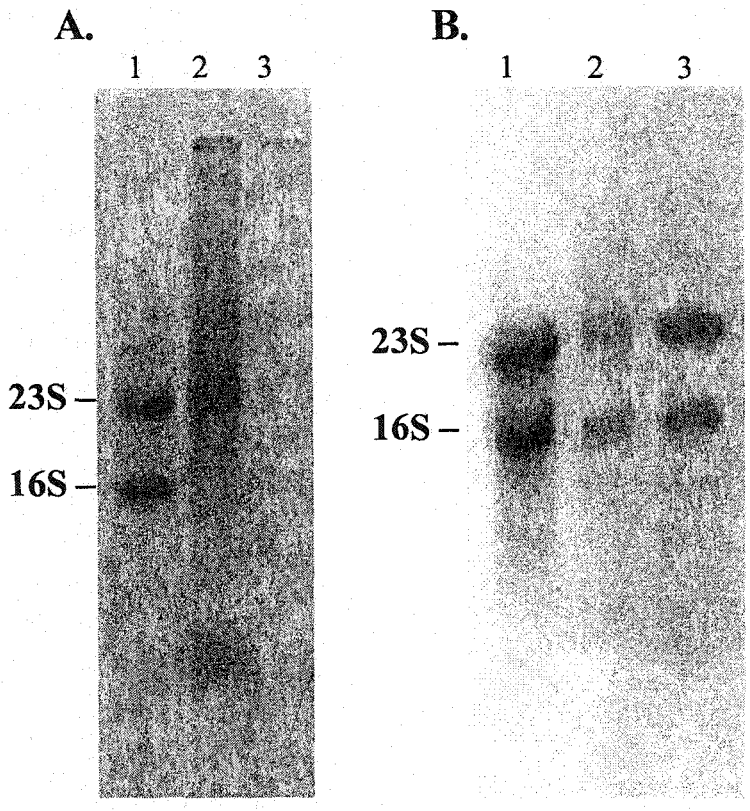

Figure 2.12: **(A)** Lysis method effects mRNA quality in 24 hrs SAM cultured *S. toyocaensis*. (1) French Pressure Cell. (2) Protoplast lysis (3) Sonication. (See Section 2.2.7.2 for detailed methods). **(B)** French pressure cell lysis of a 48 hr *S. toyocaensis* SAM culture (lane 3) and an overnight *E. coli* culture (lane 2). Lane 1: *E. coli* RNA standards.

Preliminary tests for total RNA stability after addition of 16S PCR cocktail revealed unstable RNA after French press (FP) lysis for both RNAeasy and Trizol extraction procedures (Fig. 2.13A). However, in a single experiment stable RNA was observed after additional proteinase K digestion and RNA precipitation (Fig. 2.13B) but this result could not be repeated (Fig. 2.13C).

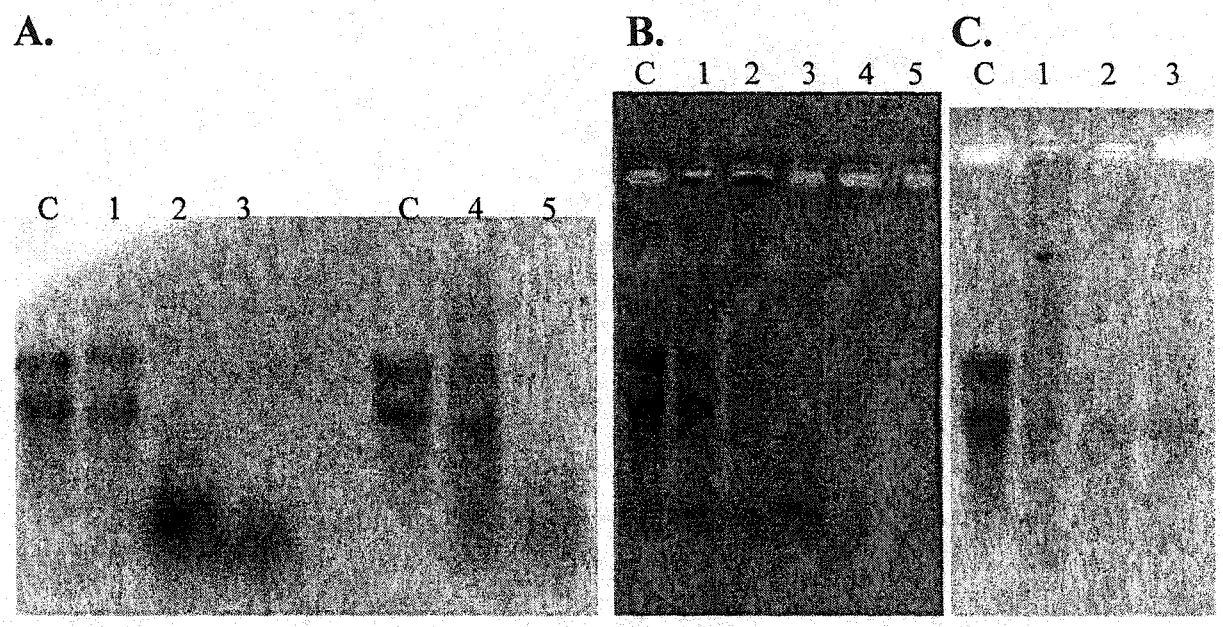

Figure 2.13: *S. toyocaensis* total RNA stability. **(A)** Comparison of RNAeasy and Trizol purification stability. Lane 1: RNAeasy extract with no incubation. Lane 2: RNAeasy extract + 16S PCR cocktail incubated 75°C for 5 min followed by 55 °C for 30 min, 58 °C for 30 min. and 60°C for 30 min. Lane 3: RNAeasy extract + 10U RNAse A incubated at room temperature for 5 min. Lane 4: Trizol extract with no incubation. Lane 5: Trizol extract + 16S PCR cocktail incubated 75°C for 5 min followed by 55 °C for 30 min, 58 °C for 30 min. and 60°C for 30 min. **(B)** Incubation of FP/Trizol extracted RNA with additional proteinase K digestion and effects of temperature cycling and PCR cocktail on RNA (4 μg) stability. Lane 1: no incubation. Lane 2: RNA only, incubated at 92 °C for 2 min., 55 °C for 30 min, 58 °C for 30 min. and 60 °C for 30 min. Lane 3: RNA + 16S PCR cocktail incubated as in lane 2. Lane 4: RNA + 16S PCR cocktail incubated as in lane 2 followed by 15 min incubation with 20U DNAse I at 37 °C. **(C)** Incubation of FP/Trizol extracted RNA from 39 hr. SAM culture (4 μg) at 55 °C for 60 min. Lane 1: no incubation, Lane 2: incubation at 55 °C for 60 min. Lane 3: incubation at 55 °C for 60 min with 16S PCR cocktail for reverse transcription (see Section 2.2.7.2). Lane C in all gels is *E. coli* control RNA.

2.3.5.2. RT-PCR Studies

Preliminary RT-PCR studies with total RNA from *S. toyocaensis* cultures failed to give any amplification products utilizing the degenerate *ddl*M primers (Figure 2.14A). Parallel control experiments using *E. coli* total RNA and primer specific for *ddl*B gave a amplification product of the correct size (Figure 2.14B lane 1 and 2). However the addition of an RNAse A incubation failed to completely abolish amplification of this product, suggesting the presence of contaminating genomic DNA even though DNase I treatment was performed.

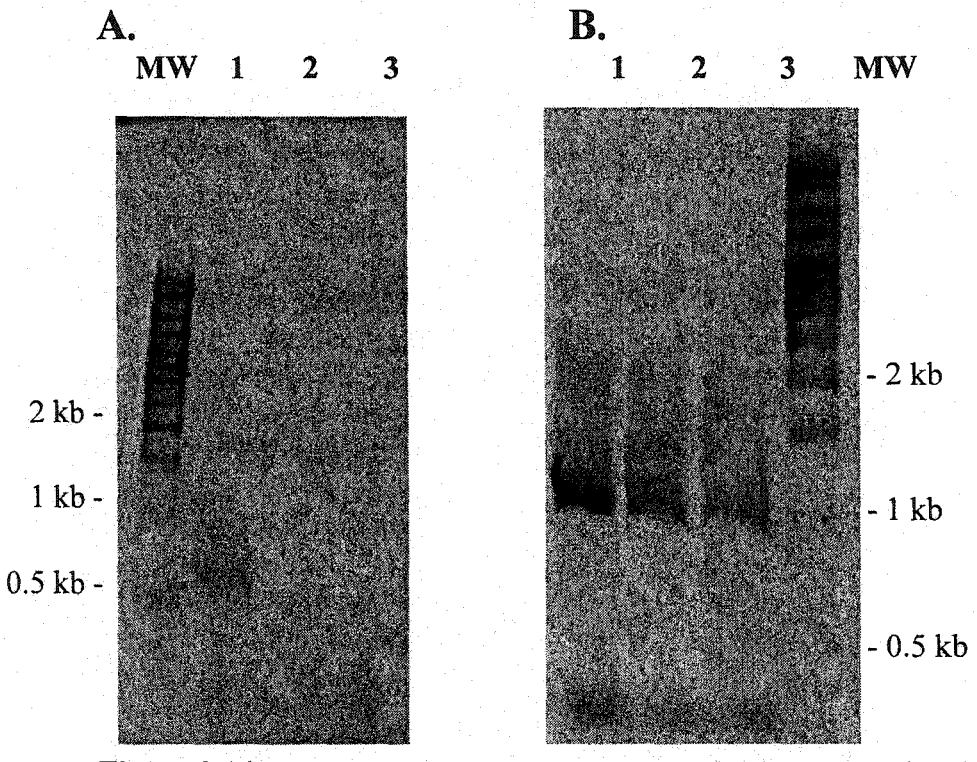

Figure 2.14: RT-PCR of *S. toyocaensis vanA*$_{ST}$ (*ddl*M) and *E. coli ddl*B. **(A)** RT-PCR of *ddl*M (prod. size = 0.6 kb) : Lane 1: PCR reaction with *S. toyocaensis* genomic DNA (0.3 μg) as template. Lane 2: *S. toyocaensis* total RNA (0.3 μg). Lane 3: *S. toyocaensis* total RNA (0.9 μg). **(B)** RT-PCR of *ddl*B (prod. size = 1 kb). Lane 1: RT-PCR reaction with *E. coli* total RNA (1.4 μg). Lane 2: RT-PCR reaction with *E. coli* total RNA (1.4 μg) + 5% DMSO. Lane 3: RT-PCR reaction with *E. coli* total RNA (1.4 μg) + 10 U RNAse A. All reactions were performed in 50 μL total volume with 25 mM manganese acetate, 2U rTth polymerase and 300 μM dNTPs with 0.5 μM of appropriate primers in EZ buffer. MW is molecular weight standards.

2.4 Discussion and Significance

2.4.1 Nutrient and Temporal Requirements

The cellular processes that control glycopeptide antibiotic production are largely unknown. Findings shown here, that A47934 production is linked temporally to late log and early stationary phase are consistent with precedent for the production of secondary metabolites in many other actinomycetes (Mertz and Doolin 1973, Kang *et al* 1998, Majumdar and Majumdar 1967, Vu-Trong *et al* 1981, Horinouchi and Beppu 1993). However, the fact that A47934 production is also highly dependent on the composition of the culture medium indicates that in addition to intracellular signals, external stimuli also likely play a role in regulating A47934 production. Thus the availability of certain nutrients such as inorganic phosphate, which play a role in secondary metabolism in other Streptomycetes (Mertz and Doolin 1973, Liras *et al* 1977) is also important in A47934 production as shown here (Table 2.2 and 2.3).

The findings presented here also suggest that A47934 production and metabolic processes that increase culture pH may be linked and may reflect a switch in cellular metabolism in the presence of limited phosphate. In this respect, phosphate may be "recruited" from external media stores through the activity of excreted phosphatases (*eg.* alkaline phosphatase) not unlike that seen in growth of the many other *Streptomyces* antibiotic producers such as *Amycalotopsis orientalis* and *Streptomyces griseus* under limiting phosphate (Mertz and Doolin 1973, Asturias *et al* 1990). The increase in pH may therefore be an adaptation for increasing alkaline phosphatase activity, a conclusion that

is supported by the measured pH optimal of 9.5 for the major phosphatase from *S. griseus* (*pho*A) (Asturias *et al* 1990).

2.4.2 Protein Phosporylation

These studies demonstrate that *S. toyocaensis* NRRL 15009 has proteins phosphorylated on His, Ser, Thr and Tyr, and the prediction that specific protein kinases are present within this organism is strongly suggested. The eukaryotic-like Ser, Thr and Tyr protein phosphorylation in *S. toyocaensis* NRRL 15009 are consistent with a growing body of evidence that demonstrates that non-His protein kinases are present in many bacteria. Recently available complete genome sequences from several bacteria demonstrate the existence of putative Ser/Thr kinases such as *Mycobacterium tuberculosis* (~11 protein kinase genes) (Cole *et al* 1998), and *Bacillus subtilis* (5 protein kinase genes) (Kunst *et al* 1997), and even *Mycoplasma genitallium* (Fraser *et al* 1995) and *Mycoplasma pneumoniae* (Himmelreich *et al* 1996), which have genomes of only 0.6 -0.8 Mb respectively, each have one predicted Ser/Thr protein kinase. Thus, Ser/Thr and even Tyr phosphorylation is not a eukaryotic-exclusive phenomenon, but occurs in many organisms.

2.4.3 Effects of Protein Kinase Inibitors

The observation that some Ser/Thr phosphorylated proteins were more prominent or exclusively found in A47934-producing culture extracts (e.g. p20, p32, and p36b), suggests that eukaryotic-like protein phosphorylation may be linked to antibiotic production. In order to determine if protein phosphorylation may play a role in the

production of A47934 in *S. toyocaensis,* several inhibitors were added to growing cultures. These included the protein kinase inhibitors staurosporine and H-9, the tyrosine kinase inhibitor genistein, and the ATPase/kinase inhibitor quercetin. None of the compounds tested appeared to have any significant effect on growth, although a small delay was observed in the presence of genistein and H-9, and all the compounds inhibited antibiotic production (Fig. 2.8). Staurosporine was not effective in inhibiting production. Genistein was also tested on agar plates (Bennett's agar) and also inhibited A47934 production (data not shown). Interestingly, in addition to blocking antibiotic production, both quercetin and genistein blocked sporulation of *S. toyocaensis*. (Fig 2.9).

A connection between sporulation and secondary antibiotic production has been observed in many other studies (Kudo *et al* 1995, Hong *et al* 1993, Hourinouchi and Beppu 1992). The observation that both antibiotic production and sporulation can be inhibited with the addition of protein kinase inhibitors without adversely affecting growth supports earlier work done in *Streptomyces griseus* (Hong *et al* 1993).

In *Streptomyces coelicolor* a global regulatory protein AfsR and its associated eukaryotic-like ser/thr kinase, AfsK control the production of the pigmented antibiotic actinorhodin (Matsumoto *et al* 1994). AfsR shows no sequence homology to any of the regulatory proteins of the classical two component regulatory system and thus the AfsK/AfsR system is thought to constitute a relay system quite different from typical two component bacterial phosphorylation systems (Matsumoto *et al* 1994).

More recent work has shown AfsK can rescue aerial mycelium-defective *S. griseus* mutants. These mutants are deficient in A-factor, a metabolite essential for the

formation of aerial mycelium, sporulation and streptomycin production (Ueda *et al* 1996). Interestingly, although AfsK can reverse aerial mycelium-defective *S. griseus* mutants, this effect is independent of secondary metabolic function (*i.*e. does not reverse loss of streptomycin production). This is in contrast to *S. coelicolor* where AfsK is only reported to be involved in secondary metabolism and not morphogenesis (Ueda *et al* 1996).

The work presented here suggests that a similar system in *S. toyocaensis* may be operating in the control of A47934 production. Protein kinase inhibitors used in the present study may be interfering with phosphorylation of a similar pathway. Of note is the observation that the isoquinoline Ser/Thr kinase inhibitor H-9 could inhibit A47934 production to a limited extent (Fig 2.7), but did not affect sporulation (data not shown). This observation parallels the division between secondary metabolism and sporulation seen with AfsK.

Genistein is a protein tyrosine kinase inhibitor with little or no activity against Ser/Thr kinases (Akiyama *et al* 1987), suggesting that transfer to tyrosine residue may be important in the A47934 pathway. In this respect, studies on AfsK from *S. coelicolor* have revealed an autophosphylation activity on tyrosine and serine residues, and a sensitivity to the kinase inhibitors staurosporine and K-252a (Matsumoto *et al* 1994). Subsequent work reported in this thesis revealed the presence of a AfsK homolog in *S. toyocaensis* of similar molecular size (~83 kDa, See Chapter 3), but only two tyrosine phosphorylated protein species (of approximately 52 kDa and a 120 kDa) appeared in any of the studies undertaken here. In addition, genistein has also been shown to have some

ability to inhibit histidine kinases (Huang *et al* 1992) and the involvement of a two component regulatory system can therefore not be ruled out. The involvement of a coupled Ser/Thr kinase and a classical two component system is also possible as recent studies in yeast have identified a two component system coupled to a MAP kinase cascade (Maeda *et al* 1994). In any event, the further study of AfsK in *S. toyocaensis* remains an attractive avenue of exploration.

2.4.4 Gene expression and subtraction library

Isolating and cloning of differentially expressed genes is very important for the study of many aspects of developmental biology. One of the most powerful techniques in isolating these differentially expressed genes is subtractive hybridization. Originally employing the use of hydroxyapatite for the separation of single and double stranded nucleic acids, subtractive hybridization has allowed the identification of several important proteins including the muscle specific transcription factor MyoD (Davis e*t al* 1987).

Subtraction hybridization requires the isolation of high quality mRNA from a driver cell population (eg. control conditions) and a tester cell population (eg. induced conditions). Driver mRNA is then subtracted from tester mRNA leaving only differentially expressed gene products behind. Three substantial problems which are faced when trying to apply such techniques to *Streptomycete* bacteria are the lack of selectable mRNA (lack of polyA tail), the high GC content (~75%) and the high prevalence of nucleases. Unfortunately, the purification techniques employed were unable to consistently overcome the instability of the isolated RNA. It remains a distinct

possibility that RNAse contamination will be a forgone conclusion with *S. toyocaensis*. Although all possible precautions were taken during the course of these experiments to ensure RNAse contamination was not externally introduced, the widespread use of RNAse in our laboratory remains an ongoing problem. However, it may be possible that changing culture conditions to prevent pigmented growth may be beneficial as there appeared to be some pigment carry over in both methods attempted. In this regard the defined media Czapek Dox shows white mycelial growth, and does induce antibiotic production.

Additional techniques that preferentially amplify RNA in the presence of genomic DNA may also be useful in the construction of *S. toyocaensis* subtractive libraries. The use of nucleotide analog such as hydroxymethyl dUTP or dITP in the amplification procedure can decrease strand separation temperatures (Levy and Teebor 1991, Karran and Lindahl 1980). Lowered strand separation temperatures has provided a method for preferentially amplifying cDNA generated from reverse transcription using r*Tth* polymerase by preventing amplification of genomic DNA through altered PCR cycling parameters (Auer *et al* 1996). This technique could be applied to differentially expressed protein kinases in *S toyocaensis* RNA with the use of degenerate PCR primers or alternatively, specific *S. coelicolor* primers, and may represent an alternate path of investigation.

2.4.5 Conclusions

Experiments shown here reveal that secondary metabolism as measured by A47934 production in *S. toyocaensis* is regulated by temporal and nutrient conditions. The minimal defined Czapek Dox media was found to support some growth as well as A47934 production and may represent a good starting media for further nutrient studies. In addition this defined media, which does not support pigment production, may also be useful in the development of techniques for isolating stable RNA in the process of constructing subtraction libraries for A47934 production.

The identification of numerous Ser, Thr and Tyr phosphorylated protein species, some of which are differentially expressed in the membrane fractions, strongly suggests the presence of eukaryotic- type protein kinases in this organism and indirectly supports a role for such proteins in regulating secondary metabolism. Furthermore, the inhibition of A47934 production and sporulation by several protein kinase inhibitors also lend credence to their regulatory role in these late growth stage events. As a result of these observations, a genetic search of the genome of *S. toyocaensis* for the kinases involved has lead to the cloning of several putative protein kinase fragments and an *afs*K homolog as outlined in the following chapters.

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Chapter 3.

Identification and Isolation of Ser/Thr Kinases in *Streptomyces toyocaensis* NRRL 15009

3.1 Background

3.1.1 Hanks' Protein Kinases

Serine, threonine and tyrosine protein kinases make up an extremely large superfamily of homologous proteins. Careful analysis has allowed the division of the protein kinase domain, which consists of 250 to 300 amino acids, into twelve conserved sequences (Hanks and Hunter 1995). This scheme was originally created by Steven Hanks and the conserved sequences are now called Hanks' domains or Hanks' consensus sequences (Hanks *et al* 1988). Figure 3.1 outlines the arrangement of these domains for Ser/Thr and Tyr kinases.

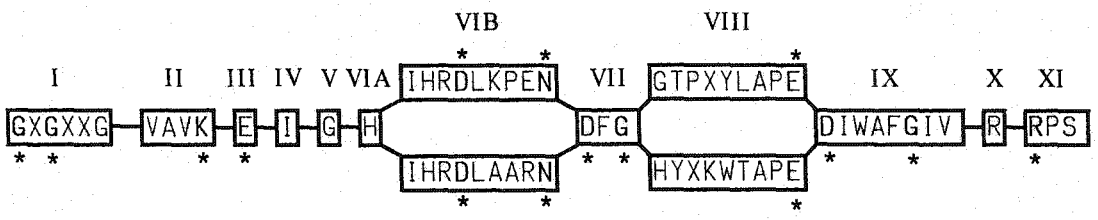

Figure 3.1: Conserved Hanks sequences in protein kinases showing the two main divisions in consensus VIB and VIII – Top: Ser/Thr kinases; Bottom: Tyr kinases. Invariant residues are marked with an asterisk.

The strict conservation of the Hanks' kinase consensus sequences in all protein kinases identified suggests their importance to the proper functioning of these enzymes. The function of each has been characterized for various protein kinases through affinity labeling (Zoller *et al* 1981, Kamps *et al* 1984), site directed mutagenesis (Wierenga and

Hol 1983, Jakobi and Traugh 1992, Yokoyama and Miller 1999), crystallography (Knighton *et al* 1991, DeBondt *et al* 1993, Zhang *et al* 1994, Carmel *et al* 1994), and peptide mapping studies (Smith and Sale 1989). A summary of the consensus sequence associated functions are given in Table 3.1.

Table 3.1: Functions of Hanks' protein kinase consensus sequences.

Subdomain	Function	Comments
I	glycine-rich loop, helps to anchor nucleotide (ATP)	consensus motif of Gly-X-Gly-X-X-Gly-X-Val
II	invariant Lys interacts with the alpha and beta phosphates of MgATP	invariant lysine important to the optimum activity of the enzyme
III	invariant Glu residue stabilize the domain II Lys	
IV	structural role	No invariant residues
V	anchors the MgATP by hydrogen bonding with the adenine or ribose rings	acts as a bridge between the large and small lobes
VIA	structural role	No invariant residues
VIB	Catalytic loop: The invariant Asp assists in phosphotransfer	substrate formation of an oxyanion, to which the γ phosphate from MgATP is transferred[1]
VII	Invariant Asp acts as a Mg^{2+} ligand	conserved triplet of Asp-Phe-Gly
VIII	recognition of the substrate, site of intermolecular autophosphorylation	conserved 'APE' motif
IX	invariant Asp acts to stabilize the catalytic loop (VIB)	
X	function is not known	No invariant residues
XI	function is not known	invariant Arg, His-X-aromatic-hydrophobic consensus 9-13 residues downstream usually defines C-terminal boundary of kinase

[1]- Recent work has disputed the actions of this conserved Asp as a general base (See Boer *et al* 2001).

As a direct consequence of the high conservation of the catalytic core, protein kinases all share the same basic 3-dimensional shape. The catalytic core folds into a two

domain structure: a smaller, NH_2-terminal lobe containing Hanks' sequences I-IV, and a larger COOH-terminal lobe which includes sequences VIA-XI (see Fig. 3.2). The basic function of each lobe is based principally on the Hanks' sequences contained within them (Hanks and Hunter 1995). The smaller domain is primarily involved in nucleotide binding and orientation (usually ATP), and is composed primarily of an antiparallel β-sheet structure. The larger lobe is mostly an α-helical structure involved in peptide substrate binding and phosphotransfer initiation (See Table 3.1). A deep cleft between the two lobes serves as the site of catalysis.

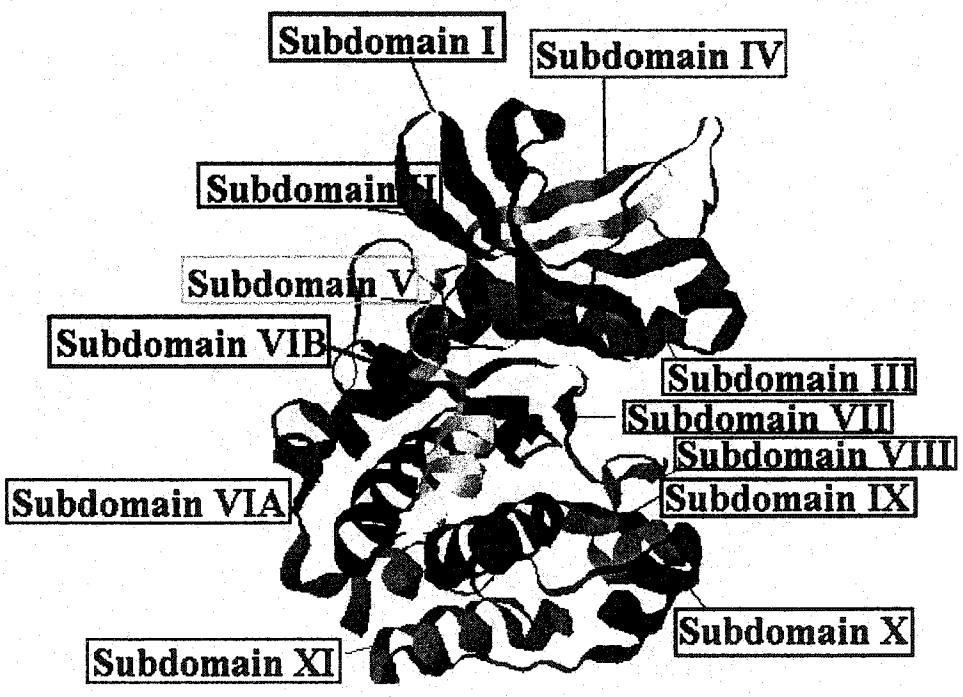

Figure 3.2: Three-dimensional structure of the Protein Kinase A Catalytic Subunit showing conserved subdomains I -XI. See text for details. Modified from "the protein kinase resource" http://pkr.sdsc.edu/html/structure.shtml#kinasestruct.

3.1.2 Hanks' Protein Kinase Calssification

Hanks and his colleagues went on to devise a classification scheme that subdivides the known members of the eukaryotic protein kinase (ePK) superfamily into distinct groups that share similar structural and functional properties. The sole consideration for the scheme was based on the amino acid sequence of the kinase domain. Groups with similar sequences also tend to have similar structural and functional properties. Thus, phylogenic trees were used as a guide for the groupings (Hanks *et al* 1991). Table 3.2 outlines Hanks' classification scheme for ePKs. Kinases are placed in four groups: 1) the AGC Group, which includes PK**A** and PK**G** (the cycle nucleotide dependent family), the PK**C** (protein kinase C) and the β-adrenergic receptor kinase families; 2) the CaMK group (calcium/calmodulin regulated); 3) the CGMC group, which includes the **C**yclin-dependent kinases, the **M**AP kinase family, the **G**SK3 (glycogen synthase 3) family, the **C**asein kinase II family and the Clk family of kinases.; 4) the conventional protein tyrosine kinases (PTK group).

Each one of these groups is further subdivided into more groups (Table 3.2). Furthermore, an OPK (Other Protein Kinases) group has been added for proteins not falling in the major groups. One of the OPK groups, group XIII, has been designated the PKN (or Pkn2) prokaryotic protein kinase group (Table 3.2). However, more recent analysis of protein kinases in bacteria and archaea suggest the presence of at least four more groups. These have been designated the ABC1, RIO1, piD261 and AQ578 families after their respective prototype members (Leonard *et al* 1998). This analysis allowed these authors to postulate the existence of an ancestral protein kinase prior to the

divergence of eukaryotes, bacteria and archaea as shown in Figure 3.3. An ancestral gene has also been alluded to by other studies (Ogawara *et al* 1999).

Table 3.2: Hanks' classification of protein kinases based on amino acid similarities.

	MAJOR GROUPS				
	ACG[1]	**CaMK[2]**	**CGMC[3]**	**PTK[4]**	**OPK[5]**
S U B D I V I S I O N	**I** Cyclic nucleotide regulated protein kinase (PKA & PKG) family **II** Diacylglycerol-activated/phospholipid-dependent protein kinase C (PKC) family **III** Related to PKA and PKC (RAC/Akt) protein kinase family **IV** Kinases that phoshorylate G protein-coupled receptors family **V** Budding yeast AGC-related protein kinase family **VI** Kinases that phosphorylate ribosomal protein S6 family **VII** Budding yeast DBF2/20 family **VIII** Flowering plant PVPK1 protein kinase homolog family **OTHER** Other AGC related kinase families	**I** Kinases regulated by Ca^{2+}/CaM and close relatives family **II** KIN1/SNF1/Nim1 family **OTHER** Other CaMK related kinase families	**I** Cyclin-dependent kinases (CDKs) and close relatives family **II** ERK (MAP) kinase family **III** Glycogen synthase kinase 3 (GSK3) family **IV** Casein kinase II family **V** Clk family **OTHER** Other CGMC related kinase families	**I** Src family **II** Tec/Atk family **III** Csk family **IV** Fes (Fps) family **V** Abl family **VI** Syk/ZAP70 family **VII** Tyk2/Jak1 family **VIII** Ack family **IX** Focal adhesion kinase (Fak) family **X** Epidermal growth factor receptor family **XI** Eph/Elk/Eck receptor family **XII** Axl family **XIII** Tie/Tek family **XIV** Platelet-derived growth factor receptor family **XV** Fibroblast growth factor receptor family **XVI** Insulin receptor family **XVII** LTK/ALK family **XVIII** Ros/Sevenless family **XIX** Trk/Ror family **XX** DDR/TKT family **XI** Hepatocyte growth factor receptor family **XII** Nematode Kin15/16 family **OTHER** Other membrane spanning kinases	**I** Polo family **II** MEK/STE7 family **III** PAK/STE20 family **IV** MEKK/STE11 family **V** NimA family **VI** wee1/mik1 family **VII** Kinases involved in transcriptional control family **VIII** Raf family **IX** Activin/TGFb receptor family **X** Flowering plant putative receptor kinases **XI** PSK/PTK "mixed lineage" leucine zipper domain family **XII** Casein kinase I family **XIII** PKN prokaryotic protein kinase family **OTHER** Other protein kinase families (each with no close relatives)

[1] PK**A**, PK**C** and PK**G** cylic nucleotide regulated kinases.
[2] calcium/calmodulin regulated (CaMK) kinase family
[3] **C**yclin-dependent, **M**AP, **G**SK3, and **C**asein Kinase II Families.
[4] **P**rotein **T**yrosine **K**inases.
[5] **O**ther **P**rotein **K**inases.

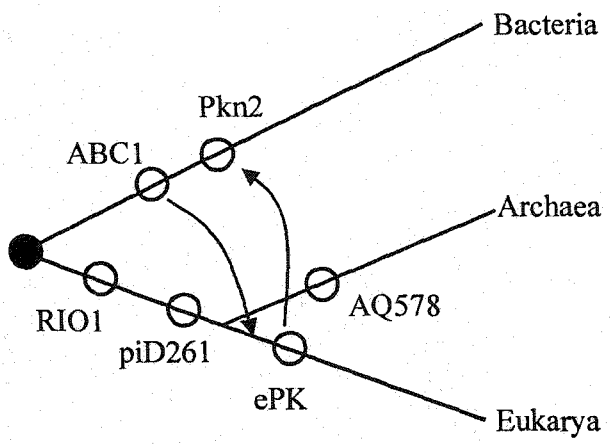

Figure 3.3: Tentative scheme for the evolution of protein kinases. Major branches of the rRNA-based phylogenic tree (heavy lines; Pace 1997) showing divergence of bacteria, archaea and eukarya. Points of emergence of kinase groups are shown as hollow circles and arrows indicate proposed horizontal transfer events. ePK – eukaryotic protein kinase. This model postulates a common ancestral gene (solid circle). Adapted from Leonard *et al* 1998.

3.1.3 Protein Kinase in Various Organisms

Unlike prokaryotes, which do not all contain Hanks' protein kinases, the genomes of all eukaryotic cell types encode Ser/Thr and Tyr kinases. For example, in the budding yeast *Saccharomyces cerevisiae*, at least 119 classical protein kinases have been identified (Hunter and Plowman 1997). These numbers increase for increasingly complex organisms such as *Caenorhabditis elegans* which has 514 (Plowman *et al* 1999). Table 3.2 outlines the total number of Serine, Threonine and Tyrosine protein kinases thought to be present in various organisms including several bacteria genera. The suggestion has been made that bacterial genera containing more than 3 protein kinases all exhibit various

morphological forms and/or have biochemical adaptations to changes in their environment and that kinases have been conserved in these species accordingly (Ogarwara *et al* 1999).

Table 3.3: Distribution of protein kinases in various prokaryotic and eukaryotic organisms.

ORGANISM (GENUS SP.)	PUTATIVE KINASES	TOTAL GENES	% OF GENES	GENOME SIZE (MB)	REFERENCES
Prokaryotic					
Bacillus subtilis	5	4,225	0.12	4.2	Kunst *et al* 1997, http://genolist.pasteur.fr/SubtiList
Mycobacterium tuberculosis	11	3924	0.28	4.4	Cole *et al.*, 1998
Streptomyces coelicolor	40	7846	0.5	8.7	This study, www.sanger.ac.uk/Projects/S_coelicolor/
Eukaryotic					
Saccharomyces cerevisiae	119	5885	2.02	12.1	Goffeau *et al* 1996, Hunter and Plowman 1997.
Neurospora crassa	70	~5000[1]	~1.5	43	www.genome.wi.mit.edu/annotation/fungi/neurospora/
Caenorhabditis elegans	514	19,099	2.7	97	Plowman *et al* 1999.
Drosophila melanogastor	251	13,601	1.85	120	Morrison *et al* 2000.
Arabidopsis thaliana	1122[2] (480)[3]	25,498	4.4 (1.9)[3]	125	Venter *et al* 2001, The Arabidopsis Initiative 2000.
Homo sapiens	501	26,588	1.88	300	Venter *et al* 2001.

[1]estimated for available raw sequence data. [2]contains duplicate genes. [3]estimated from Genbank.

3.1.4 AfsK Protein Kinase Involvement in Antibiotic Production

In *S. coelicolor* the global regulatory protein AfsR has been shown to be involved in regulation of secondary metabolism (Horinouchi *et al* 1990, Hong *et al* 1991). AfsR is thought to be activated by phosphorylation by the eukaryotic-type protein kinase AfsK of the Pkn2 type (Horinouchi and Beppu 1992). Activated AfsR is believed to increase transcription of the *act* and *red*D genes (Bibb 1996) and A-factor (2-isocapryloyl-3R-

hydroxymethyl-gamma-butyrolactone) biosynthesis (Horinouchi *et al* 1983). A-factor is a small butyrolactone signaling molecule essential for aerial mycelium formation and streptomycin production in *Streptomyces griseus* (Ueda *et al* 1996), and regulating secondary metabolism and morphogenesis in *S. coelicolor* (Onaka *et al* 1998). Other A-factor-like compounds have been shown to be important in secondary metabolism in various Actinomycetes including the regulation of rifamycin production in *Norcardia sp.* (Kawaguchi *et al* 1984), the regulation of anthracycline biosynthesis in *S. viridochromogenes* (Grafe *et al* 1982), and the regulation of virginiamycin production in *S. virginiae* (Yamada *et al* 1987).

A-factor is thought to act on its receptors, which are generally repressors of gene expression, by inhibiting their DNA binding, freeing the target genes' promoter for transcription. A proposed scheme showing AfsK and A-factor involvement in secondary metabolism (*S. coelicolor*) and aerial mycelial formation (*S. griseus*) is outlined in Figure 3.4 below. Apparently, the same gene products (AfsK and AfsR) are used in different ways between the two organisms.

In *S. coelicolor*, two secondary metabolites include actinorhodin (a blue pigment), the synthesis of which is controlled by the *act* gene, and undecylprodigiosin (a red pigment) which is controlled by the *red*D gene. Both of these genes are transcribed by the transcriptional activators actII-ORF4 and redD respectively, and AfsR may also play a role (Bibb 1996). The synthesis and buildup of A-factor is thought to trigger the release repression of A-factor dependent genes (through quorum sensing (cell density)) by decreasing A-factor receptor (CprB) DNA binding (See Figure 3.4A). Like *S. griseus*

(see below) the transcriptional activator AdpA or a related protein (*S. coelicolor* genome contains at least 8 AdpA-like proteins) may be the principle target and branching point between morphogenesis and secondary metabolism (Yamazaki *et al* 2000, Bibb *et al* 2000). A-factor responsive receptors such as AdpA may directly increase levels of actII-ORF4 and redD comparable to the *S. griseus* system where levels of the streptomycin biosynthesis cluster activator StrR are increased (See Figure 3.4) (Ohnishi *et al* 1999, Onaka *et al* 1998, Yamazaki *et al* 2000). Although the exact pathways are not clear at this time, A-factor also affects aerial mycelial formation through the *S. coelicolor* sigma factor σ^{AspA} homologue σ^{BldN} (Yamazaki *et al* 2000, Bibb *et al* 2000), but since aerial mycelial formation is not affected in Afsk/AfsR mutants, their role is unlikely in this pathway in *S. coelicolor* (Bibb 1995, Matsumoto *et al* 1994, Horinouchi *et al* 1990).

In *S. griseus*, streptomycin (Sm) and aerial mycelial formation both are under the control of the transcriptional activator AdpA, which is synthesized upon release of the A-factor receptor ArpA (CprB homolog) by increasing levels of A-factor (Figure 3.4B). Various studies have ruled out the AfsR/AfsK system in Sm biosynthesis (Ueda *et al* 1996, Umeyama *et al* 1999). However, AfsR and AfsK disruption mutants failed to form aerial mycelia on glucose containing media, implicating this kinase in regulating morphogenesis (Umeyama *et al* 1999).

