ISOLATION OF MORPHOLOGICAL MUTANTS IN *STREPTOMYCES* SPECIES

by

SARAH ELIZABETH PIOTROWSKI JORDAN

(Under the direction of Dr. Janet Westpheling)

ABSTRACT

Streptomyces are filamentous, multi-cellular soil bacteria that undergo a complicated life cycle of differentation and secondary metabolite production. However, little is known about how these pathways function in the organism. We sought to isolate new mutants in these processes in two species: *S. coelicolor* and *S. venezuelae*. This was accomplished through the use of intergeneric conjugation, *in vitro* transposition, and co-transformation. Through these methods we were able to identify 5 morphological mutants, 3 of which were caused by transposon insertion into previously uncharacterized genes. Based on sequence analysis, putative functions of these gene products are: an integral membrane protein and a tetR-family transcriptional regulator. We have not yet obtained sequence data for the third mutant gene.

INDEX WORDS: *Streptomyces*, *bld* genes, Intergeneric conjugation, *in vitro* transposition, Differentiation, Antibiotic Production

ISOLATION OF MORPHOLOGICAL MUTANTS IN *STREPTOMYCES* SPECIES

by

SARAH ELIZABETH PIOTROWSKI JORDAN

B.S., Georgia Southern University, 1999

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GA

2002

ISOLATION OF MORPHOLOGICAL MUTANTS IN *STREPTOMYCES* SPECIES

by

SARAH ELIZABETH PIOTROWSKI JORDAN

Appproved:

Major Professor: Janet Westpheling

Committee: Anna Karls
Larry Shimkets

Electronic Version Approved:

Gordhan L. Patel
Dean of the Graduate School
The University of Georgia
August 2002

DEDICATION

This work is dedicated to my family. Without their support and encouragement, this would not have been possible. A special dedication goes to my son, Harper. You may not have arrived yet, but you are already a part of me and I love you.

ACKNOWLEDGMENTS

There are lots of people who have made this work possible. Thanks to everyone in my lab that helped me through all my problems, both lab related and not. I especially need to thank Ondrej Sprusansky, without whose technical support, none of the molecular work would have ever gotten finished, and Liqin Zhou for her collaberation with this project. I also need to thank Julie Boylan who not only taught me all I needed to know in the lab, but she also kept me sane when it felt like the world was closing in on me. A special thanks to Jan Westpheling for her support, both monetary and academic. I would never have made it through without the devotion and support provided by the love of my life, my husband Spencer. I love and appreciate all you do for me more than you will ever know. Thanks to my family for supporting me all my life in every decision I've ever made. I wouldn't be here if it wasn't for you. Thank you as well to Dr. Rodney Mauricio and Dave Brown for the incredible technical support you provided throughout my time here.

TABLE OF CONTENTS

Page

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Streptomyces

Streptomyces are Gram-positive soil bacteria. They contain one long linear chromosome, approximately 8 Mb long, as well as several linear plasmids (Lin 1993). This chromosome has a high GC content, close to 73%. The completed genome sequence of *Streptomyces coelicolor* (A3(2)), the model organism for the streptomycetes, and is available online at www.sanger.ac.uk/Projects/S_coelicolor. It is believed that replication of the chromosome occurs from an *oriC* and proceeds bi-directionally (Musialowski 1994). The *oriC* is centrally located on the chromosome. Replication is completed by the priming of proteins that bind covalently to both 5' free ends (Chang 1994). The ends of the chromosome are highly unstable, often resulting in spontaneous deletions that can be up to 2 Mb long under laboratory conditions. This is the result of long terminal inverted repeats of 24-600 kb, depending on the species, and occurs rather frequently (0.1-1%) (Chen 1996).

The model organism of this group, *Streptomyces coelicolor*, displays a gene density pattern typical of bacteria (one gene per 1100 base pairs). This would suggest that *S. coelicolor* contains approximately 7000 genes. So far close to 200 have been physically mapped on the chromosome. Assuming the gene prediction is accurate, *S. coelicolor* would contain more genes than *Saccharomyces cerevisiae*, a eukaryote, by

20%. This large number of genes could be explained by the complex processes of sporulation and antibiotic production streptomycetes perform in response to environmental stimuli.

Life cycle and morphological mutants

Streptomyces undergo a complex cycle of morphological and physiological differentiation. Growth begins when a spore germinates and forms a vegetative mycelial mass (figure 1.1). Septation is not frequent in the vegetative mycelium, resulting in many multi-nucleoid compartments. When certain signals are sensed in the environment, this vegetative mycelia begins to differentiate. The response to these stimuli is mediated by cell-cell signaling through diffusible extracellular molecules (Willey 1991). Once the response is initiated, aerial hyphae begin to form on the surface of the mycelial mass. These aerial hyphae grow by tip elongation and progressively coil, then septate into uni-nucleoid compartments, which ultimately give rise to mature spores.

Genetic analysis has been the key to understanding this complex pathway. There are two classes of developmental mutants: *whi* mutants, which can form aerial hyphae, but cannot give rise to mature spores and *bld* mutants, which are blocked at the earliest stages of differentiation, failing to form aerial mycelia. Not only are *bld* mutants unable to erect the spore forming aerial mycelium, they are also defective in antibiotic production, regulation of carbon utilization and production or response to extra-cellular signals (Champness 1988; Willey 1991; Pope 1996). To date, seven *bld* mutants have been characterized at the molecular level. *bldA* encodes a tRNA that recognizes the

leucine codon UUA, which is rare in *Streptomyces* DNA (Lawlor 1987). *bldB* is the most pleiotropic of the *bld* mutants as it cannot be rescued by any of the mechanisms that can rescue the other *blds*. It is needed for catabolite repression, morphogenesis and antibiotic production and contains a putative DNA binding site (Pope 1998). *bldD* is a DNA binding protein that acts as a transcription factor, regulating it's own transcription as well that of *whiG* and *bldN* (Elliot 1998). *bldG* is an anti-anti-sigma factor (Bignell 2000) and *bldK* is an oligopeptide importer (Nodwell 1996). *bldM* encodes another transcription factor (Molle 2000) and *bldN* encodes an RNA polymerase sigma factor (Bibb 2000).

Aerial mycelium formation and spore production actually appear to be regulated by at least two different pathways. One pathway is dependant on SapB, a small hydrophobic protein, and occurs on rich medium, such as a glucose carbon source (Merrick 1976; Champness 1998; Willey 1993). This pathway is dependant on the *bld* genes. Aerial mycelia are able to grow in this pathway because SapB coats the mycelial surface and allows the aerial mycelium to break the surface tension and grow upwards (Willey 1991). All *bld* mutants studied to date are defective in production of SapB, which may explain why they fail to produce aerial hyphae. However, when grown on minimal medium with an alternate carbon source, such as mannitol, the production of aerial hyphae seems independent of most *bld* genes products and no SapB is produced. Most *bld* mutants can form aerial hyphae on poor carbon sources (exceptions are *bldB* and *bldN*) and many of the antibiotic production defects are rescued as well (Merrick 1976; Champness 1988; Champness 1994). This suggests that regulation of carbon

utilization and initiation of differentiation and antibiotic production are connected (Champness 1988).

One of the most unique and interesting aspects of the *bld* mutants is that most of them fit into an extracellular complementation cascade (Willey 1991). For certain pairs of *bld* mutants grown in close proximity, the ability to form aerial hyphae can be restored to one of the pair at the point nearest the contact. This complementation always occurs unidirectionally, indicating a hierarchy whereby each mutant can restore formation of aerial mycelium to each mutant below it in the cascade, but not to those above it (figure 1.2) (Willey 1991). This hierarchy suggests a cascade of diffusible extracellular signals that are important for erection of aerial mycelia, and that these signals are either directly or indirectly governed by *bld* gene products.

As mentioned above, the *bld* mutants are also defective in regulation of carbon utilization (Pope 1996). It has been shown that most characterized *bld* mutants have deregulated expression of *galP1*, a glucose-sensitive, galactose-dependent promoter of the galactose utilization operon. The most pleiotropic *bld* mutant, *bldB*, is globally defective in regulation of carbon utilization. This suggests that the functions of *bld* genes may not be in differentiation specifically, but rather as assessors of the nutrients available to the cell.

The other class of morphological mutants, the *whi* mutants, were first described in 1970 (Hopwood 1970). Although these mutants can initiate aerial hyphae formation, they cannot form the mature chains of spores. Currently nine *whi* loci have been identified and three have been cloned and characterized. *whiG* encodes a sigma factor

(Chater 1989), *whiE* encodes the polyketide spore pigment (Davis 1990) and *whiB* encodes a transcription-factor-like protein (Davis 1992).

whi mutants were so named because they stay white upon prolonged incubation, never developing the characteristic gray pigment of mature spores. This indicates that they are partly responsible for formation of this polyketide pigment. Analysis of double mutants indicated a pattern of epistatic interactions: *whiG* → *whiH* → *whiA*,B → *whiI*. This follows a presumed pattern of aerial hyphae development. Hyphae initially grow straight up from the vegetative mycelia (*whiG* phenotype) and then begin to curl (*whiH*), curling proceeds (*whiA* and *B*) and then they septate, round off and are released. The process is not this straight forward though, as the mutant phenotypes do not accurately represent "steps" of sporulation as visualized under the microscope. Young hyphae already curl a little to begin with, unlike the *whiG* phenotype, nor do they curl as regularly as in *whiA* and *whiB* mutants. Again we see that the process of differentiation is neither straightforward or simple.

Antibiotic production

Coincident with the complex cycle of morphological differentiation is the production of antibiotics and other secondary metabolites. As a group, the actinomycetes produce more than 70% of all known natural product antibiotics, which makes the streptomycetes a very important group of microorganisms commercially (figure 1.3). The pathways used to produce these antibiotics are highly complex, oftentimes consisting of a cluster of genes (as many as 20-30) involved in the production of one compound.

These clusters contain their own regulatory genes, which are controlled by the global regulatory genes of the cell. Initiation of antibiotic production occurs through many different factors. Things such as physiological stress, growth rate, diffusible signaling molecules and metabolic imbalances all initiate the pathways. They can also be inhibited by glucose, nitrogen sources, metabolite repression and phosphate (Demain 1992; Chater 1997).

Although the use of antibiotics has been a boon to the medical field, it also has some serious consequences. Due to misuse and over prescription of antibiotics, many of the most devastating human pathogens are developing resistance to them. Diseases that were easily treated before are now becoming threatening once again. Consequently, there is an increased need for the identification of novel antibiotics. Since streptomycetes produce so many secondary metabolites and antibiotics, they are important targets for analyzing production pathways and then manipulating these pathways to generate novel antibiotic compounds.

Streptomyces **genetics**

Streptomyces species offer many advantages as model organisms. They allow for the study of differentiation and development in a simple model, as they are prokaryotic, and they perform unique biochemistries to achieve production of a wide range of secondary metabolites. However, despite the complex biology of the process of sporulation, only seven *bld* genes have been characterized at the molecular level. One of the major reasons for this lack of progress has been the inability to perform global

screens for these mutants and clone the genes identified by these mutations efficiently. This is the result of three major problems: the need to protoplast cells to introduce DNA (this process is mutagenic in streptomycetes), the lack of an efficient transposon delivery system, and the inability to establish linkage between insertions and mutant phenotypes. Until recently, protoplasting was the method of choice to introduce foreign DNA into the cells. This process does allow for large numbers of resulting transformants in some species. However, the protocols have not been applicable to all species. Also, the ends of the *Streptomyces* chromosome are unstable, often resulting in large deletions and a high background of spontaneous mutations during protoplasting.

Transposon mutagenesis is the most efficient way to generate libraries of random mutations, as it allows for easy identification of the gene responsible for an observed mutant phenotype, as well as the location of that gene. Since the sequence of the transposon is known, primers can be designed to sequence out into the surrounding DNA and sequence analysis can be preformed to identify the location of the insertion. However, until recently transposon mutagenesis has not been useful to perform genome-wide screens for specific genes as there was no efficient system for linkage verification of the insertional mutants. Previous methods of transposon insertion in *Streptomyces* relied on the use of delivery vectors that had temperature sensitive replicons, as had been done in other bacterial genera (Muth 1989). Curing of these plasmids is mutagenic in *Streptomyces* as they are sensitive to elevated temperatures. This compounds the large background of spontaneous mutations generated by protoplasting, making it impractical to identify the real insertional mutants from this background (figure 1.4). There were

some methods available to establish linkage between a transposon insertion and an observed mutant phenotype. Most commonly, Southern analysis, random sequencing and complementation were used. However, all three methods are very tedious and expensive, especially when dealing with the large number of false positives resulting from the increased spontaneous mutation rate.

There are a several recent advances that have addressed these problems. One is a method for *in vitro* transposon mutagenesis in *Streptomyces* (Gehring 2000). This new method relies on the use of a kit for Tn*5* insertion, available through Epicentre, making it convenient and easily reproducible. A genomic library is generated and transposition takes place *in vitro,* using a hyperactive mutant transposase with an increased affinity for binding to the transposon. The mutagenized library is then introduced into wild-type *Streptomyces* and gene replacement occurs through homologous recombination. This eliminates the need for heat curing of delivery vectors. However, the problem of how to introduce these insertional libraries into the wild-type cells without mutagenic protoplasting is still a problem. This can be eliminated as well through the use of intergeneric conjugation of plasmid DNA from *Escherichia coli* to *Streptomyces* (Mazodier 1989). These methods have been successfully optimized for several different species of *Streptomyces* and are completely non-mutagenic. This further eliminates the background of spontaneous mutations, making it far easier to identify real transposon mutants. However, as there is still some background of spontaneous mutation it is necessary to establish linkage between the transposon insertion and the observed mutant phenotype. Recently, it has been established that alkali-denatured DNA is far more

efficient than untreated DNA at facilitating homologous recombination events in *Streptomyces* (Oh 1997). This enables the chromosomal DNA of a mutant to be transformed into a wild type recipient and co-transformation of the mutant phenotype with the transposon marker to be scored. If 100% of the transformants containing the transposon marker also display the same mutant phenotype, then linkage between the two occurrences is established. The combination of the reduction in false positives via *in vitro* transposition and conjugation, and the ability to use co-transformation to establish linkage between mutant phenotypes and insertions allows for the efficient use of transposons in the genome-wide mutagenesis of *Streptomyces*.

Streptomyces venezuelae

Although most of the genetic data available for *Streptomyces* is from studies of *S. coelicolor*, this species is not the best model for molecular characterization of these genes. *S. venezuelae* offers many advantages over *S. coelicolor* for these molecular studies. One advantage is that *S. venezuelae* grows and differentiates faster than *S. coelicolor* (approximately four days to complete the life cycle versus seven days for *S. coelicolor*). Another advantage is that germination of spores occurs synchronously, with approximately 99% of spores germinating simultaneously. This allows for transcriptional analyses, such as microarrays, to be performed with a much higher sensitivity. *S. venezuelae* is also able to sporulate in both liquid media and on agar plates, while *S. coelicolor* is limited to sporulation on plates (Glazebrook 1990). This will make isolations of stage specific RNA much easier for methods like microarray analysis.

Genetic studies of *S. venezuelae* have been hampered as no efficient method for protoplasting has ever been developed for this species. However, recent developments have allowed for highly efficient transfer of DNA into *S. venezuelae* via intergeneric conjugation (Green, personal communication). As little is known about the pathways of sporulation and antibiotic production in this species, it will be interesting to see how much conservation exists between *S. coelicolor* and *S. venezuelae* in these pathways.

Transposable elements

Transposable elements are a naturally occurring way of rearranging the genome of an organism. They are sequences of DNA that are able to insert into different sites in the host chromosomes, oftentimes causing major changes in the functions of the gene that it has inserted in or those genes nearby (Watson 1998). Some insert fairly randomly, while others require specific sites for re-integration (Goryshin 1998). The specificity of these elements was originally determined by examining the frequency of insertion into different loci within a short, defined region of the host chromosome. For example, based on observations of Tn*10* insertion within the *his* operon of *Salmonella typhimurium*, a strong preference for insertion into a specific region was established. Approximately 40% of the insertions occurred at the same nucleotide position, while the others were distributed among 21 other sites within the operon (Botstein 1977). This is in stark contrast to the case for Tn*5*, where 19 independent insertions into *lacZ* all mapped to different positions (Berg 1977). Originally identified in maize by Barbara McClintock (McClintock 1951), transposable elements have since been found in the genomes of most

organisms including: bacteria, plants and animals and appear to be important in evolution (Watson 1998). They have been successfully utilized and modified extensively for the mutagenesis of many bacterial species.

Transposable elements were first discovered in prokaryotes as a type of highly polar mutation in the late 1960s (Malamy 1966; Saedler 1967a and b; Jordan 1968; Adhya 1969; Shapiro 1969). For some elements this polarity can occur if the element inserts in either orientation (i.e. Tn*5*) (Berg 1980) and for others it occurs for insertion in only one orientation (i.e. Tn*3*) (Calame 1979). It was subsequently established that these mutations were caused by an insertion by physical means (Jordan 1968; Shapiro 1969; Michaelis 1969; Malamy 1970). Hybridization studies were able to show that there were only a few major classes of transposable elements (Fiant 1972; Hirsch 1972a and b; Malamy 1972). Currently, transposable elements are divided into 3 main classes based upon the different mechanisms of insertion (figure 1.5). Replicative transposable elements, like Tn*3*, insert by utilizing both replication and recombination so that the donor maintains it's copy of the transposon and a new copy is inserted into the recipient (Watson 1998). Host replicative machinery is necessary to generate the new copy of the transposon. Most of the early work on determining how transposition worked was performed with Tn*3* (Heffron 1977), and it was initially believed that this was how all transposition occurred. Retroelements, such as the Ty element in yeast, insert via an entirely different mechanism that is similar to the mechanism of retroviral insertion. The element first synthesizes an RNA transcript from the DNA template it carries. This processed RNA intermediate is then inserted into the recipient DNA by transposition

(Watson 1998). The element contains all it needs to transcribe and integrate itself. The simplest mechanism is that of "cut-and-paste" transposition, as occurs in Tn*10* and Tn*5*. The donors of these transposable elements lose their own copy upon insertion into the recipient (Watson 1998). Encoded within the element is the only enzyme needed to catalyze the reaction, transposase (Tnp). The Tnp must be supplied *in cis* for function, limiting the number of multiple insertions by nearby elements (Watson 1998). These are the simplest elements, as they often need only 1 enzyme to insert into the recipient DNA. Insertion of any element results in the duplication of a region of recipient DNA, ranging from 4-12bp in length. This occurs in an element-specific manner and the repeated regions flank the element in the host DNA (Grindley 1978). This duplication is the result of host replicative machinery filling in the single-stranded DNA sequence generated from the nicking of the DNA prior to insertion (Grindley 1978).

Transposable elements can also be placed into one of two main categories based on their configuration. There are insertion sequences (IS), which were discovered as the polar mutations mentioned above. These elements consist of 2 inverted repeats of 20-40 bp termed inside ends (IE) and outside ends (OE). The only gene(s) contained between these sequences is the enzyme(s) needed for transposition (Calos 1980). Although originally identified on the chromosome, they have since been shown to be located on bacteriophages (Brachet 1970) and plasmids (Hu 1975) as well. Transposons were first distinguished from IS elements based on the fact that they contain detectable genes, often drug resistance genes (Calos 1980). These genes are surrounded by large (800-1500 bp) direct or inverted repeats, and these repeats are actually IS or IS-like elements (Calos

1980). Both types of elements can perform several types of DNA rearrangements that could be beneficial in the evolution of the host organism. They can mediate both deletions and inversions of DNA near the insertion. This would lead to the fusion of unrelated DNA molecules. They can also contain transcriptional start and stop signals, which is part of the way that they exert the polar effects originally observed upon their insertion. They are able to precisely, near-precisely and imprecisely excise themselves from the host chromosome. As a result, there is a high reversion frequency and selection must be in place to maintain an insertion (Berg 1980). All of these functions are carried out without the use of host recombination machinery as they occur in *recA*$^-$ mutant strains of *E. coli* (Calos 1980). They can also serve as "portable" regions of homology within the host chromosome, allowing for homologous recombination events to occur and resulting in gene duplication, deletion or rearrangement via host recombination mechanisms.

Recently, a lot of work has been done *in vitro* to elucidate the mechanism of Tn*5* insertion (Goryshin 1998), which was originally identified from the R factor plasmid of *E. coli* (Berg 1975). The only sequences needed for transposition of Tn*5* are the mutant outside ends (OE) of each IS element, which are 19 bp inverted repeats that have been optimized from wild type outside ends to increase binding to Tnp (Zhou 1998). Between these ends, any gene fragment can be cloned (often a drug resistance marker in molecular studies), up to 11 Kb in length (Reznikoff personal communication). Tnp binds to specific terminal sequences at each end of the transposon and then forms a synaptic complex via dimerization between the 2 Tnp molecules. The reaction is catalyzed when

the DNA is nicked to generate a nucleophilic 3' OH group on both strands. The 3' OH then attacks the 5' strand to generate a hairpin, which is then cleaved by attack from an activated water molecule. Target DNA is then captured by the free synaptic complex, and strand transfer occurs when the 3' OH groups attack both strands of target DNA. This attack occurs with staggered spacing between insertions sites in a transposon-specific manner. For Tn*5*, this spacing is 9 base pairs (Davies 2000). Naturally occurring transposons can only be transposed by Tnp supplied *in cis*. This helps to prevent any further movement of the transposon, which may be deleterious to the host (Goryshin 1998). These hops are further prevented by a *trans*-acting inhibitor protein (Inh) produced by the element (Bick 1980). In nature, selection for transposons that are limited in their mobility would be an advantage, as too many hops would be deleterious to the host. A kit is now available from Epicentre that has modified Tn*5* to be a more efficient transposon. In this kit, Tnp has been mutated so that: it can be supplied *in trans*, Inh is not expressed, Tnp will bind to OEs with a higher efficiency and the dimerization potential of Tnp has been reduced (to avoid both Inh-Tnp dimers and non-productive Tnp-Tnp dimers) (Goryshin 1998). This results in a much higher transposition efficiency than that of wild-type Tn*5*.

Transposition as a tool to identify genes involved in differentiation

Transposition is a tool that has long been known to be useful in genetic engineering (Berg 1987; Kleckner 1977). Of particular interest is the use of transposons to identify genes involved in differentiation of prokaryotes. This has been accomplished

particularly successfully for 2 different genera of bacteria: *Bacillus* and *Myxococcus*. In 2000, Tortosa, et. al. used mini Tn*10* to generate a library of insertional mutants *in vivo* in *B*. *subtilis* and screened for mutants with defects in competence (Tortosa 2000). Through this screen, they re-isolated several previously characterized genes, but were also able to identify 2 new genes: *ylbF* and *ypuN*. Mutants in *ylbF* were also defective in sporulation, a closely related response to the competence response.

Tn*917*, a transposon originally isolated from *Streptococcus faecalis* that behaves like Tn*3*, has been extensively used for generating mutants in *Bacillus* since its usefulness was first described (Youngman 1983). It was shown that Tn*917* not only transposed efficiently in *Bacillus,* whereas all known Gram-negative derived transposons were not able to do so, but it did so fairly randomly, indicating it would be useful as a tool for isolation of sporulation genes. Tn*917* was used in a genome wide screen to isolate *spo* mutants (those defective in sporulation, but no vegetative growth) (Sandman 1987). Twenty-four insertions were located in 20 separate loci. Of these, nine were novel *spo* genes. Linkage was established by transformation and transduction experiments. Extensive modifications have been made to Tn*917*, making it easier to clone mutated DNA (Tn*917ac*1) (Chang 1994) and generate transcriptional fusions with *lacZ* to allow the identification of mutants with differential gene expression (Camilli 1990). Its use had been extended to many other genera including *Listeria* (Camilli 1990), *Lactococcus* (Israelsen 1995), *Clostridium* (Babb 1993), *Staphylococcus* (Cheung 1992), and *Enterococcus* (Clewell 1982) as well as other *Streptococcus* species (Gutierrez 1996).

Transposon mutagenesis has also been used in *Myxococcus*, a genus that makes multi-cellular fruiting bodies in response to nutritional downshift. Tn*5* has been one of the most useful transposons for this group. It has been shown that transposition can occur after transduction of P1, previously mutagenized with Tn*5* (P1::Tn*5*) (Kuner 1981). P1 is unstable in *Myxococcus,* as it is able to absorb to the cells, but unable to replicate or integrate it's DNA into the host chromosome (Kaiser 1975; Kuner 1981). This is another way to "cure" the recipient of the transposon vector in addition to the conventional method of heat shocking. This method results in clean transposition (20/20 mutants tested were linked to insertion) as well as stable transposition (phenotype stable after 23 generations without selection) (Kuner 1981). A derivative of Tn*5*, Tn*5 lac*, has been created that allows for transcriptional analysis of the transposon mutants via a *lacZ* promoter fusion (Kroos 1984). This has allowed for the study of developmentally regulated genes in this genus. Also, a method has been developed using P1::Tn*5* to integrate plasmid DNA in the absence of homologous recombination (Downard 1988). This provides a better method for analyzing *cis*-acting regulatory mechanisms.