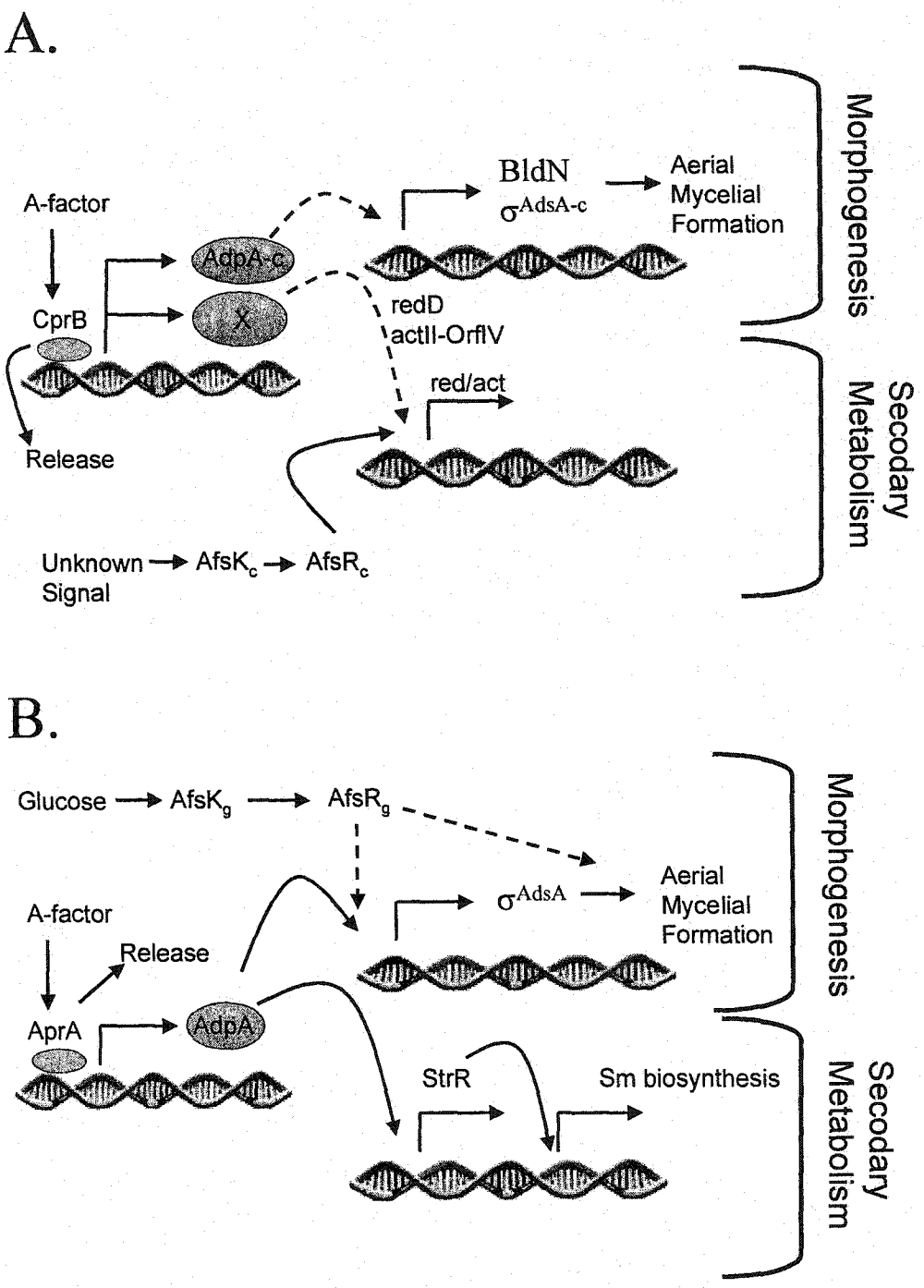

Figure 3.4: Proposed pathways involved in morphogenesis and secondary metabolism in (A) *S. coelicolor*. (B) *S. griseus*. For details see text. Solid arrows: experimentally established pathways. Broken arrows: hypothetical pathways. X – represents an unknown activator, possibly a AdpA-related protein (see text).

Like earlier studies with *Streptomyces* (Hong *et al* 1993), our work with *S. toyocaensis* had suggested that protein kinases may be involved in the regulation of differentiation, secondary metabolism, antibiotic production and resistance in *Streptomyces* (Neu and Wright 2001). We therefore attempted to detect the presence of a *S. toyocaensis* AfsK homologue, and to clone and sequence the genomic region for further study.

3.1.5 Project Strategy and Goals

At the time of our earlier work with protein kinase labeling in *S. toyocaensis* (Neu and Wright 2001), the *Streptomyces coelicolor* sequencing project had just been initiated. Our first estimates of the total number of kinase genes present in these organisms was therefore based on number present in other bacteria such as *Mycobacterium tuberculosis* and the earlier kinase labeling work (See Chapter 2). The estimates for *S. toyocaensis* were set at under 20, and our intent was to clone the majority of these and systematically disrupt each in order to study their functions. By adopting this strategy it was hoped that any kinases involved in antibiotic production or resistance could be discovered and at the very least, characterization of bacterial kinase genes would be instrumental in advancing our knowledge of this newly evolving field.

Investigations for the presence of any AfsK orthologs from *S. toyocaensis* were carried out by PCR based on the primary amino acid sequence homologies between the previously cloned *afs*K genes from *S. coelicolor* and *S. griseus* (Masumoto *et al* 1994, Ueda *et al* 1996). Using this strategy we successfully isolated and cloned a 4.2 kb fragment from *S. toyocaensis* containing the gene (designated *afs*K_t – subscript indicates

toyocaensis). In order to clone as many other protein kinases as possible, we also adapted a degenerate PCR approach utilized by others for cloning families of genes (Wilks 1989, Kamb *et al* 1989, Wilkie and Simon 1991). In addition, this strategy has been successfully employed in cloning protein kinases from various sources (Wilkie and Simon 1991, Zhang 1993, Urabe and Ogawara 1995). We designed primers based on the available gene sequences (see section 3.2.2.1) and the results generated a large amount of sequencing data from *S. toyocaensis* NRRL 15009 including forty non-kinase and four protein kinase gene fragments.

3.2 Materials and Methods

3.2.1 Genomic DNA isolation

Genomic DNA was isolated by modification of a procedure developed by Pospiech and Neumann, 1995. *S. toyocaensis* NRRL 15009 or *S. coelicolor* A3(2) were grown for 48 hrs in vegetative media and 200 μL of vegetative culture was inoculated into 15 mL of TSB + 0.5% glycine (for *S. toyocaensis*) or 15 mL of Streptomyces antibiotic medium (SAM) consisting of 1.5% soytone, 1.5% glucose, 0.5% NaCl, 0.1% $CaC0_3$, 2.5% glycerol, pH 7.0. Cultures were incubated at 30 °C and 225 rpm for 24-30 hrs. and mycelia were harvested and washed twice with 50 mM HEPES pH 7.4, 1 mM EDTA. Cells were resuspended in 5-10 mL of wash buffer and lysozyme was added to a final concentration of 2 mg/mL. The solution was incubated at 20 °C for 5-60 min until slightly viscous. Sodium dodecyl sulfate (SDS) was then added to 1.2% (w/v) followed by 600 μg/mL proteinase K and the mixture incubated at 55 °C for 2hrs, inverting occasionally. NaCl was then added to 1.25 M and the solution mixed thoroughly by

repeated gentle inversion and cooled to 37 °C. The preparation was then washed with an equal volume of chloroform with gentle inversion for 30 min followed by centrifugation at 4500 g for 16 min at 20 °C. The aqueous phase was then mixed with 0.6 volumes of isopropanol to precipitate the DNA, which was spooled, washed with 70% (v/v) ethanol and dissolved in distilled H_2O to 100-500 ng/mL.

3.2.2 Isolation of Protein Kinase Fragments

3.2.2.1 Degenerate PCR Primer Design

Initially, much focus was directed at designing two sets of degenerate PCR primers. These would be separate sets based on major substrate divisions: (1) serine/threonine and (2) tyrosine. Design of each set of primers (Table 3.4) was carried out using the available sequencing data from all major Hanks' groups. For primer set #1 (see Table 3.4), 104 Tyr kinases were examined at Hanks' Domains VI, VIII and IX, and a consensus sequence was generated (Table 3.5). Similarly, for primer set #2, 288 Ser/Thr kinase genes were examined and a primer consensus was generated for each of these domains (Table 3.6). In addition, a third primer set (set #3) based on prokaryotic homology was created using available sequences (Table 3.7). Emphasis in this case was placed on *Streptomyces* genes and included a putative *S. toyocaensis* kinase fragment (StoPK-1) isolated from a fragment isolated from a mixture of primers from set #1 and set #2 (Table 3.7).

In order to reduce primer complexity, deoxyinosine was substituted at highly degenerate nucleotides, as others have reported good results when amplifying sequences

containing amino acid residues with multiple codons (Knoth *et al* 1988, Rossolini *et al* 1994). In addition, deoxyinosine-substituted primers can reduce false positives, increase the amount of amplification products, and work over a broader range of reaction conditions than their degenerate counterparts (Rossolini *et al* 1994). Primers in Table 3.4 were designed to contain $\leq$ 20% deoxyinosine (dI), since primers proportioned higher than this are apparently not as effective (Rossolini *et al* 1994).

Table 3.4: Degenerate PCR primers used in this study designed around different Hanks' consensus sequences.

Set	Primer Designation	Subdomain	Primer Sequence	dI (%)
1	AB10093 (YF[3])	VIb	5'-CACCGIGACCT(C/G)GCI(G/A)CICGIAAC	16.7
	AB10094 (YR[4])	IX	5'-A(C/G)(C/G)A(T/C)ICCG(A/T)AIG(A/C)CCAIAC-GTC	12.5
2	AB10095 (SF[1])	VIb	5'-ACCGIGAC(G/C)TIAAGC(C/T)I(G/C)AIAAC	17.4
	AB10096 (SR[2])	VIII	5'-CTCIGGIGCI(C/A)(G/T)GTAI(T/C)(A/C)I(C/G)-GIGT	20.8
3	AB12387(SFn)	VIb	5'-CACCGIGACITIAAGCCI(G/T)(C/G)IAAC	20.8
	AB11891(SRn)	VIII	5- TC(G/C)GG(G/C)G(A/C)IAIGTAI(T/G)(A/C)I(A/G)-(C/G)(C/G)GTICC	19.2

[1] Serine Forward; [2] Serine Reverse; [3] Tyrosine Forward; [4] Tyrosine Reverse;

Table 3.5: Homology survey of Hanks' consensus sequences VIB through IX for Tyr protein kinases.

GROUP	# [2]	TYR KINASE FAMILY	DOMAIN VI	DOMAIN VIII	DOMAIN IX
PTK 1	16	Src family	H R D L R A A N I	F P I K W T A P E	D V W S F G $^{V}_{I}$ $^{I}L_{V}$
PTK II	5	Tec/Atk family	H R D L A A R N C	F P V K W $^{S}A_{C}$ $^{P}_{S}$ P E	D V W S $^{F}_{Y}$ G V L
PTK III	2	Csk family	H R D L A A R N $^{I}_{V}$	L P V K W T A P E	D V W S F G $^{V}_{I}$ L
PTK IV	3	Fes (Fps) family	H R D L A A R N C	$^{I}_{V}$ P $^{I}_{V}$ K W T A P E	D V W S F G I L
PTK V	4	Abl family	H R D L A A R N C	F P I K W T A P E	D V W A F G V L
PTK VI	3	Syk/ZAP70 family	H R D L A A R N $^{I}_{V}$	W P $^{L}_{V}$ K W Y A P E	D V W S $^{F}_{Y}$ G $^{V}_{I}$ $^{L}_{T}$
PTK VII	4	Tyk2/Jak1 family	H R D L A A R N $^{I}_{V}$	S P $^{I}_{V}$ F W Y A P E	D V W S F G V $^{T}_{V}$
PTK VIII	1	Ack family	H R D L A A R N L	V P F A W C A P E	D T W M F G V T
PTK IX	1	Food adhesion kinase	H R D I A A R N V	L P I K W M A P E	D V W M F G V C
PTK X	7	EGF receptor family	H R D L A A R N V	$^{V}_{M}$ P I K W $^{L}_{M}$ A $^{I}_{L}$ E	D V W $^{S}_{A}$ Y G V T
PTK XI	12	ph/Elk/Eck orphan receptor	H R D L A A R N I	I P I R W T A P E	D V W S $^{F}_{Y}$ G $^{I}_{V}$ V
PTK XII	4	Axl family	H R D L A A R N C	$^{M}_{L}$ P V K W I A $^{I}_{L}$ E	D V W $^{S}L_{A}$ F G V T
PTK XIII	2	Tie/Tck family	H R D L A A R N $^{I}_{V}$	L P V R W M A I E	D V W S F G V L
PTK XIV	8	PDGF receptor family	H R D L A A R N $^{I}_{V}$	L P V K W M A P E	D V W S $^{F}_{Y}$ G V L
PTK XV	5	FGF receptor family	H R D L A A R N V	L P V K W M A P E	D V W S F G V L
PTK XVI	4	Insulin receptor family	H R D L A A R N C	L P V R W M $^{A}S_{P}$ P E	D V W S F G V C
PTK XVII	2	LTK/ALK family	H R D I A A R N C	L P V K W M P P E	D $^{S}_{T}$ W S F G V L
PTK XVIII	2	Ros/Sevenless family	H R D L A $^{A}_{C}$ R N C	L P V R W M $^{A}_{S}$ P E	D V W S F G $^{V}_{I}$ $^{I}_{C}$
PTK XIX	7	Trk/Ror family	H R D L A T R N C	L P I R W M P P E	D $^{I}_{V}$ W S $^{F}_{I}$ G V $^{I}_{V}$
PTK XX	2	DDR/TKT family	H R D L A T R N C	L P I R W M $^{A}_{S}$ W E	D V W A F G V T
PTK XXI	3	Hepatocyte growth factor	H R D L A A R N C	L P V K W M A L E	D V W S F G V L
PTK XXII	2	Nematode Kin15/16	H R D L A L R N V	$^{M}_{V}$ P $^{A}_{V}$ R W M $^{A}_{S}$ P E	D V W S $^{F}_{Y}$ G $^{V}_{I}$ L
	5	Other	H R D L A A R N $^{I}C_{V}$	$^{I}R_{L}$ P $^{I}V_{L}$ $^{R}_{K}$ W $^{L}_{M}$ A P E	D V W $^{S}_{A}$ $^{F}_{Y}$ G V $^{L}_{V}$
Total:	104	Consensus:	**H R D L A** $^{A}_{T}$ **R N** $^{I}C_{V}$	$^{L}_{F}$ **P** $^{I}_{V}$ $^{R}_{K}$ **W X** $^{S}A_{P}$ **P E**	**D** $^{I}_{V}$ **W** $^{S}_{A}$ $^{Y}_{F}$ **G** $^{I}_{V}$ $^{L}_{V}$

[1] (-) 100%; superscripted and subscripted numbers represent % of clones with amino acid in consensus. Bold letters are invariant residues.

[2] Total sequences analyzed.

Table 3.6: Homology survey of Hanks' consensus sequences VIB through IX for Ser/Thr protein kinases.

G R O U P	# [2]	SER/THR KINASE FAMILY	DOMAIN VI	DOMAIN VIII	DOMAIN IX
Prokaryote	1	Streptomyces Clone	H R D V K P E N V	G T P D Y L A P E	D I Y A L A T V
Prokaryote	1	Cyanobacterium clone	H R D I K P D N I	G T P G Y M P S E	D L Y $^{S}_{A}$ L G L T
Prokaryote	1	Myxococcus clone	H R D L K P D N I	G T P E Y M A P E	D L Y A L G V I
AGC Group I	19	Cyclic nucleotide regulated	Y R D L K P E N L	G T P $^{E}_{D}$ Y $^{I}_{L_M}$ A P E	D $^{W}_{Y}$ W $^{A}_{S}$ $^{L}_{F}$ G $^{I}_{V}$ L
AGC Group II	21	Protein kinase C (PKC)	Y R D L K L D N $^{V}_{I}$	G T P $^{E}_{D}$ Y $^{F}_{M}$ A P E	D W W $^{A}_{S}$ $^{L}_{F_Y}$ G V L
AGC Group III	3	RAC(Akt) protein kinase	Y R D L K L E N L	G T P E Y L A P E	D W W G $^{L}_{T}$ V V L
AGC Group IV	8	G protein recept substrates	Y R D L K P E N $^{V}_{I}$	G T $^{V}P_H$ G Y M A P E	D $^{W}_{Y}$ $^{W}_{F}$ $^{A}_{S}$ $^{L}_{F}$ G C $^{L}_{M}$
AGC Group V	3	Budding yeast AGC-related	Y R D L K P E N I	G T $^{P}_{T}$ E Y L A P E	D $^{W}_{F}$ F $^{S}_{T}$ L G $^{I}_{V}$ L
AGC Group VI	3	Ribosomal protein S6	Y R D L K P E N I	G T $^{V}_{I}$ E Y M A P E	D W W S $^{Y}L_F$ G $^{A}_{V}$ L
AGC Group VII	2	Budding yeast DBF2/20	H R D L K P E N F	G S P D Y M A L E	D Y W S L G C M
AGC VIII	7	Flowering plant PVPK1	Y R D L K P E N V	G T H E Y L A P E	D W W T F G I F
AGC Other	6	----------	$^{H}_{Y}$ R D L K P E N $^{I}_{L}$	G T P $^{E}_{D}$ Y $^{I}_{L}$ A P E	D W W $^{A}_{S}$ $^{L}_{M}$ G $^{T}A_I$ $^{I}_{L}$
CaMK Group I	26	Ca2+/CaM	H R D L K P E N $^{I}_{L}$	$^{GT}_{Y}$ $^{TS}_{A}$ $^{F}_{A}$ $^{G}_{E}$ $^{Y}_{F}$ $^{L}_{I}$ $^{A}_{S}$ P E	D $^{I}M_L$ W $^{A}_{S}$ $^{C}_{I}$ G V $^{I}_{L}$
CaMK Group II	12	KIN1/SNF1/Nim1 family	H R D L K $^{P}_{I}$ E N $^{I}_{L}$	G S $^{P}_{L}$ $^{Y}N_H$ $^{Y}_{F}$ A A P E	D V W S $^{C}_{F}$ G $^{I}_{V}$ $^{I}_{V}$
CaMK Other	3	----------	H R D $^{L}I_V$ K $^{P}_{M}$ E N $^{I}_{L}$	$^{G}_{Y}$ $^{T}_{S}$ $^{P}_{A}$ $^{Y}_{G}$ Y $^{V}_{I}$ A P E	D $^{I}M_V$ $^{V}W_I$ $^{S}_{V}$ $^{C}_{L}$ G V I
CMGC Group I	25	Cyclin-dependent kinases	H R D $^{L}_{M}$ K P $^{Q}_{N}$ N L	V T $^{L}_{R}$ W Y R A P E	D $^{I}M_V$ W $^{S}_{G}$ $^{I}V_L$ G C I
CMGC Group II	20	ERK (MAP) kinase	H R D $^{L}_{I}$ K P $^{S}G_L$ N L	$^{A}_{V}$ T R W Y R A P E	D $^{I}M_L$ W S V G C I
CMGC Group III	7	Glycogen synthase 3	H R D I K P Q N L	C S R Y Y R A P E	D $^{V}_{I}$ W S A G C V
CMGC Group IV	10	Casein kinase II family	H R D V K P H N V	A S R Y $^{Y}_{F}$ R A P E	D $^{I}_{M}$ W S $^{L}_{V}$ G C M
CMGC Group V	8	Clk family	H $^{T}_{C}$ D L K P E N I	$^{S}_{Q}$ $^{T}_{S}$ R $^{H}_{F}$ Y R A P E	D $^{V}_{M}$ W S $^{L}_{I}$ $^{G}_{A}$ C $^{I}_{V}$
CMGC Other	10	----------	H R D $^{L}_{I}$ K $^{F}_{T}$ $^{E}_{S}$ N $^{I}_{L}$	$^{I}V_S$ T R W Y R $^{S}A_P$ P E	D $^{N}_{V}$ W $^{S}G_A$ $^{C}V_F$ G C $^{I}V_L$
Other PKs:					
OPK I	4	Polo family	H R D L K L G N F	P $^{V}_{I}$ $^{R}_{K}$ W $^{M}_{,}$ A $^{P}_{L}$ E	D I W S $^{I}_{L}$ G C $^{V}I_M$
OPK II	9	MEK/STE7 family	H R D V K P $^{S}T_Q$ N $^{I}_{V}$	G $^{T}_{C}$ $^{R}_{S}$ Y M $^{A}_{S}$ P E	D I W S $^{L}_{M}$ G L $^{S}_{T}$
OPK III	2	PAK/STE20 family	H R D I K S D N I	G T P Y W M A P E	D I W S L G I M
OPK IV	5	MEKK/STE11 family	H R D $^{I}_{V}$ K G A N F	G $^{S}_{T}$ $^{V}I_P$ F W M A P E	D I W S $^{L}_{V}$ G C X
OPK V	5	NimA family	H R D $^{I}_{L}$ K $^{S}_{P}$ $^{E}G_Q$ N $^{I}_{V}$	G T P $^{Y}_{F}$ $^{M}_{L}$ $^{S}_{A}$ P E	D $^{I}V_M$ W $^{S}_{A}$ L G C $^{I}_{V}$
OPK VI	3	wee1/mik1 family	H L D $^{I}L_V$ K P $^{S}_{A}$ N $^{I}_{V}$	G D $^{R}S_C$ $^{E}V_R$ $^{Y}_{F}$ $^{I}_{L}$ $^{P}_{N}$ E	D $^{I}_{V}$ $^{P}_{Y}$ $^{S}_{A}$ L G L $^{S}_{T}$
OPK VII	3	Transcriptional control	H R D L K P $^{SR}_{K}$ N I	G T $^{C}L_A$ $^{L}R_M$ Y $^{A}M_V$ $^{S}_{A}$ P E	D $^{L}_{M}$ Y $^{A}_{S}$ $^{L}_{V}$ G $^{I}V_L$ I
OPK VIII	6	Raf family	H R D L K S N N I	G S $^{I}_{V}$ L W M A P E	D V Y $^{A}_{S}$ $^{Y}_{F}$ G $^{I}_{V}$ $^{I}_{V}$
OPK IX	14	Activin/TGFb receptor	H R D $^{F}_{L}$ K S K N $^{I}_{V}$	G T $^{K}_{R}$ R Y M A P E	D $^{I}V_M$ $^{Y}_{W}$ $^{A}_{S}$ X G L V
OPK X	10	Flowering plant	H R D $^{STP}_{AV}$ K X $^{S}_{E}$ N $^{I}_{V}$	G T $^{L}Y_Q$ G Y $^{L}_{A}$ A P E	D V $^{I}_{F}$ S F G V $^{I}_{V}$
OPK XI	2	PSK/PTK leucine zipper	H R D L K S $^{N}_{S}$ N I	G T Y A W M A P E	D V W S $^{Y}_{F}$ G V L
OPK XII	6	Casein kinase I family	H R D I K P D N F L	G T A R Y $^{A}_{M}$ S I N	D $^{M}_{L}$ E $^{S}_{A}$ $^{I}_{L}$ G $^{Y}_{H}$ V
OPK XIII	2	PKN prokaryotic	H R D L K P $^{D}_{E}$ N $^{I}_{V}$	G T P E Y $^{M}_{L}$ $^{A}_{S}$ P E	D L Y $^{A}_{S}$ $^{L}_{F}$ G V $^{I}_{L}$
OPK Other	22	No close Relatives	H R D $^{L}_{I}$ K $^{P}_{S}$ X N $^{I}V_F$	G $^{T}_{S}$ $^{P}Y_R$ X $^{Y}W_F$ $^{N}R_L$ A P E	D $^{I}_{V}$ $^{Y}F_W$ $^{A}_{S}$ $^{L}V_F$ $^{A}_{G}$ $^{V}I_C$ $^{I}_{L}$
		[1] **AminoAcid Consensus** (% of sequences)	$^{64}_{36}$ 96 - $^{5}_{94}$ $^{88}_{12}$ 95 - - -	$^{57}_{19}$ $^{81}_{17}$ $^{43}_{40}$ - $^{90}_{10}$ $^{41}19_8$ 92 - -	- $^{11}20_{13}$ 95 $^{23}_{61}$ $^{31}16_{12}$ 99 $^{42}9_{27}$ -
Total	288	Consensus:	$^{H}_{Y}$ R **D** $^{I}_{L}$ $^{K}_{L}$ P $^{ED}_{QH}$ **N** $^{I}L_V$	$^{G}_{V}$ $^{T}_{S}$ $^{P}_{R}$ X $^{Y}_{F}$ $^{R}L_M$ **A P E**	**D** $^{W}I_M$ **W** $^{A}_{S}$ $^{L}F_C$ **G** $^{V}I_C$ $^{I}V_L$

[1] (-) 100%; superscripted and subscripted numbers represent % of clones with amino acid in consensus. Bold letters are invariant residues.

[2] # of sequences analyzed.

Table 3.7: Homology survey of Hanks' consensus sequences VIB through IX for prokaryotic protein kinases.

SER/THR KINASE	DOMAIN VI	DOMAIN VII	DOMAIN VIII	DOMAIN IX
Streptomyces				
S. toyocaensis Stopk1	H R D M K P E N	D F G	G T V S Y L A P E	D V Y A C G I L
S. griseus PKSG1	H R D L K P A N	D F G	G T P A Y V A P E	D I Y G A G I L
S. griseus PKSG2	H R D V K P E N	D F G	G T P D Y L A P E	D I Y A L A T V
S. coelicolor AfsK	H R D L K P S N	D F G	G T P A Y M S P E	D V F S L G S M
S. griseus AsfKG	H R D L K P S N	D F G	G T P A Y M S P E	D I F S L G S T
S. coelicolor PKaA	H R D L K P A N	D F G	G T P A Y V A P E	D V Y G A G I L
S. coelicolor PKaB	H R D V K P E N	D F G	G T P D Y L A P E	D I Y A L A T V
S. coelicolor PK3	H C D V K P G N	D F G	G T P L Y M A P E	D I Y G T G V V
M. tuberculosis				
MYPROTKIN	H R D L K P S N	D F G	G T P A Y M P P E	
MTCY28.05	H R D V K P E N	D F G	G T L Y Y M A P E	D I Y A L T C V
MTCY28.09	H R D V K P A N	D F G	G T V S Y A A P E	D Q Y A L A A T
PknA	H R D V K P G N	D F G	G T A Q Y I A P E	D V Y S L G V I
PknB	H R D V K P A N	D F G	G T A Q Y L S P E	D V Y S L G C V
pknD	H R D V K P E N	D F G	G T Y N Y M A P E	D I Y A L A C V
MTCY50.16	H R D V K P G N	D F G	G T W K Y M A P E	D I Y A L A C V
MTCY338.02c	H R D V N P A N	E F G	S Q P S Y P A P E	D Q Y A L A L T
MTCY49.28	H R D I K P A N	D F G	A T L A Y A A P E	D L Y S L G C A
MTCY22G10.06c	Y R D L K P E N	D L G	G T P G F Q A P E	D I Y T V G R T
Bacilus				
B. subtilis YLOP	H R D I K P H N	D F G	G S V H Y L S P E	D I Y A L G I V
Cyanobacterium clone	H R D I K P D N	D F G	G T P G Y M P S E	D L Y $^{S}_{A}$ L G L T
Myxococcus clone	H R D L K P D N	D F G	G T P E Y M A P E	D L Y A L G V I
[1]AminoAcid Consensus (21)[2]	$^{H}_{Y}$ $^{R}_{C}$ **D** $^{I}V_{L}$ **K P** $^{ED}A_{GSH}$ **N**	$^{D}_{E}$ $^{F}_{L}$**G**	$^{S}G_{A}$ $^{Q}T_{S}$ $^{V}P_{LN}$ $^{ML}_{V}$ $^{Y}_{F}$ $^{V}L_{M}$ A_{S} P E	D ^{V}I $^{Y}_{F}$ $^{AS}_{S}$ $^{LA}_{CT}$ G_{A} $^{VT}I_{S}$ $^{V}_{LTM}$

[1] superscripted and subscripted numbers represent % of clones with amino acid in consensus. Bold letters are invariant residues.

[2] Total sequences analyzed.

3.2.2.2 PCR Cloning Strategy

Taq polymerase DNA fragments generated from YF, YR, SR, SF, SFn, and SRn (Table 3.7) combinations with a Touchdown PCR protocol (Don *et al.*, 1991 See below). Primer combinations employed included set #1, set #2, set #3 and mixed sets of SF and YR, and, SF and SRn (Table 3.4). Gene fragments were subsequently designated according to the primer pairs used for amplification (eg. SFYR35 used prmers SF and YR and was clone #35). Amplification reactions consisted of 1-1.5 μg of genomic DNA template, 0-20% (v/v) dimethylsulfoxide, 2.5 mM $MgCl_2$, 250-500 mM dNTPs, 1-2 μM

of each oligonucleotide primer and 1 U Taq DNA polymerase in 10 mM Tris/HCL pH 8. The reaction sequence consisted of template denaturation for 1 min at 94 °C, 1.5 min annealing at 46-55 °C (5 cycles at 52-55 °C, 5 cycles at 50-52 °C and 20 cycles at 46-50 °C), and followed by 1 min at 72 °C for primer extension. A final 72 °C incubation was performed for 15 min after cycling was complete in order to ensure the addition of the terminal adenine for subsequent cloning. PCR reactions were run on 5% native polyacrylamide gels or 1.5% agarose gels and fragments of the appropriate size (150-200 bp) were isolated, purified over silica beads (Qiagen) and cloned into pGEMT following the manufacturer's instructions (Promega). The pGEMT ligations were transformed into *E. coli* SURE2 competent cells (Stratagene) and plated on Luria-Bertani plates containing 100 μg/mL ampicillin and 100 μL 100 mM IPTG plus 20 μL X-gal (in dimethylformamide) spread on the surface for blue/white colony selection.

3.2.2.3 Sequencing Methods

Sequencing of isolated pGEMT clones was carried out after restriction mapping of isolated plasmids from white colonies to confirm the presence of the correct sized insert. Plasmids were isolated using a standard miniprep procedure. Briefly, 1.5 mL of bacteria grown overnight at 37°C in Luria-Bertani broth supplemented with 100 μg/mL ampicillin were resuspended in 100μL 25 mM Tris/HCl pH 8, 50 mM glucose, 10 mM EDTA and 50 μg/mL RNase A. Cells were subject to lysis by addition of 200 μL 200 mM NaOH with 1% SDS, and proteins and DNA were extracted from the lysate with 150 μL of a solution containing 3M potassium acetate and 10% acetic acid. The supernatant was then extracted with 400 μL chloroform and DNA precipitated with 1 volume of

isopropyl alcohol. The pellet was washed with 1 mL of 70 % ethanol followed by drying and suspended in 30 μL of sterile distilled water.

A two fold sequencing strategy was adapted: (1) initial screening using a cycle sequencing kit identified identical sequences between clones. (2) a second sequencing of unique clones was then performed by dye termination methodology at the central facility of the Institute for Molecular Biology and Biotechnology, McMaster University. Cycle sequencing was carried out with a cycle sequencing kit following the manufacturer's instructions (CircumVent, NEB). Reactions contained 200-300 ng of plasmid template, 20 μCi α[^{35}S]-dATP (NEN) and 1 μM M13 forward sequencing primer. Reactions were run at 85-90W on a 5-7% polyacrylamide gels (19:1 acrylamide:bis solution) containing 7 M urea made up in TBE (Tris, boric acid and EDTA). Bands were visualized on dried gels (60°C for 2 hrs) by exposing to X-ray film overnight (Kodak Biomax-MR). In total, over 100 DNA inserts were sequenced and the clean data reads were translated to protein sequences in all six reading frames and analyzed for protein kinase homology using the BLAST algorithm (Altschul *et al.*, 1990).

3.2.2.4 Nucleotide Sequence accession numbers

The nucleotide sequences of *sto*PK-1 and flanking DNA (accession no. AF233851), *stoPK-2* (accession no. AF234272), *stoPK-3* (accession no. AF234273), and *stoPK-4* (accession no. AF234274), have been deposited in GenBank.

3.2.3 Isolation and Cloning of *afs*K_t

3.2.3.1 PCR Primer Design

Analysis of AfsK protein sequences from *S. coelicolor* and *S. griseus* ($AfsK_c$ and $AfsK_g$ respectively) showed strong homology in the NH_2-terminus. Therefore, primers were designed around the 5' end of the gene to give a fragment under 1 kilobase pair which is ideal for PCR. The nucleotide sequence used was specific for the *afs*K_c gene, in this way *S. coelicolor* genomic DNA could be used as a template control. Primer sequences were designated AB18920: CGA ATT CAC GCC GAC GCC GTA CCG CTG T) and AB18921: GCG AAT TCG GGG AGG TGG CAG GCA AGT GG (*Eco* RI restriction sites underlined) and amplify a 880 base pair fragment.

3.2.3.2 PCR Cloning Strategy

PCR reactions with *S. coelicolor* and *S. toyocaensis* genomic DNA were carried out in 100 μL reactions consisting of 1-1.5 μg of genomic DNA template, 5% (v/v) dimethylsulfoxide, 5 mM $MgSO_4$, 500 μM dNTPs, in 10 mM KCl, 20 mM Tris-HCl (pH 8.8 at 25°C), 10 mM $(NH_4)_2SO_4$, 0.1% Triton X-100 buffer with 2 U Vent DNA polymerase and 1-2 μM of each oligonucleotide primer. The reaction sequence consisted of template denaturation for 1 min at 95 °C, 1 min annealing at 60°C, followed by a 1 min extension at 75°C. PCR products of the correct size (~900 bp) were subsequently excised from 1% agarose gels and purified with silica beads (Quiex II, Quiagen) and ligated into *Eco* RI digested pUC19 treated with 0.1 U of calf intestinal alkaline phosphatase (CIP). The CIP reaction was carried out with the 4 hr. digestion reaction in the supplied *Eco*RI

buffer (MBI Fermentas). Ligation was carried out using T4 DNA ligase following the manufacturer's instructions (MBI Fermentas). Ligation products were transformed into *E. coli* SURE2 competent cells (Stratagene) (see Section 3.2.2.3 for plating methods), and isolated clones were restriction mapped and sequenced by dye termination methodology at the central facility of the Institute for Molecular Biology and Biotechnology, McMaster University.

3.2.3.3 Genomic Cloning Strategy

The isolated *afs*K_t PCR fragment (above) was used as a probe in Southern blots of restriction digested *S. toyocaensis* genomic DNA (See Section 3.2.1). Genomic DNA was digested with a variety of restriction enzymes including *Pst*I, *Kpn*I, *Sac*I, and *Eco*RI, transferred to Hybond-N+ charged nylon (Amersham Pharmacia), and equilibrated for 1 hr at 60 °C in hybridization buffer containing 0.5M Na_2PO_4, 7% SDS and 10 mg/mL BSA Fraction V. The blot was probed with the α^{32}P-ATP-labeled *afs*K_t PCR fragment overnight at 7.5 x 10^5 cpm/mL overnight at 60°C in a hybridization oven. Radio-labeled *afs*K_t PCR probe was generated using a random hexamer labeling kit (Gibco BRL), 3 μg *afs*K_t PCR DNA and 1U Klenow (MBI Fermentas) incubated at 37°C for 1 hr. The probe was purified over silica beads (Qiagen) and 1 μL mix with 3 mL scintillation fluid and the specific activity determined in a scintillation counter (Beckman LS 3801).

A 4.2 kb *Pst* I fragment containing the *afs*K_t gene was then isolated by cloning the 4-4.5 kb region from a 1% agarose gel into *E. coli* SURE2 cells (Stratagene) with *Pst* I cut and Calf Intestinal Phosphatase (CIP, MBI Fermentas) treated pUC19 to create pUC19-AfsKtg. This plasmid was isolated by colony hybridization using the α^{32}P-ATP-

labeled *afs*K_t PCR fragment. Transformed *E. coli* colonies from six Luria-Bertani plates containing 100 μg/mL ampicillin were transferred to charged nylon filters (Hybond N+, Amersham), dried and soaked in 0.4 M NaOH for 5 min. followed by washes in 1 M Tris/HCl pH 7.5 and 0.5M Tris/HCl with 1.25 M NaCl pH 7.5. The nylon discs containing the colony DNA was then hybridized with the α^{32}P-ATP-labeled *afs*K_t PCR fragment in hybridization buffer at 65°C overnight. Two low stringency washes were then carried out with 50 mL of 40 mM Na_2HPO_4, 5% SDS, 1 mM EDTA, and 5 mg/mL BSA Fraction V for 10 min each at room temperature. Two high stringency washes were performed at 65°C for 20 min. each using 50 mL of 40 mM Na_2HPO_4, 1% SDS and 1 mM EDTA. The discs were then exposed to X-ray film (Kodak X-OMAT) for 4 hrs to visualize hybridization. Positive colonies were picked from original plates, grown overnight and isolated plasmids were screened by restriction digests and PCR (5' end of *afs*K_t, see Section 3.2.3.1).

3.2.3.4 Sequencing Methods

The sequencing of pUC19-AfsKtg proved to be problematic. Several different sequencing facilities across Ontario were utilized in an attempt to generate a sequence for the 4.2 kb fragment. However, good sequencing data was not obtained. In order to circumvent this problem, a detailed restriction map of the fragment allowed deletions of various regions, decreasing the size of the insert. It was hoped that smaller insert sizes would facilitate clean sequencing reactions. This strategy was successful, and Figure 3.5 shows a restriction map of pUC19-AfsKtg and the deletion clones ΔAfsKtg(Eco_Xho) and ΔAfsKtg(SphI). The deletion strategies that were used to obtain the whole sequence

were as follows: (1) digestion with *Eco*RI and *Xho*I, filled overhangs with Klenow and religated with T4 DNA ligase; (2) digestion with *Sph*I and intramolecular ligation with T4 DNA ligase. Sequencing from the M13 primer sites as well as the use of the specific primers shown in Table 3.8 were carried out on the deletion clones by dye termination methodology at the central facility of the Institute for Molecular Biology and Biotechnology, McMaster University.