In vitro transposition

Although these methods have been useful in understanding the biology of *Bacillus* and *Myxococcus*, they rely on *in vivo* mutagenesis. Recently, several advances have been made in the use of *in vitro* transposon mutagenesis. *In vitro* transposon mutagenesis offers many advantages over conventional, *in vivo* mutagenesis: i) no special bacterial strains are needed, ii) potential host effects on transposition are avoided, and iii)

the system is amenable to biochemical alteration and parameter optimization (Devine 1994). High efficiency systems are now available for Tn*7* (Bainton 1993), Ty*1* (Devine 1994), Tn*5* (Goryshin 1998), *mariner* (Lampe 1998), Tn*552* (Griffin 1999), Tn*10* (Chalmers 1994), and Mu (Haapa 1999). The Ty-*1* system has been modified to enable generation of artificial transposons able to insert with only purified Ty-*1* integrase (Devine 1994). Tn*7* naturally requires 4 proteins to insert into the recipient DNA and displays an insertional specificity for *att*Tn*7* sites. A mutant form of Tn*7* is now available that is mutated to remove the site specificity, but 3 proteins are still needed for transposition (Stellwagen 1997). *Mariner* activity has been examined extensively *in vitro* (Lampe 1998). It has been shown that although the sequence specificity of *mariner* is low, it does display a preference for bent or bendable DNA. Also, the frequency increases with temperature (which can be problematic for some bacteria) and decreases with larger insertion sizes. These issues limit its usefulness for some strains. Tn*5* offers advantages over the Tn*7* and Mu systems in that it is far less complicated, needing only 1 protein for transposition (Goryshin 1998). Some of these systems have been used in generating insertional mutants in species of naturally competent bacteria, such as *Haemophilus influenzae* (Gwinn 1997; Akerley 1998; Reich 1999) and *Streptococcus pneumoniae* (Akerley 1998). For example, Ty-*1* and *mariner* have been used in genome-wide screens to identify essential genes in *H. influenzae* and *S. pneumoniae*, which has been helpful in determining new drug targets for increasingly resistant bacterial strains.

Transposition in *Streptomyces*

An efficient system of transposition would be beneficial to *Streptomyces* genetics for several reasons: i) tagging of spore and antibiotic production genes, ii) physical mapping studies, iii) insertion of cloned genes into the chromosome, iv) insertion of promoters into the chromosome, v) upregulating antibiotic production, and vi) inserting 'portable' regions of homology or restriction sites (Baltz 1992). Several transposon systems have been reported for used in *Streptomyces* (Olson 1988; Baltz 1992; Volff 1997; Herron 1999), but all have been unable to generate large numbers of random insertions. In only 2 cases has genome-wide mutagenesis resulted in mutants that were linked to transposon insertions: isolation of strains of *S. avermitilis* defective in oligomycin production with Tn*4560* (Ikeda 1993) and isolation of strains of *S. roseosporus* defective in daptomycin production with Tn*5099* (McHenney 1998). This is partially the result of utilizing delivery techniques like those used for *Bacillus* and other bacteria, delivery vectors with temperature sensitive replicons. Elevated temperatures are mutagenic in *Streptomyces*, resulting in a high background of spontaneous mutation.

Gehring, et. al. were recently able to show that use of Tn*5 in vitro* transposition could decrease this background to a far more manageable level (Gehring 2000). A genome-wide screen for genes involved in morphological differentiation was performed and seventeen new genes were identified. About 1 in 4 of the mutant phenotypes were caused by transposon insertion.

Intergeneric conjugation

It has long been known that conjugation is a natural way for bacteria to transfer DNA and maintain genetic variation. Intimate contact between the two cells is necessary for this process to occur, often mediated through a pilus, as well as transfer genes (*tra*) and an origin of transfer (*oriT*) on the plasmid to be moved (Griffiths 1996). This process has long been utilized by scientists to mediate specific exchanges of plasmid and chromosomal DNA for linkage analyses and strain construction.

However, it had long been assumed that Gram-negative and Gram-positive bacteria could not exchange plasmids with each other in this manner since contact would be compromised due to differences in the cell walls. Trieu-Cuot, et. al. were able to demonstrate conjugal transfer between *E. coli* (Gram-negative) and *Streptococcus agalactiae, Streptococcus lactis, Enterococcus faecalis, Bacillus thuringiensis, Listeria monocytogenes* and *Staphylococcus aureus* (all Gram-positive) (Trieu-Cout 1988). Subsequently, Mazodier, et. al. were able to generate exconjugants from matings between *E. coli* and *Streptomyces lividans* (Mazodier 1989). All that is needed is for the *E. coli* strain to possess the *tra* genes of RP4 and for the plasmid to be transferred to contain an *oriT* (Giebelhaus 1996). The method has since been optimized for many *Streptomyces* strains, including *S. venezuelae*. It allows for DNA transfer into previously unmanipulable strains and is non-mutagenic.

References

1. Adhya, S.L., and Shapiro, J.A. (1969). "The galactose operon of *E. coli* K-12. I. structural and pleiotropic mutations of the operon." *Genetics* 62: 231-247.

2. Akerley, B.J., Rubin, E.J., Camilli, A., Lampe, D.J., Robertson, H.M., and Mekalanos, J.J. (1998). "Systematic identification of essential genes by *in vitro mariner* mutagenesis." *Proc. Natl. Acad. Sci. USA* 95: 8927-8932.

3. Babb, B.L., Collett, H.J., Reid, S.J., and Woods, D.R. (1993). "Transposon mutagenesis of *Clostridium acetobutylicum* P262: isolation and characterization of solvent deficient and metronidazole resistant mutants." *FEMS Microbiol. Lett.* 114: 343-348.

4. Bainton, R.J., Kubo, K.M., Feng, J., and Craig, N.L. (1993). "Tn7 transposition: target DNA recognition is mediated by multiple Tn7-encoded proteins in a purified *in vitro* system." *Cell* 72: 913-943.

5. Baltz, R.H., Hahn, D.R., McHenney, M.A., and Solenberg, P.J. (1992). "Transposition of Tn5096 and related transposons in *Streptomyces* species." *Gene* 115: 61-65.

6. Berg, D.E. (1980). "Control of gene expression by a mobile recombinational switch." *PNAS* 77: 4880-4884.

7. Berg, C.M. and Berg, D.E. (1987). "Uses of transposable elements and maps of known insertions." *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology. Washington, D.C. p 1071-1109.

8. Berg, D.E., Davies, J., Allet, B., and Rochaix, J.D. (1975). "Transposition of R factor genes to bacteriophage lambda." *PNAS* 72: 3628-3632.

9. Berg, D.E., Weiss, A. and Crossland, L. (1980). "Polarity of Tn5 insertion mutants in *Escherichia coli*." *J. Bacteriol*. 142: 439-446.

10. Bibb, M.J., Molle, V., and Buttner, M.J. (2000). "An extracytoplasmic function RNA polymerase sigma factor required for aerial mycelium formation in *Streptomyces coelicolor* A3(2)." *J. Bacteriol*. 182(16): 4606-4616.

11. Biek, D., and Roth, J.R. (1980). "Regulation of Tn5-transposition in *Salmonella typhimurium*." *PNAS* 77: 6047-6051.

12. Bignell, D.R.D., Warawa, J.L, Strap, J.L., Chater, K.F., and Leskiw, B.K. (2000). "Study of the *bldG* locus suggests that an anti-anti-sigma factor and an anti-sigma factor may be involved in *Streptomyces coelicolor* antibiotic production and sporulation." *Microbiology* 146: 2161-2173.

13. Botstein, D., and Kleckner, N. (1977). DNA insertion elements, plasmids and episomes. Cold Spring Harbor Laboratory. New York. pp 185-203.

14. Brachet, P., Eisen, H., and Rambach, A. (1970). "Mutations of coliphage lambda affecting the expression of replicative functions O and P." *Mol. Gen. Genet*. 108: 266-267.

15. Calame, K.L., Yamada, Y., Shanblatt, S.H., and Nakada, D. (1979). "Location of promoter sites on plasmid NTP1 which contains the ampicillin resistance transposon Tn1701." *J. Mol. Biol*. 127: 397-409.

16. Calos, M.P., and Miller, J.H. (1980). "Transposable elements." *Cell* 20: 579-595.

17. Camilli, A., Portnoy, D.A., and Youngman, P. (1990). "Insertional mutagenesis of *Listeria monocytogenes* with a novel Tn917 derivative that allows direct cloning of DNA flanking transposon insertions." *J. Bacteriol*. 172: 3738-3744.

18. Chalmers, R.M. and Kleckner, N. (1994). "Tn10/IS10 transposase purification, activation and *in vitro* reaction." *J. Biol. Chem*. 269: 8029-8035.

19. Champness, W.C. (1988). "New loci required for *Streptomyces coelicolor* morphological and physiological differentiation." *J. Bacteriol*. 170: 1168-1174.

20. Champness, W.C., and Chater, K.F. (1994). "Regulation and integration of antibiotic production and morphological differentiation in *Streptomyces* spp." American Society for Microbiology, Washington, D.C.

21. Chang, L.-K., Chen, C.-L., Chang, Y.-S., Tschen, J.S.-M., Chen, Y.-M., and Lui, S.-T. (1994). "Construction of Tn917*ac*1, a transposon useful for mutagenesis and cloning of *Bacillus subtilis* genes." *Gene* 150: 129-134.

22. Chang, P.-C., and Cohen, S.N. (1994). "Bi-directional replication from an internal origin in a linear *Streptomyces* plasmid." *Science* 265: 952-954.

23. Chater, K.F., Bruton, C.J., Plaskitt, K.A., Buttner, M.J., Mendez, C., and Helmann, J.D. (1989). "The developmental fate of *S. coelicolor* hyphae depends upon a gene product homologous with the motility sigma factor of *B. subtilis*." *Cell* 59: 133-143.

24. Chater, K.F., and Bibb, M.J. (1997). "Regulation of bacterial antibiotic production." Biotechnology. H.A.D. Kleinhauf, H. Weinheim, Germany, VCH Press. 7: 57-105.

25. Chen, C.W. (1996). "Complications and implications of linear bacterial chromosomes." *TIG* 12: 192-195.

26. Cheung, A.L., Koomey, J.M., Butler, C.A., Projan, S.J., and Fischetti, V.A. (1992). "Regulation of exoprotein expression in *Staphylococcus aureus* by a locus (*sar*) distinct from *agr*." *Proc. Natl. Acad. Sci. USA* 89: 6462-6466.

27. Clewell, D.B., Tomich, P.K., Gawron-Burke, M.C., Franke, A. E., Yagi, Y., and An, F.Y. (1982). "Mapping of *Streptococcus faecalis* plasmids pAD1 and pAD2 and studies relating to transposition of Tn917." *J. Bacteriol*. 152: 1220-1230.

28. Davies, D.R., Goryshin, I.Y., Reznikoff, W.S., and Rayment, I. (2000). "Three-dimensional structure of the Tn5 synaptic complex transposition intermediate." *Science* 289: 77-85.

29. Davis, N.K., and Chater, K.F. (1990). "Spore color in *Streptomyces coelicolor* A3(2) involves the developmentally regulated synthesis of a compound biosynthetically related to polyketide antibiotics." *Mol. Microbiol*. 4: 1679-1691.

30. Davis, N.K., and Chater, K.F. (1992). "The *Streptomyces coelicolor whiB* gene encodes a small transcription factor-like protein dispensable for growth but essential for sporulation." *Mol. Gen. Genet*. 232: 351-358.

31. Demain, A.L. (1992). "Microbial secondary metabolism: a new theoretical frontier for academia, a new opportunity for industry." Ciba Foundation Symposia.

32. Devine, S.E., and Boeke, J.D. (1994). "Efficient integration of artificial transposons into plasmid targets *in vitro*: a useful tool for DNA mapping, sequencing and genetic analysis." *Nucleic Acids Res*. 22: 3765-3772.

33. Downard, J.S. (1988). "Tn5-mediated transposition of plasmid DNA after transduction to *Myxococcus xanthus*." *J. Bacteriol*. 170: 4939-4941.

34. Elliot, M., Damji, F., Passantino, R., Chater, K., and Leskiw, B. (1998). "The *bldD* gene of *Streptomyces coelicolor* A3(2): a regulatory protein involved in morphogenesis and antibiotic production." *J. Bacteriol*. 180: 1549-1555.

35. Fiant, M., Szybalski, W., and Malamy, M. (1972). "Polar mutations in *lac, gal* and phage lambda consist of a few IS-DNA sequences inserted with either orientation." *Mol. Gen. Genet*. 119: 223-231.

36. Gehring, A., Nodwell, J.R., Beverley, S.M. and Losick, R. (2000). "Genomewide insertional mutagenesis in *Streptomyces coelicolor* reveals additional genes involved in morphological differentiation." *Proc. Natl. Acad. Sci. USA* 97: 9642-9647.

37. Giebelhaus, L.A., Frost, L., Lanka, E., Gormley, E.P., Davies, J.E., and Leskiw, B. (1996). "The *tra2* core of the IncPα plasmid RP4 is required for intergeneric matings between *Escherichia coli* and *Streptomyces lividans*." *J. Bacteriol*. 178: 6378-6381.

38. Glazebrook, M.A., Doull, J.L., Stuttard, C., and Vining, L.C. (1990). "Sporulation of *Streptomyces venezuelae* in submerged cultures." *J. Gen. Microbiol.* 136: 581-588.

39. Goryshin, I.Y., and Reznikoff, W.S. (1998). "Tn5 *in vitro* transposition." *J. Biol. Chem*. 273: 7367-7374.

40. Green, B. Personal communication.

41. Griffiths, A.J.F., Miller, J.H., Suzuki, D.T., Lewontin, R.C., and Gelbar, W.M. (1996). An introduction to genetic analysis, sixth edition. W.H. Freeman and Company. New York.

42. Griffin, T.J., IV, Parson, L., Leschziner, A.E., DeVost, J., Derbyshire, K.M., and Grindely, N.D.F. (1999). "*In vitro* transposition of Tn552: a tool for DNA sequencing and mutagenesis." *Nucleic Acids Res*. 27: 3859-3865.

43. Grindley, N.D.F. (1978). "IS1 insertion generates duplication of a nine base pair sequence at its target site." *Cell* 13: 419-426.

44. Gutierrez, J.A., Crowley, P.J., Brown, D.P., Hillman, J.D., Youngman, P., and Bleiweis, A.S. (1996). "Insertional mutagenesis and recovery of interrupted genes of *Streptococcus mutans* by using transposon Tn917: preliminary characterization of mutants displaying acid sensitivity and nutritional requirements." *J. Bacteriol*. 178: 4166-4175.

45. Gwinn, M.L., Stellwagen, A.E., Craig, N.L., Tomb, J.-F., and Smith, H.O. (1997). "*In vitro* Tn7 mutagenesis of *Haemophilus influenzae* Rd and characterization of the role of *atpA* in transformation." *J. Bacteriol.* 179: 7315-7320.

46. Haapa, S., Taira, S., Heikkinen, E., and Savilahti, H. (1999). "An efficient and accurate integration of mini-Mu transposons *in vitro*: a general methodology for functional genetic analysis and molecular biology applications." *Nucleic Acids Res*. 27: 2777-2784.

47. Heffron, F., Bedinger, P., Champoux, J., and Falkow, S. (1977). DNA insertion elements, plasmids and episomes. Cold Spring Harbor Laboratory. New York. pp. 161-167.

48. Herron, P.R., Evans, M.C., and Dyson, P.J. (1999). "Low target site specificity of an IS6100-based mini-transposon, Tn1792, developed for transposon mutagenesis of antibiotic-producing *Streptomyces*." *FEMS Microbiol. Lett*. 171: 215-221.

49. Hirsch, H., Saedler, H., and Starlinger, P. (1972a). "Insertion mutations in the control region of the galactose operon of *E. coli*. II. physical characterization of the mutations." *Mol. Gen. Genet*. 115: 266-276.

50. Hirsch, H., Starlinger, P., and Brachet, P. (1972b). "Two kinds of insertions in bacterial genes." *Mol. Gen. Genet*. 119: 191-206.

51. Hopwood, D.A., Wildemuth, H., and Palmer, H.M. (1970). "Mutants of *Streptomyces coelicolor* defective in sporulation." *J. Gen. Microbiol*. 61: 397-408.

52. Hu, S., Ohtsubo, E., and Davidson, N. (1975). "Electron microscopic heteroduplex studies of sequence relations among plasmids of *Escherichia coli*: structure of F13 and related F-primes." *J. Bacteriol*. 122: 749-763.

53. Ikeda, H., Takada, Y., Pang, C.-H., Tanaka, H., and Omura, S. (1993). "Transposon mutagenesis by Tn4560 and applications with avermectin-producing *Streptomyces avermitilis*." *J. Bacteriol*. 175: 2077-2082.

54. Israelson, H., Madsen, S.M., Vrang, A., Hansen, E.B., and Johansen, E. (1995). "Cloning and partial characterization of regulated promoters from *Lactococcus lactis* Tn917-*lacZ* integrants with the new promoter probe vector pAK80." *Appl. Environ. Microbiol*. 61: 2540-2547.

55. Jordan, E., Saedler, H., and Starlinger, P. (1968). "O^o and strong-polar mutations in the *gal* operon are insertions." *Mol. Gen. Genet*. 102: 353-363.

56. Kaiser, D., and Dworkin, M. (1975). "Gene transfer to Myxobacterium by *Escherichia coli* phage P1." *Science* 187: 653-654.

57. Kleckner, N., Roth, J., and Botstein, D. (1977). "Genetic engineering in vivo using translocatable drug-resistance elements. New methods in bacterial genetics." *J. Mol. Biol*. 116: 125-159.

58. Kroos, L., and Kaiser, D. (1984). "Construction of Tn5 *lac*, a transposon that fuses *lacZ* expression to exogenous promoters, and its introduction into *Myxococcus xanthus*." *Proc. Natl. Acad. Sci. USA* 81: 5816-5820.

59. Kuner, J.M. and Kaiser, D. (1981). "Introduction of transposon Tn5 into *Myxococcus* for analysis of developmental and other non-selectable mutants." *Proc. Natl. Acad. Sci. USA* 78: 425-429.

60. Lampe, D.J., Grant, T.E., and Robertson, H.M. (1998). "Factors affecting transposition of the Himar1 *mariner* transposon *in vitro*." *Genetics* 149: 179-187.

61. Lawlor, E.J., Baylis, H.A., and Chater, K.F. (1987). "Pleiotropic morphological and antibiotic deficiencies result from mutations in a gene encoding a tRNA-like product in *Streptomyces coelicolor* A3(2)." *Genes Dev*. (1): 1305-1310.

62. Lin, Y.-S., Kieser, H.M., Hopwood, D.A., and Chen, C.W. (1993). "The chromosomal DNA of *Streptomyces lividans* 66 is linear." *Mol. Microbiol*. 10: 923-933.

63. Malamy, M. (1966). *Cold Spring Harbor Symp. Quant. Biol*. 31: 189-201.

64. Malamy, M. (1970). The lactose operon. Cold Spring Harbor Laboratory. New York. pp. 359-373.

65. Malamy, M., Fiant, M., and Szybalski, W. (1972). "Electron microscopy of polar insertions in the *lac* operon of *Escherichia coli*." *Mol. Gen. Genet*. 119: 207-222.

66. Mazodier, P., Petter, R., and Thompson, C. (1989). "Intergeneric conjugation between *Escherichia coli* and *Streptomyces* species." *J. Bacteriol*. 171: 3583-3585.

67. McClintock, B. (1951). *Cold Spring Harbor Symp. Quant. Biol*. 16: 13.

68. McHenney, M.A., Hosted, T.J., Dehoff, B.S., Rosteck, P.R., Jr., and Baltz, R.H. (1998). "Molecular cloning and physical mapping of the daptomycin gene cluster from *Streptomyces roseosporus*." *J. Bacteriol*. 180: 143-151.

69. Merrick, M.J. (1976). "A morphological and genetic mapping study of bald colony mutants of *S. coelicolor*." *J. Gen. Microbiol*. 96: 299-315.

70. Michaelis, G., Saedler, H., Zenkov, P., and Starlinger, P. (1969). "Two insertions in the galactose operon having different sizes but homologous DNA sequences." *Mol. Gen. Genet*. 104: 371-377.

71. Molle, V., and Buttner, M.J. (2000). "Different alleles of the response regulator gene *bldM* arrest *Streptomyces coelicolor* development at distinct stages." *Mol. Microbiol.* 36(6): 1265-1278.

72. Musialowski, M.S., Flett, F., Scott, G.B., Hobbs, G., Smith, C.P., and Oliver, S.G. (1994). "Functional evidence that the principal DNA replication origin of the *Streptomyces coelicolor* chromosome is close to the *dnaA-gyrB* region." *J. Bacteriol.* 176: 5123-5125.

73. Muth, G., Nussbaume, B., Wohlleben, W., and Puhler, A. (1989). "A vector system with temperature sensitive replication for gene disruption and mutational cloning in *Streptomyces*." *Mol. Gen. Genet*. 219: 341-348.

74. Nodwell, J.R., McGovern, K., and Losick, R. (1996). "An oligopeptide permease responsible for the import of an extracellular signal governing aerial mycelium formation in *Streptomyces coelicolor*." *Mol. Microbiol*. 22: 881-893.

75. Oh, S.H., and Chater, K.F. (1997). "Denaturation of circular or linear DNA facilitates targeted integrative transformation of *Streptomyces coelicolor* A3(2): possible relevance to other organisms." *J. Bacteriol*. 179: 122-127.

76. Olsen, E.R. and Chung, S.-T. (1988). "Transposon Tn4556 of *Streptomyces fradiae*: nucleotide sequence of the ends and the target sites." *J. Bacteriol*. 170: 1955-1957.

77. Pope, M.K., Green, B., and Westpheling, J. (1996). "The *bld* mutants of *Streptomyces coelicolor* are defective in the regulation of carbon utilization, morphogenesis and cell-cell signaling." *Mol. Microbiol*. 19: 747-756.

78. Pope, M.K., Green, B. and Westpheling, J. (1998). "The *bldB* gene encodes a small protein required for morphogenesis, antibiotic production, and catabolite control in *Streptomyces coelicolor*." *J. Bacteriol*. 180(6): 1556-1562.

79. Reich, K.A., Chovan, L., and Hessler, P. (1999). "Genome scanning in *Haemophilus influenzae* for identification of essential genes." *J. Bacteriol*. 181: 4961-4968.

80. Reznikoff, W.S. Personal communication.

81. Saedler, H., and Starlinger, P. (1967a). "O degree mutations in the galactose operon in *E. coli*. I. genetic characterization." *Mol. Gen. Genet*. 100: 178-189.

82. Saedler, H., and Starlinger, P. (1967b). "O degree mutations in the galactose operon in *E. coli*. II. physiological characterization." *Mol. Gen. Genet*. 100: 190-202.

83. Sandman, K., Losick, R., and Youngman, P. (1987). "Genetic analysis of *Bacillus subtilis spo* mutations generated by Tn917-mediated insertional mutagenesis." *Genetics* 117: 603-617.

84. Shapiro, J.A. (1969). "Mutations caused by the insertion of genetic material into the galactose operon of *Escherichia coli*." *J. Mol. Biol*. 40: 93-105.

85. Stellwagen, A.E. and Craig, N.L. (1997). "Avoiding self: two Tn7-encoded proteins mediate target immunity in Tn7 transposition." *EMBO J* 16: 6832-6834.

86. Tortosa, P., Albano, M., and Dubnau, D. (2000). "Characterization of *ylbF*, a new gene involved in competence development and sporulation in *Bacillus subtilis*." *Mol. Microbiol*. 35: 1110-1119.

87. Trieu-Cout, P., Carlier, C., and Courvalin, P. (1988). "Conjugative plasmid transfer from *Enterococcus faecalis* to *Escherichia coli*." *J. Bacteriol*. 170: 4388-4391.

88. Volff, J.-N. and Altenbuchner, J. (1997). "High frequency transposition of the Tn5 derivative Tn5493 in *Streptomyces lividans*." *Gene* 194: 81-86.

89. Watson, J.D., Witkowski, J., Gilman, M., and Zoller, M. (1998). Recombinant DNA, second edition. W.H. Freeman and Company. New York.

90. Willey, J., Santamaria, R., Guijarro, J., Geislich, M., and Losick, R. (1991). "Extracellular complementation of a developmental mutation implicates a small sporulation protein in aerial mycelium formation by *S. coelicolor*." *Cell* 65: 641.

91. Willey, J., Schwedock, J., and Losick, R. (1993). "Multiple extracellular signals govern the production of a morphogenetic protein involved in aerial mycelium formation by *Streptomyces coelicolor*." *Genes Dev*. 7: 895-903.

92. Youngman, P.J., Perkins, J.B., and Losick, R. (1983). "Genetic transposition and insertional mutagenesis in *Bacillus subtilis* with *Streptococcus faecalis* transposon Tn917." *Proc. Natl. Acad. Sci. USA* 80: 2305-2309.

93. Zhou, M., Bhasin, A., and Reznikoff, W.S. (1998). "Molecular genetic analysis of transposase-end DNA sequence recognition: cooperativity of three adjacent base-pairs in specific interaction with a mutant Tn5 transposase." *J. Mol. Biol.* 276: 913-925.