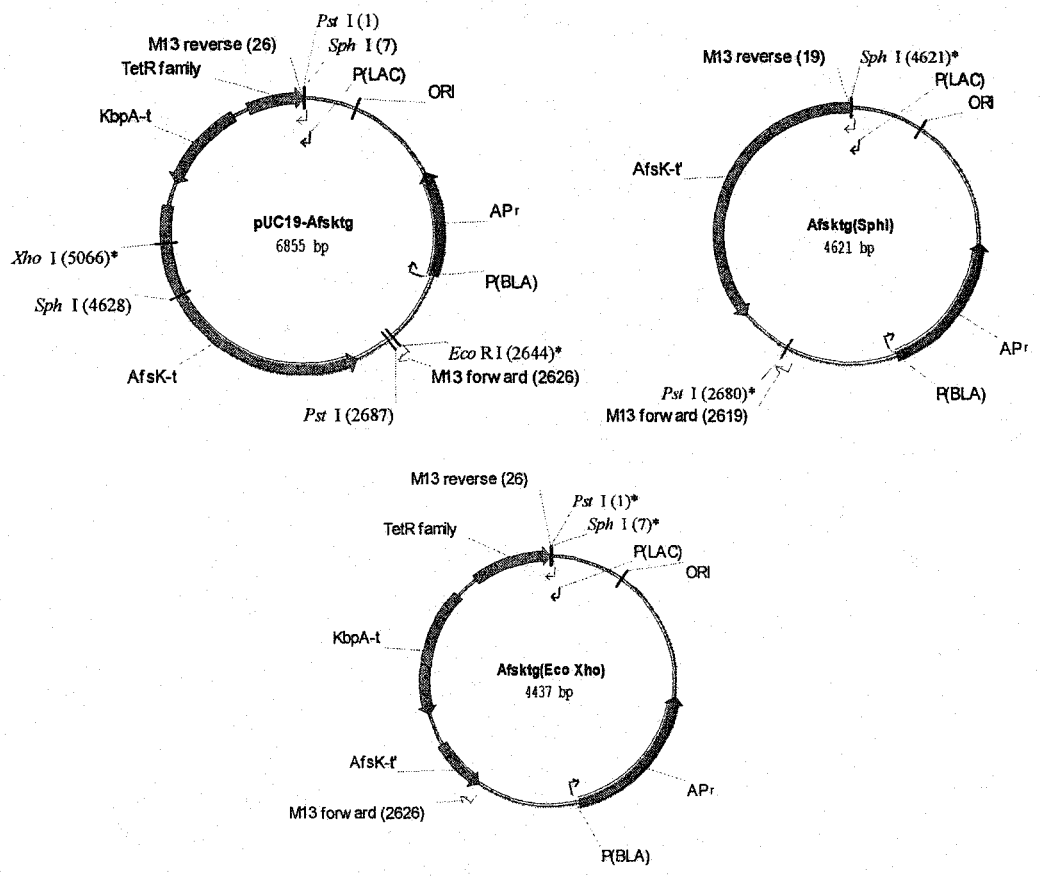

Figure 3.5: Restriction map of pUC19-AfsKtg showing key sites for deletion strategy and the deletion plasmids used for sequencing the entire *Pst* I fragment.

Table 3.8: Specific *afs*K_t sequencing primers used in this study.

Primer Designation	Sequence (5'-3')
AB25893	GGC CGC CAC CTG GTC CAG AC
AB25894	TAC ACG CAG CGG TCC TTG CT
AB25895	GAT TCG GCC GAC TGC AAA TG

3.3 Results

3.3.1 Degenerate PCR

In all degenerate PCR reactions products of the expected hypothetical sizes (~150 base pair) were observed. Figure 3.6 shows a typical agarose gel for fragments amplified in the presence of increasing DMSO using SFSR primers. In this case optimal reactions conditions occurred without the presence of DMSO. However, other primer combinations gave better amplification of the 150-200 base pair range in the presence of DMSO (data not shown).

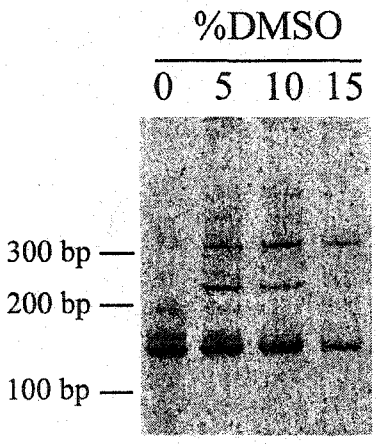

Figure 3.6: Degenerate PCR agarose gel of amplified products from *S toyocaensis* genomic DNA in the presence of increasing DMSO concentrations. Primers used were AB10095 and AB10096 (SFSR, Table 3.6). See section 3.2.2.2 for reaction conditions.

After restriction mapping and sequencing of over 100 clones, analysis of initial cycle sequencing reads revealed 70 readable reactions (Table 3.9 and Table 3.10). A total of 57 sequences were non-protein kinase sequences (Table 3.9) representing 72% of the

readable reactions. Conversely, only 28% (or ~1 in 5) of all clones were bona fide protein kinases as defined by the presence of Hanks' sequence VII (or 'DFG') which is not contained in the primer sequences (Figure 3.7).

Many of the non-kinase sequences showed significant homology with fragments identified in the *S. coelicolor* genome sequencing project (http://www.sanger.ac.uk/Projects/S_coelicolor/). A total of 19 fragments showed no significant homology to any known DNA fragments (Table 3.9) and analysis of these for predicted protein sequences in all six reading frames failed to show significant homology for all but one in the *S. colicolor* or Genbank databases (data not shown). Oddly, the human protein kinase pseudogene fragment *tyro*3P showed up in the "non-kinase" sequencing data (clone YFYR11, Table 3.9). This gene fragment was cloned using the tyrosine kinase primers YF and YR, and although no other human kinase fragments were isolated, its presence suggests the existence of contaminating human genomic DNA in these reactions.

In total, four protein kinase fragments were isolated for *S. toyocaensis* with all the primer pair combinations employed (Table 3.10). From a grand total of 70 readable sequences 18.6% or 13 cloned PCR fragments appeared to be protein kinases. This ratio improves to 13/34 or 38.2% if the products generated using only tyrosine primers are eliminated as these primers were unsuccessful in amplifying any *S. toyocaensis* kinase gene fragments (Table 3.9). All four kinase sequences isolated show significant DNA homology to *S. coelicolor* genes (Table 3.10), and considerable protein homology to putative *S. coelicolor* protein kinases (Figure 3.8).

Table 3.9: Summary of cloned non-kinase degenerate PCR DNA fragments from *S. toyocaensis* showing clones isolated using tyrosine primer (YF-YR), and Serine/Threonine primer combinations (SF-YR, SF-SR, SF-SRn, SFn-SR and SFn-SRn)

Clone Designation	# of clones	DNA Homology (Blastn)	% similar at NT level
Tyrosine primers:			
YFYR 1	3	possible lipoprotein, SCH22A.16c, ACCESSION 7210989	22/23 (95%)
YFYR 2	1	unknown	----
YFYR 2	1	UNKNOWN	----
YFYR 3	2	UNKNOWN	----
YFYR 4	1	ATP synthase Gene="atpA" ACCESSION 8568778	56/61 (91%)
YFYR 5	1	UNKNOWN	----
YFYR 5	2	2SCD4.01, possible membrane protein ACCESSION 10178320	18/18 (100%)
YFYR 7	1	UNKOWN	----
YFYR 9	1	hypothetical protein SCL6.02	87/100 (87%)
YFYR 11	3	H.sapiens TYRO3P mRNA ACCESSION X72887	177/182 (97%)
YFYR 14	1	2SCK36.07c, probable aminotransferase ACCESSION 14285271	54/58 (93%)
YFYR 15	1	UNKNOWN	----
YFYR 17	2	SCGD3.09, hypothetical protein ACCESSION 5457234	86/96 (89%)
YFYR 18	1	SCK7.21, possible secreted protein, ACCESSION 9967654	99/118 (83%)
YFYR 22	1	CZA382.31, probable peptide synthetase ACCESSION 5051792	34/38 (89%)
YFYR 23	2	SCJ9A.28c, possible monooxygenase ACCESSION 5763880	25/27 (92%)
YFYR 26	2	UNKNOWN	----
YFYR 29	1	gene="rrnF", probable 16S rRNA ACCESSION 5123647	88/90 (97%)
YFYR 33	1	UNKNOWN	----
YFYR 36	2	2SC6G5.15, atpA, ATP synthase ACCESSION 8568778	35/36 (97%)
YFYR 39	1	SC2H12.28c, possible membrane protein ACCESSION 8546912	22/23 (95%)
YFYR 41	1	UNKNOWN	----
YFYR 42	1	SC3A7.04, hypothetical protein ACCESSION 3367738	31/33 (93%)
YFYR 47	1	SCE66.18, possible sodium:solute symporter ACCESSION 8894754	116/136 (85%)
YFYR s3g	2	hypothetical protein SCD10.01c ACCESSION 8894718	249/315 (79%)
YFYR 3g	1	SC8E7.06, TetR-family regulator ACCESSION 9843813	27/66 (40%)
Serine/Threonine Primers:			
SFYR SDR	1	UNKNOWN	----
SFYR 250_7	1	SCE9.28c, hypothetical protein ACCESSION 4808332	31/34 (91%)
SFSR250_1	1	UNKNOWN	----
SFSR250_4	1	SC1G2.03, probable glutamine synthetase ACCESSION 6714650	124/146 (84%)
SFYR250_4	2	UNKNOWN	----
SFYR450_2	1	UNKNOWN	----
SFSR1	1	UNKNOWN	----
SFSR7	5	UNKNOWN	----
SFSR16	1	histidinol-phosphate amidotransferase (hisC) ACCESSION M31628	63/68 (92%)
SFSR18	1	pep1, possible member of the alpha amylase family ACCESSION 4580329	106/120 (88%)
SFSRn20	1	UNKNOWN	
SFSRn27	1	gene="ECs0397" beta-D-galactosidase ACCESSION 13359705	48/50 (96%)
SFSRn32	1	UNKNOWN	----
SFSRn37	1	UNKNOWN	----
SFSRn38	1	UNKNOWN	----
SFnSRn7	1	UNKNOWN	----
Total clones = 57			

Table 3.10: Table of DNA and protein homologies for protein kinase cloned degenerate PCR sequences including Genbank accession numbers.

Clone Designation	DNA or Protein Homology	% similar at NT level	% similar at AA level
stoPK-1: SFYR 13 or SFYR 34 or SFYR 35 or SFSR19n	pKaF, ACCESSION 5689916	117/133 (87%)	31/36 (86%)
stoPK-2: SFSR9N or SFSR30n or SFSR33n or SFSR36n	pKaB, ACCESSION D86821	128/139 (92%)	51/57 (89%)
stoPK-3: SFSR29n or SFSR31n or SFSR35n or SFSR41n	pKaJ, ACCESSION 7320887	113/123 (91%)	30/49 (61%)
stoPK-4: SFSR8nn	pKaA ACCESSION CAB87324.1	77/143 (53%)	26/52 (50%)

Total clones = 13.

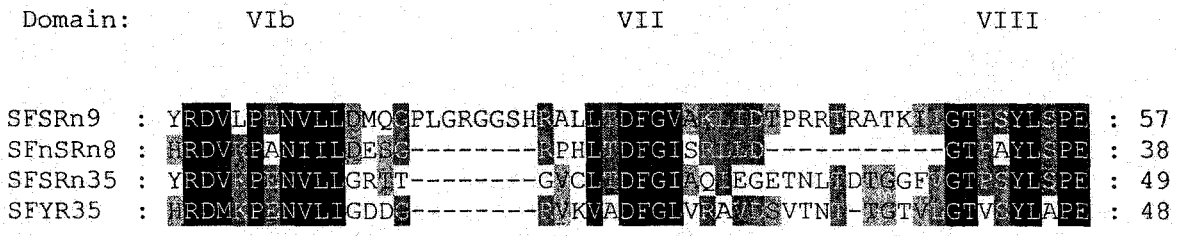

Figure 3.7: Alignment of protein translations for four putative protein kinase clones from *S. toyocaensis* degenerate PCR showing conserved Hanks' sequences.

Figure 3.8: Alignment of *S. toyocaensis* protein kinase amino acid sequences with known putative *S. coelicolor* A3(2) protein kinase catalytic fragments.

3.3.2 Cloning of *afs*K_t

Initial PCR reactions of *S. coelicolor and S. toyocaensis* genomic DNA with the PCR primers specific for *afs*K_C (subscript indicates species) amplified products of the correct molecular size in both cases (Figure 3.9A). Sequencing of the *S. toyocaensis* PCR fragment revealed an *afs*K_C homolog which hybridized to a ~4.5 kb *Pst* I fragment (Fig. 3.10) in Southern blot experiments (Figure 3.9B). Cloning and subsequent sequencing of the genomic *Pst*I fragment revealed the striking DNA homology of 78% to the *afs*K_C gene cluster and the same genomic organization as that in *S. coelicolor* (Figure 3.11). Analysis of protein homology between AfsK_T and AfsK_C, as well as the other open reading frames (ORFs) in the genetic neighborhood are shown in Table 3.10.

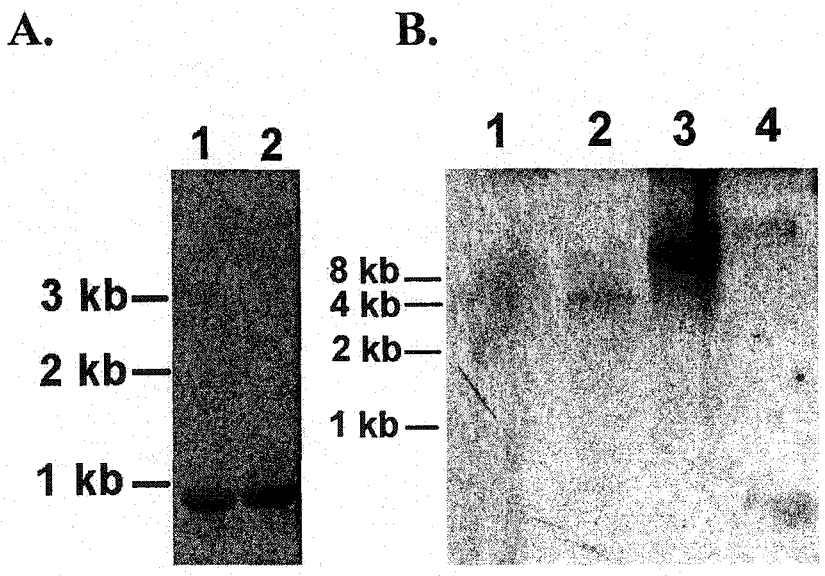

Figure 3.9: **(A)** PCR of *afs*K_C (Lane 1) and *afs*K_T (Lane 2). **(B)** Southern hybridization of *S. toyocaensis* genomic DNA using the *afs*K_t PCR fragment. Lane 1: *Sac* I, Lane 2: *Pst* I, Lane 3: *Kpn* I, Lane 4: *Eco* RI. See text for methods.

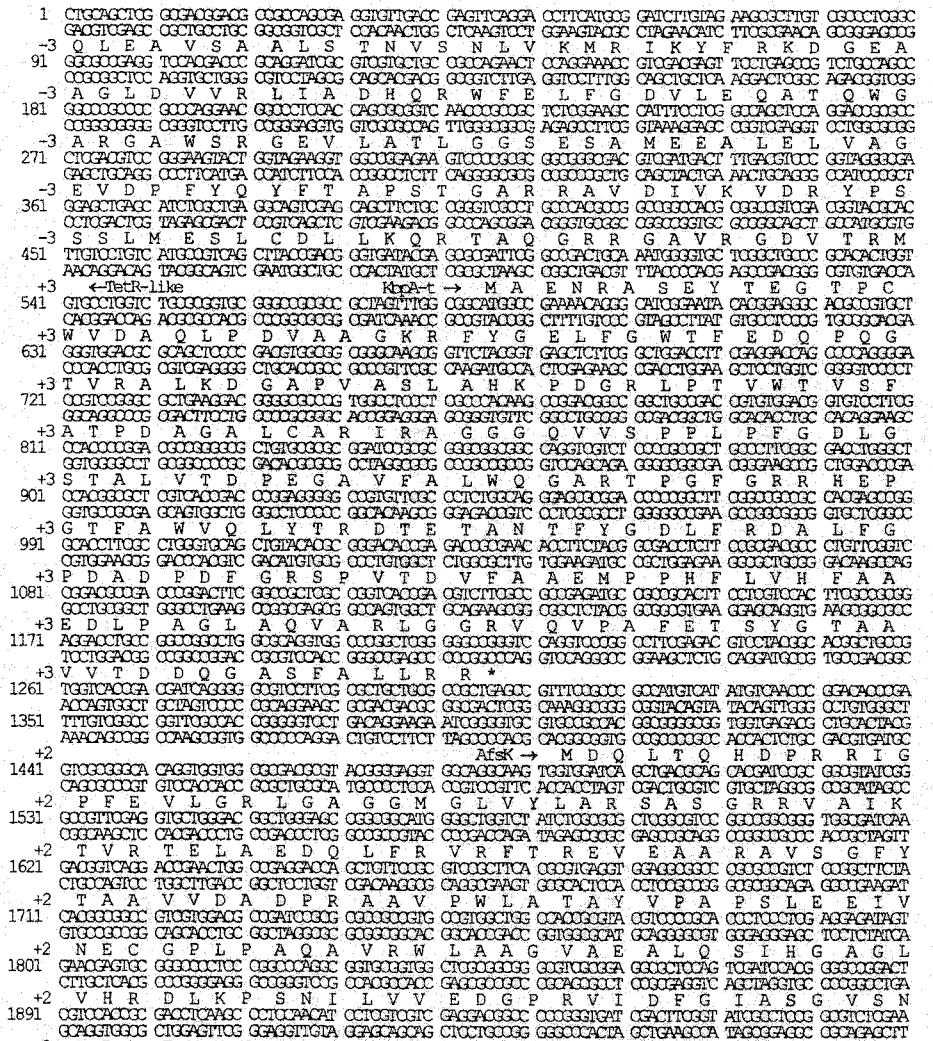

Figure 3.10: Sequence of 4.2 kb *Pst* I genomic DNA from *S. toyocaensis* encoding *afs*K_t. Putative open reading frames are numbered and translated.

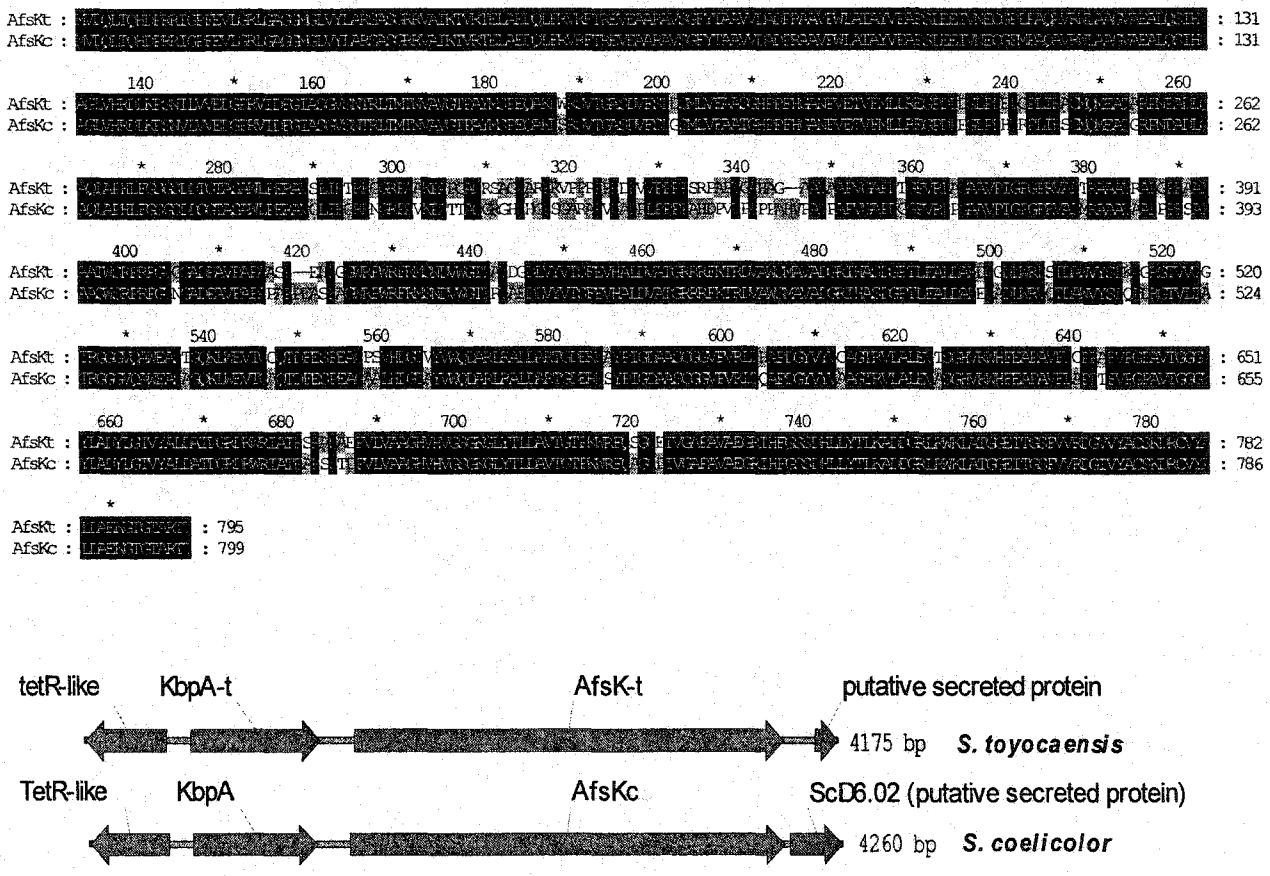

Figure 3.11: Sequence homology and genomic arrangements between S. coelicolor A3(2) and S. toyocaensis NRRL 15009 afsK regions.

Table 3.11: Protein homologies (% similarity) for open reading frames from the cloned *Pst* I genomic fragment from *S. toyocaensis* NRRL 15009.

Protein Designation	Size (# of amino acids)	Homology to:	% Similarity
TetR-like (fragment)	>150	TetR-like[1]	138/150 (92%)
KbpA-t	252	KbpA	194/252 (76%)
AfsK-t	799	AfsKc	708/799 (88%)
Putative secreted protein (fragment)	>42	ScD.02	35/42 (82%)

[1]- TetR-like protein from *S. coelicolor* cosmid Sc6F11.

3.3.3 Survey of *S. coelicolor* protein kinases

An analysis of the entire genome sequence of *S. coelicolor* A3(2) revealed the presence of 40 putative Hanks' protein kinases (Table 3.12). Further analysis of each of the predicted individual protein sequences was carried out for molecular weight, location of catalytic domains, and the predicted presence of transmembrane domains. The analysis reveals a striking number with predicted membrane spanning regions (36 to 38), although these predictions should be approached with caution. Unlike membrane-bound protein kinases found in eukaryotic organisms which contain Hanks' subdomains in the C-terminal portion of the protein, the majority of protein kinases found in *S. coelicolor* (33/40) contain N-terminal domains and only a single gene predicts a C-terminal catalytic domain (pk3, Table 3.12). Analysis of predicted molecular weights shows a range from 31 to 169 kDa, with an average of 70 kDa.

Table 3.12: Survey of 40 Hanks' protein kinases in the genome of *Streptomyces coelicolor* A3(2).

Protein Desigantion		Catlytic Domain Location[1]	Transmembrane domains[2] #	to	from	Direction	Size (AA)	MW[3] (kDa)
1	PKaA	N-terminus	1	488	510	i-o	543	58.2
2	PKaB	N-terminus	1	376	395	i-o	417	43.3
3	PKsC	N-terminus	1	408	429	i-o	556	58.3
4	PKaD	N-terminus	1	380	399	i-o	599	63.9
5	PKaE	N-terminus	1	367	385	i-o	487	53.3
6	PkaF	N-terminus	1	595	616	i-o	667	70.8
7	PKaG	N-terminus	1	194	214	i-o	592	61
			2	344	364	o-i		
8	PKaH	N-terminus	1	475	496	i-o	717	74.1
9	PKaI	N-terminus	1	204	222	i-o	380	39.2
			2	304	326	o-i		
10	PKaJ	N-terminus	1	352	371	i-o	548	58.1
11	AfsK	N-terminus	1	191	209	i-o	799	83.8
			2	630	658	o-i		
12	SC7A12.07	N-terminus	None			----	745	81.2
13	SCD65.24	N-terminus	1	198	217	i-o	632	64.1
			2	347	372	o-i		
14	2SCK31.03	N-terminus	1	365	381	o-i	670	71.1
15	SCE7.11	N-terminus	1	335	355	i-o	720	75.4
16	SC66T3.32c	N-terminus	1	595	616	i-o	783	75.6
17	SCI11.13	N-terminus	1	338	359	i-o	550	55.6
18	SCH69.30	N-terminus	1	554	573	i-o	576	59.8
19	SCH69.18	N-terminus	1	350	367	i-o	673	71.5
20	SCGD3.21c	N-terminus	1	366	384	i-o	522	53.5
21	SC2A6.02c	N-terminus	1	324	341	i-o	452	49.1
22	SCD10.09	N-terminus	1	117	135	i-o	580	59.6
			2	370	392	o-i		
23	SC5F8.01 (SC5H1.01)	N-terminus	1	303	324	i-o	538	56.3
24	SCC53.11c	N-terminus	1	221	240	i-o	294	31
25	SCD69.08	N-terminus	1	202	221	o-i	626	63.1
			2	348	367	o-i		
26	SCD35.33	N-terminus	none			----	435	47.5
27	SCD35.14	N-terminus	1	198	217	i-o	586	58.8
			2	419	442	o-i		
28	SCL11.07	N-terminus	1	202	221	i-o	493	51.4
			2	436	455	o-i		
29	SC1G2.06c	N-terminus	1	317	335	i-o	686	71.7
30	SC9G1.09	N-terminus	1	26	48	i-o	689	73.6
31	SCH35.50c	N-terminus	1	7	25	i-o	433	43.8
32	SCP1.273c	N-terminus	1	11	32	i-o	1039	110.1
33	SCD63.08	N & Central (590-680)	1	749	766	i-o	979	100.7
34	SC2A6.05c	Central (295-481)	1	408	426	i-o	712	74.6
			2	507	526	o-i		
35	SCC24.21	Central (611-750)	1	463	484	i-o	1349	145.7
36	SCL6.25c	Central (131-380)	1	127	148	i-o	774	83.4
			2	400	418	o-i		
37	SC6D10.09	Central (206-457)	None			----	903	94.2
38	SC1F2.23	Central (530-811)	None			----	1557	169.2
39	SC5A7.31	Central (268-457)	1	448	466	o-i	930	100.1
			2	579	603	i-o		
			3	724	743	o-i		
			4	888	910	i-o		
40	Pk3	C-terminus	none			----	565	60.4

1- amino acid numbering according to predicted protein.
2- Predicted from TMpred (http://www.ch.embnet.org/software/TMPRED_form.html); i-inside, o- outside.
3- Predicted molecular weight from VectorNTI Suite software.

3.4 Discussion and Significance

In this study two approaches to cloning protein kinase gene fragments were undertaken. First, a degenerate PCR strategy using primers designed from available kinase sequences was employed to isolate four *S. toyocaensis* DNA fragments. Secondly, a set of PCR primers designed for a known *S. coelicolor* kinase were utilized to clone a *S. toyocaensis* homologue. Both strategies were successful to some extent.

Degenerate PCR strategies used by others for cloning novel kinase genes have met with varying degrees of success. Shishido *et al* (1991) isolated 9 different tyrosine kinase fragments which collectively appeared 28 times in 155 clones for a success rate of 11%. Sells and Chernoff (1995) achieved a rate of 33% of cloned fragments appearing as kinase fragments with only 3 different sequences appearing, and Wilks was able to successfully isolate 133 kinase fragments containing 6 different genes from a total of 200 cloned degenerate PCR fragments for a rate of 66% (Wilks 1991). A comparable success rate of 38% was seen in this study for the serine/threonine kinase primers (section 3.3.1).

The isolation of a human gene (*tyro*3P, Table 3.9) with the tyrosine kinase primers YF and YR supports the contention of others that a contamination of only a few molecules of foreign DNA can produce a significant amount of amplified products in the degenerate reactions (Sells and Chernoff 1995). These authors suggest employing separate tubes, pipettes and tips exclusively for PCR work to eliminate any contamination. Such a strategy may be prudent given the results seen here, and the fact that previously isolated gene fragments could contaminate subsequent reactions.

In designing degenerate PCR primers for amplifying genes of a specific family or class several factors must be considered. These include (1) the degree of conservation of the protein domains between family members, (2) the total degeneracy of the primers, (3) primer length, (4) amplified product length, and (5) the number of suitable conserved domains for designing the primers. Ideally, optimal primers cover a stretch of from six to eight invariant amino acid residues and have low (<1064-fold) degeneracy. Primer should also be ~50% GC:AT in content. The distance between conserved domains should also be short (<1 kilobase) since larger fragments can prove difficult to amplify efficiently. In addition, more than one set of primers should be designed around different conserved stretches, since conservation around each area may be different, and quite often conservation will hold true for only one set of primers (Sells and Chernoff 1995). In this respect the results observed in this study show that although *sto*PK-2 and *sto*PK-3 were not isolated with primers based on Hanks' subdomains VI and IX (YF and SR, Table 3.10), they were amplified using two primers designed around subdomains VI and VIII (SF and SRn, Table 3.10).

The primers designed in this study met most of the criteria discussed above for ideal primers, but due to the high GC content (codon usage) of *Streptomyces* in which DNA typically contains about 70-80% GC, the degenerate primers contained high GC levels. In order to counteract the reduced primer specificity and increased secondary structure in the template that is inherent in high GC DNA, the addition of DMSO to the PCR reactions was utilized (Varadaraj and Skinner 1994). These researchers have found that improved specificity can be achieved with glycerol or DMSO, and in PCR

experiments carried out in our laboratory, the utility of DMSO (typically 5%) is indispensable for amplifying high GC sequences.

The successful amplification of the *afs*K_c homologue *afs*K_t from *S. toyocaensis* using specific primers for *afs*K_c shows the utility of this approach for cloning genes in this organism. The entire genome of *S. coelicolor* A3(2) has now been completed and as such represents a wealth of sequencing knowledge which can be directly applied to *S. toyocaensis*. The high degree of similarity between the two close cousins should allow the isolation of other genes in *S. toyocaensis*. This prospect is also supported by studies carried out in this thesis on *sto*PK-1 and the genomic region encoding it (presented in Chapter 4).

3.4.1 Conclusions

Although the degenerate PCR strategy employed here met with limited success, it should be emphasized that the primer design was primarily based on sequences available from eukaryotic organisms. At the time of their design, only a few prokaryotic protein kinase sequences were available (Table 3.7), and the most successful primer used in this study (SRn, in combination with SF or SFn, Table 3.6) was designed based on these prokaryotic sequences. However, the initial goal of this study to perform an exhaustive search for protein kinases, was later modified and emphasis was placed on the cloning, characterization and disruption of a single kinase gene product (StoPK-1, see Chapter 4) in the interest of time constraints. This was based on the rapidly increasing number of putative protein kinase sequences being reported by the *S. coelicolor* sequencing project. As a result of this modified strategy, several primer combinations (eg. SFn-SR and

SFnYR) were not attempted. Regardless of the utility of such combinations, it now seems very likely, that given the complete *S. coelicolor* genome sequence, a highly successful new set of degenerate PCR primers could be constructed based on these sequences. In addition, the high similarity between *S. toyocaensis* and *S. coelicolor* can allow the direct cloning of *S. toyocaensis* genes based on *S. coelicolor* sequences. Unfortunately, *S. coelicolor* does not contain any glycopeptide biosynthesis genes, although it does possess glycopeptide resistance genes (See Chapter 5). Thus genes relating to the regulation of a glycopeptide biosynthesis cluster may be absent in *S. coelicolor*. However, a strategy that focuses on genetic differences may be useful for isolating regulatory genes in *S. toyocaensis* that are not present in *S. coelicolor*. For instance, the isolation of novel protein kinase genes in *S. toyocaensis* could be preferentially amplified in the presence of RNA specific for *S. coelicolor* protein kinases. Such an inhibitory degenerate PCR technique has shown considerable promise for selectively amplifying genes not sharing homologous sequences with the extraneous kinase RNA (Yuen *et al* 2001). Gene fragments amplified in such a study could be the hybridized to a kinase degenerate RT-PCR library created from drug producing cultures of *S. toyocaensis*.

Alternatively, subtractive libraries generated from subtractive hybridization between *S. toyocaensis* and *S. coelicolor* could be useful in isolating unique *S. toyocaensis* genes if the subtractive methodology could be perfected (See Chapter 2). In combination with a degenerate PCR approach, protein kinases not homologous to *S. coelicolor* genes could be isolated and studied further. Although the evidence for a protein kinase involvement in *S. toyocaensis* glycopeptide production is circumstantial and may be an indirect effect, analysis of these unique kinases would represent a good

first step in addressing the involvement of individual protein kinases in the biosynthesis process. Contrarily, the suggestion that the shared kinase AfsK may be involved in regulating glycopeptide biosynthesis similar to that of *S. coelicolor*, is tempting given the high similarity of the genomic regions encoding the two homologues. Future studies that address the function of $AfsK_t$ will help to clarify this and expand our knowledge of prokaryotic protein kinases.

3.5 References

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Chapter 4.

StoPK-1, a Serine/Threonine Protein Kinase from the Glycopeptide Antibiotic Producer *Streptomyces toyocaensis* NRRL 15009 affects Oxidative Stress Response

4.1. Background

4.1.1. Cell Signalling

The growth, maintenance, and general well being of living cells is highly dependent on sensing and responding to external environmental stimuli. All cells carry out these processes through intracellular signalling by transferring phosphates by specific protein kinases. In eukaryotes, the most familiar protein kinases (PK), including for example a large number of oncogene products, catalyze the ATP-dependent phosphorylation of serine (Ser), threonine (Thr), or tyrosine (Tyr) (Hunter 1995). Until relatively recently, prokaryotic signal transduction was primarily thought to be mediated through autophosphorylation of sensor kinases on histidine (His). These kinases then transfer phosphate to Asp residues of cognate response regulators (Hoch and Silhavy 1995). However, over the past 10 years, it has become increasingly apparent that prokaryotes also use Ser, Thr, and Tyr kinases (Kennelly and Potts 1996, Zhang 1996). Ser/Thr PKs genes and proteins that share many of the classical Hanks sequences have been described in a growing number of bacteria including the actinomycetes *Streptomyces coelicolor* A3(2) (Hirakata *et al* 1998, Matsumoto *et al* 1994, Ogawara *et al* 1999), *Streptomyces granaticolor* (Nadvornik *et al* 1999, Vomastek *et al* 1998), *Streptomyces griseus* (Umeyama *et al* 1999) and *Mycobacterium tuberculosis* (Cole *et al* 1998, Peirs *et al* 1997). In addition, our analysis of the recently sequenced *S. coelicolor* genome has revealed at least 40 protein kinases containing classical Hanks consensus sequences (see Chapter 3 section 3.3.3).

4.1.2. Study Purpose and Strategy

Earlier studies with kinase inhibitors had suggested the involvement of protein kinases in the regulation of antibiotic production and resistance, and sporulation in *Streptomyces toyocaensis* NRRL 15009 (Neu and Wright 2001). In order to search for the protein kinase(s) involved, a cloning strategy for kinase gene fragments in *S. toyocaensis* was adapted using degenerate PCR amplification of DNA fragments to conserved Hank's consensus sequences. Initially the expectation was that less than 20 kinase genes would be present in this organism based on the genome sequencing of *Mycobacterium tuberculosis,* which only has 11. With the degenerate PCR strategy the intent was to clone most or all of the kinase genes and initiate a plan to systematically disrupt these genes and characterize the resulting phenotypes. Using this strategy it was hoped that a definitive link between specific protein kinases and glycopeptide antibiotic production or resistance in *S. toyocaensis* as suggested by the earlier work, could be firmly established (Neu and Wright 2001). Initially, 4 putative protein kinase gene fragments were isolated in the degenerate PCR screens (Chapter 3). In order to establish the necessary techniques in the laboratory for gene disruption in *S. toyocaensis*, a single gene was cloned in its entirety (*sto*PK-1) and protein analyses and genetic disruption studies carried out. The results of these studies are presented in this chapter.

4.2. Materials and Methods

4.2.1. Bacteria and cell growth

S. toyocaensis NRRL 15009 spores were maintained and grown as described in a previous Chapter (Chapter 2 section 2.1.1). *E. coli* were grown in Luria-Bertani broth.

4.2.2. *S. toyocaensis* NRRL 15009 genomic DNA isolation

Genomic DNA was isolated as described in Chapter 3 section 3.2.1.

4.2.3. Cloning of StoPK-1

The degenerate PCR fragment SFYR35 (stoPK-1) was used as a probe in Southern blots of restriction digested *S. toyocaensis* genomic DNA as previously described (Marshall *et al* 1998). *S. toyocaensis* genomic DNA was cut with a variety of restriction enzymes including *Hind* III, *Kpn* I, *Sal* I, *Sac* I, *Bam*H I and *Nde* I and transferred to Hybond-N+ charged nylon (Amersham Pharmacia), equilibrated for 1 hr at 60 °C in 10 mL hybridization buffer containing 0.5 M Na_2PO_4, 7% SDS and 10 mg/mL BSA Fraction V (Sigma). The blot was probed with the $\alpha[^{32}P]$-ATP-labeled SFYR35 PCR fragment overnight (See Chapter 3 for sequence) with 7.5 x 10^5 cpm/mL at 60 °C in a hybridization oven. Two low stringency washes were subsequently carried out with 50 mL of 40 mM Na_2HPO_4, 5% SDS, 1 mM EDTA, and 5 mg/mL BSA Fraction V for 10 min each at room temperature. A single high stringency wash was performed at 65 °C for 20 min. using 50 mL of 40 mM Na_2HPO_4, 1% SDS and 1 mM EDTA. Labeled probe was generated using a random hexamer labeling kit (Gibco BRL), 3 μg SFYR35 DNA and 1U

Klenow (MBI Fermentas). The probe was purified over silica beads (Qiagen) and counted in a scintillation counter (Beckman LS 3801).

A 3.2 kb *Bam*H I fragment was isolated by cloning the 3-3.5 kb region from a 1% agarose gel into *E. coli* SURE cells (Stratagene) with *Bam*H I cut and calf intestinal phosphatase (CIP) treated pUC19 to create pUC19-SFYR35g. This plasmid was isolated by colony hybridization using the $\alpha[^{32}P]$-ATP-labeled SFYR35 PCR fragment above. Transformed *E. coli* colonies from six Luria-Bertani plates containing 100 µg/mL ampicillin were transferred to charged nylon filters, dried and soaked in 0.4 M NaOH for 5 min. followed by washes in 1 M Tris/HCl pH 7.5 and 0.5 M Tris/HCl with 1.25 M NaCl pH 7.5. The nylon discs containing the colony DNA was then hybridized with the $\alpha^{32}P$-ATP-labeled SFYR35 PCR fragment in hybridization buffer at 75 °C overnight. Washes were then performed as for Southern blotting above except the high stringency wash was performed at 75 °C. This screening gave rise to one positive colony. The isolated plasmid was then completely sequenced by dye termination methodology at the central facility of the Institute for Molecular Biology and Biotechnology, McMaster University using a nested deletion approach.

Nested deletions were made through linearization and end protection by 3' overhang using a double stranded nested deletion kit (Pharmacia). Briefly, 7 µg of pUC19-SFYR35g was digested with 10 U *Xba* I and 10 U *Pst* I (3'-overhang) 2-4 hrs, heat inactivated at 70 °C for 20 min. followed by exonuclease III digestion. Samples were removed from this digest at 4 min intervals and placed in S1 nuclease and digested a further 30 min at room temperature. Half of these digests were then separated by agarose

gel electrophoresis and the other half recircularized using T_4 DNA ligase. Appropriately sized deleted plasmids were then transformed in *E. coli* SURE cells, restriction mapped and sequenced using the M13 forward sequencing primers. Sequencing was also supplemented using specific primers to fill any gaps in the sequencing data (see Table 4.1). Analysis of the sequencing data for open reading frames was carried out using FramePlot software (generous gift from Mervyn Bibb) and by analysis of protein coding homology using the BLAST algorithm (Altschul *et al* 1990).

4.2.4. Overexpression of StoPK-1 in *Escherichia coli*

The *sto*PK-1 gene was subcloned into the expression vector pET22b using the PCR primers shown in Table 4.1. Complete DNA sequencing verified sequence integrity, and the expression of StoPK-1 was achieved in *E. coli* BL21/pLysS (Novagen). Six histidine residues were also added to the C-terminal end of StoPK-1 using a PCR approach (see Table 4.1) and histidine-tagged genes were utilized for expression and complementation studies. The *sto*PK-1 gene was also subcloned into the plasmid pT7-7 (Tabor and Richardson 1985).

E. coli BL21/pLysS/pET22b-*sto*PK-1 and pT7-7-*stop*K-1 cultures (50 mL, in Luria-Bertani broth) were grown at 30 °C to an absorbance of 0.6 at 595 nm and induced with 1 mM isopropyl β-D-thiogalactopyranoside for 2 hrs. Cells were harvested by low speed centrifugation, washed 3 times in 20 mL of 50 mM HEPES pH 8.0, 1 mM EDTA. Washed cells were resuspended in 5 mL 2× kinase buffer consisting of 100 mM Tris pH 7.5, 100 mM NaCl, 2 mM EDTA, 2 mM dithiothreitol, 10% glycerol and 1 mM freshly prepared phenylmethanesulfonyl fluoride. Cells were immediately lysed by three

passages through a French Pressure Cell at 1100 psi. Cellular debris was removed by centrifugation at 20,000 ×g for 15 min. and supernatant was centrifuged at 100,000 ×g for 1 hr to isolate membranes. The membrane pellet was washed once with 2× kinase buffer and resuspended in the same buffer to a final protein concentration of 5 mg/mL after protein concentration was determined by Bradford protein assay (Bio-Rad).

Table 4.1: PCR and sequencing primers used in this study.

Primer Designation	Sequence (5'→ 3')	Comments
Degenerate PCR Primers		
YF	CACCGIGACCT(C/G)GCI(G/A)CICGIAAC	Tyr kinase forward
YR	A(C/G)(C/G)A(T/C)ICCG(A/T)AIG(A/C)CCAIACGTC	Tyr kinase reverse
SF	ACCGIGAC(G/C)TIAAGC(C/T)I(G/C)AIAAC	Ser/Thr kinase forward
SR	CTCIGGIGCI(C/A)(G/T)GTAI(T/C)(A/C)I(C/G)GIGT	Ser/Thr kinase reverse
SFn	CACCGIGACITIAAGCCI(G/T)(C/G)IAAC	Ser/Thr kinase forward
SRn	TC(G/C)GG(G/C)IAIGTAI(T/G)(A/C)I(A/G(C/G)(C/G)GTICC	Ser/Thr kinase reverse
PCR primers		
AB11416	GGAATTCCATATGGACACGACCCTTCAGGACCCTC	*sto*PK-1 forward
AB11414	GGAATTCAAGCTTCGGTCAGCGGATCTCGATGGTGAT	*sto*PK-1 reverse
AB16708	GGAATTCACCGGTGTTTATCACCACCGACTATTTGC	Am^R forward
AB16709	GGAATTCGACGTCAGCGAGCTGAAGAAAGACAATC	Am^R reverse
AB21460	GCCTGCAGTCTAGATGTCGACCCGGGAGTTCAGTGAC	His Tag forward
AB21461	GCGAATTCAGTGGTGGTGGTGGTGGTGGCGGATCTCGAT GGTGATCGTCG	His Tag reverse
AB21462	*GTTCGTGCACCGG**GCC**ATGAAGCCCGAG	D141A reverse
AB21463	*CTCGGGCTTCAT**GGC**CCGGTGCACGAAC	D141A forward
AB21347	GCTGGAGCATATGCGACCCGGCGTT	*pka*F forward
AB21348	GATGCGGAGATCTTGTCGGCACCAAGGATGCCA	*pka*F reverse
AB26028	GTCTAGAAGCTTGTGCGGCACGCCTACAGCGA	3' *pka*F forward
AB26029	GGCTGCAGAATTCGGATCAGCGAATCGTGATGGTG	3' *pka*F reverse
Sequencing primers		
AB11330	CGGCGGGCACGGAGTC	*sto*PK-1
AB11331	GGTGTGGGAGCCGAAGTTGA	*sto*PK-1
AB12928	CGGTGGACTCGGTGACGAACAC	*sto*PK-1
AB25927	TCGCCGCGGAGCACGACAAC	*pka*F
AB25928	TCTGCTTGGCGTCGTCCACG	*pka*F
AB25956	GGGCGGCCTGAAGGTGAAG	*pka*F

* - Asp141Ala codon in bold.

4.2.5. StoPK-1 Histidine Tagged Western Blots

Isolated membrane fractions from overexpressed StoPK-1 proteins including wild type and histidine fusion proteins were applied and separated on 10% SDS-polyacrylamide gels (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. Transfer of proteins to PVDF membranes was accomplished at 70V for 50 min. in a transfer buffer consisting of 25 mM Tris, 15% methanol and 192 mM glycine. Western Blots were then carried out with primary mouse IgG2a anti-His Ab according to the manufacturer's instructions (Amersham Pharmacia). Blots were then probed with donkey anti-mouse HRP conjugated secondary Ab (1/5000 dilution, Jackson Immunological) and developed with HRP chemiluminescent substrate (Pierce, Rockford Il.). Blots were exposed to Kodak X-OMAT film for 5-30 s to visualize labeled proteins.

4.2.6. StoPK-1 Autophosphorylation, Substrate and Inhibitor studies

^{32}P-Labeling of StoPK-1 was carried out in the presence of 5 μg protein extract with 10 μCi [γ-^{32}P]-ATP, 10 mM $MgCl_2$, 2 mM $MnCl_2$, in a volume of 10 μL and incubated at room temperature for 10 min. The reaction was stopped by the addition of 10 μL of 1 mM DTT, 4% SDS, 2 mM EDTA, 100 mM Tris pH 8.8, 10% glycerol, 0.01% bromophenol blue, applied to an 11% SDS-PAGE gel and separated by electrophoresis and exposed to Kodak X-OMAT film for 2-3 hrs.

Proteins were then transferred to polyvinylidene difluoride membranes and the position of ^{32}P-labeled bands determined by exposure to X-Ray film (Kodak X-OMAT film). ^{32}P-Labeled proteins were excised and placed in 500 μL 6 M HCl, heated to 110 °C

for 60 min, dried under vacuum and the residue resuspended in 10 μL distilled water and 5 μL spotted onto 20 cm × 20 cm glass-backed cellulose thin layer plates (Merck) with 5 μL of 1 mM phosphoamino acid standards. Two-dimensional electrophoresis was then performed as described elsewhere (Ausubel *et al* 1994).

The ability of StoPK-1 to transfer phosphate to various substrates was examined using a phosphocellulose paper radioassay (Glass *et al* 1978). Substrates tested (33 μM – 2.5 mM) included kemptide (33 μM), myelin basic protein (MBP) (33 μM), CREBtide (33 μM), Histone H1 (33 μM), MARCKS (2.5 mM), casein (1 mg/mL), $p60^{SRC}$ substrate II (33 μM), and polyGluTyr (1 mg/mL). Substrates were incubated at room temperature for 5 min. in a total reaction volume of 10 μL containing 5 μg protein extract, 10 μM ATP (1.7 μCi [γ-^{32}P]-ATP per reaction), and 10 mM $MgCl_2$. Reactions carried out in triplicate, were stopped with 3 μL glacial acetic acid and applied to phosphocellulose paper strips (Whatmann P81). Strips were allowed to dry and then washed in 300 mL 30 % acetic acid for 15 min and twice in 15% acetic acid followed by a single 5 min. rinse in acetone. Dried papers were then immersed in 3 mL scintillation fluid (ACS, Amersham) and Cherenkov radiation counted (Beckman LS 3801). Results were expressed as a percentage of the control counts per min. (StoPK-1 autophosphorylation) after subtracting the background ([γ-^{32}P]-ATP + MBP).

MBP appeared to be highly phosphorylated and further inhibitor studies were carried out utilizing this substrate. The effects of prospective inhibitors of StoPK-1 kinase activity were examined using 11% SDS-polyacrylamide gels. In this assay ^{32}P-labeled MBP was visualized with X-OMAT film. The potential inhibitors screened included

genistein (100 μM), roscovitine (140 μM), staurosporine (20 μM), apigenin (800 μM) and erbstatin analog (260 μM). In addition, IC_{50} values were determined for compounds showing significant inhibition of phosphotransfer by using direct scintillation counting of excised ^{32}P-MBP.

4.2.7. Disruption of *sto*PK-1

A pBluescript KS+-derived vector pBlutsr was constructed specifically for *Streptomyces* disruption studies (Fig. 4.1). pBlutsr carries thiostrepton resistance (*tsr*) so that single and double cross-over events can be differentiated when a second resistance cassette is used to disrupt a gene of choice. pBlutsr was constructed by using the unique *Afl* III site in pBluescript KS+ (Stratagene) to create a *Pst* I compatible *Mph* 1103I site. A *Pst* I fragment containing thiostrepton resistance cassette (containing *tsr*) from pIJ702 (Hopwood *et al* 1985) was then inserted into this new site creating pBlutsr.

*sto*PK-1 gene disruptions were carried out using pBlutsr-SFYR35g-Am. The disruption plasmid was created by cloning a 3.2 kb *Bam*H I genomic fragment containing *sto*PK-1 into pBlutsr at the *Bam*H I site. Disruption of *sto*PK-1 was achieved by insertion of the PCR amplified apramycin resistance cassette (Am) from pOJ260 (Bierman *et al.*, 1992). The 1 kb fragment Am fragment was generated with specific PCR primers containing *Age* I and *Aat* II restriction sites engineered into the 5' ends (Table 4.1). Using these sites, the amplified fragment was inserted into the 3.2 kb *Bam*H I genomic fragment between the unique *Aat* II and *Age* I sites (Fig. 4.1).

S. toyocaensis protoplasts were prepared as described previously (Matsushima and Baltz 1996). Vector constructs were first transformed into *E. coli* GM48, a *dcm*-,

dam- cell line. Transformation of *S. toyocaensis* protoplasts was carried out using alkaline denatured plasmid DNA (up to 3 μg) as described by Oh and Chater (1997). Regeneration of transformed protoplasts was allowed to take place overnight after which appropriate amounts of antibiotics were overlaid in 500 μL of TSB and individual colonies were picked after 3-4 days. Generation of the *sto*PK-1::Am mutant was confirmed by PCR and Southern analyses using a [α^{32}P]-ATP labeled full length *sto*PK-1 probe (Ausubel *et al* 1994).

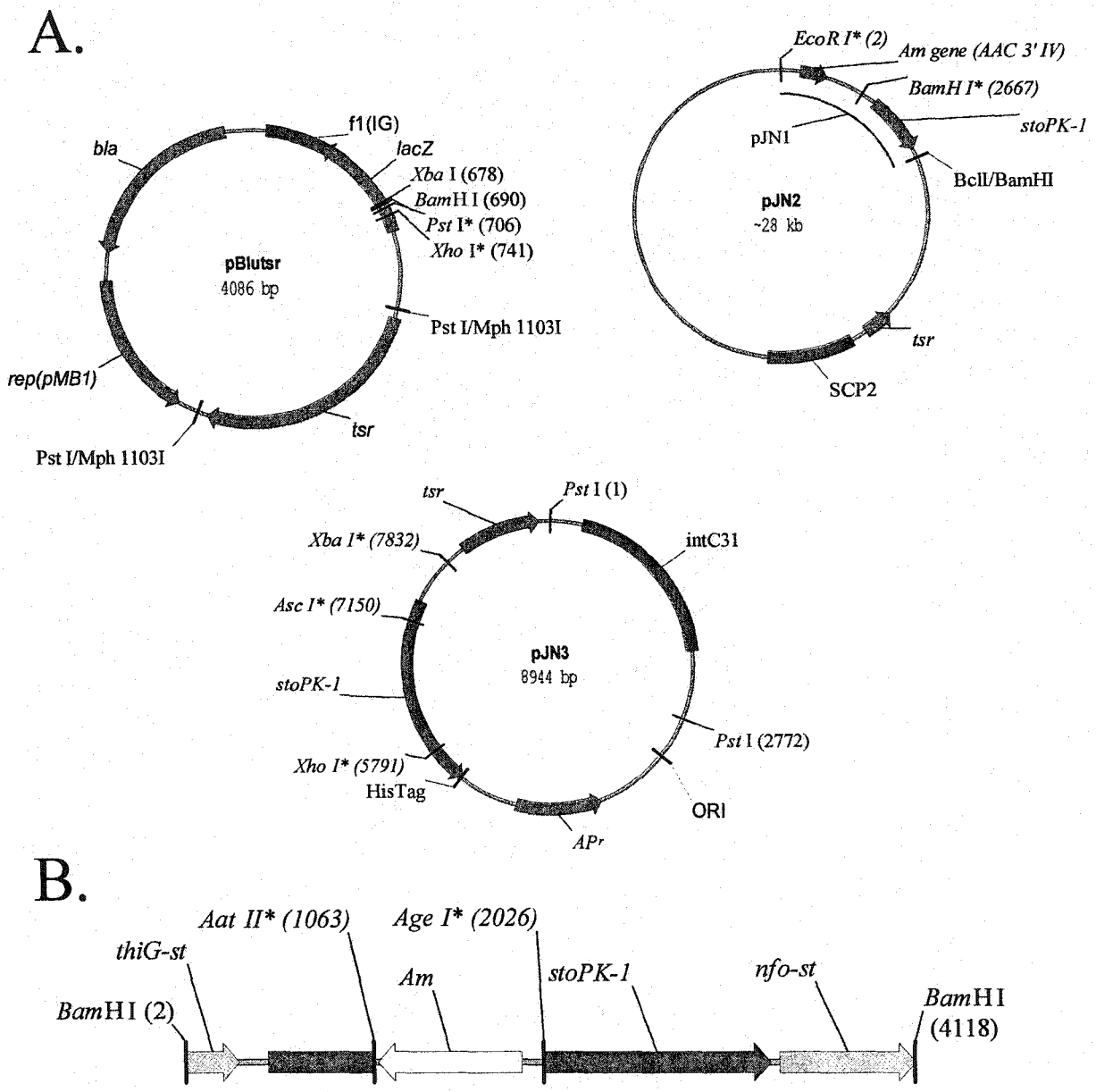


Figure 4.1: Diagram of plasmids and constructs used in this study. **(A)** Streptomyces gene disruption plasmid pBlutsr and *sto*PK-1 complementation plasmids pJN2 and pJN3. Note: pJN4 is identical to pJN3 but contains a Asp141Ala mutation in the *sto*PK-1 gene. **(B)** Arrangement of the insertional disruption of *sto*PK-1 using the apramycin resistance gene (Am).

4.2.8 Complementation of *sto*PK-1

*sto*PK-1 was provided in *trans* on the low copy plasmid pIJ922 (Hopwood *et al* 1985), which was derived from the plasmid pJRM10 (McCormick *et al* 1994). The 3.2 kb genomic *Bam*H I fragment containing *sto*PK-1 was first subcloned into pOJ260 which contains an apramycin resistance marker (Bierman *et al* 1992). The resulting plasmid, pJN1, was digested with *Eco*R I and *Bcl* I and ligated to pIJ922 at *Eco*R I and *Bam*H I creating pJN2 (Fig. 4.1). The resulting *E. coli/Streptomyces* shuttle vector only contained the *sto*PK-1 gene from the original genomic clone, including 467 bp upstream from the predicted start site. pJN2 was subsequently used to transform protoplasts of *sto*PK-1 disruption mutants. This strategy was repeated for a C-terminal hexa-histidine tagged version of *sto*PK-1 which was placed in an integration vector constructed from the *Pst* I fragment from pSET152 containing the ϕC31 integrase (Bierman *et al* 1992) and pUC19 containing *sto*PK-1HisTag creating pJN3 (Fig. 4.1). An Asp131Ala mutation was then made in this construct by a three way ligation with two separate PCR products creating the plasmid pJN4. This ligation took advantage of the presence of a unique *Alw* 44I site 3 bp upstream of the catalytic aspartate. Thus the ligation was carried out between the unique 5' *Asc* I to the *Alw* 44I site and the *Alw* 44I site to the unique 3' *Xho* I site. Complete sequencing of the gene was used to confirm the fidelity of each construct.

4.2.9. Complementation of *sto*PK-1 using *pka*F

In addition to *sto*PK-1, we provided the *S. coelicolor pka*F gene *in trans* in an attempt to complement the disruption phenotype. In order to eliminate upsteam promoter

differences, we fused the *sto*PK-1 upstream region to the *pka*F gene at the 5' *Kpn* I site. (The translated gene products of *sto*PK-1 and *pka*F are identical from the putative start sites to the *Kpn* I site). A PCR fragment containing the *pka*F gene was kindly provided by Justin Nodwell in the plasmid pCRScript (Stratagene). However, sequencing of this construct revealed a single mutation in the 3' end of *pka*F. In order to circumvent this error, we first subcloned the *Sac* II/*Kpn* I fragment from this construct into pUC19-SFYR35g to generate the *sto*PK-1-*pka*F fusion plasmid pUC19kinF*35gp. The 3'end of *pka*F was then amplified by PCR from *S. coelicolor* genomic DNA and cloned using the pCR4Blunt-TOPO cloning system (Invitrogen). After confirmation of the fidelity of this construct by sequencing, the 641 bp *Bsp* EI/*Eco* RI 3' fragment was subcloned into pUC19kinF*35gp effectively replacing the 3' end of the *pka*F gene. The resulting plasmid pUC19kinF35gp was then ligated to pJN4 utilizing the unique *Sca* I and *Xba* I sites to create the integrating complementation plasmid pJN5 (Figure 4.2).

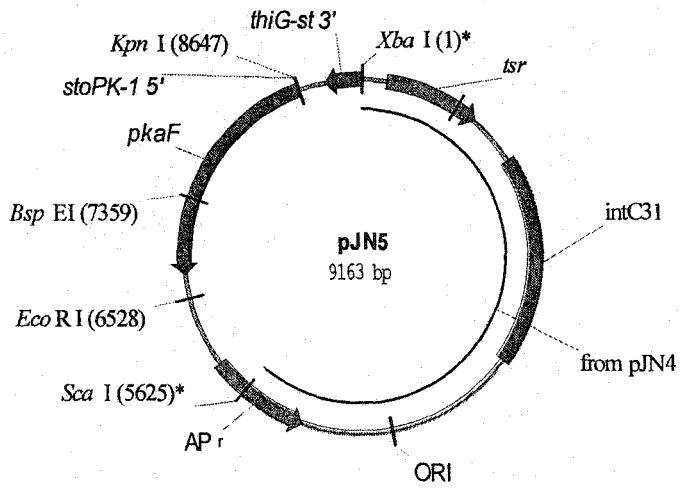


Figure 4.2: Construction of pJN5 complementation vector containing *sto*PK-1 promoter–*pka*F fusion. For construction details see text.

4.2.10. *sto*PK-1::Am Phenotype Characterization

Wild type *S. toyocaensis* and *stoPK-1*::Am disruption strains were examined for A47934 production, resistance to A47934 glycopeptide and sporulation essentially as describe previously (Neu and Wright 2001). Carbon source utilization was examined on agar plates supplemented with 1% glucose, maltose, lactose or sorbitol and 1.5% soytone. Spores were plated (100 μL of 10^3 -10^4 cfu/mL) and colony growth examined after 3-4 days. Colony surface area (SA) was measured from scanned photographs taken with a Nikon 810S SLR camera fitted with a 60 mm micro lens. Electronic images were analyzed for colony areas by counting the number pixels occupied by a particular colony. The average surface area of ten randomly picked colonies for each plate was then compared directly between strains and difference examined using a paired student's T-test.

4.2.11. Chemical Gradient Plates

The effects of oxygen radical generators such as paraquat, nitrofurantoin, *t*-butylhydroperoxide and hydrogen peroxide were assessed on gradient plates consisting of TSB with and without glucose as solid medium. The highest concentrations of chemicals in the plates were: paraquat (400 μg/mL without glucose and 1.5 mg/mL with glucose), nitrofurantoin (4 μg/mL without glucose and 10 μg/mL with glucose), *t*-butylhydroperoxide (1.5 mM without glucose) and hydrogen peroxide (10 mM without glucose). Square 9 x 9 cm gradient plates were prepared according to Cunningham *et al* (1986) and 100 μL of spore suspension (1×10^6 cfu/mL) was transferred to the edge of a

8.5 cm glass plate and touched to the surface of the TSB agar across the chemical gradient. Plates were incubated at 37°C and growth examined after 3-4 days. Disc assays were also performed on TSB supplemented with 2% glucose by placing 15 µL of the appropriate concentrations of paraquat (35 – 250 mg/mL), H_2O_2 (8.8 – 880 mM), nitrofurantoin (0.1 – 10 mg/mL), menadione (1 -100 mg/mL), sodium nitrite (10 – 400 mM) and diamide (10 – 500 mM) on a 7 mm filter paper disc on a 100 µL lawn of spores (~10^8 cfu/mL) each *S. toyocaensis* strain. Zones of inhibition were compared after 3-4 days growth at 37 °C.

4.2.12. Survival curves

Spore suspensions were diluted in Luria-Bertani broth to $3x10^3$ – $1x10^4$ CFU/mL on TSB agar. Spores were plated on TSB agar with and without 2% glucose and increasing concentrations of oxidizing agent. Colonies were counted and colony numbers expressed as a percentage of control plates following incubation at 37 °C for 3-4 days. Reductions in colony numbers on paraquat or nitrofurantoin were plotted using Grafit (ver 4.0.10, Erithacus Software Limited) and IC_{50} values estimated from fitting data to the curve $y = 100/(1+(X/IC_{50})^S)$ and differences examined using a paired student's T-test.

4.2.13. StoPK-1 expression in *S. toyocaensis*

4.2.13.1. Northern Analysis

In order to examine StoPK-1 expression in *S. toyocaensis*, attempts were made to carry out Northern blots on RNA isolated from the wild type, *sto*PK-1 disruption and

complementation strains. For Northern analysis, total RNA was isolated according to Hopwood *et al* 1985. *S. toyocaensis* spores were seeded into a defined vegetative medium with appropriate antibiotics as described previously (Marshall and Wright 1996). Cells were grown for 48 hrs at 30°C and 225 rpm and 100 µL subsequently inoculated into spring baffled 50 mL flasks containing 15 mL of TSB with and without 2% glucose and 250 µg/mL paraquat for *sto*PK-1::Am strains and 500 µg/mL paraquat for wild type and pJN3 complementation strains. Cultures were harvested by centrifugation at 4 °C at various time points and washed once in chilled 0.4% DEPC treated distilled water. Mycelia was then transferred to 20 mL screw cap universal bottles containing 5 mL of Kirby mixture which contained 1% sodium triisopropylnaphthalene sulphonate (Eastman), 6% sodium 4-amino salicilate, 6% phenol mixture (500 g phenol (Sigma) and 0.5 g 8-hydroxyqinolone equilibrated with 50 mM Tris-HCl pH 8.3) all combined in 50 mM Tris-HCl pH 8.3.

Approximately 14 g of 0.5 mm glass beads (Biospec) were then added to the mycelium/Kirby mixture and the bottle contents mixed vigorously for 2 min on a vortex mixer. 5 mL of phenol/chloroform mixture (phenol mixture:chloroform:isoamyl alcohol, 50:50:1) was then added and the bottle contents mixed vigorously a further 1 min. The homogenate was then transferred to a 50 mL polypropylene tube and centrifuged at 5,000 ×g for 5 min at 4 °C. The upper aqueous phase was carefully removed and transferred to a fresh 50 mL polypropylene tube containing 5 mL of phenol/chloroform mixture, mixed and phases separated as described above. This extraction was then repeated again after which RNA was precipitated by adding 1/10 volume of 3 M sodium acetate (pH 6) and

an equal volume of isopropanol, and incubated at room temperature for 10 min.. Nucleic acids were pelleted at 7,000 ×g for 10 min. and washed with 5 mL 95% ethanol. The pellet was dried and resuspended in 500 µL of 0.4% DEPC treated distilled water. A sample (50 µL) was removed for Northern analysis (see below).

In order to obtain DNA free RNA, 45 µL of 10× DNase buffer was combined with 450 µL total nucleic acids and 70 U RNase free DNase I (Boehringer) and incubated 1 hr at room temperature. The mixture was extracted once with phenol/chloroform mixture, and precipitated with 50 µL 3 M sodium acetate (pH 6) and 500 µL isopropanol in a microcentrifuge. The purified RNA was then washed with 1 mL of 80% ethanol, dried and resuspended in 100 µL of 0.4% DEPC treated distilled water. RNA quantity and quality was assessed by spectrophotometry (Abs 280/260) on a Cary 3E.

Purified RNA was prepared for Northern analysis by mixing 11 µL of RNA (10-15 µg) with 5 µL 10X MOPS running buffer (0.4 M MOPS, 0.1 M sodium acetate, 0.01 M EDTA pH to 7.0), 9 µL 12.3 M formaldehyde, 25 µL formamide and 0.02% bromophenol blue) and heated for 15 min at 55 °C. Duplicate sample were examined on 1% agarose/formaldehyde gels containing 1× MOPS running buffer and 2.2 M formaldehyde. Gels were run at 5 V/cm in 1× MOPS until the dye front was ½ to ¾ the gel length. Removal of formaldehyde from the portion of gel to be stained was carried out by two 20 min. washes in 0.5 M ammonium acetate. Gels were then stained with 0.5 µg/mL ethidium bromide in 0.5 M ammonium acetate for 40 min.

RNA was transferred to Hybond N+ charged nylon (Amersham Pharmacia) from the duplicate gels after 3 washes in 0.4% DEPC treated distilled water followed by

soaking in 10 gel volumes of 20× saline-sodium citrate buffer (SSC, 3 M NaCl + 0.3 M Sodium Citrate pH 7.4) for 45 min.. Transfer was carried out by standard capillary transfer in 20× SSC overnight (Ausubel *et al* 1994). In some cases, 1μL of pUC19-SFYR35g containing the intact *sto*PK-1 gene was mixed with 1 μL 0.8 M NaOH and spotted in the corner of the blot as a control.

RNA on dried membranes were immobilized using UV light (auto setting, UV Statalinker 1800, Stratagene) and prepared for hybridization by wetting in 6× SSC. Membranes were then transferred to hybridization tubes containing 1 mL hybridization solution (5× SSC, 5× Denhardt's, 50% formamide and 1% SDS) per cm^2 of membrane. Hybridization tubes were transferred to 50 °C hybridization oven for 1 hr for prehybridization. Hybridization was carried out with fresh hybridization buffer and the denatured (10 min at 100 °C) ^{32}P-labelled probe was added to 1×10^6 cpm/mL. Labelled probe was generated using a random hexamer labelling kit (Gibco BRL), 10 μCi [α^{32}P]-ATP, 3 μg of the 3' end of PCR-generated *sto*PK-1 DNA and 1U Klenow (MBI Fermentas). The PCR reaction was carried out with Taq polymerase (1.5 U) over 30 cycles with 1.5 pmol primers (AB21460 and AB21461, Table 4.1), 500 μM dNTPs, 2.5 mM $MgCl_2$, and 5% DMSO in a total volume of 100 μL. ^{32}P-labeled probe was purified over silica beads (Qiagen) and counted in a scintillation counter (Beckman LS 3801).

Hybridization was carried out overnight at 50 °C and membranes were washed twice for 5 min. each at room temperature with 0.2 × SSC and 0.1% SDS. Two further high stringency washes were carried out at 68 °C for 15 min each using 0.1 × SSC and

0.1% SDS. A final rinse was then performed with 2 × SSC and the blot exposed to Kodak X-OMAT film for up to 24 hrs.

4.2.13.2. Reverse Transcriptase PCR

We also examined the expression of *sto*PK-1 in the pJN3 complementation strain utilizing a RT-PCR based approach. Total RNA isolated as in Northern analysis (see above section 4.2.13.1), was subject to PCR using the Qiagen One Step RT-PCR kit. Up to 10 μg of total RNA was subject to reverse transcription and PCR following the manufacturer's instructions. 0.6 μM of each primer was used to amplify the 3' end of the *sto*PK-1 gene (AB21460 and AB21461, Table 1). In order to remove any contaminating genomic DNA total RNA was incubated 1 hr. and overnight with DNase I (see section 4.2.13.1). Results were visualized on 1% agarose gels after ethidium bromide staining.

4.2.13.3. Histidine Tag Western Blots

In addition, we also examined StoPK-1 protein expression in *sto*PK-1 disruption and complementation strains using anti histidine tag antibodies. Membrane fractions from each strain under oxidative stress conditions (section 4.2.13.1) were isolated in the same fashion as for *E. coli* (see section 4.2.4). Western blots were carried out as described in section 4.2.5.

4.2.14. Glucose 6-Phosphate Dehydrogenase Levels in *S. toyocaensis*

Glucose 6-phosphate dehydrogenase (G6PDH) levels were monitored in crude cell free extracts using gel visualization (zymograms). Cell free extracts were prepared from

cells lysed in a French Pressure cell. Briefly, cells were harvested by low speed centrifugation and washed 3 times in 20 mL of 100 mM Tris pH 7.5. Washed cells were resuspended in 5 mL of 100 mM Tris pH 7.5, 100 mM NaCl, 1 mM DTT, 5% glycerol and 1 mM freshly prepared phenylmethanesulfonyl fluoride. Cells were immediately lysed by three passages through a French Pressure Cell at 1100 psi. Cellular debris was removed by centrifugation at 20,000 ×g for 15 min. Native 5% polyacrylamide gels (1 mM DTT, 50 mM Tris, 50 mM tricine, pH 7.4) were electrophoresed at 75V and equilibrated in reaction buffer for 15 min (100 mM Tris (pH 7.2), 100 mM NaCl, 1 mM DTT, 5 mM $MgCl_2$, 10 mM glucose 6-phosphate and 1 mM $NADP^+$. Gels were stained in 0.2 mM phenazine methosulfate (PMS) and 0.2 mM nitroblue tetrazolium (NBT) (Lessmann *et al* 1975). Activity levels were also monitored by following production of NADPH at 340 nm in a 1 mL reaction containing 1 mM glucose-6-phosphate, 0.4 mM NADP+, and 0.3-0.4 mg crude cell extract on a Cary 3E spectrophotometer (Ma *et al* 1998). G6PDH from *Leuconostoc mesenteroides* (Roche) was used as a control in each experiment.

4.3. Results

4.3.1. Genetic Organization of *sto*PK-1

Southern blots of restriction digested *S. toyocaensis* genomic DNA using the radio-labelled *sto*PK-1 degenerate PCR fragment hybridized to a 3-3.5 kb *Bam*H I fragment (Fig. 4.3). Cloning of this region revealed the entire *sto*PK-1 sequence (Fig. 4.4). The *sto*PK-1 gene is 1929 bp in length, encoding a predicted 642 amino acid

protein. The *S. toyocaensis* StoPK-1 protein is 87 % identical to PkaF from *S. coelicolor* A3(2) (Ogawara *et al* 1999), and lacks a 14 amino acid insert found in PkaF at positions 326 and 340. Furthermore, the genes flanking *stoPK-1* in *S. toyocaensis*, *thi*G and *nfo* (endonuclease IV), also flank *pka*F in *S. coelicolor* A3(2) (Fig. 4.5A). In addition, both *S. coelicolor* A3(2) orthologues share high homology (88% identity at the nucleotide level) to their *S. toyocaensis* counterparts.

The protein kinase domain of *sto*PK-1 is located in the N-terminus with regions homologous to all 11 Hanks consensus sequences (Fig. 4.4). The protein has a predicted membrane spanning sequence (Hofmann and Stoffel 1993) from amino acid 355-372 that divides the protein into N-terminal and C-terminal domains. A predicted coiled-coil region often associated with protein/protein interactions, is located between Asp518 to Ala551 in the C-terminal domain (Berger *et al* 1995).

Analysis of the nucleotide sequence upstream from the putative *sto*PK-1 translational start site revealed similarities with known *Streptomyces* promoter regions and gene fragments. Homology searches with this +1 to –50 region showed strong similarity (>50%) to the promoter regions in *nsh*P which encodes a 23S RNA methylase (Li *et al* 1990). Both regions contain a putative Class A element (Bourn and Babb 1995) and a –35 region similar to the *galP1* –35 region, which may be recognized by an alternative sigma factor (Brawner *et* al 1997). In addition, homology to a fragment of *S. coelicolor* genomic DNA (cosmid F12) containing a putative glucose-6-phosphate dehydrogenase and upstream of a D-3-phosphoglycerate dehydrogenase was also noted (shown in Fig. 4.5B).

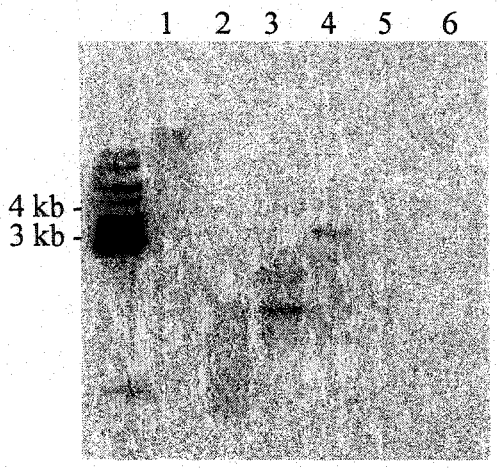


Figure 4.3: Southern blot of S. toyocaensis genomic DNA digested with (1) *Hin* d III (2) *Kpn* I (3) *Sal* I (4) *Bam*H I (5) *Sac* I (6) *Nde* I.

4.3.2. Overexpression of StoPK-1

4.3.2.1. Localization of stoPK-1 to Membrane Fractions

Expression of StoPK-1 in *E. coli* generated a protein of the expected size (68 kDa) in cell free extracts. Following cell fractionation, StoPK-1 was found to be restricted to the membrane fraction only, consistent with the prediction of transmembrane topology from its primary sequence. Incubation of membrane extracts with $\gamma[^{32}P]$-ATP revealed that StoPK-1 was an active protein kinase with autophosphorylation activity (Fig. 4.6A). Phosphoamino acid analysis of ^{32}P-labeled *sto*PK-1 revealed only Thr phosphorylation (Fig. 4.6B).

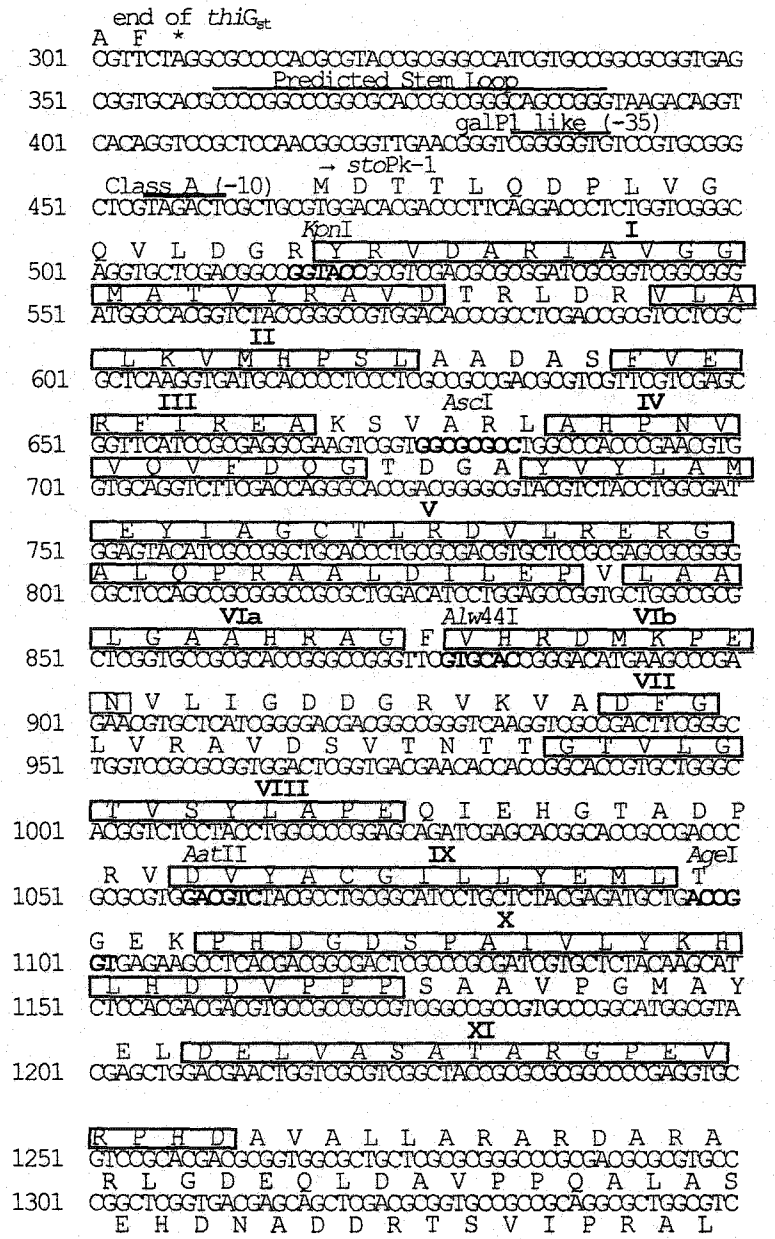
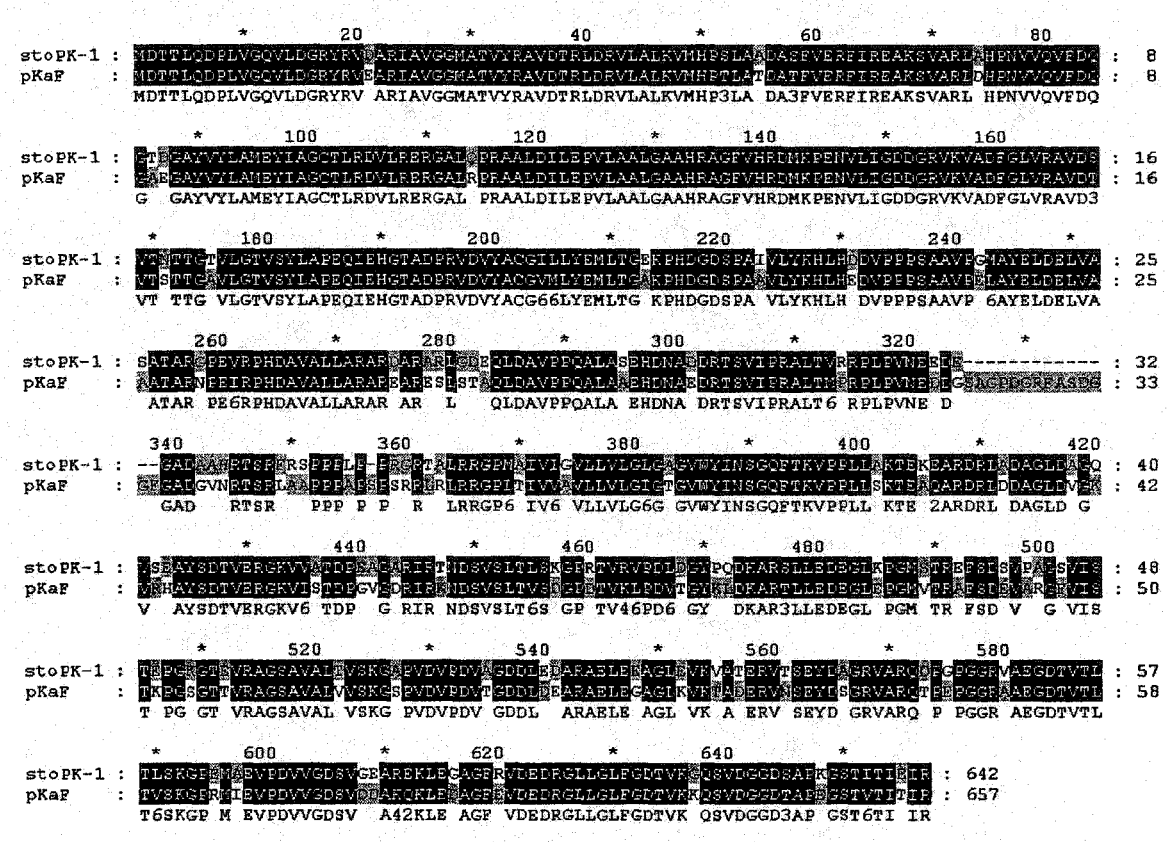


Figure 4.4: Arrangement of the 3.2 kb genomic *Bam*HI genomic fragment cloned from *S. toyocaensis* NRRL 15009 showing translation and key restriction sites. Hanks consensus sequences and putative coiled-coil regions are boxed. The predicted transmembrane domain is underlined, and putative promoter elements are highlighted.