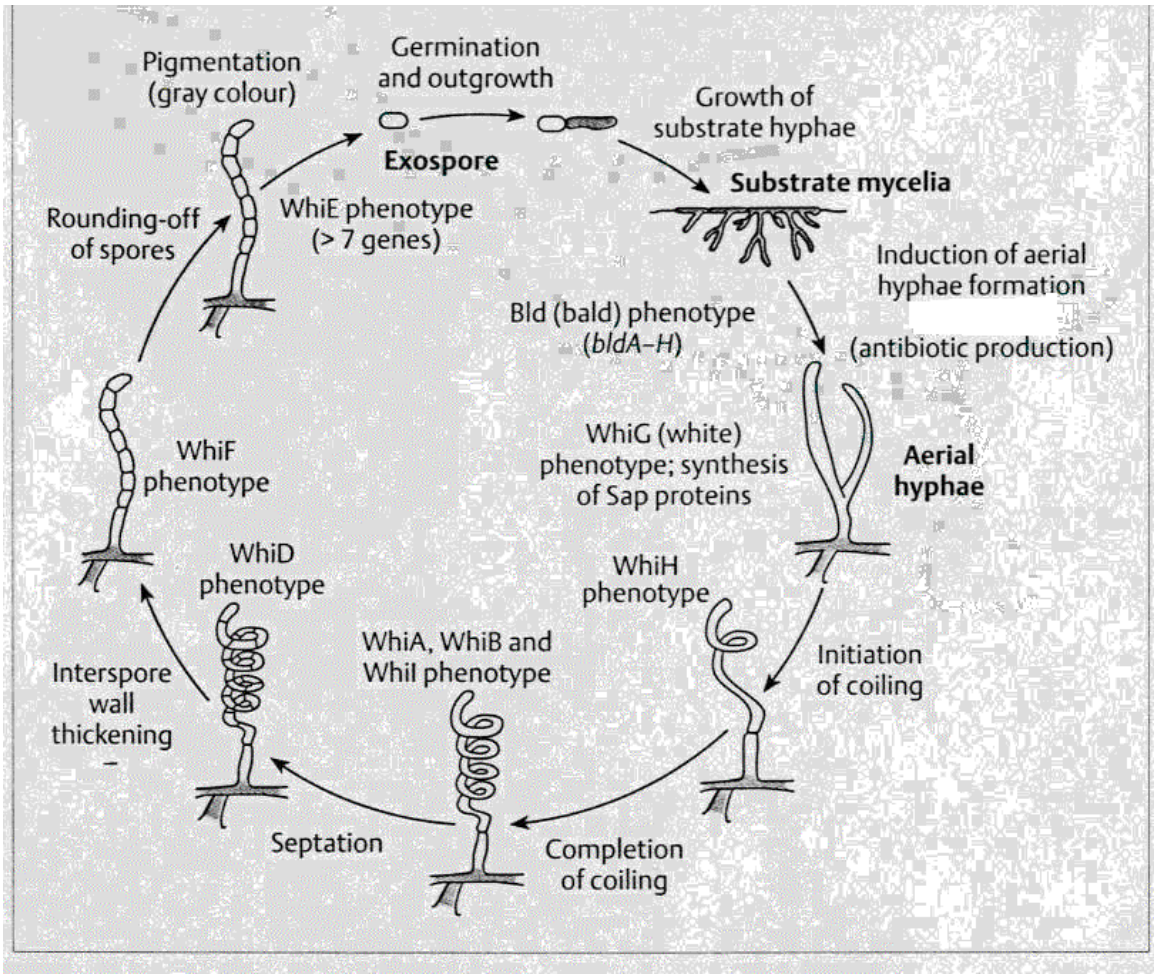


Figure 1.1. Developmental Cycle of *Streptomyces*.

A. *bldJ*(261) < *bldK*, *bldL* < *bldA*, *bldH* < *bldG* < *bldC* < *bldD*, *bldM*

B.

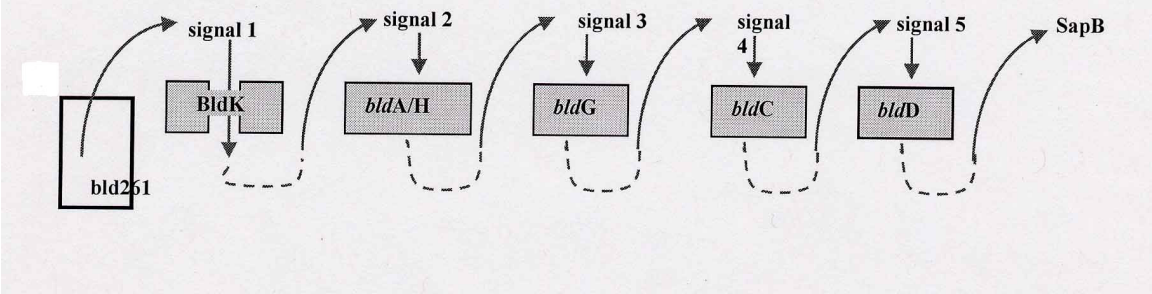
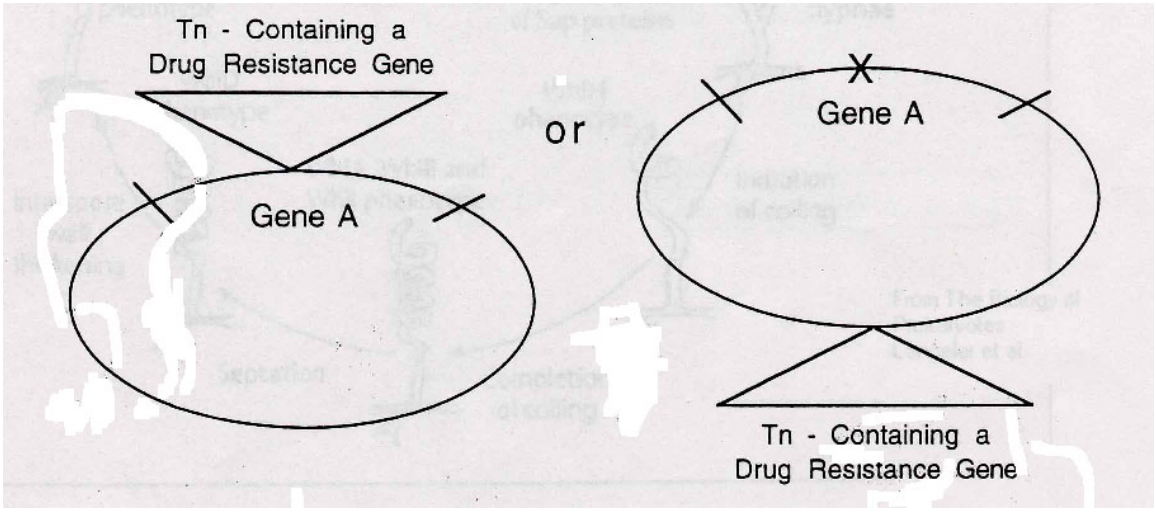


Figure 1.2. Model of the Extracellular Cascade of Regulatory Signals in *S. coelicolor*. (a) The hierarchy of extracellular complementation of the *bld* mutants. (b) Proposed cascade of signals responsible for the initiation of aerial mycelium production. *bldJ* is involved in the production of signal one, which accumulates extracellularly and is imported by the *bldK* permease. Signal 2 is then triggered, is imported, and initiates the *bldA*/H-dependant production of signal 3 and so on until the *bldD*-dependant production of SapB and the rest of the events necessary for aerial mycelium formation.

Antibiotic	Producer	Chemical Class	Target	Application
Actinomycin D	*Streptomyces spp*	Peptide	Transcription	Antitumor
Avermectin	*S. avermitilis*	Polyketide	Chloride ion channels	Antiparasitic
Bialaphos	*S. hygroscopicus*	Peptide	Glutamine synthetase	Herbicidal
Bleomycin	*S. verticillus*	Glycopeptide	DNA strand breakage	Antitumor
Erythromycin	*Sac. erythraea*	Polyketide	Protein synthesis	Antibacterial
Mitomycin C	*S. caespitosus* *S. verticillatus*	Benzoquinone	DNA cross-linking	Antitumor
Nystatin	*S. noursei*	Polyketide	Membrane	Antifungal
Rapamycin	*S. hygroscopicus*	Polyketide	Binds to FK protein	Immunosuppressant
Rifamycin	*A. mediterranei*	Polyketide	RNA polymerase	Antibacterial
Tetracycline	*S. aureofaciens*	Polyketide	Protein synthesis	Antibacterial
Tobramycin	*S. tenebrarius*	Aminoglycoside	Protein synthesis	Antibacterial
Vancomycin	*A. orientalis*	Glycopeptide	Peptidoglycan	Antibacterial

Figure 1.3. Table of Useful Antibiotics Produced by *Streptomyces*.



A. B.

Figure 1.4. Background of Transposition. Transposition could occur: (a) into a gene, knocking out the function of that gene and conveying the transposon-born drug resistance, or (b) it could insert into an already mutated strain at another location, conveying the drug resistance but not causing the observed mutation.

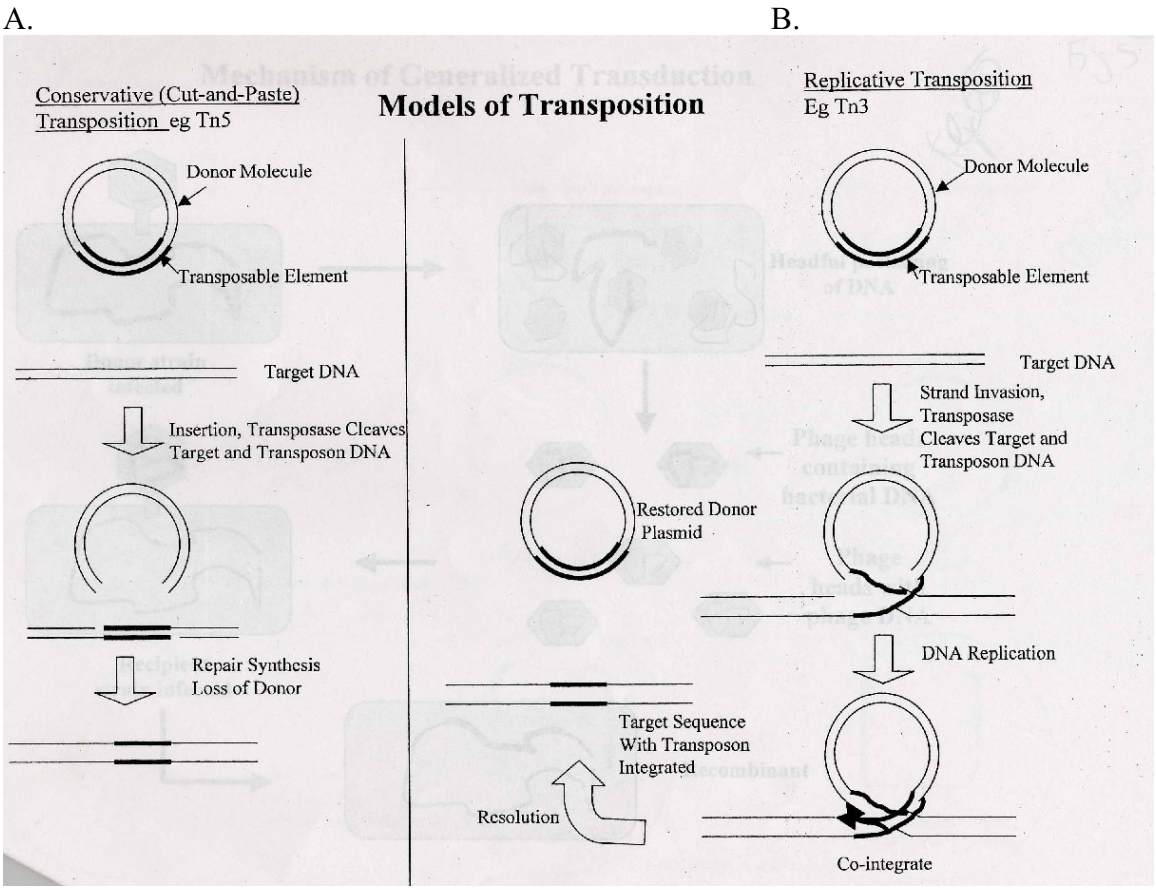


Figure 1.5. Models of Transposition. (a) Cut-and-paste mechanism. The transposon excises itself from the donor and inserts into the recipient, resulting in loss of donor copy. The transposon contains all needed enzymes for these processes. (b) Replicative mechanism. The transposon captures the target, new DNA is synthesized and resolution occurs leaving both donor and recipient with identical copies of the transposon. The transposon needs the host DNA replication machinery to carry out transposition.

CHAPTER 2

IDENTIFICATION OF TWO NEW GENES INVOLVED IN MORPHOGENESIS AND ANTIBIOTIC PRODUCTION IN *STREPTOMYCES COELICOLOR*[1]

[1] Jordan, S.E., Zhou, L., Sprusansky, O., and Westpheling, J. To be submitted to *Journal of Bacteriology*.

Abstract

We report the isolation and partial characterization of two new mutants of *Streptomyces coelicolor* that are defective in morphogenesis and antibiotic production. The genes identified by these mutations were cloned using a combination of Tn*5 in vitro* mutagenesis, co-transformation and genetic complementation. Mutant SE293 is defective in morphogenesis (*bld*) and actinorhodin production on poor carbon source (MYM) media and fails to grow on minimal media with glucose. This gene identifies a protein with similarity to the TetR-family of transcriptional regulators. Mutant SJ175 sporulates sooner and more abundantly than the wild type and overproduces antibiotics. It identifies a protein with regions of predominantly hydrophobic residues similar to integral membrane proteins.

Introduction

Streptomyces is a Gram-positive soil bacterium ubiquitous in nature. Upon sensing starvation, signals are released that initiate a complex process of differentiation (Willey 1991). Aerial hyphae are produced which coil and ultimately septate to form uni-nucleoid mature spores. Coincident with these complex morphological changes, is the production of a number of secondary metabolites, including many medically important antibiotics. Most of what is known about the pathways that contribute to morphogenesis and antibiotic production comes from the study of mutants defective in one or both of these processes. Bald (*bld*) mutants are blocked at the earliest stages of

morphogenesis, failing to erect aerial hyphae and often also failing to produce some or all of the antibiotics.