A.



B.

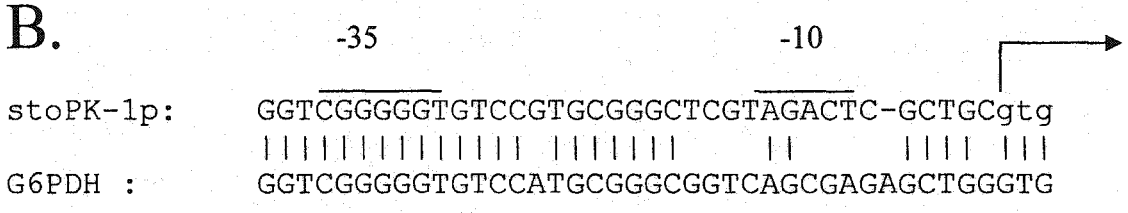


Figure 4.5: StoPK-1 sequence homology. **(A)** Protein homology between StoPK-1 and PkaF from *S. coelicolor* cosmid 6E10. **(B)** *sto*PK-1 upstream DNA homology with *Streptomyces coelicolor* glucose 6-phosphate dehydrogenase (G6PDH) DNA fragment (arrow show putative translational start site).

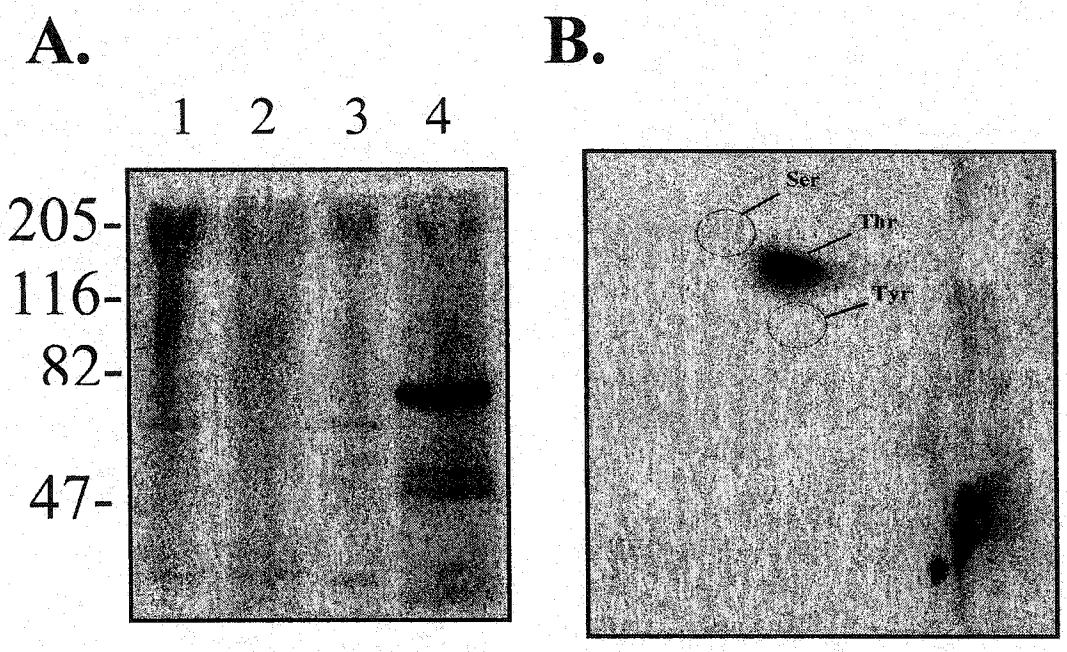


Figure 4.6: Autophosphorylation of StoPK-1. **(A)** ^{32}P-ATP-labeling of cytosolic (Lanes 1 and 3) and membrane fractions (Lanes 2 and 4) from *E. coli* BL21 (DE3) pLysS-pET22b-*sto*PK-1 reveals expression of a phosphoprotein of 68 kDa only in IPTG induced cells (Lanes 3 and 4), and specifically only in the membrane fraction (Lane 4). Uninduced fractions did not show the presence of this phosphoprotein (Lanes 1 and 2). **(B)** Phosphoamino acid analysis of the 68 kDa phosphoprotein in (A) showing phosphorylation exclusively on threonine residues.

4.3.2.2 Substrate and Inhibitor Studies

A survey of protein kinase substrates frequently used in analysis of eukaryotic kinases, demonstrated that StoPK-1 catalyzed phosphoryltransfer to myelin basic protein (MBP) and Histone H1 (Fig. 4.7). Phosphoryltransfer to MBP could be inhibited by apigenin, a flavanoid that is an established inhibitor of MAP kinase (Fig. 4.8A). In these assays the calculated IC_{50} value of 60 μM was approximately 10-fold more potent than the inhibition observed with the apigenin regioisomer genistein (Fig. 4.9). No apparent effect was seen with roscovitine, staurosporine and an erbstatin analog (Methyl 2,5-

Dihydroxycinnamate 2,5-Dihydroxycinnamic Acid Methyl Ester). Phosphoamino acid analysis of MBP and Histone H1 phosphorylation revealed phosphotransfer occurred primarily on Thr for Histone H1 and on Ser and Thr residues for MBP (Fig. 4.8B and 4.8C).

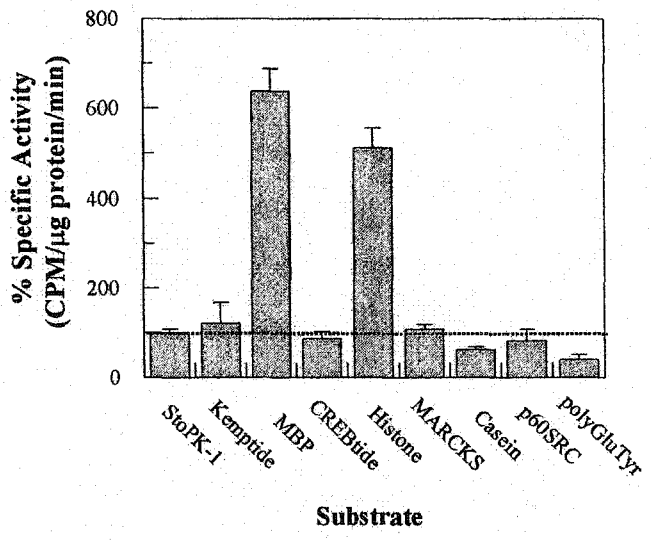


Figure 4.7: StoPK-1 phosphorylates MBP and Histone H1 *in vitro* and is inhibited by apigenin. Examination of phosphotransfer by phosphocelluloase paper radioassay reveals high levels of MBP and Histone H1 phosphorylation by StoPK-1. For methods see section 4.2.6.

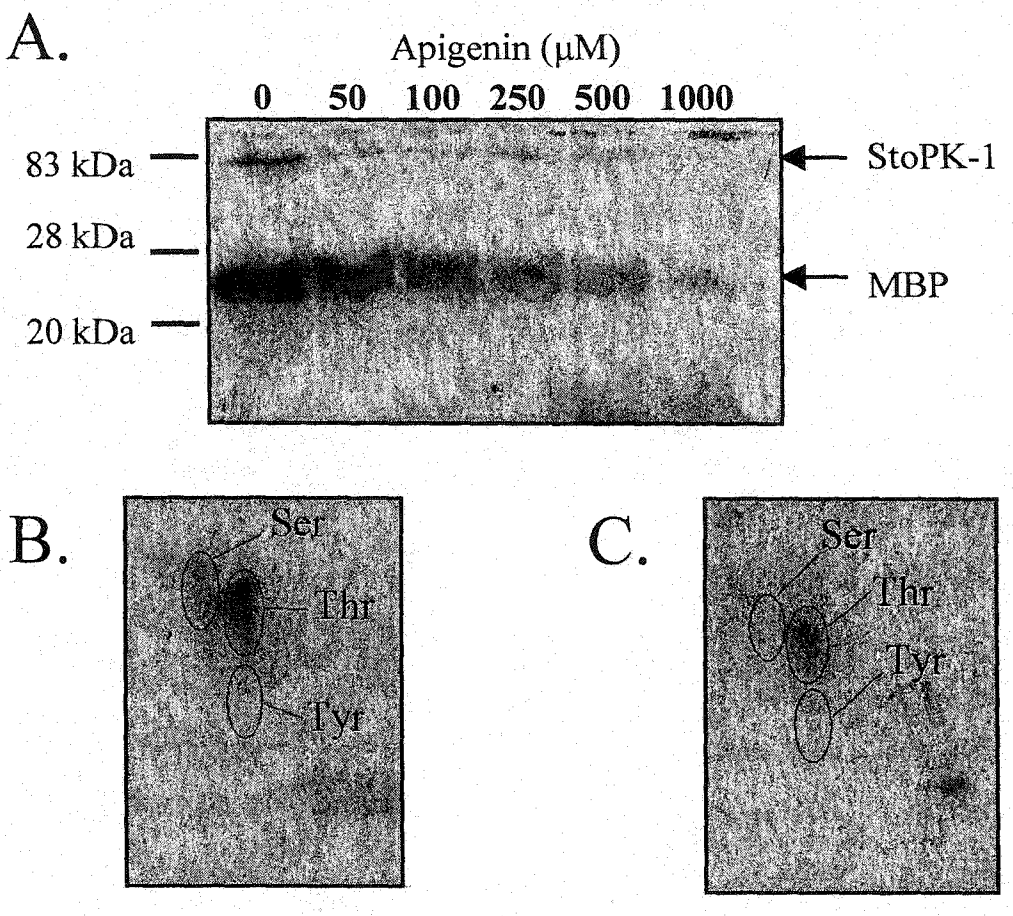


Figure 4.8: (A) StoPK-1 phosphorylation of MBP is inhibited by apigenin (IC_{50} = 60 μM). **(B)** Phosphoamino acid analysis of phosphorylated Histone H1 reveals transfer to threonine residues. **(C)** Phosphoamino acid analysis of phosphorylated MBP reveals transfer to threonine and serine residues.. For methods see section 4.2.6.

4.3.2.3 Hexa-histidine Tagged StoPK-1 Studies

Examination of autophosphorylation of *E. coli*-expressed hexa-histidine tagged *sto*PK-1 showed that the kinase activity was not effected by the extra C-terminal histidines (Fig. 4.10, lane 1) and the Asp141Ala mutant was incapable of autophosphorylation as predicted (Fig. 4.10, lane 2). These were the same protein fusions used for complementation of the *S. toyocaensis* disruption mutant with the plasmids pJN3

and pJN4 (see below). Western blots of the *E. coli*-expressed proteins using anti-his Ab showed the presence of mutant Asp141Ala *sto*PK-1 confirming the lack of kinase activity (Fig. 4.10, lane 4).

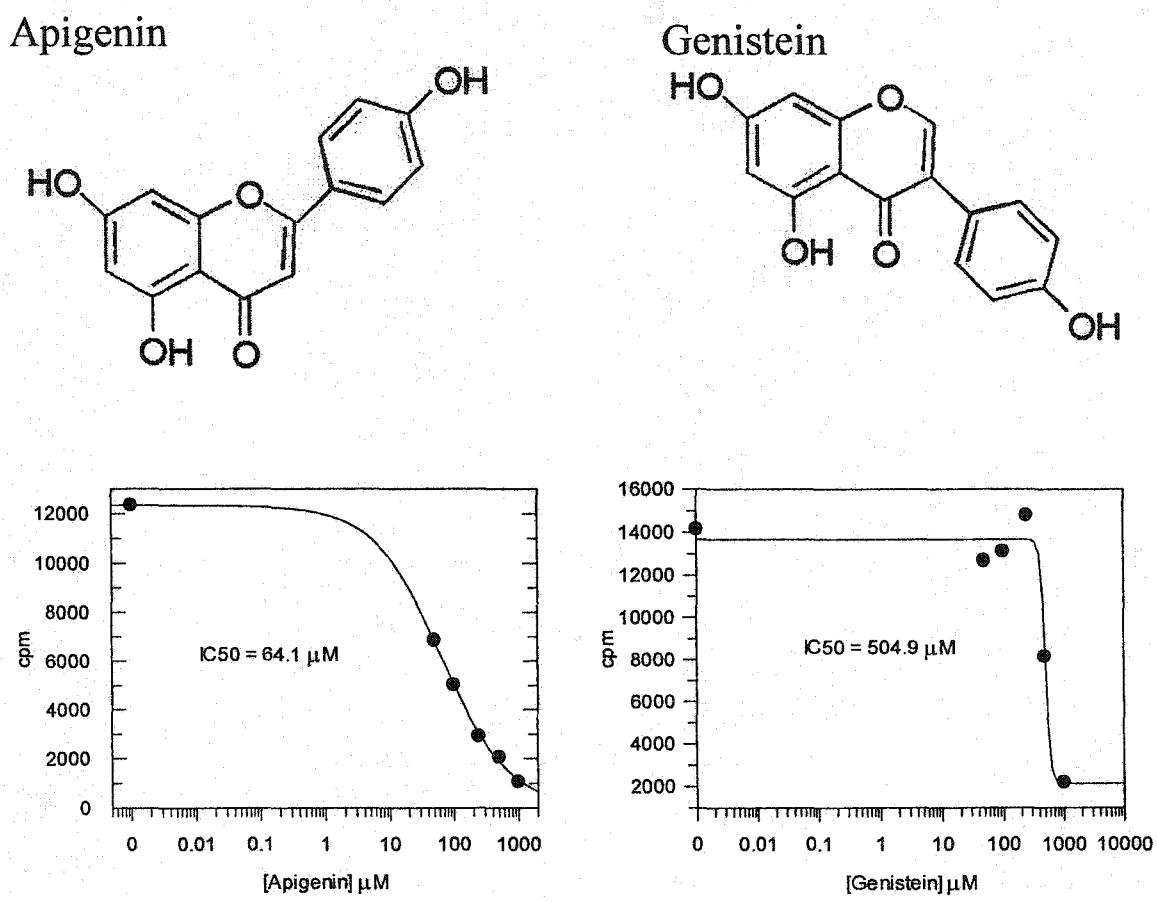


Figure 4.9: Structure of regeoisomers apigenin and genistein and their effects on transfer of ^{32}P from StoPK-1 to myelin basic protein.

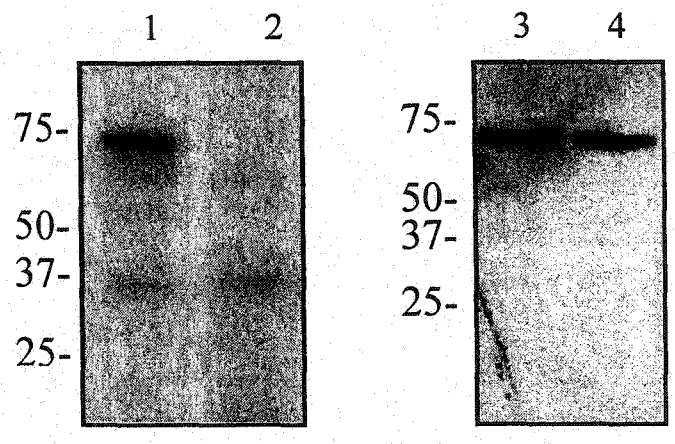


Figure 4.10: ^{32}P-ATP-labeling of histidine tagged StoPK-1 membrane proteins showing autophosphorylation of histidine tagged wild type StoPK-1 (Lane 1), and lack of autophosphorylation on the Asp131Ala histidine tagged mutant (Lane 2). Anti-His Western blots confirm the presence of the Asp141Ala mutant. Lane 3: wild type histidine fusion and Lane 4: Asp141Ala histidine fusion.

4.3.3. Disruption and Complementation of stopK-1 in S. toyocaensis

4.3.3.1. Confirmation of Genotypes

The *sto*PK-1 gene in *S. toyocaensis* NRRL 15009 was insertionally inactivated by partial gene replacement with the apramycin resistance gene. Introduction of the antibiotic resistance gene within *sto*PK-1 was confirmed by PCR and Southern analyses (Figure 4.11A and B).

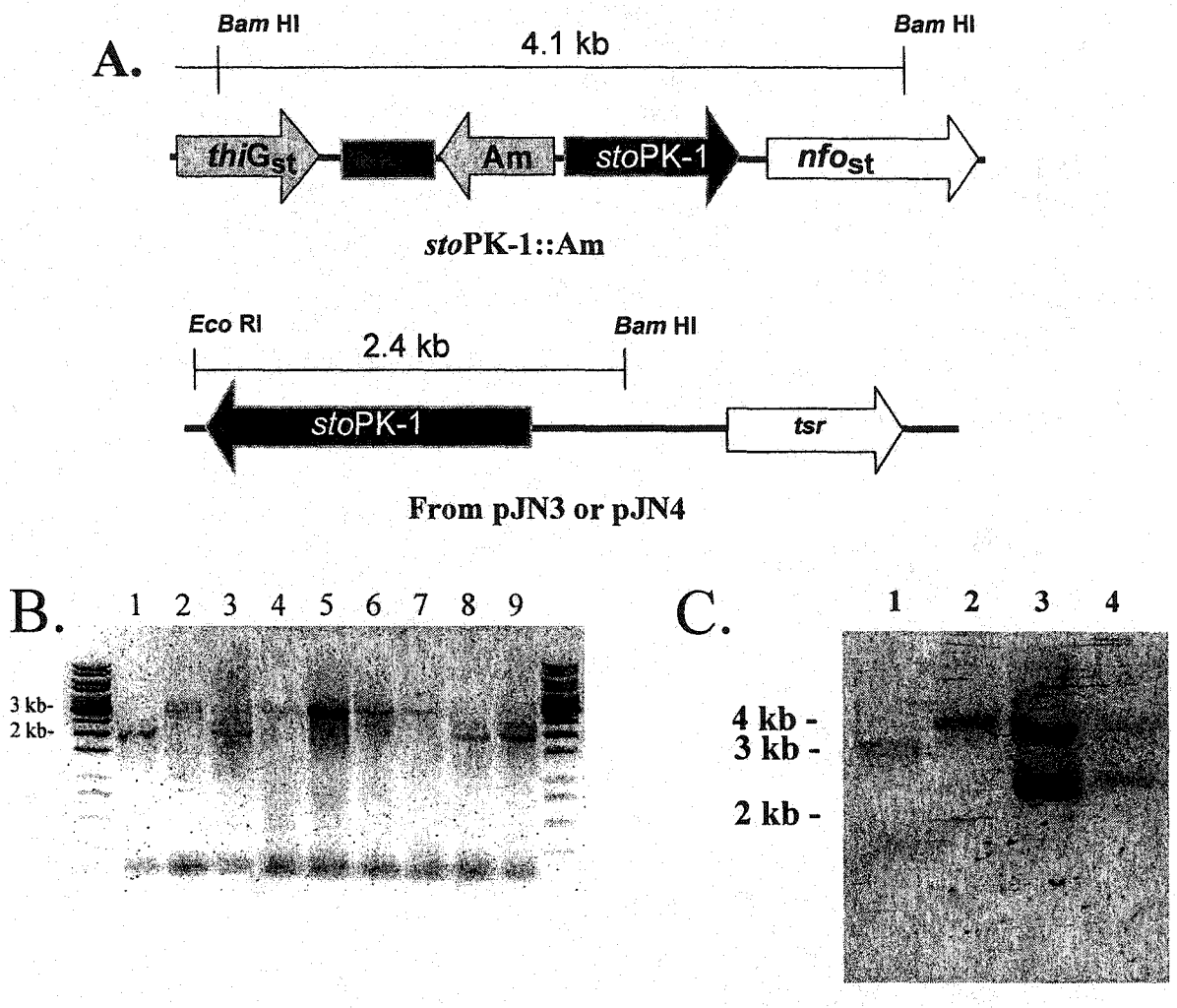


Figure 4.11: Genotype confirmation in *sto*PK-1 mutants. **(A)** Maps of genomic arrangement for disruption mutant and complementation strains. **(B)** PCR of several transformants showing amplification of the correct fragment for double crossovers. Templates: **1**: *sto*PK-1; **2**: *sto*PK-1::Am; **3**: 1 + 2; **4-7** genomic DNA from double cross-over transformants; **8-9**: genomic DNA from single cross-over transformants. **(B)** Southern blots of wild type (Lane 1), *sto*PK-1::Am (Lane 2), *sto*PK-1::Am + pJN3 (lanes 3), and *sto*PK-1::Am + pJN4 (Lane 4).

4.3.3.2. Effects of Carbon Source

Disruption of *sto*PK-1 had no effect on A47934 production, glycopeptide antibiotic resistance, sporulation, or carbon source utilization. Furthermore, colony morphology on various solid and liquid media including R2YE, TSB, SAM, and SVM also appeared normal. However, a difference in colony surface area was noted on solid TSB media. This difference was enhanced when cells were grown at elevated temperatures (37 vs 30 °C) (Fig. 4.12A). Electron micrographs of these colonies also revealed differences in the mycelial morphology (Fig 4.12B) with *sto*PK-1::Am mutants having different mycelial branching patterns and what appear to be disintegrating mycelium, reminiscent of a *pkg*2 kinase mutant phenotype in *S. granaticolor* (Nadvornik *et al* 1999). Addition of the *sto*PK-1 gene in *trans* restored the wild type mycelial patterns (Fig 4.12B.4). Furthermore, supplementation of solid media with glucose alone, but not maltose, lactose, or sorbitol, returned the mycelial form to wild type (Fig 4.12B.3). One of the complementation plasmids used, pJN2, was a low copy number vector and included only the *sto*PK-1 and not downstream genes. This suggested that the effects of the *sto*PK-1 insertional inactivation were not due to polar effects on adjacent genes. To confirm that this was the case, we also transformed the *sto*PK-1::Am disruption mutant with the integrating plasmids pJN3 and pJN4 which contain *sto*PK-1 with and without the Asp141Ala active site mutation and a histidine tag on the C-terminus (see below; Fig. 4.1).

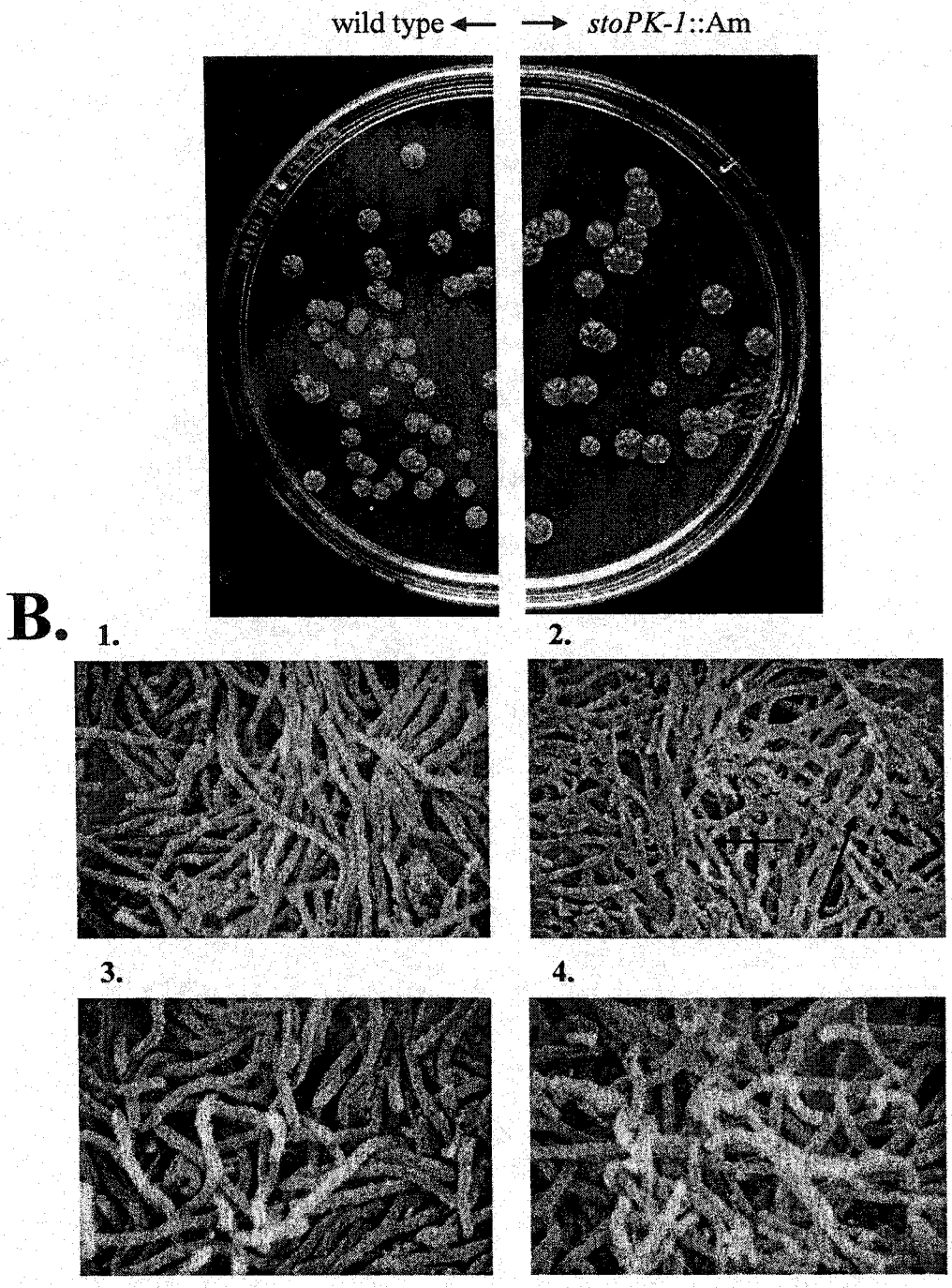


Figure 4.12: Morphology of *sto*PK-1 disruption mutants on TSB agar. **(A)** Colony morphology of wild type (left) and *sto*PK-1::Am (right) on TSB grown at 37 °C. **(B)** Mycelial morphology by scanning electron micrography. Mycelial photomicrographs (×5000) of wild type cells on TSB alone (**1**), *sto*PK-1::Am on TSB alone; arrows indicate branching not seen in wt (**2**), *sto*PK-1::Am on TSB + 2% glucose (**3**) and *sto*PK-1::Am + pJN2 on TSB alone (**4**).

4.3.3.3. Effects of Oxidative Stress

Both *sto*PK-1 and the *S. coelicolor* A3(2) orthologue, *pka*F are located adjacent to a putative endonuclease IV gene (*nfo*-st and Sc6E10.05 respectively), which encodes a DNA repair enzyme responsive to oxidative stress in other organisms (Chan and Weiss, 1987; Cunningham *et al.*, 1986; Greenberg *et al.*, 1990) (Fig. 4.6A). The proximity of these genes to one another and the fact that this arrangement is conserved in two streptomycetes suggested the possibility that *sto*PK-1 might have a role in the response to oxidative stress. Therefore, sensitivity of the wild-type (wt) *S. toyocaensis,* the *sto*PK-1::Am disruption mutant, and complementation strains (vs. wt) to DNA damaging agents capable of inducing oxidative stress was examined. Agents tested included H_2O_2, paraquat, nitrofurantoin, menadione, diamide and sodium nitrite. All strains exhibited similar sensitivities to H_2O_2, menadione, diamide and sodium nitrite.

Initial studies with paraquat and nitrofurantoin gradient plates without glucose supplementation revealed only minor differences on solid TSB. A preliminary spore survival experiment also showed only small differences between wt, *sto*PK-1::Am and pJN2 complementation strains on TSB without added glucose (Table 4.2). However, growth on chemical gradient TSB plates with glucose supplementation revealed an increase in sensitivity to paraquat and nitrofurantoin of the *sto*PK-1::Am disruption mutant which was reversed with the pJN2 and pJN3, but not pJN4 complementation (Fig. 4.13). In addition, *sto*PK-1 appears to be effectively replaced by the *S. coelicolor* gene *pka*F supplied on pJN5 (Fig. 4.13C and D). These results confirmed our earlier results with pJN2, that increased sensitivity to these agents was indeed due to the functional kinase.

We further explored the sensitivity of the wt, *stoPK-1* disruption mutant and complementation strains to paraquat by generating survival curves in repeated experiments ($n \geq 3$) and determining IC_{50} values (Fig. 4.14). In the presence of glucose, a nearly 2-fold increase in sensitivity to paraquat (46% decrease in IC_{50}), was observed for the disruption mutant compared to the wt ($P = 1.3 \times 10^{-4}$, paired T-test) and this sensitivity could be reversed by providing *stoPK-1* in *trans* on pJN3 but not by the catalytically inactive Asp141Ala provided on pJN4 (Fig. 4.14). These results support the conclusion that the increased sensitivity of the *stoPK-1* disruption mutant to paraquat in the presence of glucose was linked to a loss of functional StoPK-1 kinase.

Table 4.2. Effect of paraquat and nitrofurantoin exposure on S. toyocaensis NRRL 15009 spore survival on TSB plates without glucose.

Strain	IC_{50} [b] (mg/mL)	
	Paraquat	Nitrofurantoin
wt	0.17	0.004
*sto*PK-1::Am	0.12	0.003
*sto*PK-1::Am + pJN2[a]	0.14	0.003

a- pJN2 is a low copy number plasmid containing *sto*PK-1.
b- Means of duplicates from a single experiment.

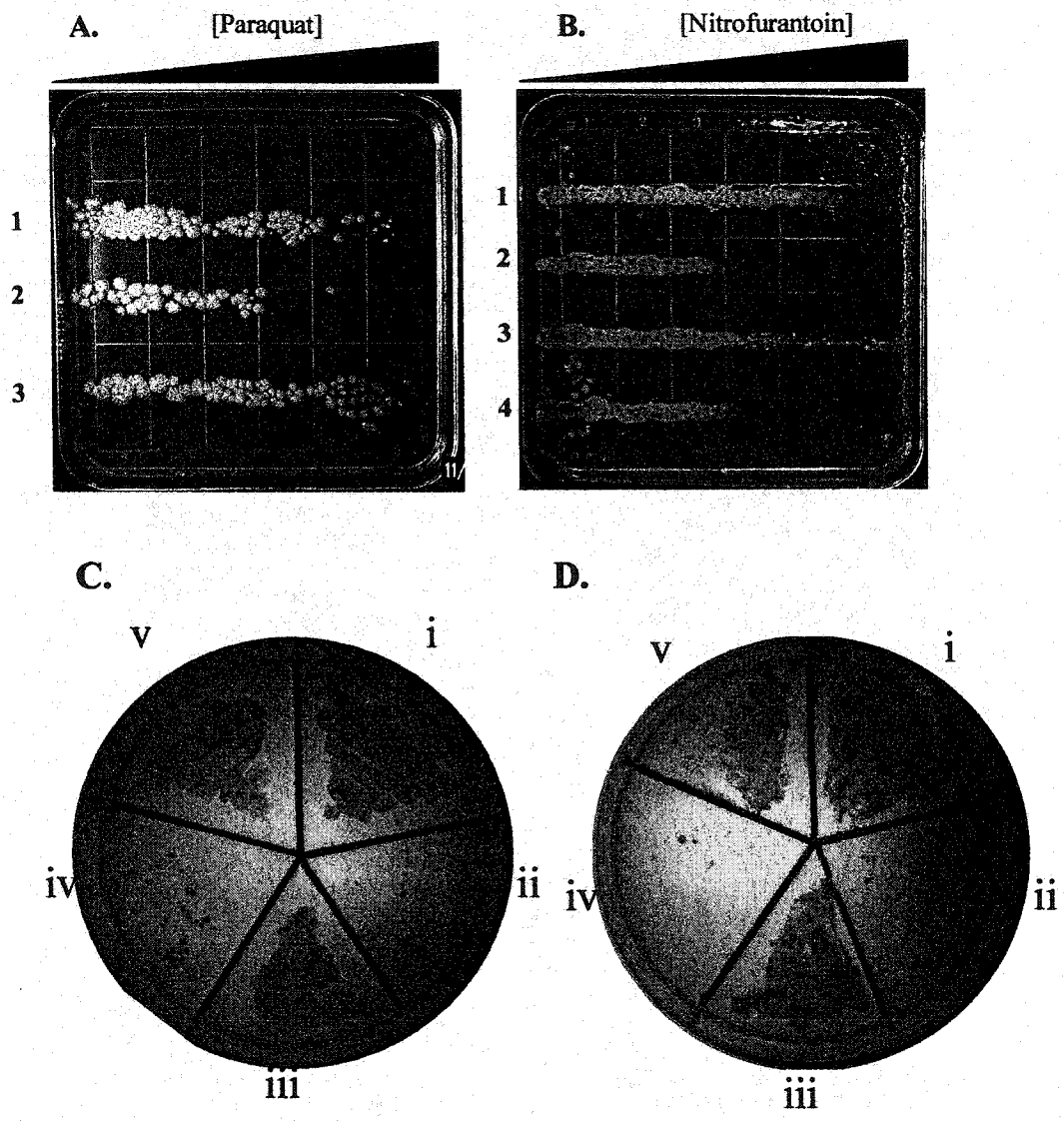


Figure 4.13: *sto*PK-1 regulates responses to oxidative stress. Gradient plates were inoculated with spores from each strain: *S. toyocaensis sto*PK-1::Am mutants show an increased sensitivity to paraquat (**A**, Lane 2) and nitrofurantoin (**B**, Lane 2) compared to the wild type (A and B, Lane 1) and the complementation strain with a single copy of functional *sto*PK-1 from pJN3 (**A** and **B**, Lane 3). Phosphotransfer is critical for this effect as complementation with an Asp131Ala mutant form of the *sto*PK-1 (pJN4) is ineffective at reversing sensitivity to nitrofurantoin (**B**, Lane 4). **C** and **D**: Plates were inoculated with 2×10^6 spores from each strain: *S. toyocaensis sto*PK-1::Am mutants show an increased sensitivity to paraquat (800 µg/mL)(**C**, ii) and nitrofurantoin (10 µg/mL)(**D**, ii) compared to the wild type (**C** and **D**, i) and the complementation strain with a single copy of functional *sto*PK-1 from pJN3 (**C** and **D**, iii). Phosphotransfer appears critical for this effect as complementation with an Asp141Ala mutant form of the *sto*PK-1 (pJN4) is ineffective at reversing sensitivity to paraquat (**C**, iv) and nitrofurantoin (**D**, iv). In addition, *sto*PK-1 can be effectively replaced by the *S. coelicolor* gene *pka*F supplied on pJN5 (**C** and **D**, v).