To date only seven *bld* mutants have been characterized at the molecular level. *bldA* is a tRNA that recognizes a rare leucine codon (Lawlor 1987). *bldB* is the most pleiotropic of the *bld* mutants and is required for catabolite repression, morphogenesis and antibiotic production (Pope 1998). *bldD* is a DNA binding protein that acts as a transcriptional regulator of it's own transcription as well as that of *whiG* and *bldN* (Elliot 1998). *bldG* is an anti-anti-sigma factor (Bignell 2000) and *bldK* is an oligopeptide importer (Nodwell 1996). *bldM* encodes a putative response regulator required for morphogenesis (Molle 2000) and *bldN* encodes an RNA polymerase sigma factor (Bibb 2000). No clear pathway emerges from the description of these genes, although, for certain pairs of *bld* mutants grown in close proximity, the ability to form aerial hyphae can be restored uni-directionally, suggesting a cascade of extracellular signals. Since *bldN* and *bldB* do not fit into this cascade, the interaction between these genes and the pathways to which they belong is likely to be more complicated (Bibb 2000; Willey 1993).

The complexity of these pathways and the fact that several of the *bld* genes were identified by single alleles suggests that many more genes are involved that have yet to be identified. In fact, a recent genome-wide screen for morphological mutants led to the isolation of several previously uncharacterized genes (Gehring 2000). A limitation in the identification and characterization of morphological mutants and the genes they identify has been the lack of an efficient system for utilizing transposon mutagenesis in

Streptomyces to perform a genome-wide screen for morphological mutants. Previous methods for *in vivo* delivery of transposons resulted in a background of spontaneous mutation that was difficult to distinguish from those caused by transposon insertion. Recent advances by Gehring, et. al. have led to the description of an effective method of *in vitro* transposon mutagenesis in *Streptomyces* using a Tn*5* derivative (Goryshin 1998; Gehring 2000). We have used this method to perform a genome-wide screen for genes involved in morphogenesis and antibiotic production. In addition to the version of Tn*5* used by Gehring, et. al., which contained only a drug resistance marker, we have constructed two new transposons containing *EGFP* and *xylE* reporter genes to allow for the generation of transcriptional fusions upon insertion of the transposon. The *xylE* gene encodes a catechol dioxygenase, which converts colorless catechol to a yellow oxidation product. The *EGFP* gene encodes a protein that fluoresces green when exposed to UV light (Chalfie 1994; Sun 1999).

Our screen led to the discovery of two new mutants with morphogenic and/or antibiotic production phenotypes. Linkage between the drug resistance marker contained within the transposon and the mutant phenotype was established by co-transformation. Attempts to establish linkage by co-transduction were unsuccessful because germlings (required in transduction experiments) were hypersensitive to apramycin and selection of transductants using apramycin was not possible. Primers homologous to the ends of Tn*5* were used to determine the chromosomal DNA sequence adjacent to the insertion and then used to identify the address of the insertion in the genome. In each case, the

transposon had inserted into an open reading frame. The wild type allele of each mutant was cloned and used to complement the mutant phenotype.

Mutant SJ175 was caused by an insertion into a gene predicted to have integral membrane spanning domains based on pockets of hydrophobic residues with the protein sequence. This mutant is an early producer of spores and antibiotics. Mutant SE293 was the result of an insertion into a putative TetR-family transcriptional regulator based on sequence similarity within the conserved helix-turn-helix region of the TetR-family. This mutant displayed a *bld* phenotype.

Materials and methods

Strains and growth conditions. *S. coelicolor* M145 (SCP1$^-$ SCP2$^-$) genomic DNA was used for *in vitro* mutagenesis with Tn*5*. *S. coelicolor* M145 and A3(2) were used as recipients of the M145 genomic library with Tn*5* insertions. Growth and manipulation of these strains were as described (Hopwood 2000). R2YE or MYM were used for growth on solid medium and YEME was used for growth in liquid medium (Hopwood 2000). When appropriate, the medium was supplemented with 50 μg/ml apramycin or 150 μg/ml spectinomycin. *E. coli* strain XL-10 gold was used for preparation of plasmid DNA as described (Maniatis 1989). Plasmids used for transformation into *S. coelicolor* were passaged through *E. coli* SCS110 (*dam*$^-$ *dcm*$^-$). *E. coli* ET12567/pUB307 (*dam*$^-$ *dcm*$^-$) was used for conjugation with *S. coelicolor*. *E. coli* DH5a was used for the Tn*5EGFP* insertion library, *E. coli* XL-1 blue was used for Tn*5xylE*, and *E. coli* strain XL-10 gold was used for Tn*5apr*.

Construction of modified Tn5 transposons for use in vitro. Three versions of Tn*5* were used in this study, Tn*5apr* (Gehring 2000), Tn*5xylE*, and Tn*5EGFP*. To construct Tn*5xylE,* the 2.66kb *Hind*III-*Nru*I fragment of pXE4 was ligated into pBluescript II KS (+) that had been digested with *Hind*III and *Sma*I resulting in plasmid pLQ1. The 2.4 kb *Bam*HI fragment of pLQ1 was cloned into the *Bam*HI site of pMODApr (Gehring 2000) to insert a promoterless copy of the *xylE* gene immediately adjacent to the 19 bp end of Tn*5*, resulting in plasmid pLQ2 (Figure 2.1a). To construct Tn*5EGFP,* the 1.8 kb *Eco*RI-*Eco*RV fragment of pIJ8668 (Sun 1999) was ligated into pBluescript II KS (+), resulting in plasmid pLQ4. The 1.8 kb *Bam*HI fragment of pLQ4, containing a promoterless copy of the *EGFP* gene, ligated into pMODApr to insert the *EGFP* gene immediately downstream of the 19 bp end of Tn*5* to generate pLQ6 (Figure 2.1b).

In vitro transposon mutagenesis. For insertion of Tn*5xylE* and Tn*5EGFP*, a library of M145 genomic DNA in plasmid pSpec*oriT* was used for *in vitro* mutagenesis. The genomic library was kindly provided by Amy Gehring and its construction is described elsewhere (Gehring 2000). In addition to the library containing Tn*5apr* insertions constructed and described by Gehring, et. al. (Gehring 2000), Tn*5xylE* and Tn*5EGFP* insertional libraries were constructed by incubation of either pLQ2 or pLQ6 with the pSpec*oriT* M145 genomic library as described by the manufacturer (Epicenter Technologies, Madison, WI). After incubation, each transposon-containing plasmid library was transformed into *E. coli* and transformants were selected on LB agar

(Maniatis 1989) containing 50 μg/ml apramycin sulfate (Sigma) and 200 μg/ml spectinomycin (Sigma). The resulting transformants were pooled and plasmid DNA was transformed into *E. coli* SCS110, with selection as above. Transformants were pooled and plasmid DNA isolated. Plasmid DNA isolated from *E. coli* SCS110 was used for transformation into *Streptomyces* protoplasts and this DNA was alkali-denatured (Oh 1997) prior to transformation. Generation and transformation of *Streptomyces* protoplasts was as described (Hopwood 2000). Transformants were selected on R2YE media overlaid with apramycin (20 μg/ml for A3(2) and 50 μg/ml for M145) after 16hrs incubation at 30^{o}C. Transformants were retested on MYM containing apramycin (50 μg/ml) and screened for spectinomycin sensitivity (150 μg/ml) and defects in antibiotic production and/or morphogenesis. Spectinomycin resistant strains with obvious morphological defects were serially passaged on MYM containing 50 μg/ml apramycin and screened for spectinomycin sensitivity.

Co-transformation. Genomic DNA from the putative transposon generated mutants was prepared (Hopwood 2000), alkali-denatured (Oh 1997), and transformed into M145 protoplasts. Transformants were selected on R2YE overlayed with 50 μg/ml apramycin after 16 hrs incubation at 30^{o}C. Morphological and antibiotic production phenotypes were scored on MYM (Tn*5apr*) or MM with glucose (Tn*5EGFP* and Tn*5xlyE*).

Identification of the site of transposon insertion. Genomic DNA was isolated from the verified transposon mutants (Hopwood 2000) and digested with *Apa*I (M145 mutants) or

*Sac*II(A3(2) mutants) and ligated into pBluescript II KS (+). The ligated DNA was then used to transform *E. coli* XL-10 gold. Transformants were selected on LB agar containing 50 μg/ml apramycin (apr). Plasmid DNA was recovered from the apr^R transformants and sequenced with primers complementary to the ends of the transposon: (5'TCGACCTGCAGGCATGCAAGCTT3' and 5'GGGTACCGAGCTCGAATTCA TCG3') using the ABI Prism Sequencer according to the manufacturer's instructions (cycle S).

Complementation of insertional mutants. As shown in figure 2.2, plasmid pHyg*oriT* was constructed by ligation of the hygromycin resistance gene (hygB) from pUH19a into pSET152. The 1.98 kb fragment from pUH19a resulting from *Sap*I-*Alw*NI digestion was treated with Klenow polymerase (Maniatis 1989) to generated blunt ends and then ligated to the 4.84kb *Sac*I fragment of pSET152, also treated with Klenow polymerase.

PCR primers were designed to obtain wild-type copies of each gene from *S. coelicolor* A3(2). For SJ175 primers 5' ACAAGCGCCTGCGAGGCAAGGA 3' and 5' TCACCGCGTAGTACCACAAGCTG 3' were used; for SE293, 5'TCGACGTCATC CCACGGCTGCG3' and 5'GACGGCGAGGTTCAGGCAGGCG3' were used. PCR was performed with the DyNAzyme EXT PCR kit according to the manufacturers instructions (MJ Research). PCR cycle was 94°C for 5 minutes, then 35 cycles of 94°C for 45 seconds, 68°C for 30 seconds and 72°C for 2 minutes, followed by an incubation at 72°C for 10 minutes and holding at 4°C. Reactions were supplemented with 5% DMSO and 5mM $MgCl_2$. The PCR products were subjected to electrophoresis, extracted from

the agarose gel, digested with *Sac*II (SJ175) or *Bcl*I (SE293) and ligated into pHyg*oriT* that had been digested with *Sac*II and *Eco*RV (SJ 175) or *Bam*HI and *Eco*RV (SE293) (figure 2.3a and b). The ligation reaction was transformed into *E. coli* XL-10 gold and transformants selected on LB media containing 50 μg/ml hygromycin (Sigma). Plasmid DNA was recovered and used to transform *E. coli* strain SCS110 (Stratagene). Conjugation was as described (Hopwood 2000). The conjugation mixture was plated on MYM containing 10 mM $MgCl_2$ and incubated for 18 hrs at 30°C. Nalidixic acid (0.5 mg) and hygromycin (2 mg) were applied to the plates in a liquid solution. The phenotypes of the resulting exconjugants were examined MYM and MM + glucose.

Southern hybridization will be performed as described (Hopwood 2000). Probe DNA for *attP* was the 1.82 kb *Nhe*I-*Hind*III fragment from pSET152. Genomic DNA from complemented strains will be digested with *Eco*RI, *Xho*I or *Xba*I prior to probing.

Results and Discussion

Construction of Tn5 insertions in the S. coelicolor chromosome using in vitro transposition and marker replacement. Three transposons based on Tn*5* were used in these experiments. Tn*5apr* was constructed by Gehring, et. al., (Gehring 2000) and contains an apramycin resistance gene flanked by the 19 bp direct repeats of Tn*5*. Tn*5xylE* is a derivative of Tn*5apr* that contains a promoterless copy of the *xylE* reporter immediately downstream of the 19 bp repeat at the end of the transposon so that insertion of Tn*5xylE* downstream of a regulatory region would generate a transcriptional or translations fusions between the regulatory region and the *xylE* gene. Tn*5EGFP* is a

derivative of Tn*5apr* that would generate similar fusions to the *EGFP* reporter gene. A library of *Streptomyces coelicolor* M145 genomic DNA was constructed in pSpec (for Tn*5apr* insertion) or pSpec*oriT* (for Tn*5xylE* and Tn*5EGFP* insertion), which have no origin of replication for *Streptomyces* but which do contain an *E. coli* origin that allows both replication in *E. coli* and transfer of the plasmid by mating between *E. coli* and *S. coelicolor*. These genomic libraries were exposed to one of the three transposons under conditions that would allow the transposons to insert into the genomic DNA *in vitro* (Gehring 2000). Protoplasts of *S. coelicolor* M145 and A3(2) were transformed with these mutagenized genomic libraries and transformants were selected using the apramycin resistance (apr^R) gene located within the transposon. Apramycin resistant transformants arose at a frequency of approximately 10^{-3} transformants/μg of DNA. Since pSpec and pSpec*oriT* do not contain an origin of replication for *S. coelicolor*, transformants arise either by integration of the entire vector, via a single cross-over event, or by marker replacement of the chromosomal DNA with that contained on the vector, via a double cross over event. These two events are distinguished from each other in that a single cross-over resulting in insertion of the entire vector would give transformants that were apr^R, contained within the transposon, and spectinomycin resistant (spc^R), contained on the vector. On the other hand, marker replacement between the vector and the chromosome via a double cross-over would result in transformants that were apr^R and spc^S. These transformants would contain a replacement of the region of the chromosome contained on the vector with a fragment that contained a transposon insertion. Such transformants were screened for morphological or antibiotic production phenotypes

potentially arising from insertions of the transposon into genes required for those functions (Table 2.1).