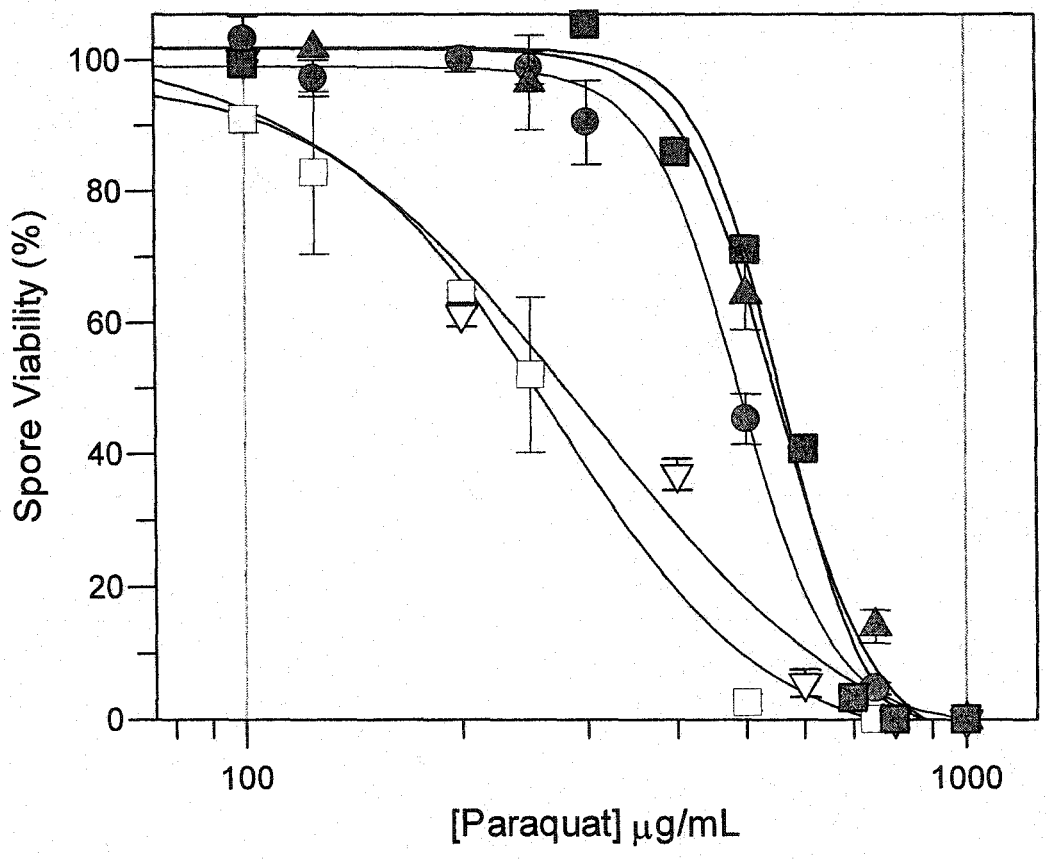


Figure 4.14: *S. toyocaensis* spore survival on TSB + glucose in the presence of increasing concentrations of paraquat reveals an significantly increased sensitivity for the *stoPK-1*::Am disruption mutant (□, IC_{50} = 0.27 mg/mL) compared to wt (● , IC_{50} = 0.49 mg/mL)($p<0.01$). This effect can be reversed by the addition of *stoPK-1* in *trans* from pJN2 (■ , IC_{50} = 0.56 mg/mL) or pJN3 (▲ , IC_{50} = 0.56 mg/mL) but not by supplying the Asp141Ala *stoPK-1* mutant on pJN4 (∇ , IC_{50} = 0.29 mg/mL). Curves were repeated at least three times with similar results.

4.3.4. StoPK-1 expression in *S. toyocaensis*

Analysis of StoPK-1 expression levels using Northern blots, RT-PCR and Western blots proved unfruitful. Most Northern blots showed no hybridization, and some produced bands of unusual size (data not shown). Examination of the effects of DNase I treatment revealed probe hybridization of contaminating genomic DNA but failed to reveal any RNA hybridization (Fig. 4.15). The utility of Northern blots in *Streptomyces* is not recommended by others (Hopwood *et al* 1985, Kieser *et al* 2000). Analysis by RT-PCR also failed. However in this case failure to remove contaminating genomic DNA fragments was indicated, which interfered with the results (Fig. 4.16). In the case of histidine tag Western blots, a band of correct size was not visualized even after addition of glucose and paraquat, suggesting that the StoPK-1 protein is present at low levels or a posttranslational modification has removed the tag (not shown).

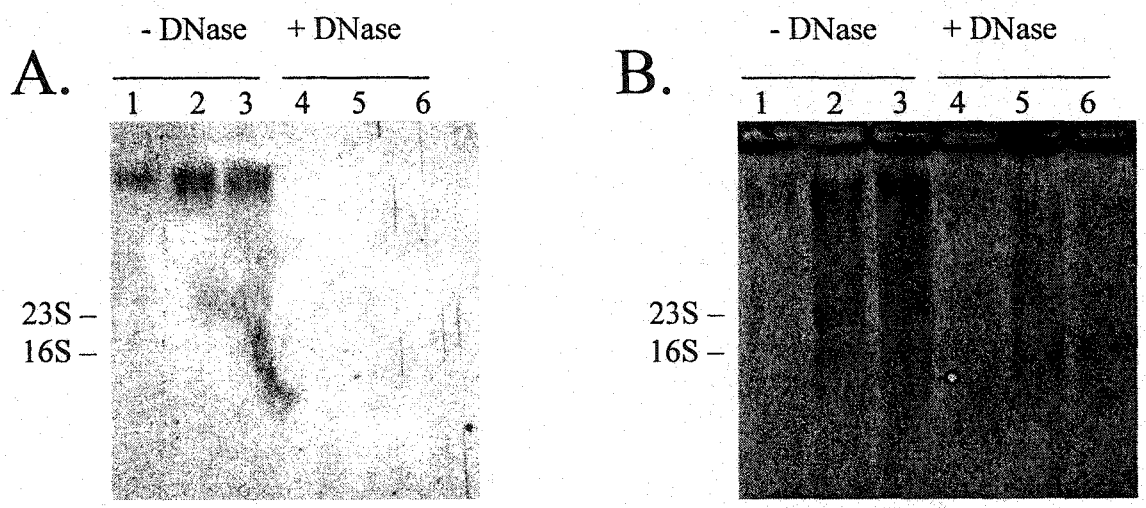


Figure 4.15: Northern analysis of *sto*PK-1 expression in the *S. toyocaensis* pJN3 complementation strain. **(A)** Northern blot of *sto*PK-1 expression: lane 1: grown in TSB alone; lane 2: grown in TSB + 2% glucose; lane 3: **(B)** Ethidium bromide stained duplicate of gel in (A).

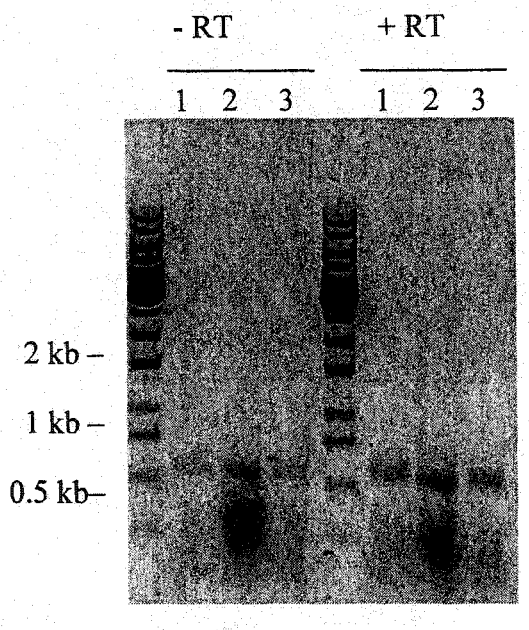


Figure 4.16: *sto*PK-1 RT-PCR of total RNA from 24 hr cultures of the pJN3 complementation strain plus and minus reverse transcriptase (RT) prior to the PCR reaction . Lane 1: TSB alone; Lane 2: TSB + 2% glucose; Lane 3: TSB + 2% glucose + 500 μg/mL paraquat.

4.3.5. Glucose 6-Phosphate Dehydrogenase Levels in *S. toyocaensis*

Examination of glucose 6-phosphate dehydrogenase (G6PDH) levels in wild type, stoPK-1::Am and complementary strains grown for 46 hrs in the presence and absence of paraquat showed no definite pattern (Fig. 4.17 and 4.18). On native polyacrylamide gels a second higher molecular weight band was noted in addition to a band migrating at a similar molecular weight to the *L. mesenteroides* G6PDH (Fig 4.17). Levels of G6PDH as measured by the coupled assay showed definite increases with the addition of glucose to the growth media (Fig. 4.17). In the single experiment we performed, increased levels were noted for the complementation strain in the presence of paraquat, whereas a

decrease was seen for the *sto*PK-1::Am mutant. However, this pattern was not confirmed in the wild type strain (Fig. 4.18).

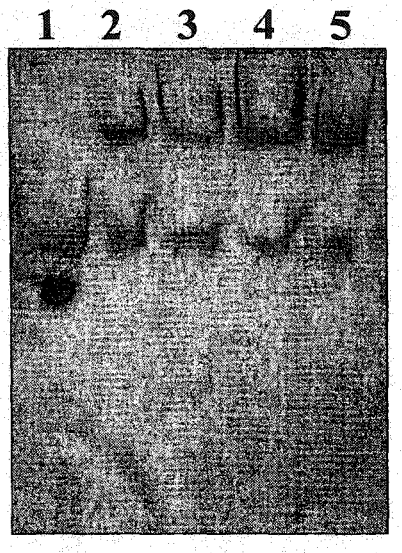


Figure 4.17: Gel labelling of glucose 6-phosphate dehydrogenase in *S. toyocaensis* StoPK-1 mutants grown in the presence of 2% glucose. Lane **(1)**: *L. mesenteroides* G6PDH (0.1U). Lane **(2)**: 30 μg *sto*PK-1::Am protein extract. Lane **(3)**: 30 μg protein extract from *sto*PK-1::Am grown in 150 μg/mL paraquat. Lane **(4)**: 30 μg *sto*PK-1::Am + pJN2 protein extract. Lane **(5)**: 30 μg extract from *sto*PK-1::Am + pJN2 grown in 500 μg/mL paraquat.

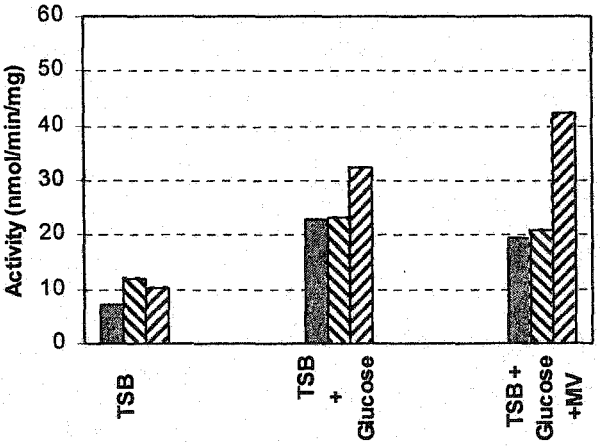


Figure 4.18: Analysis of glucose 6-phosphate dehydrogenase activity in *S. toyocaensis* StoPK-1 mutants. (■) Wild Type, (▨) *sto*PK-1::Am, and (▨) *sto*PK-1::Am + pJN2 were grown for 24 hrs at 30 °C in TSB, TSB + 2% glucose and TSB + 2% glucose + 500 μg/mL paraquat (methylviologen MV).

4.4 Discussion and Significance

4.4.1. Polar Effects

It had been suggested that the insertion of the apramycin resistance gene in the opposite direction to the transcription of *sto*PK-1 and the downstream endonuclease IV gene could lead to the paraquat sensitivity seen by affecting its transcription (polar effect). That is, the predicted endonuclease IV (*nfo*-st) start codon is only a 48 bp downstream from the end of *stoPK-1*, suggesting the possibility of co-ordinate regulation (*ie.* an operon), and the complementation plasmid could complement this sensitivity by crossover events at the 3' end of *sto*PK-1 placing *nfo*-st back under the control of the upstream *stoPK-1* promoter contained in the complementation plasmid (see Fig. 4.19).

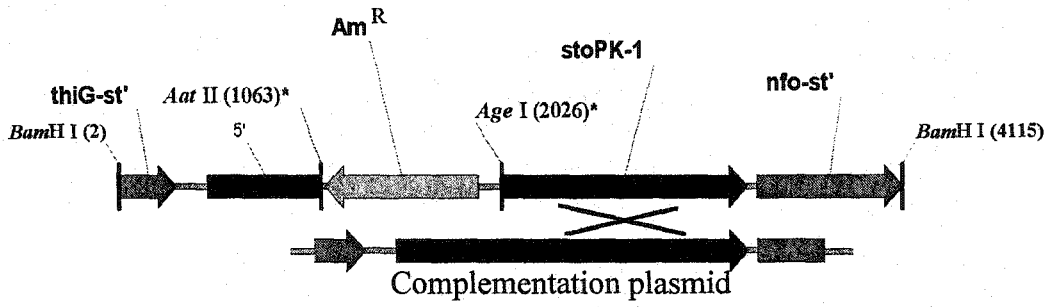


Figure 4.19: Organization of *stoPK-1::Am* *Bam*H I genomic fragment from *S. toyocaensis* showing proposed crossover event which could lead to complementation of polar effect on nfo_{st}.

As a result of this possibility, two new plasmids were constructed, pJN3 and pJN4. These integrating plasmids contained C-terminal histidine tags of *stoPK-1* (pJN3) and the inactive mutant *sto*PK-1Asp141Ala (pJN4; see Fig. 4.1). Both versions of *sto*PK-

1 contain approximately 400 bp of upstream DNA and presumably the native promoter region.

4.4.2. Oxidative Stress

The lack of complementation with inactive mutant *sto*PK-1Asp141Ala (pJN4) strongly indicates a role for this membrane-bound protein kinase in pathways sensitive to oxidative stress and/or glucose metabolism. Although the results do not indicate the specific pathways involved, they clearly implicate *stoPK-1* in regulating a response to certain redox cycling agents in *S. toyocaensis* NRRL 15009. It should be noted here that using a kinase for responding to oxidative stress has been observed in plants (Kovtun *et al* 2000). This group found a MAP kinase cascade with the MAPKKK ANP1 responding to oxidative stress (H_2O_2) which could also cross-talk with cycle controls (Hirt 2000).

The involvement of protein kinases in oxidative stress responses in yeast and mammalian cells (called stress-activated protein kinases or SAPK/JNK) is also well documented (Gustin *et al* 1998, Kyriakis and Avruch, 1996, Hirt 2000). In mammalian cells, SAPK can be activated by oxidative stress agents such as H_2O_2 and paraquat (Chun *et al* 2001), and DNA damage is thought to be the dominant stimulus for SAPK activation by chemical mutagens (Kyriakis and Avruch 1996). However, the evidence presented here that the protein kinase *sto*PK-1 is involved in oxidative stress response is unprecedented in bacteria.

Differences in growth for mutant and wild type colonies were only seen with the redox cycling agents paraquat (methyl viologen-MV) and nitrofurantoin, and were not seen with H_2O_2, menadione (2-methyl-1,4-naphthoquinone), or the thiol oxidizers NO

(from sodium nitrite) and diamide (Nunoshiba *et al* 1993, Kosower and Kosower 1995). Redox cycling agents such as methyl viologen can undergo one electron reductions to produce radicals that go on to react with molecular oxygen as shown in Figure 4.20.

$$MV^{2+} + e^- \rightarrow MV^{\bullet+}$$

$$MV^{\bullet+} + O_2 \rightarrow MV^{2+} + O_2^{\bullet-} \quad \text{(Spontaneous)}$$

$$2O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2 \quad \text{(Superoxide dismutase)}$$

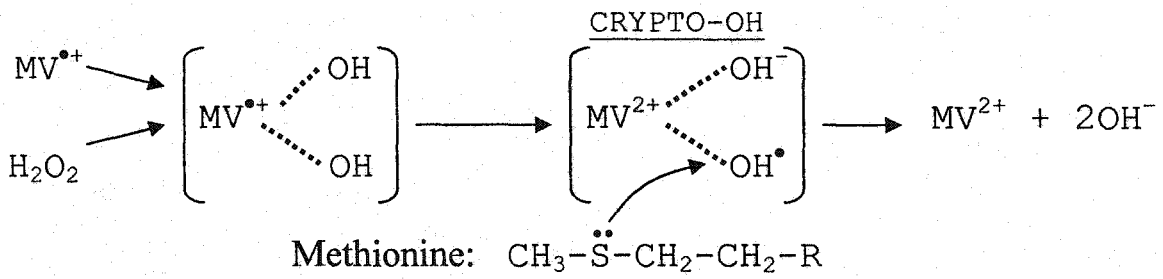


Figure 4.20: Redox cycling agents like methyl viologen (MV) can undergo one electron reductions to form radicals reactive with molecular oxygen and the crypto-OH radical (Youngman and Elstner 1981). This scheme also applies to nitrofurantoin (Youngman *et al* 1982).

These reactive species of oxygen can disrupt proteins, cause DNA damage and lipid peroxidation. In *E. coli* a general defence strategy is to replace oxidation-sensitive enzymes (eg. *fum*AB and aconitase B) with oxidation-stable ones (*fum*C and *acn*A) (Liochev and Fridovich 1992). These proteins are under transcriptional control of the SoxRS regulon (see Fig. 4.21). This strategy is also deployed as a defence against redox nitro compounds and quinones. For instance, oxygen-sensitive nitroreductases (type II) carry out univalent reductions of nitrocompounds such as nitrofurantoin, which are safely reduced in a bivalent fashion by SoxRS inducible oxidation-stable Type I nitroreductase

A (Liochev *et al* 1999). Also, with quinones like menadione, induction of a FMN-dependent divalent reduction enzyme menadione reductase (DT diaphorase or NADH-quinone reductase EC 1.6.99.2) safely reduces this compound to menadiol avoiding univalent reduction and generation of superoxide by enzymes such as lipoamide dehydrogenase (Youn and Kang 2000, Hayashi *et al* 1990). However in this case induction occurs based on structural determinants in the quinone and not through the SoxRS regulon (Unemoto *et al* 1992).

The picture is slightly different for responses to paraquat as three enzymes are believed to be responsible for single electron reductions, including thioredoxin reductase, NADPH:ferredoxin reductase (*fpr*) and sulfite reductase (Liochev and Fridovich 1994a). However, the sulfite reductase is thought to be predominantly responsible for diverting electrons to oxygen since this enzyme has the highest turnover rate and disruption of encoding gene increases resistance to paraquat (Gaudu and Fontecave 1994).

Induction of the NADPH:ferredoxin reductase (*fpr*), which is also a member of the SoxRS regulon, and has lower specificity to paraquat compare to the oxygen-sensitive enzyme (pyruvate:ferredoxin reductase), increases the availability of reduced ferredoxin (Liochev *et al* 1994b). This can again be viewed as a replacement of a oxygen-sensitive enzyme with an oxygen-stable one (as with fumerase), and has a positive effect since disruption of *fpr* increases sensitivity to paraquat (Bianchi *et al* 1995). Thus the strategy for responding to paraquat is a more generalized response to superoxide generation in *E. coli* and not a specific divalent reduction to lower toxicity, like menadione or nitrofurantoin. These cellular responses are summarized in Figure 4.21 below.

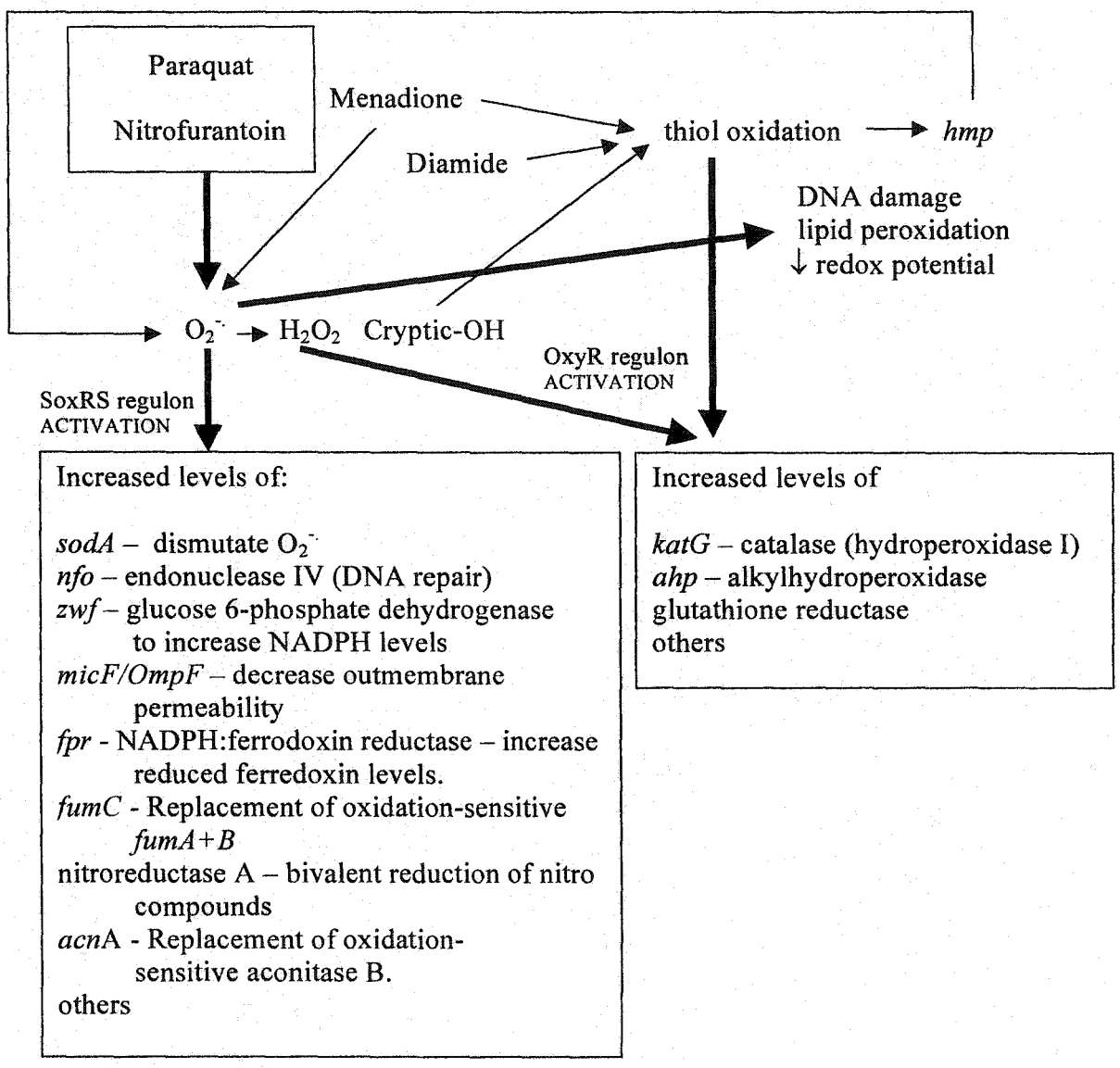


Figure 4.21: Response of *E. coli* to various oxidative stress generating agents. Gene products induced by superoxide and hydrogen peroxide are given together with their functional activities. Induction of the SoxRS regulaon occurs through the oxidation of SoxR (by superoxide) and is a separate pathway from the oxyR regulon which responds to hydrogen peroxide and thiol oxidation.

In addition to superoxide effects, redox cycling agents also exert toxic effects by diverting electrons from various reducing agents through enzymes like sulfite reductase back to themselves and repeat this by autoxidation (Figure 4.20). This lowers the "reducing" environment of the cell interfering with many processes. The SoxR protein senses this disruption in redox balance through oxidation of its [2Fe-2S] center, activating it own transcription and that of SoxS, a positive regulator of transcription of the regulon (Gaudu and Weiss 1996, Wu and Weiss 1991). As shown in Figure 4.21, increased production of NADPH (through *zwf*) and reduced ferredoxin (through *fpr*) counteracts this effect.

4.4.3. Oxidative Stress in *Streptomyces*

Unlike *E.coli,* response to oxidative stress in *Streptomyces* has not been well studied. It remains unclear if a similar Sox or OxyR systems exists in these organisms. However, a homolog (61% identity) of SoxR exists in *S. coelicolor* (ScI30A.18c) with all four C-terminal cysteines responsible for Fe-S binding in SoxR completely conserved (Wu and Weiss 1991, Gaudu and Weiss 1996), but no SoxS homolog has been detected. In addition, an OxyR-like protein has characterized in *S. viridosporus* and a homolog exists in *S. coelicolor,* and like its counterpart in *E. coli*, this protein activates peroxidases in response to H_2O_2 (Ramachandran *et al* 2000). A single study has been carried out which looked at the expression of several stress proteins in *S. coelicolor* in response to hydrogen peroxide challenge. These authors found that H_2O_2 caused increases in catalase, and glutathione reductase activities as would be expected (OxyR), but unexpectedly also

cause a marked increase in glucose 6-phosphate dehydrogenase activity (SoxRS). This suggested to the authors that makeup of the peroxide inducible regulon in *Streptomyces* is somewhat different than *E. coli* (Lee *et al* 1993).

Other studies in *S. coelicolor* have examined the effects of paraquat and quinones on expression of superoxide dimutase (SOD) (Kim *et al* 1996). These researchers found a less than two-fold increase in SOD activity after treatment with either agent. Thus SOD in the strict aerobe *S. coelicolor* is apparently not inducible by oxidative stress as in facultative bacteria like *E. coli*. The "non-inducible" nature of SOD in *Streptomyces* may be due to relatively less variations in environment encountered compared to facultative bacteria (Kim *et al* 1996).

One response to oxidative stress in *S. coelicolor* that has been well characterized is effects of the oxidation of thiols by agents such as diamide (Paget *et al* 1998, Kang *et al* 1999, Paget *et al* 2001). This system relies on an antisigma factor RsrA which binds under reducing conditions to σ^R, and blocks its activity. σ^R is a sigma factor responsible for the transcription of thioredoxin and thioredoxin reductase. RsrA controls the systems just like oxyR : through the formation of a disulfide bond (oxidation) it function is activated, releasing repression of transcription by σ^R (Kang et al 1999).

4.4.4. Conclusions

Given the results of the few studies in *Streptomyces*, it seems that significant differences with the systems of *E. coli* may exist. The work presented in this report also suggests an increased level of complication and control exists in the responses to

oxidative stress in *Streptomyces*. Assuming that similar base enzyme systems are used as defence in both *E. coli* and *Streptomyces* (*eg.* glucose 6-phosphate dehydrogenase for NADPH generation) then only regulatory differences should exist. Indeed this seems to be the case for responses to paraquat and thiol oxidation as given above. The evidence suggests that *stoPK-1* is a regulatory protein involved in an adaptive response to paraquat and nitrofurantoin and this is probably not through induction of SOD activity, since SOD has not been shown to be inducible in *Streptomyces* (Kim *et al* 1996). Also, the *RsrA*/σ^R system is not likely to be involved since differences to the thiol oxidizer diamide were not evident. Given the observation that growth of the *stoPK-1*::Am mutant is only affected on glucose containing media, and the general strategy of bacteria to replace oxygen-sensitive enzymes with stable isoforms, a logical role for *stoPK-1* is the regulation of some stable glucose metabolising enzyme (*eg.* G6PDH). Although our G6PDH studies were inconclusive, StoPK-1 may directly regulate G6PDH levels. In this respect, better design of experiments to measure G6PDH levels in each strain challenged with various redox cycling agents may be fruitful. Alternatively, given the close genetic proximity of *stoPK-1* with the endonuclease IV gene, and the observation that this gene is involved in responses to oxidative stress (Cunningham *et al* 1986, Chan and Weiss 1987), StoPK-1 may be involved in its regulation. However, although paraquat is a potent inducer of endonuclease IV in *E. coli*, nitrofurantoin had no effect (Chan and Weiss 1987). Therefore the exact regulatory role of StoPK-1 remains to be elucidated.

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Chapter 5.

Glycopeptide Resistance in *Streptomyces*

5.1. Background

5.1.1. History and use of Glycopeptide Antibiotics

Glycopeptides are an important class of antibiotics effective in the treatment of gram positive infections. Vancomycin, the archetype glycopeptide, was first isolated from soil of India and Indonesia in 1953 by Eli Lilly and Company and is produced by the Streptomycete bacterium *Amycalotopsis orientalis* C329.2 (formally *Streptomyces orientalis*). By 1958, it was being administered to adults for the treatment of gram-positive bacterial infections (Perl 1999, Lundstrom *et al* 2000). Vancomycin is active against most gram positive bacteria including Staphylococci, Streptococci and Enterococci, Corynebacteria, Clostridia, Listeria, and Bacillus species (Lundstrom *et al* 2000) and is bactericidal to susceptible bacteria at levels ranging from 0.5 - 5 mg/L (French 1998, Lundstrom *et al* 2000). Vancomycin must be administered intravenously because it is not readily absorbed through the gastrointestinal tract. However oral vancomycin is effective therapy against *Clostridium difficile*, a cause of antibiotic-associated colitis (French 1998).

5.1.2. Emergence of Vancomycin Resistance in *Enterococcus*

Enterococci have developed into important nosocomial pathogens although they were originally viewed as organisms of a minimal clinical importance. Because they are inherently resistant to penicillin (French 1998), aminoglycosides were originally used for treating infections caused by these enteric bacteria. Subsequently, it was found that a

combination of penicillin or ampicillin and streptomycin had a synergistic effect and this combination became the standard treatment strategy despite the high toxicity of streptomycin (Murray 2000). Unfortunately, the development of high levels of streptomycin resistance rapidly appeared in some Enterococcal strains, but the successful synergistic treatment strategy was temporarily maintained by the commercial development of gentamicin. However with the emergence of resistance to streptomycin and gentamicin as well as any synergistic effects with penicillins, vancomycin became a popular drug to combat infections. Vancomycin is now the only generally effective treatment for enteroccal infections (Moellering 1998).

Given the past patterns of drug introduction followed by the rapid emergence of resistance seen with all other drugs used to treat enterococcal infections, it is not surprising that vancomycin resistance has emerged. Vancomycin and teichoplanin resistant *Enterococcus faecium* (VRE, VanA type) was first reported in 1986 in patients from France and England (Leclercq *et al* 1988, Uttley *et al* 1988) and vancomycin-resistant, teicoplanin-sensitive *Enterococcus faecalis* (VRE, VanB type) were isolated in the USA in 1987 (Sahm *et al* 1989). Since that time, the incidence of the VRE phenotype had grown to 21-23% of all hospital isolates by 1998. (Perl 1999).

Recently, the newly approved antimicrobials quinipristin-dalfopristin (Synercid) and linezolid are being used for the treatment of non-responsive infections caused by VRE. However, Synercid is only effective against *Enterococcus faecium*, with *E. faecalis* showing inherent resistance (Johnson *et al* 2000). In addition, although the effectiveness Synercid is fair (68% response rate), treatment can be associated with a substantial

frequency of serious side effects (Raad *et al* 2001). Similarly, linezolid is 67% efficacious, but has shown a high incidence for the development of resistance *in vitro* and only limited clinical data is available on its use (Noskin *et al* 1999, Zeana *et a*l 2001, Gonzales *et al* 2001). Thus, neither treatment strategy appears to be an ideal one, and the effective treatment of VRE infections remains problematic.

5.1.3. Development of Antibiotic Resistance in *S. aureus*

Staphylococcal infection was originally treated with penicillin. Today, up to ninety percent of Staphylococcal strains are resistant to penicillin. By the late 1950s, almost 50% of all clinically isolated *S. aureus* strains had become resistant to penicillin (Edmond *et al* 1996). In order to combat the penicillin resistance in *S. aureus*, methicillin was introduced. However, only one year later the first methicillin-resistant *S. aureus* (MRSA) strain had been reported (Hryniewicz 1999). Many more MRSA strains have emerged in the past 40 years and are increasingly more prevalent as nosocomial pathogens (Edmond *et al* 1996). *S. aureus* and *S. epidermidis*, including both methicillin-susceptible or resistant-species, are usually sensitive to vancomycin with minimum inhibiting concentrations (MIC) less than 1.5 mg/L. Thus the use of vancomycin is now indicted for the treatment of MRSA infections and represents the only available treatment against these pathogenic bacteria.

The recent acquisition of vancomycin resistance by *Enterococcus* has been a cause of great concern as high-level vancomycin resistance has been experimentally transferred from *Enterococcus faecalis* to *S. aureus* in both *in vitro* and *in vivo* models.

(Noble *et al* 1992). This has lead to the belief that the emergence of a VRSA "super bug" from transfer of glycopeptide resistance from *Enterococcus* to MRSA is inevitable since in the hospital setting, VRE and MRSA frequently co-mingle.

5.1.4. Glycopeptide Antibiotic Resistance in *Streptomyces*

The vancomycin resistance cluster, which is present in glycopeptide producers such as *Streptomyces toyocaensis* and *Amycalotopsis orientalis* as well as VRE strains, contains the operon *van*HAX (Figure 1.7, Chapter 1). Its presence in *S. coelicolor* was first revealed by a simple blast search of the *Streptomyces coelicolor* genome project database at the Sanger Centre (http://www.sanger.ac.uk/Projects/Scoelicolor/index.shtml). Comparisons with the resistance cluster from *Enterococcus* shows the presence in *Streptomyces* of the five genes believed to be necessary to confer resistance: *van*R, *van*S, *van*H, *van*A, and *van*X (reviewed in Chapter 1). In addition, two new **g**lycopeptide **r**esistance **a**ssociated genes not seen before appear in the cluster: Sc66T3.03 (*gra*1) and Sc66T3.04 (*gra*2) (Figure 1.7, also see Chapter 6).

5.1.5. Project Strategy

The medical dangers of VRE and the possible acquisition of resistance in *S. aureus* encourages the study of vancomycin resistance not only in *Enterococcus* but also in other organisms utilizing the same resistance genes. Consequently, a comprehensive study of resistance in *Streptomyces* should be insightful. However, in order to utilize *Streptomyces* species for cloning and genomic disruptions, a detailed knowledge of their

resistance profiles for several antibiotics under different growth conditions is necessary. Therefore, initial studies were carried out to examine antibiotic resistance in several *Streptomyces* including *S. toyocaensis* and *S. coelicolor* on typical media developed for the growth of these organisms. This information is not readily available for some species and a comparison of resistance profiles between species on various media would be informative.

Although the utility of the *van*HAX cluster in VRE has been clearly shown as conveying glycopeptide resistance in these organisms, no such studies have been carried out in *Streptomyces*. As shown in Table 5.1, these genes share significant homology to their enterococcal counterparts. Therefore, disruption and complementation of the pivotal ligase *van*A_{ST} (*ddl*M) gene in *S. toyocaensis* NRRL 15009 was performed to confirm the importance of the *van*HAX cluster in conferring glycopeptide resistance. In addition, the presence of a two component system in *Streptomyces toyocaensis* and *Streptomyces coelicolor* closely associated with putative glycopeptide biosynthesis and resistance genes and with significant homology to the two component system shown to be critical for regulating resistance in *Enterococcus* (see Table 5.1) strongly suggests that the *Streptomyces* system may play a comparable role. Therefore the systematic disruption and complementation of the *van*R-like homologue in *S. coelicolor* A3(2) was also undertaken.

Table 5.1: Homologies between *Enterococcus* and putative *Streptomyces* glycopeptide resistance proteins.

Putative Streptomyces Resistance Protein	% similarity with *Enterococus* protein	% similarity between *S. toyocaensis* and *S. coelicolor* proteins
$VanH_{ST}$	66%	73%
$VanH_{SC}$	77%	
$VanA_{ST}$	79%	87%
$VanA_{SC}$	78%	
$VanX_{ST}$	78%	85%
$VanX_{SC}$	75%	
$VanR_{ST}$	49%	77%
$VanR_{SC}$	48%	
$VanS_{ST}$	46%	74%
$VanS_{SC}$	38%	

5.2. Materials and Methods

5.2.1. Media and Bacterial strains.

In all studies, 200 µL of *S. coelicolor* A3(2) and *S. toyocaensis* NRRL 15009 spores were first inoculated into a vegetative medium to produce a seed cultures for subsequent studies (see section 2.1.1). Cells for protoplasts were subsequently inoculated into baffled 50 mL or 250 mL flasks containing 15 or 50 mL respectively of Tryptone Soy Broth (TSB; Oxoid) for *S. toyocaensis* or YEME (3 g/L yeast extract, 5 g/L peptone, 3 g/L malt extract, 10 g/L glucose and 340 g/L sucrose supplemented with 5 mM $MgCl_2 \cdot 6H_2O$) for *S. coelicolor* with 0.5% or 0.4% glycine (w/v) respectively. Other *Streptomyces* utilized are given in Table 5.2 with a list of plasmids and constructs. All *E. coli* strains were grown in Luria Bertani medium.

Antibiotic resistance in *S. toyocaensis* NRRL 15009, *S. coelicolor* A3(2), *S. lividans* 66, and *S. fradiae* were assayed on various solid media typically used to culture *Streptomyces* (Kieser *et al* 2000) including (1) minimal media (MM) containing 0.5 g/L L-asparagine, 0.5 g/L K_2HPO_4, 0.2 g/L $MgSO_4 \cdot 7H_2O$, 0.01 g/L $FeSO_4 \cdot 7H_2O$, 1.2% agar and 0.5% D-glucose, (2) mannitol soy media (MSM) composed of 2% soyaflour, 2% mannitol, 100 mM $MgCl_2$, and 1.6% agar, and (3) R2YE media containing 10.3% sucrose, 1.01% $MgCl_2 \cdot 6H_2O$, 1% D-glucose, 0.1 g/L casaminoacids, 0.25 g/L K_2SO_4, 0.5% yeast extract, 0.573% TES (pH 7.2), 0.005% KH_2PO_4, 0.368% $CaCl_2 \cdot 6H_2O$, 0.3% L-proline, 7 mM NaOH, 2.2% agar, and 2 mL/L of trace elements containing 40 mg/L $ZnCl_2$, 200 mg/L $FeCl_3 \cdot 6H_2O$, 10 mg/L $CuCl_2 \cdot 2H_2O$, 10 mg/L $MnCl_2 \cdot 4H_2O$, 10 mg/L $Na_2B_4O_7 \cdot 10H_2O$ and 10 mg/L $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$.

Antibiotic resistance profiles for *S. toyocaensis* NRRL 15009, *S. coelicolor* A3(2), *S. lividans* 66, and *S. fradiae* on various media (see above) were generated using a disc diffusion method. 25 μL of antibiotic was placed on a 7 mm sterilized filter disc on a lawn of 100 μL freshly spread spores ($1\text{-}5 \times 10^8$ cfu/mL) and zones of inhibition analyzed after 3-4 days at 30°C.

Table 5.2: Bacterial strains and plasmids used in this study.

Plasmid or Strain	Characteristics	Source
Bacterial Strains		
Streptomyces		
S. toyocaensis NRRL 15009	A47934-producing strain	NRRL.
S. coelicolor M145	*S. coelicolor* A3(2)	ATCC.
S. lividans 66		ATCC
S. fradiae		ATCC
E. coli		
Novablue	General cloning strain	Novagen.
SURE	General cloning strain	Stratagene.
GM48	*thr leu thi*-1 *lac*Y *gal*K *gal*T *ara ton*A *tsx* dam dcm supE44	Stratagene.
Base Plasmids and Cosmids		
pStBlue-1	Neo^r, Ap^r, general cloning vector	Novagen.
pRB374	*Bacillus* shuttle plasmid	Bruckner 1992.
pSET152	Streptomyces integrating vector	Bierman *et al* 1992.
pBlutsr	*Streptomyces* disruption plasmid containing thiostrepton resistance gene.	*stoP*K-1 study Chapter 3.
pRB374-Am	Apramycin cassette contained on a *Pst* I fragment.	This study.
pSTBlue-tsr	Thiostrepton cassette contained in pSTBlue-1	This study.
SuperCos66T3	S. coelicolor cosmid containing resistance genes.	
pFD666-ddl3.0	*Streptomyces* shuttle plasmid containing a 3.0 *Sac* I fragment with *van*Ast.	Marshall and Wright 1997.
pBlutsr-ddl3.0	pBlutsr containing 3.0 *Xba* I / *Hind* III fragment from pFD666-ddl3.0.	This study
pGEMC329van3.5	3.5 kb *van*HAX cluster from *Amycalotopsis orientalis*	CG Marshall
pSTBlue-RR	1.9 kb *Xho* I / *Eco* RI PCR fragment in pST1Blue1	This study
Disruption Plasmids		
pBlutsr-ddl3.0-Am	Disruption plasmid containing apramycin resistance gene inserted into $vanA_{ST}$.	This study.
pSTBlue-vanR-Am	pSTBlue-vanRsc with apramycin resistance gene inserted in 5' *Pst* I site of $vanR_{SC}$.	This study.
Complementation Plasmids		
pJN6	Thiostrepton resistant shuttle plasmid containing *S. toyocaensis* resistance genes *van*HAX and integration region from pSET152	This study.
pJN7	Thiostrepton resistant shuttle plasmid containing *A. orientalis* resistance genes *van*HAX and integration region from pSET152.	Marshall and Wright 1997.
pSET-vanRS'sc	$vanR_{SC}$ complementation vector created from ligating pSET152 to pSTBlue-vanRS'sc at *Xba* I / *Xho* I sites.	This study.