Using the library mutagenized with Tn*5apr*, 276 apr^R transformants were selected, all of which were also spc^R. Of these, 69 had phenotypes of interest and were serially passaged on apramycin and tested for spectinomycin sensitivity. Ultimately, 11 apr^R, spc^S strains were generated.

Using the library mutagenized with Tn*5xylE,* 5216 apr^R transformants were selected, of those 151 were spc^S and of those 14 had phenotypes of interest. Using the library mutagenized with Tn*5EGFP,* 8096 apr^R transformants were selected, of those 158 spc^S were and of those 35 had phenotypes of interest.

Establishment of linkage between mutant phenotypes and the apramycin resistance marker contained in the transposon. Protoplasting, especially of *S. coelicolor,* results in a significant level of spontaneous morphological mutation. To distinguish this background from mutations caused by transposon insertion, linkage between the apramycin resistance marker and the phenotype of interest was examined by co-transformation. Genomic DNA was isolated from each apr^R, spc^S strain that displayed either an antibiotic production or morphological phenotype different from wild type and used for transformation of *S. coelicolor* M145 protoplasts. Apr^R transformants were then screened for the mutant phenotype of the donor. Only those that showed 100% co-transformation between the apr^R marker and the phenotype of the donor were studied further.

Of the 11 potential mutants generated by Tn*5apr*, 7 yielded apr^R transformants and of those 1 (SJ175) was linked to the transposon (4/4). Of the 14 potential mutants generated by Tn*5xylE*, and the 35 potential mutants generated by Tn*5EGFP*, 37 yielded transformants. No mutant phenotypes were linked to Tn*5xylE* insertions and 4 (SE69 (15/15), SE145 (1/1), SE220 (8/8), and SE293 (2/2)) were linked to Tn*5EGFP* insertions.

Location of the transposon insertions and associated phenotypes. Primers complementary to the 19 bp repeats at the end of Tn*5* were used to sequence out from the transposon insertion in both directions into the neighboring chromosomal DNA. Attempts to sequence directly from chromosomal DNA were unsuccessful because of random priming of sequences outside the region of the transposon insertion. To eliminate this problem the transposon and chromosomal DNA adjacent to it were cloned into a plasmid vector prior to sequencing. Approximately 100 base pairs of DNA from each primer were used to locate the site of the transposon insertion on the *S. coelicolor* genome and the locations of the insertions are indicted on the map of the chromosome shown below. The sequence obtained was then used to as a query to BLAST the *S. coelicolor* genome.

Mutant SJ175 produces spores and the pigments associated with antibiotic production at least 24 hours sooner than wild-type on both MM+glucose and MYM (Figure 2.4). The sequence obtained from the DNA surrounding the SJ175 insertion identified the location of the insertion on the *S. coelicolor* genome (SCD66.12c). The

annotated region of the chromosome at this address is shown in figure 2.5. The open reading frame identified by this insertion is postulated to be an integral membrane protein based on regions of hydrophobic residues, often found in these proteins. BLAST searches identified no homology to any proteins in the database.

Mutant SE145 fails to produce aerial hyphae on MM + glucose (*bld*) and also fails to produce the red pigment associated with undecylprodigiosin (Rd) production. It does, however, still produce the blue pigment associated with actinorhodin (Ar) production. On MYM, SE145 exhibits accelerated differentiation, similar to that seen for SJ175. The sequence surrounding SE145 yielded conflicting results. With primer 1, the insertion appeared to be located at SC2G61.01c. The open reading frame from this insertion is a putative protein of no known function. However, sequence obtained with primer 2 predicted the insertion was located in cosmid SCI35. This open reading frame is postulated to be a glutamate N-acetyltransferase by sequence similarity. These 2 regions are located over 250 kb apart, indicating that a chromosomal rearrangement occurred in either the original mutant, or during cloning of the region for sequencing.

SE220 was *bld* and produced neither the red or blue pigments associated with Ar and Rd production on MM + glucose. On MYM, this mutant still failed to produce any pigments and was able to produce a reduced number of spores only after prolonged incubation (greater than two weeks). Sequence from this mutant identified the insertion location at SCE59.12c. This was a re-isolation of *rsuA*, recently described by Gehring, et. al. (Gehring 2001). Our insertion was located approximately 300 bp upstream of that determined by Gehring, et. al.

Mutant SE293 is completely defective in morphogenesis (*bld*) on MYM and it also fails to produce the blue pigment associated with Ar production but still produces the red pigment associated with Rd production. This mutant failed to grow on MM+glucose, indicating that it is an auxotroph as well. The sequence obtained from the DNA surrounding the SE293 insertion identified the location of the insertion to 2SCG38.28. Figure 2.6 shows a diagram of the annotated genome in that region and the site of the integration. The open reading frame identified by this insertion shows sequence similarity to the TetR-family of transcriptional regulators, particularly in the conserved TetR helix-turn-helix region, responsible for binding to target DNA sequences (figure 2.7).

SE69 fails to produce mature spores from the aerial hyphae that it erects on MYM (*whi*) and fails to produce even aerial hyphae on MM+glucose (*bld*) as well as displaying an interesting orange pigmentation. No sequence data has yet been obtained from this mutant.

The location of the inserts was randomly distributed around the chromosome (figure 2.8). This indicates that there are probably no hot spots for Tn*5* insertion with respect to general areas of the genome. This correlates well with the results of Gehring, et. al. (Gehring 2000).

Complementation of the mutants. To confirm that the mutant phenotypes observed were caused by the transposon insertions and to test whether the mutants were dominant or recessive, a wild-type copy of each gene was obtained by PCR amplification of A3(2)

genomic DNA, cloned into pHyg*oriT*. As shown in figure 2.3a, plasmid pHO-175 contains a 2 kb PCR fragment containing an intact copy of the open reading frame identified by SJ175 and the two overlapping genes and as shown in figure 2.3b, plasmid pHO-293 contains a 1.5 kb PCR fragment containing an intact copy of the open reading frame identified by SE293. Each of these plasmids was introduced in the corresponding mutant strain as well as the wild type by mating. As the vector contains no *Streptomyces* origin of replication, exconjugants contain integrated copies of the plasmid via either integration at the ϕC31 attachment site contained on the plasmid or at the chromosomal location corresponding to the insert DNA. Southern analysis will be undertaken to assure that insertion has occurred via the attachment site, as homologous recombination at the locus would result in plasmid integration and could cause polar effects. As seen in table 2.2, complementation was achieved for SJ175, with introduction of pHO-175 restoring cells to wild type levels of sporulation. However, pHO-293 was not able to restore the ability to sporulate to SE293, indicating that this mutation may be dominant.

Conclusions

Using the newly developed system of *in vitro* Tn*5* mutagenesis for *Streptomyces*, we performed a genome-wide screen for genes involved in morphogenesis and antibiotic production. In order to cross the transposon insertions, which had been generated *in vitro,* into *S. coelicolor*, it was necessary to generate protoplasts. Protoplasting is a mutagenic process in *Streptomyces*, leading to a high background of spontaneous mutation. It was therefore necessary to establish linkage between the observed mutant

phenotypes and transposon insertions. This linkage was established via co-transformation of the observed mutant phenotype and the drug resistance marker within the transposon. Initially, we attempted to use co-transduction to establish linkage, which would have been far more efficient than co-transformation. However, the germlings used as recipients for transduction in *Streptomyces* were hypersensitive to apramycin, making selection of transductants impossible. Primers were then designed to sequence the chromosomal DNA region around the verified transposon insertions. Based on the sequence results, 2 of the mutants were assigned putative functions, SJ175 and SE293. To further confirm that the observed mutant phenotypes were caused by Tn*5*, each mutant was complemented with a wild type copy of the gene that was shown, from the sequence data, to have received the insertion. Complementation was carried out by insertion into the chromosome via the *attP* site, with the wild type promoter to control for gene dosage effects. If the mutation causing the observed phenotype is recessive, then insertion of a wild type copy of the same gene should restore the mutant to wild type. Complementation will not be observed if the mutation is dominant.

SJ175 shows no homology to any proteins in the database using any of several standard matrices with several sets of parameters. Mutations in this gene result in abundant sporulation and antibiotic production, indicating that this protein may be a negative regulator of initiation of differentiation. There are several stretches of hydrophobic residues within this protein, which could allow the protein to span the cell membrane. Since this gene appears to be co-transcribed with 2 other genes of unknown function (figure 2.4), it is possible that the observed phenotype is due to polar effects of

the insertion, rather than directly to loss of the SJ175 gene product. It was also likely that the SJ175 gene did not have it's own promoter. Therefore, as we wanted to complement with wild type levels of expression, this mutant was complemented with the wild type copies of all three genes, as they appear on the chromosome. This construct of all three genes was able to complement the SJ175 and restore it to wild type levels of spore producion. This further confirms that the proposed gene does cause the mutant phenotype. However, to fully determine the effect of Tn*5* insertion necessary for the observed phenotype, complementation will need to be performed with only the SJ175 gene, as the observed effects might be polar. Also, an analysis of the membrane should be undertaken to determine if SJ175 really is an integral membrane protein.

SE293 shows significant homology to the TetR-family of transcriptional regulators (Beck 1982), particularly with the helix-turn-helix region, which is part of the conserved TetR-like domain. Proteins in this family are usually repressors, often targeting genes involved in cell envelope permeability (Kojic 2001). If this is also the function of SE293, it could explain why mutations in this gene result in a lack of sporulation. This protein could regulate a gene responsible for the uptake of some extracellular signal involved in sporulation and antibiotic production. *bldK* has been shown to be involved in the uptake of some extracellular factor(s) (Nodwell 1996), and the cascade described by Willey, et. al. relies on the production and uptake of extracellular signals (Willey 1993). Unlike many of the *bld* mutants, SE293 does not appear to be rescued by growth on poor carbon sources, as is seen for *bldB* (Willey 1993). It will be interesting to see if SE293 is complemented by any of the other *bld*

mutants as *bldB* is not. Introduction of a single wild type copy of this gene did not restore sporulation to the SE293 mutants. This would suggest that the mutation is dominant, and may be the result of the need for this protein to operate in *cis*. It would be beneficial to perform S1 nuclease mapping experiments to determine if SE293 regulates the transcription of several nearby genes, and if this regulation occurs only in *cis*.

Sequence analysis of SE69 is still being attempted. Primers will be generated that complement the apramycin resistance and *EGFP* genes instead of the primers used for the other mutants, which complemented the multiple cloning site of the transposon. Also, the gene region will PCR amplified and sequenced directly as opposed to sequencing the whole plasmid. Hopefully, these methods will result in sequence for this mutant, which can then further characterized with SJ175 and SE293.

Streptomyces are very interesting prokaryotes in that they perform a complex cycle of morphological differentiation as well as producing greater than 70% of known natural product antibiotics currently used in human and veterinary medicine. Understanding of how these processes occur is imperative, especially in this time of increasing development of resistance to currently prescribed mediations by deadly pathogens. Further characterization of the two new genes isolated here will add greatly to our understanding of this bacteria.

References

1. Beck, C.F., Mutzel, R., Barbe, J., and Muller, W. (1982). "A multifunctional gene (*tetR*) controls the Tn10-encoded tetracycline resistance." *J. Bacteriol*. 150: 633-642.

2. Bibb, M.J., Molle, V., and Buttner, M.J. (2000). "An extracytoplasmic function RNA polymerase sigma factor required for aerial mycelium formation in *Streptomyces coelicolor* A3(2)." *J. Bacteriol*. 182: 4606-4616.

3. Bignell, D.R.D., Warawa, J.L., Strap, J.L., Chater, K.F., and Leskiw, B.K. (2000). "Study of the *bldG* locus suggests that an anti-anti-sigma factor and an anti-sigma factor may be involved in *Streptomyces coelicolor* antibiotic production and sporulation." *Microbiol*. 146: 2161-2173.

4. Chalfie, M., Tu, Y., Euskirchen, G., Ward, W., and Prasher, D.C. (1994). "Green fluorescent protein as a marker for gene expression." *Science* 263: 802-805.

5. Elliot, M., Damji, F., Passantino, R., Chater, K., and Leskiw, B. (1998). "The *bldD* gene of *Streptomyces coelicolor* A3(2): a regulatory protein involved in morphogenesis and antibiotic production." *J. Bacteriol*. 180: 1549-1555.

6. Gehring, A. M., Nodwell, J.R., Beverley, S.M, and Losick, R. (2000). "Genomewide insertional mutagenesis in *Streptomyces coelicolor* reveals additional genes involved in morphological differentiation." *Proc. Natl. Acad. Sci.* 97: 9642-9647.