5.2.2. Polymerase Chain Reaction.

Standard PCR reactions were performed by designing primers based on melting temperatures of 63-65°C. Amplification of *van*A_{ST}, *van*R_{SC}, and other *Streptomyces* high-GC DNA fragments were performed with Vent polymerase (NEB). 100 µL reactions contained up to 1 µg template DNA, 1.2 µM each of the specific primers (Table 5.3), 20 mM Tris (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 0.1 % Triton X-100, 2.5 mM $MgSO_4$, 500 µM dNTPs and 5% DMSO. Reactions were incubated for 1 minute at each of 95 °C, 60 °C and at 1 min/kb DNA at 75 °C for 30 cycles in a Techne thermocycler (Mandel Scientific). Reactions (10-15 µL) were visualized on 1% agarose gels. Primers used in this study are summarized in Table 5.3 and Figure 5.1 below.

Table 5.3: PCR primer used in this study. See Figure 5.2 below for locations.

Primer	**Sequence (5' – 3')**	**Location (see Fig. 5.2)**
AB9363	GAGATATACATATGGCCAGACTGAAGATCGG	*van*A_{ST} forward
AB9364	TGACATAAGCTTCAGAGCGAGGAGACGGAGA	*van*A_{ST} reverse
AB18592	GACGTTGTGGGAGGCCATGGTGAAT	*van*R_{SC} forward
AB18593	GGTGATCCGAGGGCGAGGGTTACG	*van*S_{SC} reverse
AB18787	CCGGATGCGGTGGGAGAGGG	*van*S_{SC} reverse
AB18788	TGACGCATACGCCCAGTGAAC	*van*R_{SC} forward

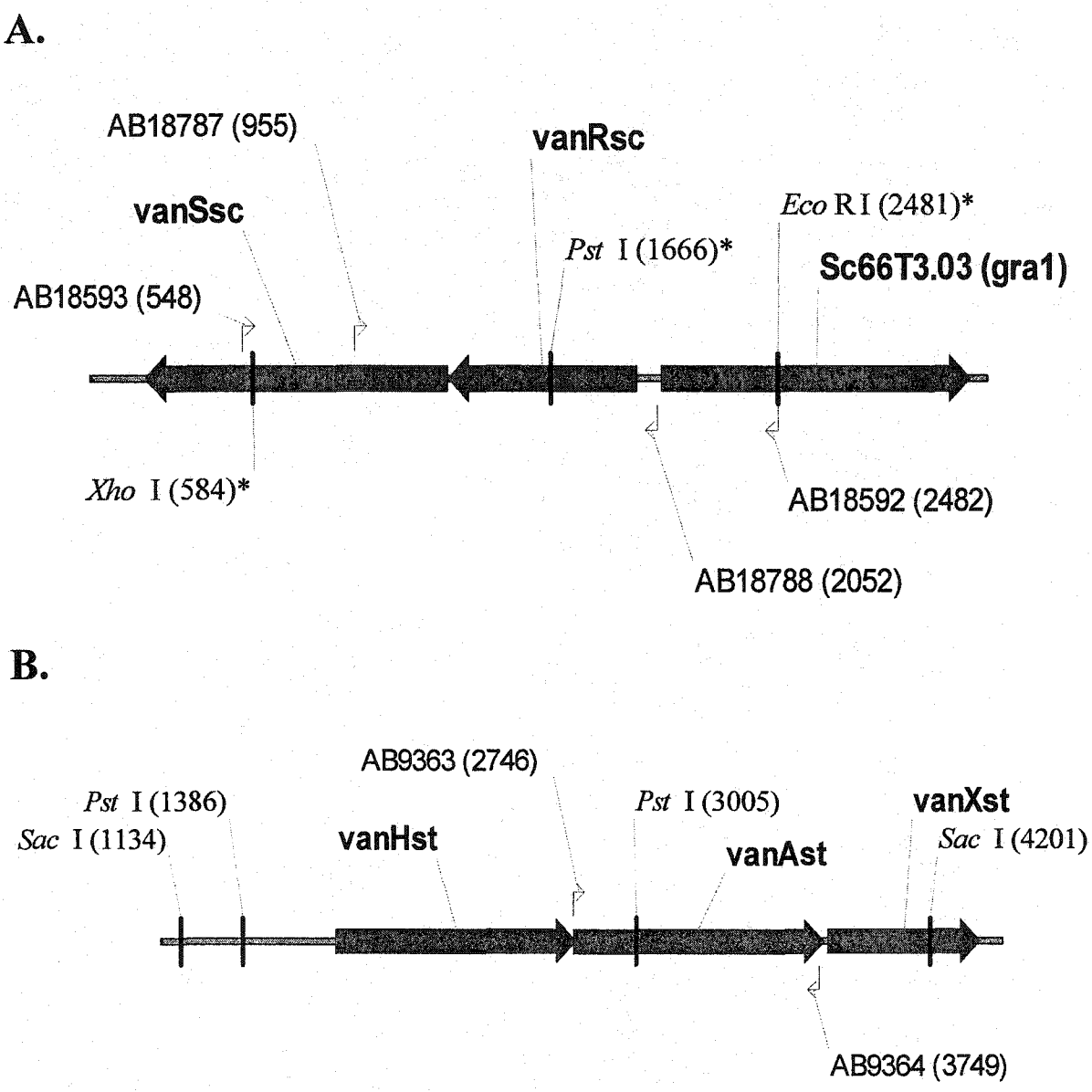

Figure 5.1: (A) Map of primer locations for *S. coelicolor* A3(2) used in this study. **(B)** Map of primer locations for *S. toyocaensis* NRRL 15009 used in this study.

5.2.3. Genomic DNA isolation.

S. coelicolor M145 and *S. toyocaensis* NRRL 15009 were grown for 48 hrs in vegetative media and 200 μL of vegetative culture was inoculated into 15 mL of YEME + 0.4% glycine or TSB + 0.5% glycine respectively, and incubated at 30 °C and 225 rpm for 24-36 hrs. The genomic DNA was then isolated by the same procedure as in Chapter 3 section 3.2.1.

5.2.4. Gene Disruptions.

5.2.4.1. *van*A_{ST} (*ddl*M)

A pBluescript-derived vector pBlutsr was previously constructed specifically for *Streptomyces* disruption studies (Chapter 4). This vector was utilized for the disruption of *van*A_{ST}. A 3 kb *Sac* I genomic fragment previously cloned from and *S. toyocaensis* NRRL 15009 (pFD666-ddl3.0, Marshall and Wright 1997) was inserted into pBlutsr and the apramycin resistance cassette from pRB374-Am inserted in the 5'end of *van*A_{ST} at the *Pst* I site resulting in the disruption plasmid pBlutsr-ddl3.0-Am (Fig. 5.2).

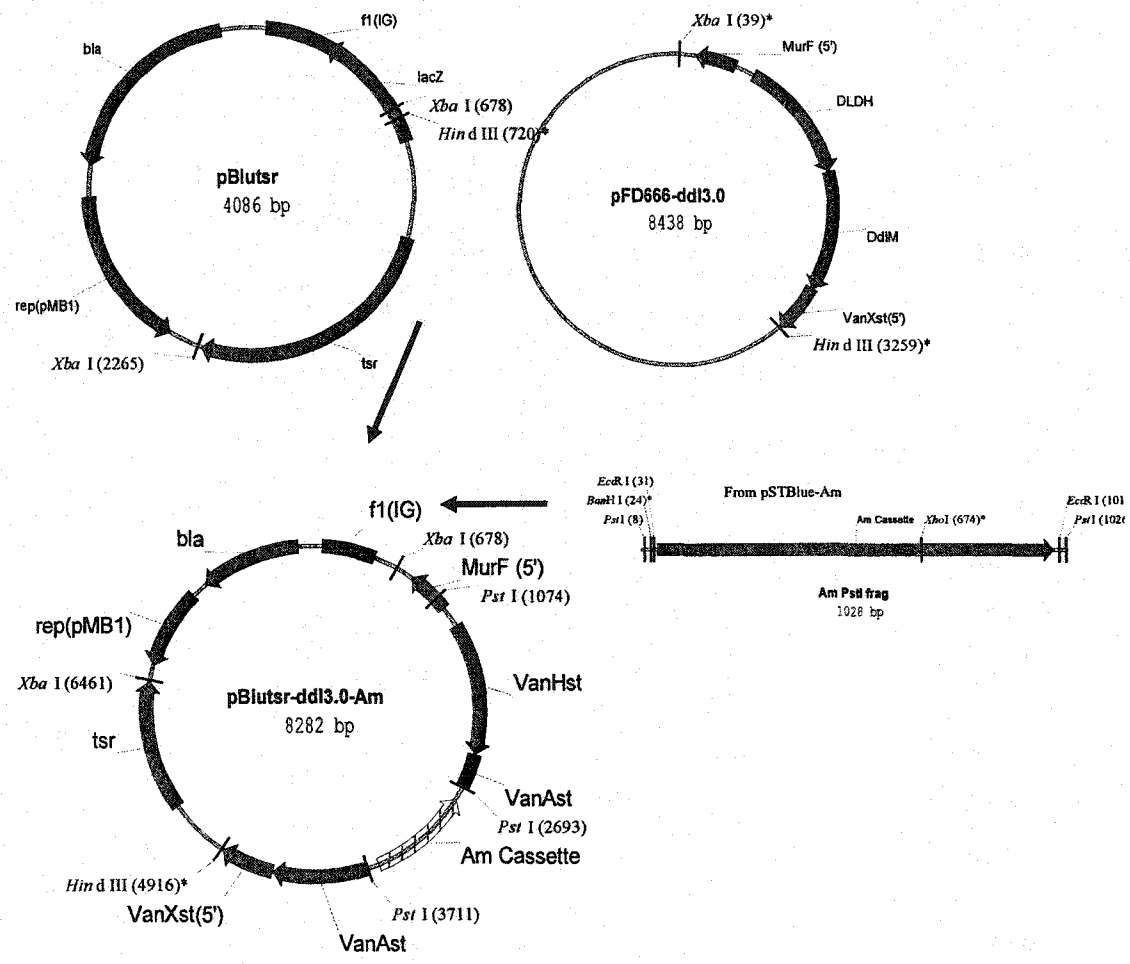

Figure 5.2: Construction of *van*A_{ST} disruption plasmid pBlutsr-ddl3.0-Am. See text for details.

5.2.4.2. *vanR*$_{SC}$ Disruption

For *vanRsc* disruption experiments, the commercially available plasmid pSTBlue-1 (Novagen), which contains a kanamycin resistance gene was utilized. A 1.9 kb *Eco* RI / *Xho* I PCR fragment generated from genomic *S. coelicolor* A3(2) DNA was cloned into pSTBlue-1. This PCR fragment was generated using the standard PCR protocol (section 5.2.2) with the specific primers AB18592 and AB18593 (Table 5.2, Fig. 5.1). The apramycin resistance gene was inserted into a *Pst* I site located in the *vanRsc* coding region creating pSTBlue-vanR-Am (Fig. 5.3). This disruption plasmid was transformed through the *dam*$^-$, *dcm*$^-$ *E. coli* cell line GM48 and subsequently used to transform *S. coelicolor* protoplasts in single stranded form (up to 3 μg) as previously described by Oh and Chater (1997). Regeneration of transformed protoplasts occurred overnight, after which 1 mg of apramycin sulfate was overlaid in 500 μL of TSB. Plates were examined for apramycin resistant colonies after 3-4 days. Transformants exhibiting the correct phenotype (*ie.* apramycin resistant and sensitive to high levels of neomycin) were subsequently examined for the correct genotype by PCR with the primers AB18787 and AB18788, and by Southern blot. Resistance to glycopeptides was also examined on R2YE and media.

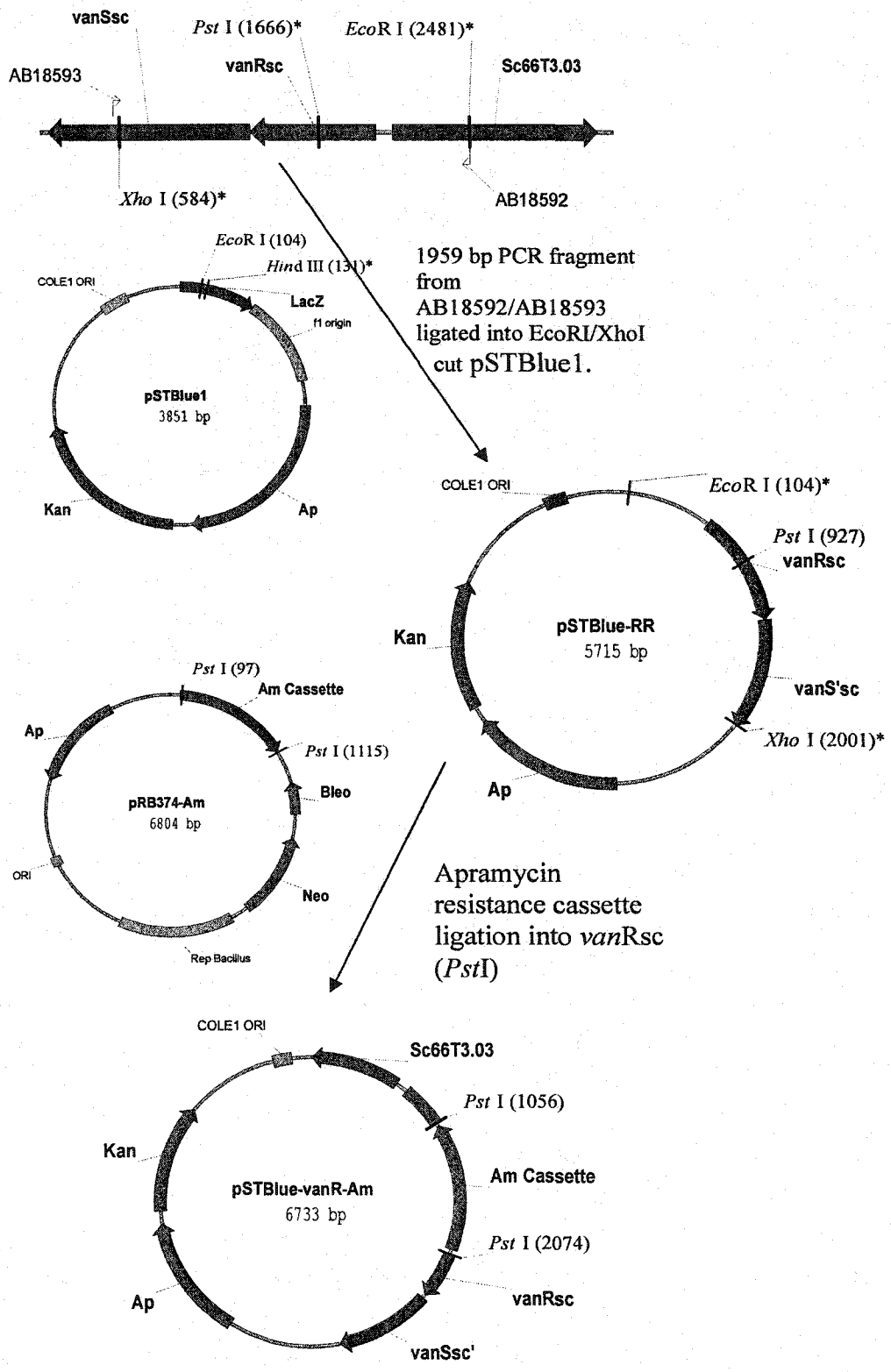

Figure 5.3: Disruption plasmid construction for *van*R_{SC}. See text for details.

5.2.5. Complementation of *van*A_{ST} (*ddl*M)

In order to complement the *van*A_{ST} disruption two plasmids were designed and constructed. These were designated pJN6 and pJN7. The construction of pJN6, a pFD666-based plasmid was carried out by joining pFD666-ddl3.0 and a *Not* I/*Hin* d III fragment from pRB374-vanX5.4, both of which were previously made by Gary Marshall. However, careful mapping failed to confirm the fidelity of the final construct. The rearrangement of large *Streptomyces* genomic fragments has been noted in our laboratory and it is thought that certain fragments are unstable in *E. coli*, including this one. Therefore, the construction of an integrating plasmid, pJN7, as shown in Figure 5.4, was carried out. pJN7 contains the *van*HAX operon and upstream elements from the vancomycin-producer *Amycalotopsis orientalis* C329.2 and the thiostrepton resistance gene as well as the ϕC31 integrase from pJN4 (See Chapter 4).

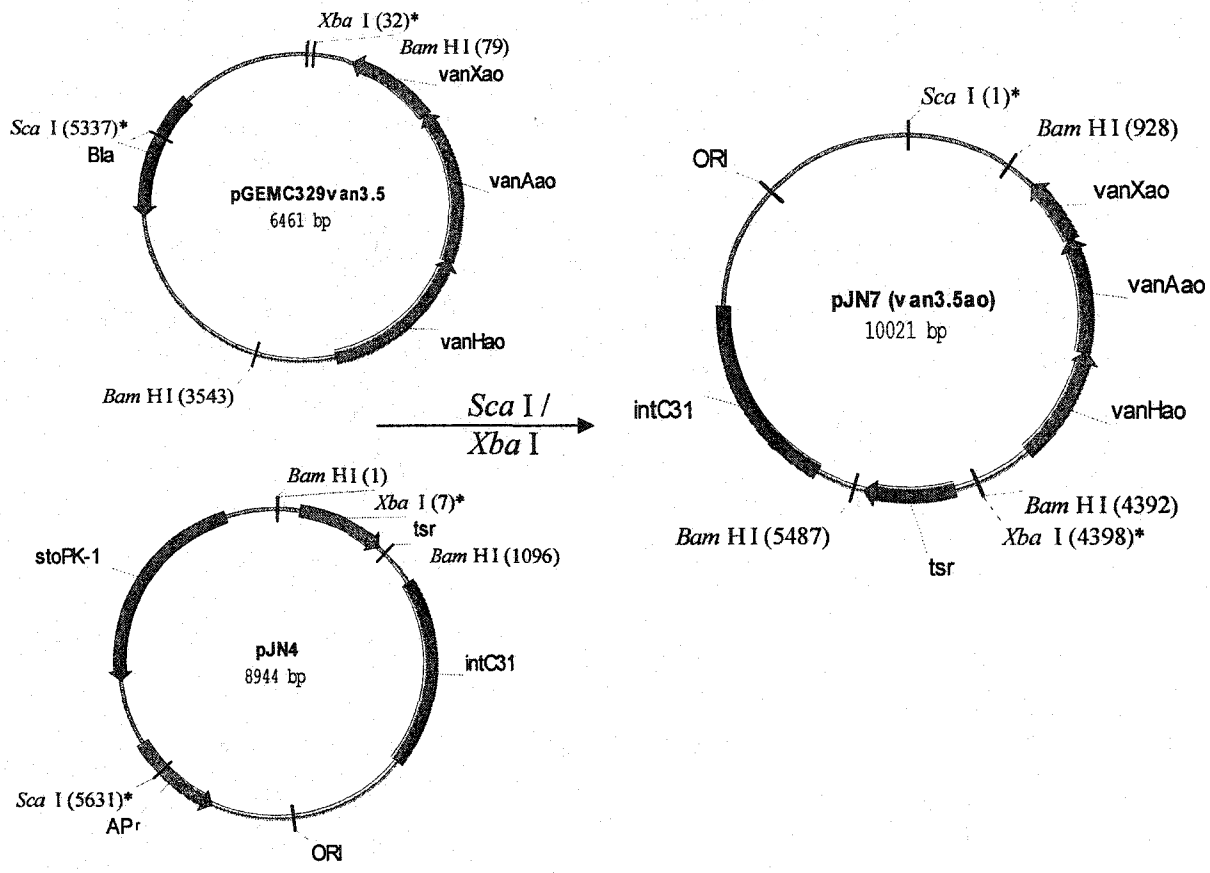

Figure 5.4: Construction of pJN7. Ligation of the *Sca* I/*Xba* I fragments from pGEMC329van3.5 and pJN4 create a *E. coli/Streptomyces* shuttle plasmid carrying the thiostrepton resistance marker and the ϕC31 integrase for insertion into the genome.

5.2.6. Complementation of *van*R_{SC}::Am.

The *van*R_{SC} gene was supplied *in trans* utilizing the site specific integration region of pSET152 (Bierman *et al* 1992). Ligation of the entire pSTBlue-RR plasmid containing the intact *van*Rsc gene and upstream region with the integrase was achieved with *Xho* I/*Xba* I restriction to generate pSET-vanRS'sc (Figure 5.5). Approximately $1x10^8$ *van*R_{SC}::Am protoplasts were transformed with 1 μg of this plasmid after passage through the *dam*$^-$, *dc*m$^-$ cell line GM48. Regeneration of transformed protoplasts occurred overnight, after which 3 mg of neomycin sulfate was overlaid in 500 μL of SAM. Plates were examined for neomycin resistant colonies after 3-4 days. Transformants exhibiting the correct phenotype (*ie.* apramycin resistant and neomycin resistant) were subsequently examined for the correct genotype by Southern blot and were subsequently tested for resistance to vancomycin.

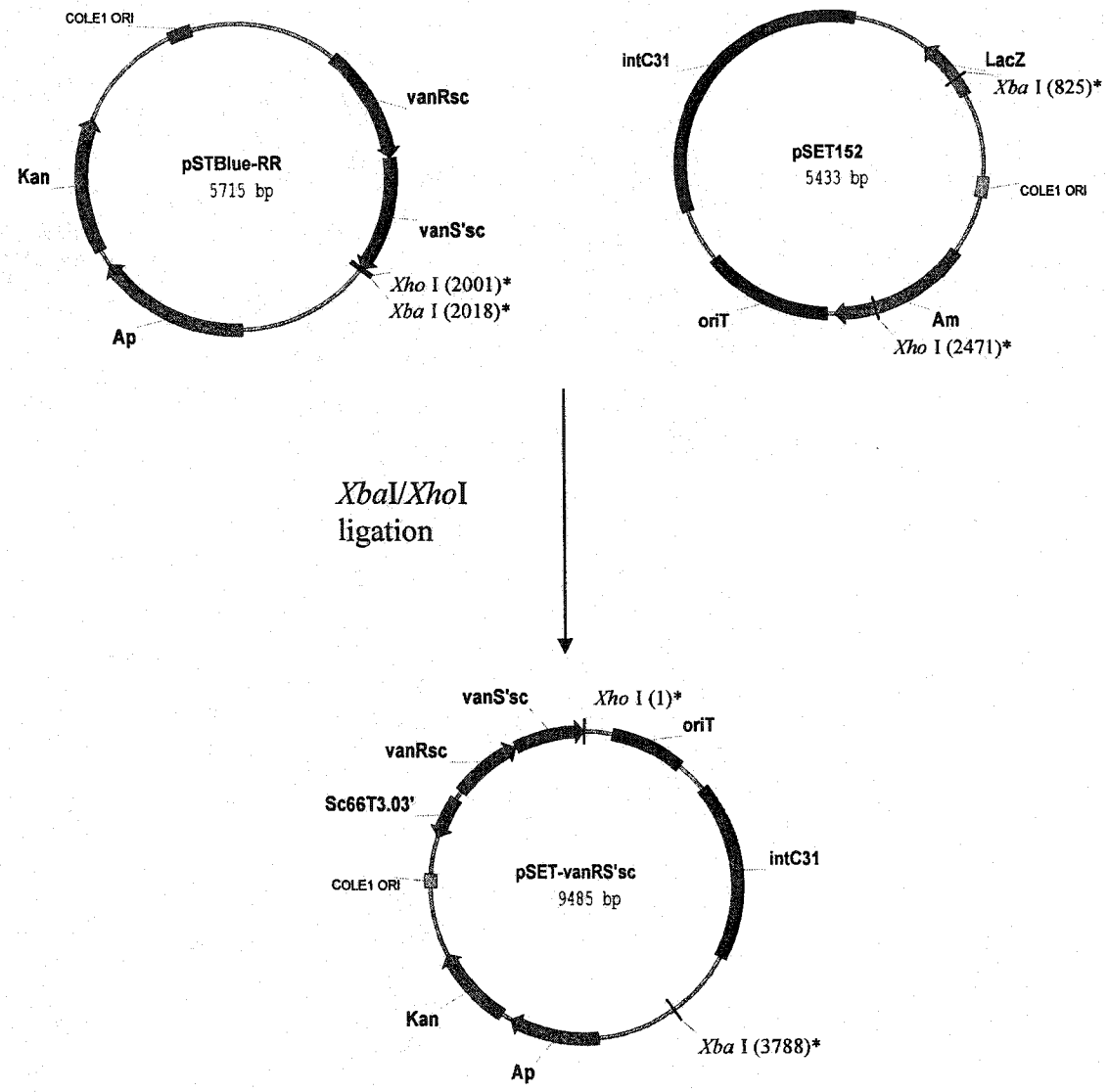

Figure 5.5: Construction of pSET-vanRS'sc, an integrating complementation vector for *van*R_{SC}

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5.2.7. Southern Blots.

Southern blots were carried out by standard techniques. *S. coelicolor* genomic DNA was cut with a variety of restriction enzymes and transferred to Hybond-N+ charged nylon (Amersham Pharmacia), equilibrated for 1 hr at 60 °C in hybridization buffer containing 0.5M Na_2PO_4, 7% SDS and 10 mg/mL BSA Fraction V. The blots were probed with a 700 bp α^{32}P-ATP-labeled *vanRsc* PCR fragment (generated using primers AB18787 and AB18788, Table 5.2) overnight in a 60°C hybridization oven. Two low stringency washes were then carried out with 50 mL of 40 mM Na_2HPO_4, 5% SDS, 1 mM EDTA, and 5 mg/mL BSA Fraction V for 10 min each at room temperature. A single high stringency wash was performed at 65 °C for 20 min. using 50 mL of 40 mM Na_2HPO_4, 1% SDS and 1 mM EDTA. Labelled probe was generated using a random hexamer labelling kit (Gibco BRL), 0.5 µg PCR DNA and 1U Klenow (MBI Fermentas). The probe was purified over silica beads (Qiagen) and counted in a scintillation counter (Beckman LS 3801). Blots were probed with 5-10 × 10^5 cpm/mL and exposed to X-ray film (X-omat, Kodak) for 1-4 hrs.

5.3. Results

5.3.1. Resistance profiles of various *Streptomyces* sp.

Examination of antibiotic resistance in *S. toyocaensis* NRRL 15009, *S. coelicolor* A3(2), *S. lividans* 66, and *S. fradiae* on different solid media revealed different resistance profiles (Table 5.4). The glycopeptide producer *S. toyocaensis*, showed no resistance to the glycopeptides vancomycin, teicoplanin or ristocetin, and only *S. coelicolor* and *S. lividans* showed glycopeptide resistance, with all of the test organisms sensitive to teicoplanin.

Table 5.4: Resistance profiles for *S. toyocaensis* NRRL 15009, *S. coelicolor* A3(2), *S. lividans*, and *S. fradiae* on minimal media (MM), maltose soy media (MS) and R2YE (R2) solid media.

Drug (µg/mL) – 25 uL per disc	*S. toyocaensis*			*S. coelicolor*			*S. fradiae*			*S. lividans 66*	
	MM	MS	R2	MM	MS	R2	MM	MS	R2	MM	R2
1. Vancomycin (100)	-	-	-	+	+	+	-	-	-	+	+
2. Teicoplanin (100)	-	-	-	-	-	-	-	-	-	-	-
3. Ristocetin (100)	-	-	-	±	+	+	-	-	-	+	+
4. Neomycin (250)	-	-	-	-	-	-	±	+	+	-	-
5. Kanamycin (250)	-	-	+	-	-	-	±	+	+	-	+
6. Quinupristin (100)	-	-	-	+	+	+	±	±	+	+	+
7. Dalfupristin (100)	-	-	-	+	+	+	±	±	+	+	+
8. Ampicillin (250)	+	+	+	+	+	+	+	+	+	+	+
9. Apramycin (250)	-	-	-	-	-	-	-	-	+	-	±
10. Synercid (100)	-	-	-	-	-	-	-	-	-	+	+
11. Erythromycin (250)	-	-	-	±	-	-	-	-	-	+	+
12. Tetracycline (250)	-	-	+	+	-	+	+	-	+	+	+
13. Spetinomycin (300)	+	-	+	+	+	+	+	±	+	+	+
14. Streptomycin (300)	-	-	+	-	-	-	-	-	+	-	+
15. Chloramphenicol (250)	-	-	+	+	+	±	+	-	-	+	+
16. Trimethoprim (250)	+	+	+	+	+	+	+	+	+	+	+
17. Naladixic acid (250)	+	+	+	+	+	+	+	+	+	+	+
18. Cephoxitin (250)	+	+	+	+	+	+	±	-	-	+	+
19. Sterile Water	+	+	+	+	+	+	+	+	+	+	+
20. Thiostrepton (100)	-	-	-	-	-	-	-	-	-	-	-

(-) No Growth around disc
(+) Good Growth around disc
(±) Some growth around disc

For *S. toyocaensis*, R2YE media appeared to support growth in the presence of several antibiotics that were inhibitory on the other media, for example kanamycin, tetracycline and chloramphenicol (Table 5.4). In the case of kanamycin, this trend was also seen for *S. lividans* (Figure 5.6), but not for *S. coelicolor* (Table 5.4).

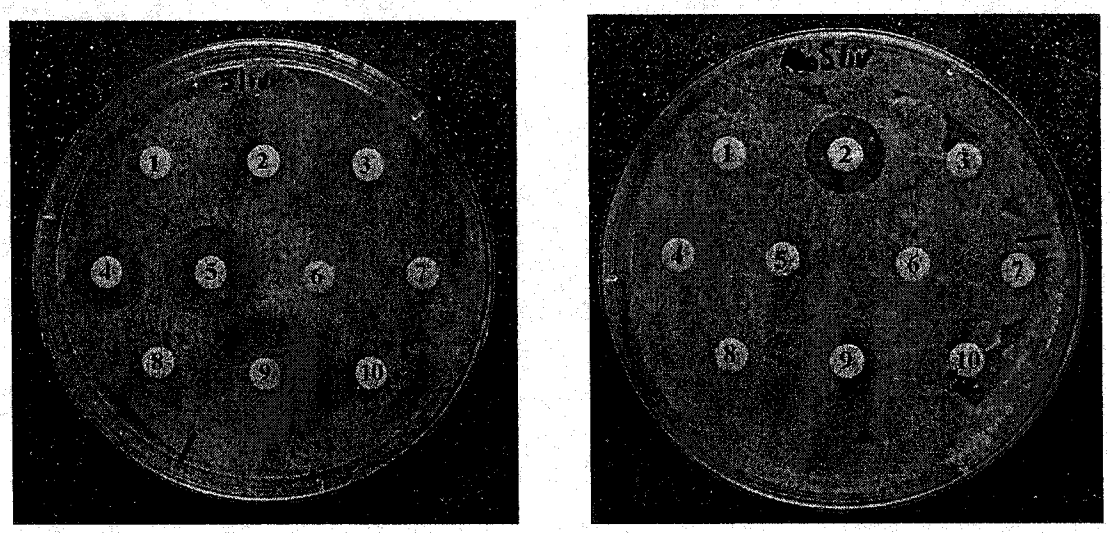

Figure 5.6: Resistance to kanamycin in *S. lividans* is media dependent. Antibiotic disc assays reveal a sensitivity to kanamycin (#5) on minimal media (left) but not R2YE (right). Antibiotics are numbered according to Table 5.4.

5.3.2. Disruption and Complementation of *van*A_{ST} (*ddl*M) in *S. toyocaensis* NRRL 15009

Transformation of *S. toyocaensis* NRRL 15009 protoplasts with denatured pBlutsr-ddl3.0-Am produced ~40 apramycin resistant colonies. Six transformants were chosen for further study. As seen in previous studies with *S. toyocaensis*, the majority of these were characteristic of double crossover events, being sensitive to thiostrepton (5 out of 6). Examination of genotype by polymerase chain reaction revealed amplification of products of the expected size (Figure 5.7A) These results were consistent with the transformants' sensitivity to thiostrepton. Analysis of glycopeptide resistance of *van*A_{ST}::Am spores revealed a sensitivity to A47934 which was not seen in the wild type (Figure 5.7B). Complementation of the *van*A_{ST}::Am mutant with pJN7 resulted in complete reversal of the vancomycin sensitivity (Fig. 5.8). This result confirms the function of the *van*HAX cluster in *S. toyocaensis* and shows the ability of *van* gene clusters from other glycopeptide producers function in the same fashion.

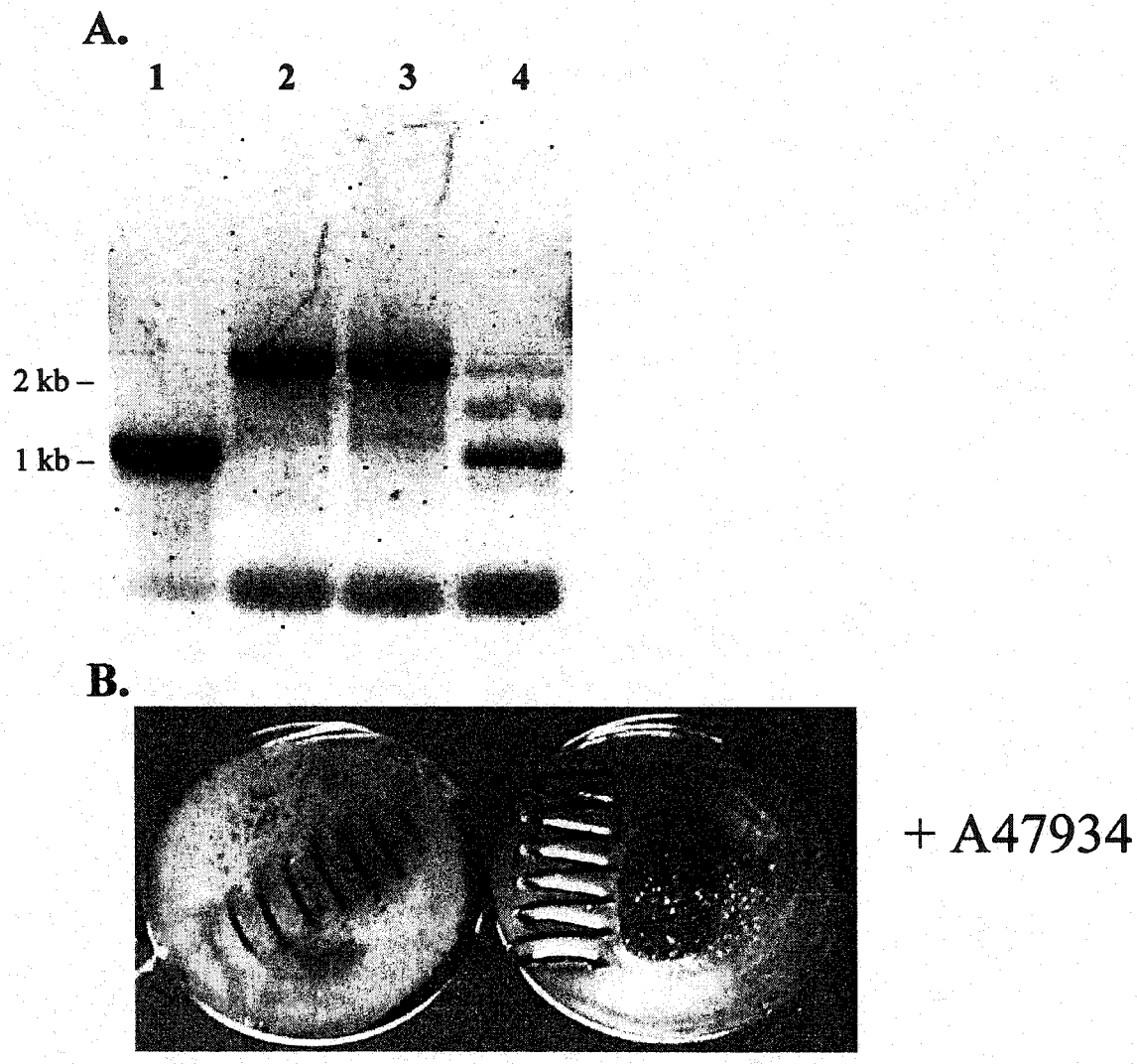

Figure 5.7: Disruption of *van*A_{ST} (*ddl*M) results in sensitivity to A47934. **(A)** Genotype characterization of *van*A_{ST}::Am knockouts. PCR of vanAst genomic region showing wild type (lane 1), two double cross over transformants (Lane 2 and 3) and a single cross-over transformant (Lane 4). PCR was performed by standard protocol with primers AB9363 and AB9364 (Table 5.2). **(B)** Phenotype characterization of *van*A_{ST}::Am double-cross mutant shows sensitivity to 50 μg/mL A47934 (right) in liquid culture, whereas the wild type is resistant (left).

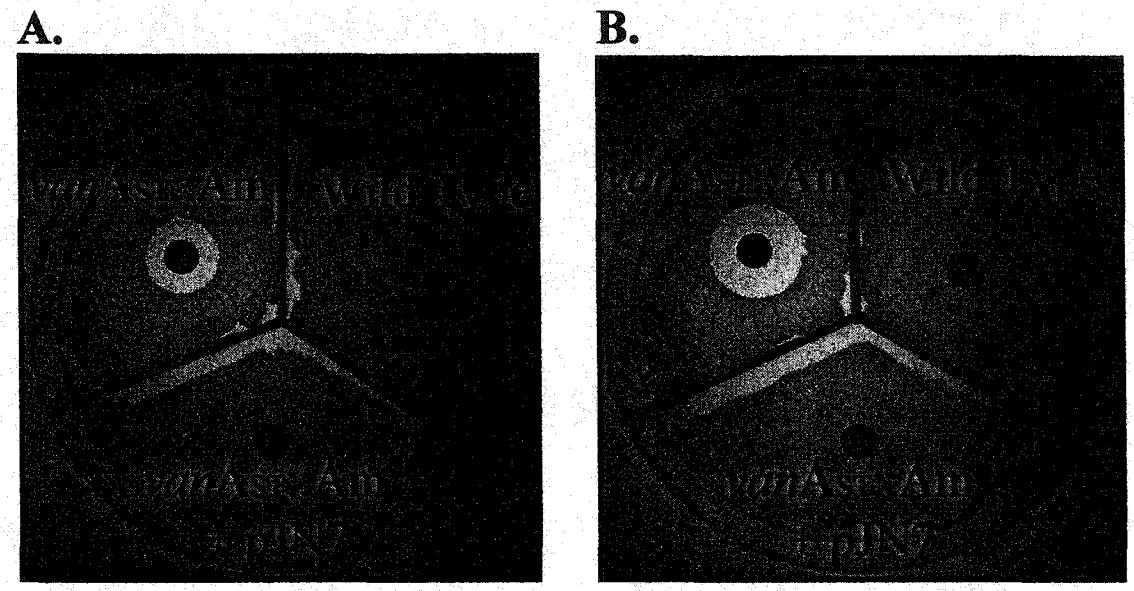

Figure 5.8: Effects of *van*A_{ST} disruption on *S. toyocaensis* sensitivity to glycopeptides. Addition of 15 µL of 500 µg/mL A47934 **(A)** or 15 µL of 300 µg/mL vancomycin + 50 µg/mL A47934 **(B)** inhibits growth of the *van*A_{ST}::Am disruption mutant but not wild type *S. toyocaensis*, and the sensitivity can be reversed by supplying the *van*HAX cluster *in trans* on pJN7.

5.3.3. Disruption and Complementation of *van*R_{SC} in *S. coelicolor* A3(2).

Transformation of *S. coelicolor* protoplasts with single stranded pSTBlue-RR-Am produced approximately 75 colonies on R2YE regeneration media. Unlike *S. toyocaensis*, all transformants from *S. coelicolor* showed high levels of resistance to the secondary marker (neomycin). This result is consistent with other work performed in this organism which shows a high ratio of single to double crossover events (Justin Nodwell, personal communication). In order to generate double recombinants, six transformants where

apramycin. Spores isolated at the end of this treatment lost their high level resistance to neomycin and were mapped using PCR and Southern hybridization. Results of mapping experiments showed the correct genotype (Figure 5.9 and Figure 5.10). Complementation of these mutants with pSETvanRS'sc also showed the expected genotype on Southern hybridization mapping (Figure 5.10). Growth tests on R2YE with amparmycin (250 μg/mL) and vancomycin (100 μg/mL) utilizing a disk assays revealed a loss of resistance in the *van*R_{SC}::Am spores (Fig. 5.11) which could be reversed upon addition of a single copy of the *van*R_{SC} gene on the integration vector pSETvanRS'sc (Fig. 5.11).

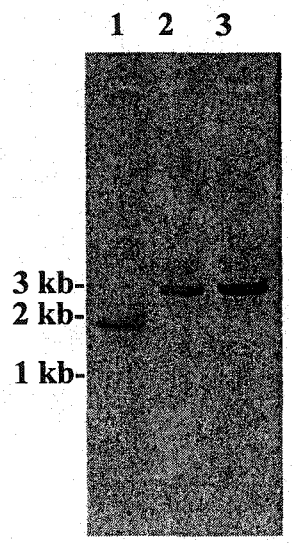

Figure 5.9: PCR characterization of *van*R_{SC}::Am genotypes. PCR amplification of 1.9 kb genomic fragment from wild type S. coelicolor (Lane 1) and a 2.9 kb fragment containing the apramycin resistance insert from two double cross-over transformants (Lane 2 and 3).

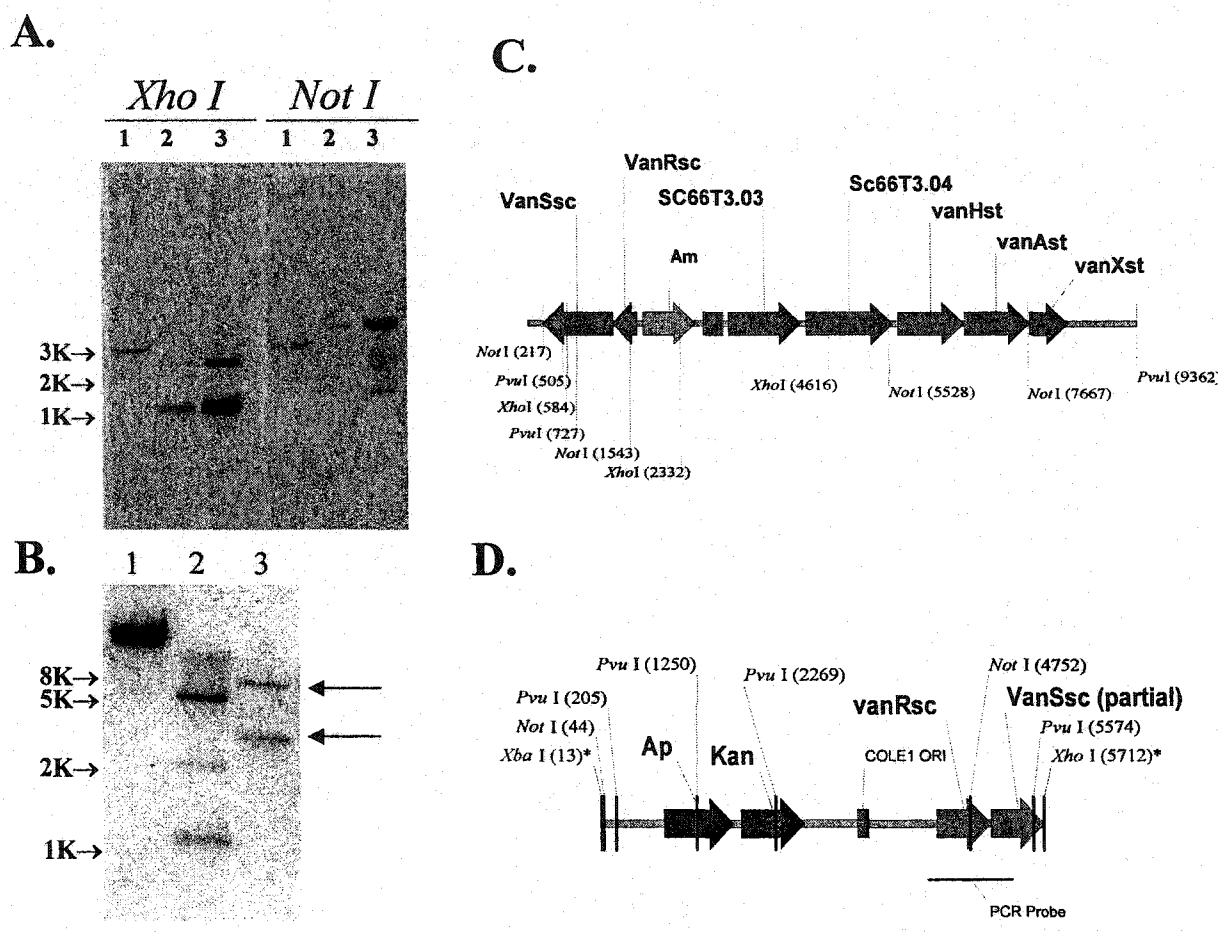

Figure 5.10: Southern blot genotype characterization of *van*R$_{SC}$ mutants. **(A)** Restriction digests with *Xho* I and *Not* I showing wild type *S. coelicolor* (Lane 1) and two double cross-over *van*R$_{SC}$::Am transformants (Lane 2 and 3). **(B)** pSETvanRS'sc complementation strains showing genomic DNA (Lane 1) and *Xho* I / *Xba* I digested (Lane 2) and Pvu I digested DNA (Lane 3). Detailed restriction maps of the relevant genomic areas are shown in **(C)** and **(D)**.Upper arrow shows DNA fragment associated with the *van*HAX cluster (in **C**) and lower arrow is fragment associated with pSETvanRS'sc integration (in **D**)

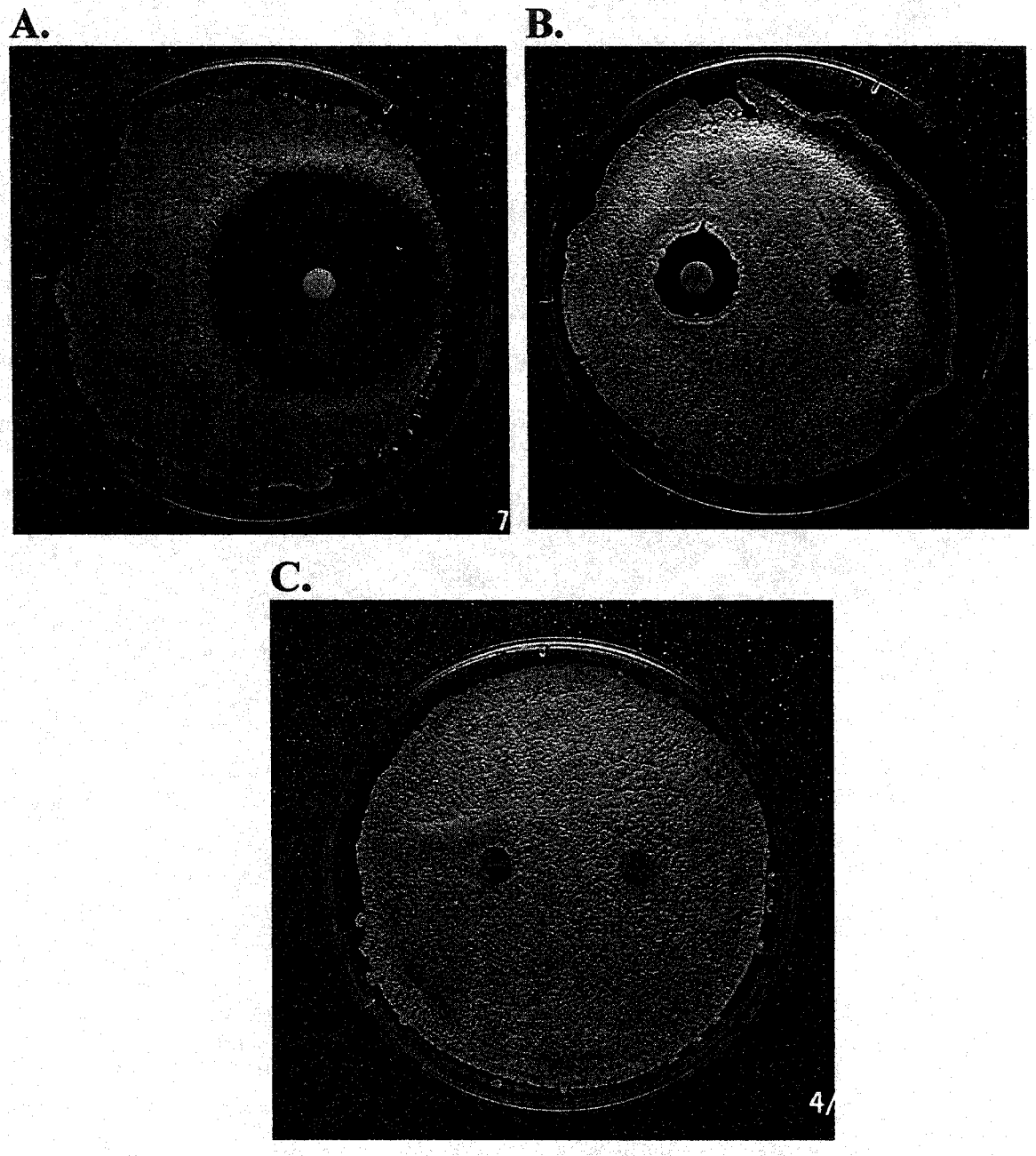

Figure 5.11: Disruption of *van*R_{SC} results in a loss of vancomycin resistance in *S. coelicolor*. **(A)** Wild type *S. coelicolor* A3(2) shows resistance to vancomycin (left disc) and sensitivity to apramycin (right disc). **(B)** *van*R_{SC}::Am mutant shaows sensitivity to vancomycin (left disc) and resistance to apramycin (right disc). **(C)** Complementation of *van*R_{SC}::Am mutant with pSETvanRS'sc restores vancomycin resistance (left disc) and resistance to apramycin is maintained (right disc).

5.4. Discussion

5.4.1. Resistance Profiles

All of the Streptomyces species examined here showed sensitivity to several of the antibiotics tested (Table 5.4). These included agents popularly used for *Steptomyces* genetic work such as apramycin and thiostrepton (Hopwood *et al* 1985). Of note is the observed media differences seen with some antibiotics and species. For instance, *S. lividans* and *S. toyocaensis*, both showed sensitivity to kanamycin on some media but not others (Table 5.4, Fig. 5.6). In fact, most of the organisms tested showed media-dependent effects in the presence of several antibiotics, with *S. toyocaensis* showing clear media-dependent effects to five compounds, *S. fradiae* three, *S. lividans* two and *S. coelicolor* showing no clear media-dependent effects (Table 5.4).

S. lividans and *S. coelicolor* are very closely related and could even be strains of the same organism (Hopwood *et al* 1985). However, with respect to resistance profiles these organisms differed in some significant ways. For instance, erythromycin and Synercid resistance appear in *S. lividans* but not *S. coelicolor* (Table 5.4). In addition, a media-dependent effect on R2YE was seen for *S. lividans* in the presence of the aminoglycosides kanamycin and streptomycin but was not seen for *S. coelicolor* (Table 5.4). Such differences may reflect the presence of mechanisms that are dependent on the expression of genes not normally employed for resistance, or may be a sign of permeability differences in the organism under the different conditions. Regardless, the differing resistance profiles between the two closely related *Streptomyces* show there are important differences between them.

The resistance profile of *S. toyocaensis* to glycopeptides revealed this organism cannot grow in the presence of glycopeptides other than the one it produces (*ie.* A47934, also see Chapter 2). This result suggests that the inducing system in this organism may have important differences to the systems employed by other *Streptomyces* such as *S. coelicolor*. In addition, the studies carried out here suggest that the presence of glycopeptide resistance in non-glycopeptide producers (*eg. S. coelicolor* A3(2) and *S. lividans* 66) may be a relatively common phenomenon. In all cases however, teicoplanin sensitivity was evident and is reminiscent of the VanB *Enterococcus* phenotype (See Chapter 1). The VanB-like phenotype is also supported by results with late stage cell wall inhibitors such as bacitracin and penicillin, which induce resistance in VanA-type but fail to induce resistance in VanB-type *Enterococcus* and in *S. toyocaensis* (see Chapter 1, section 1.3.3 and Chapter 2 section 2.3.4). This observation may reflect a similar resistance induction mechanism for VanB-type *Enterococcus* and *Streptomyces*, which in *Enterococcus* is thought to be through direct interaction of the glycopeptide with some kind of "receptor" (Baptista *et al* 1999).

5.4.2. Significance and summary

Evidence is presented in this Chapter that clearly support the service of the *van*HAX cluster in *Streptomyces* as bona fide glycopeptide resistance genes utilized in a similar fashion to those of *Enterococcus*. Furthermore, complementation with a *van*HAX cluster from the related organism *Amycalotopsis orientalis*, which produces vancomycin, supports the utilization of this cluster in other Actinomycetes for glycopeptide resistance.

The disruption of response regulator linked closely to the *van*HAX cluster in *S. coelicolor* A3(2) revealed a clear role for this gene in glycopeptide resistance. Therefore, the role of the encoded protein in regulation probably occurs in a manner not unlike enterococcal *van*R. For this reason we have named the two component system $vanR_{SC}$ and $vanS_{SC}$. Although our knowledge of vancomycin resistance in *Enterococcus* is fairly substantial, including its inducible regulation and biochemistry, the method by which the bacteria sense the presence of vancomycin to turn on the resistance genes is still a matter of some debate. A noteworthy contradiction exists in current thought as to which molecules are inducers and what consequences this has for the induction/sensing system (see Chapter 1 section 1.3.3). With this respect, *Streptomyces* glycopeptide resistance appears similar to the enterococcal VanB system in that only glycopeptides are inducers and both these systems are unable to detect teicoplanin.

The strong similarities between *Streptomyces* and *Enterococcus* with respect to glycopeptide resistance suggests there may be benefits in studying resistance in *Streptomyces*. Given these similarities, knowledge of the regulation of resistance in *Streptomyces* may be directly applicable to *Enterococcus* and careful examination of the *Streptomyces* system should produce a better understanding of the resistance mechanisms in VRE. Therefore, the detailed study of glycopeptide resistance in *Streptomyces* could be an extremely fruitful endeavour.

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Chapter 6.

General Conclusions and Future Directions

6.1 Protein Kinases in *Streptomyces*

This thesis has introduced a broad series of studies on glycopeptide antibiotic production and resistance in *Streptomyces*. The relatively recent discovery of Hank's type protein kinases in various bacterial genera including these organisms has lead us to focus on their presence in a glycopeptide producer *S. toyocaensis* NRRL 15009. Examination of protein phosphorylation and the effects of protein kinase inhibitors suggested that there is evidence to connect these regulatory proteins to glycopeptide antibiotic production and resistance. In order to provide more substantial and specific evidence, cloning of putative protein kinase fragments using a degenerate strategy was then initiated. Although it was hoped that this evidence would be forthcoming, in retrospection, the large number of these genes as judged by the recently sequenced *S. coelicolor* A3(2) genome (40 genes) suggests that a cloning/disruption strategy as employed here is an extremely large undertaking and may not be feasible for our laboratory. In any event, the work provided in this thesis, in addition to establishing the general techniques in our laboratory to undertake such analyses, provides direct evidence for these genes as bona fide protein kinases involved in regulatory functions in these organisms. Specifically, the isolation and characterization of StoPK-1 supports this contention through its autophosphorylation and phosphotransfer to various model substrates, and a requirement for an active protein in responding to oxidative stress in the presence of glucose.

In addition to providing evidence for a regulatory role in *Streptomyces* physiology, the work presented here also suggests other avenues of exploration. One of

these is born out of the observation that all of the *S. toyocaensis* protein kinase fragments isolated here have highly similar homologues in *S. coelicolor* A3(2). The isolation of $afsK_t$ from *S. toyocaensis* by a PCR strategy employing primers specific for the *S. coelicolor* A3(2) gene shows the utility of this approach and is a result of the high sequence conservation between the two organisms (typically >80%). This close relationship could allow the isolation of most if not all protein kinases from *S. toyocaensis* for further characterization possibly through expression studies utilizing oligonucleotide microarrays (Schulze and Downward 2001, Wu 2001). Furthermore, genetic differences could also be exploited to isolate protein kinase genes from *S. toyocaensis* that are not present in *S. coelicolor* (and therefore may be involved in regulating glycopeptide production) through the use of an inhibitory PCR strategy (Yuen *et al* 2001).

6.2 Vancomycin Resistance in *Streptomyces*

The similarity between the regulation of VRE and *Streptomyces* resistance genes could allow the study of *Streptomyces* regulation as a model for VRE. The aim of these studies is the complete elucidation of the regulation vancomycin resistance, which may lead to the development of alternate treatment strategies through the restoration of vancomycin sensitivity in resistant organisms. Toward this end, analysis of upstream DNA elements of *van*HAX revealed a highly similar region just upstream of the ORF Sc66T3.03 (*gra*1) and Sc66T3.04 (*gra*2) which were subsequently shown to have significantly increased expression after challenge with vancomycin (Mark Buttner

unpublished observation). The predicted protein sequence of Gra1 shares no homology with any known gene products, but is predicted to be a membrane associated protein and contains a LPXTG motif shown to be a cell wall anchor signal sequence in gram positive bacteria including *Streptomyces* (Navarre and Schneewind 1999). One the other hand, Gra2 shares significant homology (34%) with the FemA proteins from *Staphylococcus*, which are involved in pentaglycine interpeptide bridge formation in the growing peptidoglycan layer, specifically adding glycine residues 2 and 3 (Ehlert *et al* 1997). We are interested in the function of these novel proteins by examining whether their presence is necessary for vancomycin resistance. For this reason, the following additional work has been carried out.

6.2.1. Disruption of *gra*1 and *gra*2

In order to disrupt *gra*1 the disruption plasmids pSTBluevanR'66T3.03Am (Figure 6.1) and pSTBluevanR'66T3.03Am2 (Figure 6.2) were constructed. pSTBluevanR'66T3.03Am was constructed by cloning a genomic *Pst* I-*Hind* III PCR fragment (from AB18787 and AB21325, Table 5.2 and 6.1) containing the entire *gra*1 (Sc66T3.03) gene and inserting the apramycin resistance gene (Am) into the unique *Eco* RI and *Aat* II sites of *gra*1 (Sc66T3.03)(see Figure 6.1). I have transformed wild type *S. coelicolor* A3(2) and isolated apramycin resistant colonies for further examination. PCR analysis of the genomic DNA from two colonies reveals the presence of the apramycin cassette. However, these appear not to represent double recombination events and several passages of these clones and isolation of new colonies from individual spores did not

appear to effect this genotype (data not shown). For this reason the second disruption plasmid pSTBluevanR'66T3.03Am2 was constructed. This plasmid was constructed from pSTBluevanR'66T3.03Am by insertion of the downstream region containing *gra*2 (Sc66T3.04) by a three-way T4 ligation (see Figure 6.2 for details). This effectively increases the size of the recombination area and should hopefully increase the rate of double recombination events.

Table 6.1: Primers and plasmid constructs used in these studies.

Primer	**Sequence 5'-3'**	**Comments**
AB18593	GGTGATCCGAGGGCGAGGGTTACG	*gra1*sc reverse
AB21324	CGGAATTCATATGCTCGGCGACAAGTCAGGCG	*gra1*sc forward
AB21325	CGGAATTCAAGCTTCTCACCAGCTGACCCCCGCC	*gra1*sc reverse
AB22423	CCCGCACGTCGGACTGATCAAG	*gra*2sc forward
AB27103	TTCAAGGCGCCTCGGTGTTG	*gra*2sc reverse
AB27104	CTCGGCGACCTGAACGGCAC	*gra*2sc forward
AB27105	CCGAGCACGGCGAACATGGT	*gra*2sc reverse

Plasmid Construct	**Description**	**Source**
pSTBlue-vanR'66T3.03	1.5 kb PCR fragment in pST1Blue1	This study.
pSTBlue-66T3.04	2.3 kb *Eco* RI/*Mph* 1103I fragment from SuperCos66T3 cloned into pSTBlue1	This study.
pSTBlue-vanR'66T3.03-04'	2 kb *Eco* RI / *Not* I fragment from pSTBlue-66T3.04 cloned into pSTBlue-vanR'66T3.03	This study.
pSTBlue-vanR'66T3.03Am	Disruption plasmid for Sc66T3.03: apramycin resistance gene inserted in *Eco*RI / *Aat*II sites.	This study.
pSTBlue-vanR'66T3.03Am2	Same as pSTBlue-vanR'66T3.03Am with 1.2 kb additional downstream coding region	This study.
pSTBlue-vanR'66T3.03-04tsr	Disruption plasmid for Sc66T3.04: thiostrepton resistance gene inserted in *Xho* I / *Sma* I sites.	This study.

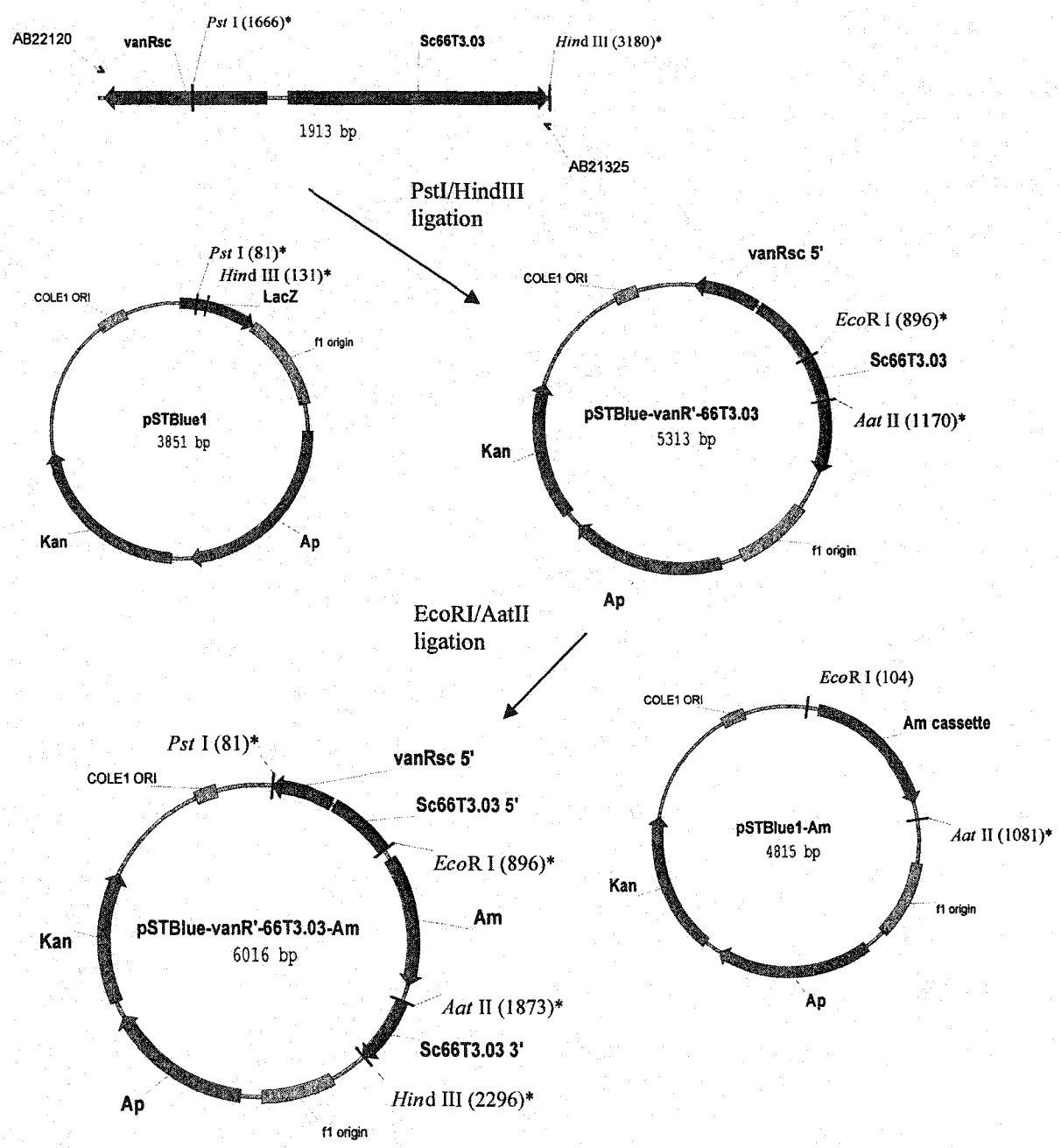

Figure 6.1: Construction of pSTBluevanR'66T3.03Am (See text for details).

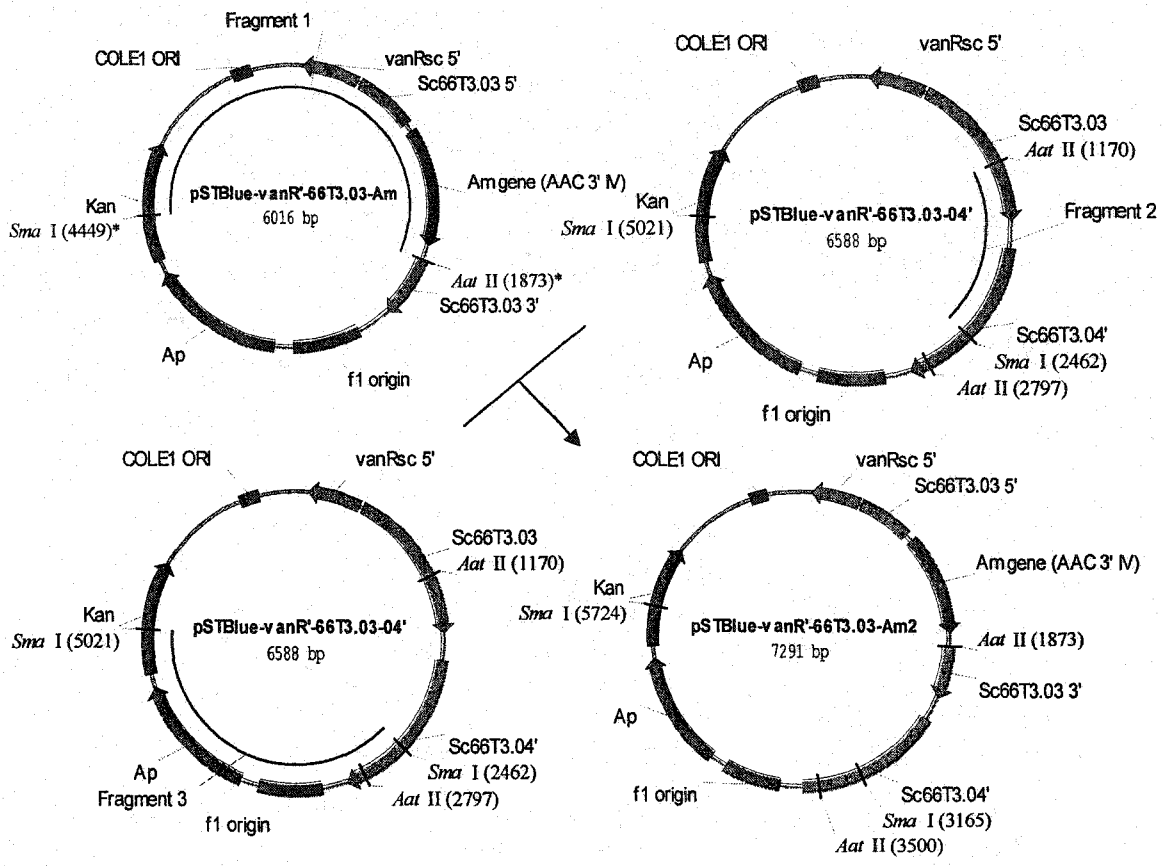

Figure 6.2: Construction of pSTBluevanR'66T3.03Am2. Three DNA fragments were joined with T4 DNA ligase: Fragment 1, Fragment 2 and Fragment 3. *Sma*I and *Aat*II restriction was carried out for each fragment and dephosphorylation with Shrimp Alkaline Phosphatase (SAP, MBI Fermentas) was performed on Fragment 3 to prevent circularization.

The fidelity of pSTBluevanR'66T3.03Am and pSTBluevanR'66T3.03Am2 were confirmed by restriction mapping and sequencing across the ligation junctions. The non-methylated plasmids isolated from *E. coli* GM48 were transformed into wild type *S. coelicolor* A3(2) in single stranded form (up to 3 μg) as previously described by Oh and Chater (1997). Regeneration of transformed protoplasts occurred overnight, after which 1 mg of apramycin sulfate was overlaid in 500 μL of SAM. Plates were examined for

apramycin resistant colonies after 3-4 days. Transformants exhibiting the correct phenotype (*ie.* apramycin resistant and neomycin sensitive) were subsequently isolated and grown for further analysis. Genotype and phenotype analysis of these is ongoing in our laboratory.

The disruption of *gra*2 was carried out with the plasmid pSTBluevanR'66T3.03-04tsr. Construction of this plasmid utilized the plasmid pSTBluevanR'66T3.03-04' and the thiostrepton cassette from pSTBlue-tsr (Figure 6.3). A three-way ligation similar to pSTBlue-vanR'66T3.03-Am2 was carried out with *Sma* I and *Sma* I/*Xho* I digestion products as outlined in Figure 5.7 below. The resulting plasmid contains a thiostrepton resistance gene inserted into the unique *Xho* I – *Sma* I sites of *gra*1. The fidelity of pSTBluevanR'66T3.03-04tsr was confirmed by restriction mapping and sequencing across the ligation junctions. The non-methylated plasmids isolated from *E. coli* GM48 were transformed into wild type *S. coelicolor* A3(2) in single stranded form (up to 3 μg) as previously described by Oh and Chater (1997). Regeneration of transformed protoplasts occurred overnight, after which 1 mg of thiostrepton was overlaid in 500 μL of SAM. Plates were examined for thiostrepton resistant colonies after 3-4 days. Transformants exhibiting the correct phenotype (*ie.* thiostrepton resistant and neomycin sensitive) were subsequently chosen and grown for further analysis. Genotype and phenotype analysis of these is also continuing in our laboratory.

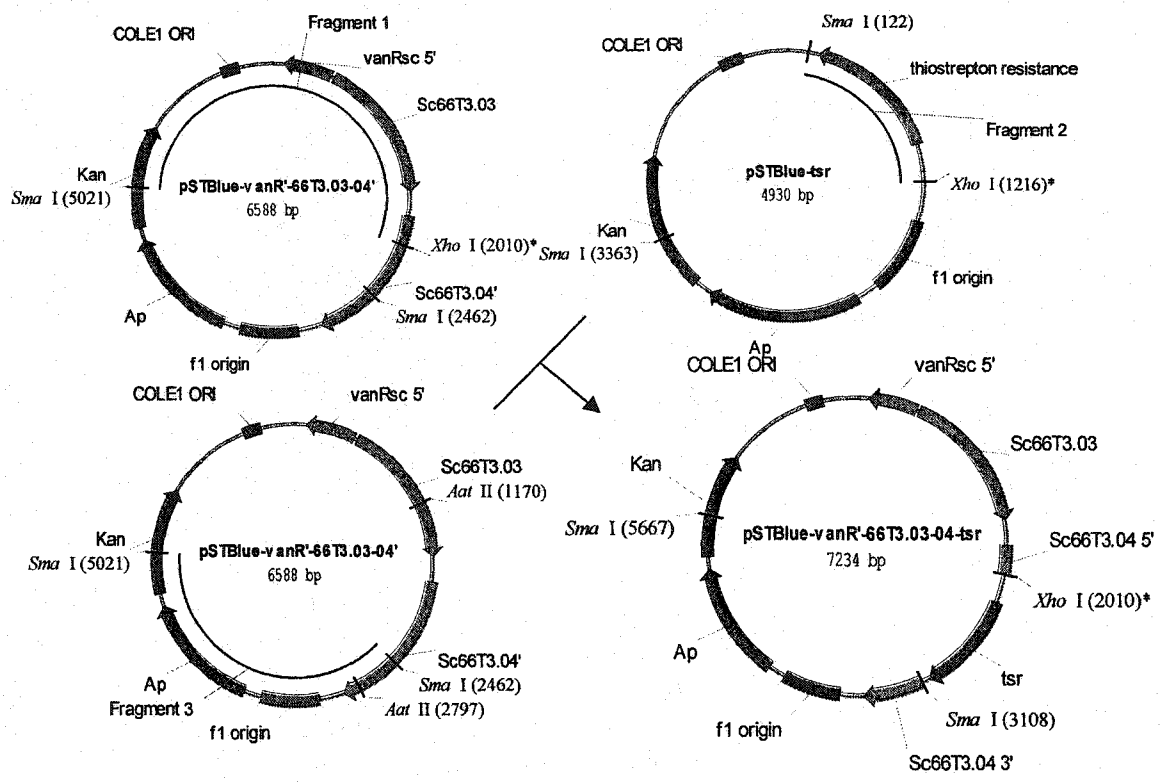

Figure 6.3: Construction of disruption plasmid pSTBluevanR'66T3.03-04tsr. Three DNA fragments were joined with T4 DNA ligase: Fragment 1, Fragment 2 and Fragment 3. SmaI and *Xho* I restriction digestion was carried out for each fragment and dephosphorylation with Shrimp Alkaline Phosphatase (SAP, MBI Fermentas) was performed on Fragment 3 to prevent circularization.

The discovery of a VRE-like resistance system in the non-glycopeptide producing organism *Streptomyces coelicolor* as outlined here, raises hope of gaining detailed knowledge of the induction/sensing system. As shown in Figure 6.4, this discovery strongly suggests that the vancomycin resistance genes originated in a glycopeptide producer and transfer of these genes occurred to other "non-producers" such as *Streptomyces coelicolor* and VRE. Therefore, a careful study of resistance in *S. coelicolor* A3(2) could lead to significant advancements in understanding the VRE

system. In this regard the functional analysis of genes associated with the resistance cluster as undertaken here, is a logical expansion of the studies carried out to date. Additionally, studies involving the transfer of glycopeptide resistance genes to sensitive *Streptomyces* species such as *S. fradiae* could also be very informative. *Streptomyces* are level one organisms and have established cloning vectors, mutation protocols etc., and determination of the minimal gene complement for inducible glycopeptide resistance should be a feasible undertaking.

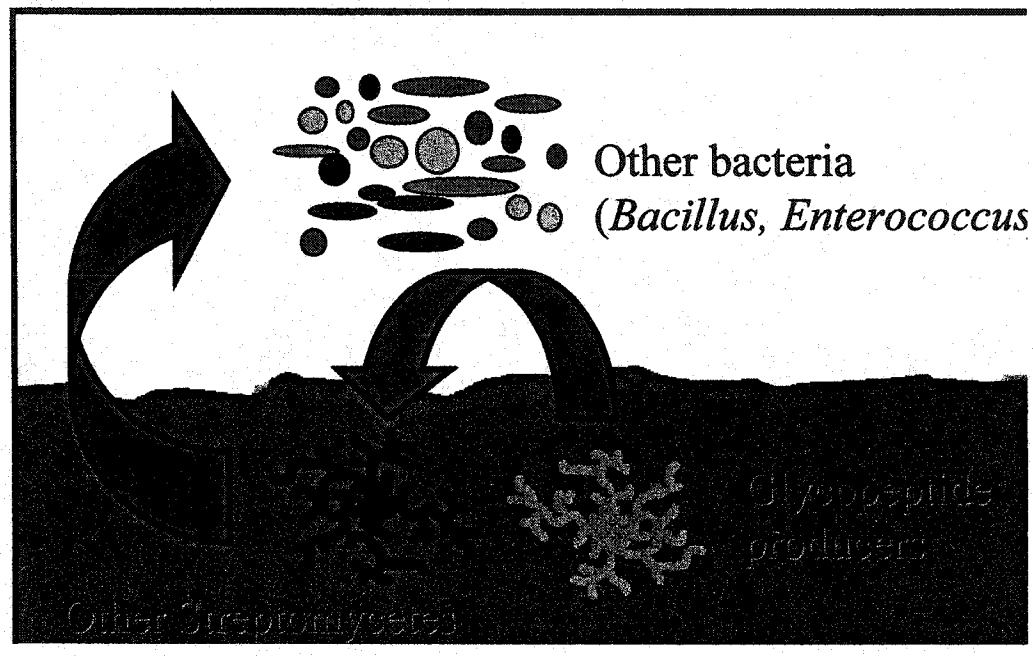

Figure 6.4: Model for horizontal transfer of glycopeptide resistance genes.

6.3 References

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