7. Gehring, A.M., Yoo, N.J., and Losick, R. (2001). "RNA polymerase sigma factor that blocks morphological differentiation by *Streptomyces coelicolor*." *J. Bacteriol.* 183: 5991-5996.

8. Goryshin, I.Y., and Reznikoff, W.S. (1998). "Tn5 *in vitro* transposition." *J. Biol. Chem.* 273: 7367-7374.

9. Ikeda, H., Takada, Y., Pang, C.-H., Tanaka, H., and Omura, S. (1993). "Transposon mutagenesis by Tn4560 and applications with avermectin-producing *Streptomyces avermitilis*." *J. Bacteriol.* 175: 2077-2082.

10. Kaneko, T., Nakamura, Y., Sato, S., Asamizu, E., Kato, T., Sasamoto, S., Watanabe, A., Idesawa, K., Ishikawa, A., Kawashima, K., Kimura, T., Kishida, Y., Kiyokawa, C., Kohara, M., Matsumoto, M., Matsuno, A., Mochizuki, Y., Nakayama, S., Nakazaki, N., Shimpo, S., Sugimoto, M., Takeuchi, C., Yamada, M., and Tabata, S. (2000). "Complete genome structure of the nitrogen-fixing symbiotic bacterium *Mesorhizobium loti*." *DNA Res.* 7: 331-338.

11. Kieser, T., Bibb, M.J., Buttner, M.J., Chater, K.F., and Hopwood, D.A. (2000). Practical *Streptomyces* Genetics. The John Innes Foundation, Norwich, U.K.

12. Kojic, M., and Venturi, V. (2001). "Regulation of *rpoS* gene expression in *Pseudomonas*: involvement of a TetR family regulator." *J. Bacteriol*. 183: 3712-3720.

13. Lawlor, E.J, Baylis, H.A., and Chater, K.F. (1987). "Pleiotropic morphological and antibiotic deficiencies result from mutations in a gene encoding a tRNA-like product in *Streptomyces coelicolor* A3(2)." *Genes Dev*. 1: 1305-1310.

14. Maniatis, T., Sambrook, J., and Fritsch, E.F. (1989). Molecular cloning: a laboratory manual, second edition. Cold Spring Harbor Laboratory Press.

15. McHenney, H.A., Hosted, T.J., Dehoff, B.S., Rosteck, P.R.Jr., and Baltz, R.H. (1998). "Molecular cloning and physical mapping of the daptomycin gene cluster from *Streptomyces roseosporus*." *J. Bacteriol*. 180: 143-151.

16. Molle, V., and Buttner, M.J. (2000). "Different alleles of the response regulator gene *bldM* arrest *Streptomyces coelicolor* development at different stages." *Mol. Microbiol.* 36: 1265-1278.

17. Molnar, I., Aparicio, J.F., Haydock, S.F., Khaw, L.E., Schwecke, T., Konig, A., Staunton, J., and Leadlay, P.F. (1996). "Organisation of the biosynthetic gene cluster for rapamycin in *Streptomyces hygroscopicus*: analysis of genes flanking the polyketide synthase." Gene 169: 1-7.

18. Nodwell, J.R., McGovern, K., and Losick, R. (1996). "An oligopeptide permease responsible for the import of an extracellular signal governing aerial mycelium formation in *Streptomyces coelicolor*." *Mol. Microbiol*. 22: 881-893.

19. Oh, S.H. and Chater, K.F. (1997). "Denaturation of circular or linear DNA facilitates targeted integrative transformation of *Streptomyces coelicolor* A3(2): possible relevance to other organisms." J. Bacteriol. 179: 122-127.

20. Pope, M.K., Green, B., and Westpheling, J. (1998). "The *bldB* gene encodes a small protein required for morphogenesis, antibiotic production and catabolite control in *Streptomyces coelicolor*." *J. Bacteriol*. 180: 1556-1562.

21. Redenbach, M., Kieser, H.M., Denapaite, D., Eichner, A., Cullum, J., Kinashi, H., and Hopwood, D.A. (1996). "A set of ordered cosmids and a detailed genetic and physical map for the 8Mb *Streptomyces coelicolor* A3(2) chromosome." *Mol. Microbiol*. 21: 77-96.

22. Salanoubat, M., Genin, S., Artiguenave, F., Gouzy, J., Mangenot, S., Arlat, M., Billault, A., Brottier, P., Camus, J.C., Cattolico, L., Chandler, M., Choisne, N., Claudel-Renard, C., Cunnac, S., Demange, N., Gaspin, C., Lavie, M., Moisan, A., Rober, C., Saurin, W., Schiex, R., Weissenbach, J., and Boucher, C.A. (2002). "Genome sequence for the plant pathogen *Ralstonia solanacearum*." Nature 415: 497-502.

23. Sun, J., Kelemen, G.H., Fernandez-Abalos, J.M., and Bibb, M.J. (1999). "Green fluorescent protein as a reporter for spatial and temporal gene expression in *Streptomyces coelicolor* A3(2)." Microbiol. 145: 2221-2227.

24. Willey, J., Schwedock, J., and Losick, R. (1993). "Multiple extracellular signals govern the production of a morphogenetic protein involved in aerial mycelium formation by *Streptomyces coelicolor*." Genes Dev. 7: 895-903.

25. Wood, D.W., Setubal, J.C., Kaul, R., Monks, D., Chen, L., Wood, G.E., Chen, Y., Woo, L., Kitajima, J.P., Okura, V.K., Almeida Jr., N.F., Zhou, Y., Bovee Sr., D., Chapman, P., Clendenning, J., Deatherage, G., Gillet, W., Grant, C., Guenthner, D., Kutyavin, T., Levy, R., Li, M., Saenphimmachak, C., Wu, Z., Gordon, D., Eisen, J.A., Paulsen, I., Karp, P., Romero, P., Zhang, S., Yoo, H., Tao, Y., Biddle, P., Jung, M., Krespan, W., Perry, M., Gordon-Kamm, B., Liao, L., Kim, S., Hendrick, C.,

Zhao, Z., Dolan, M., Tingey, S.V., Tomb, J., Gordon, M.P., Olson, M.V., and Nester, E.W. (2001). "The genome of the natural genetic engineer *Agrobacterium tumefaciens* C58." Science 294: 2317-2323.

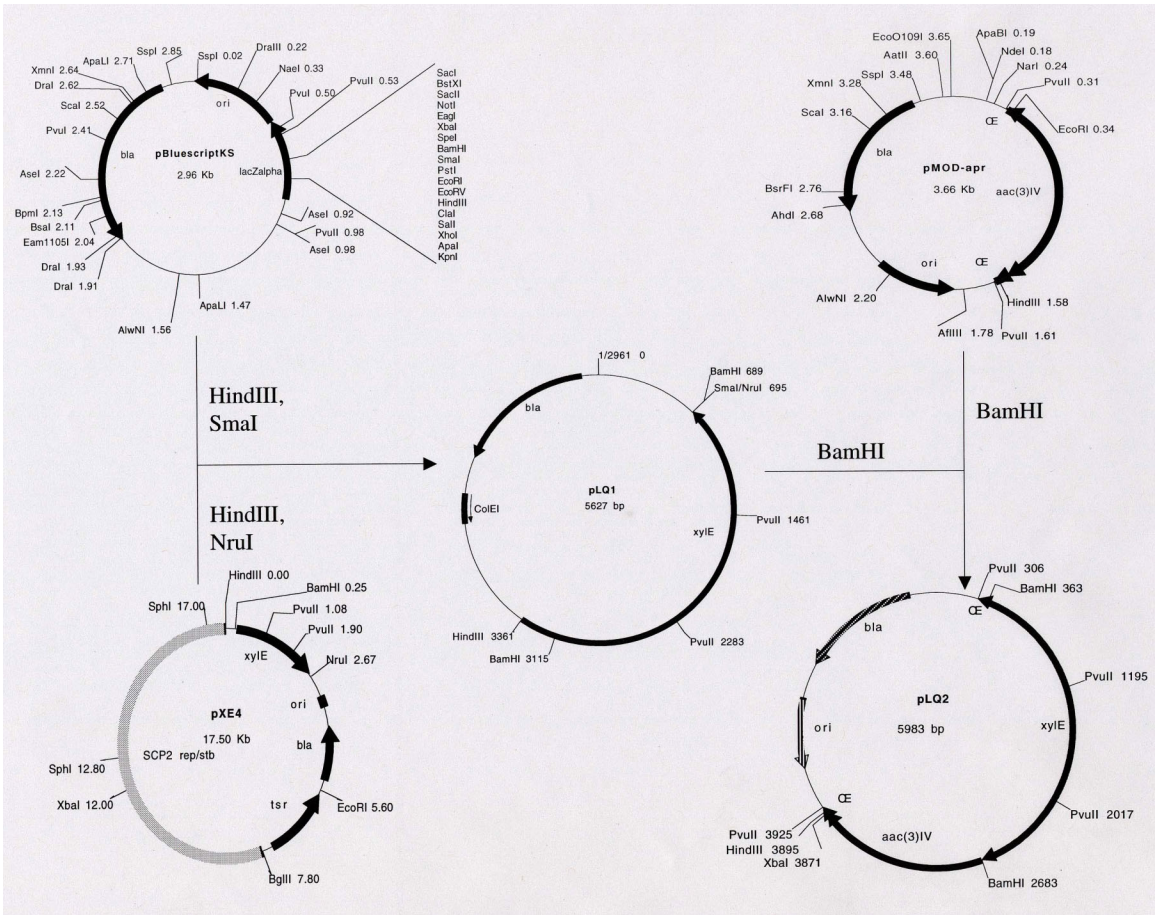


Figure 2.1a. Construction of Tn5*xylE*.

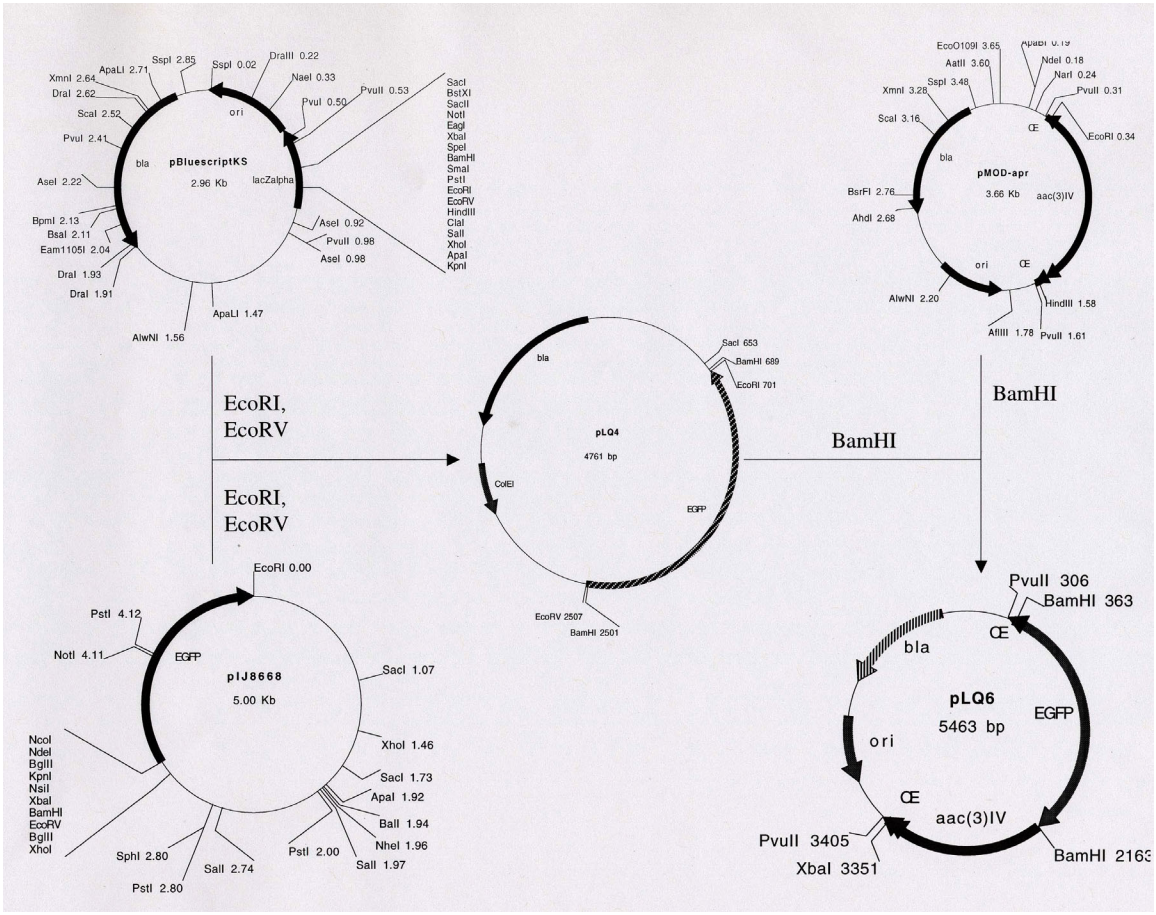


Figure 2.1b. Construction of Tn5*EGFP*.

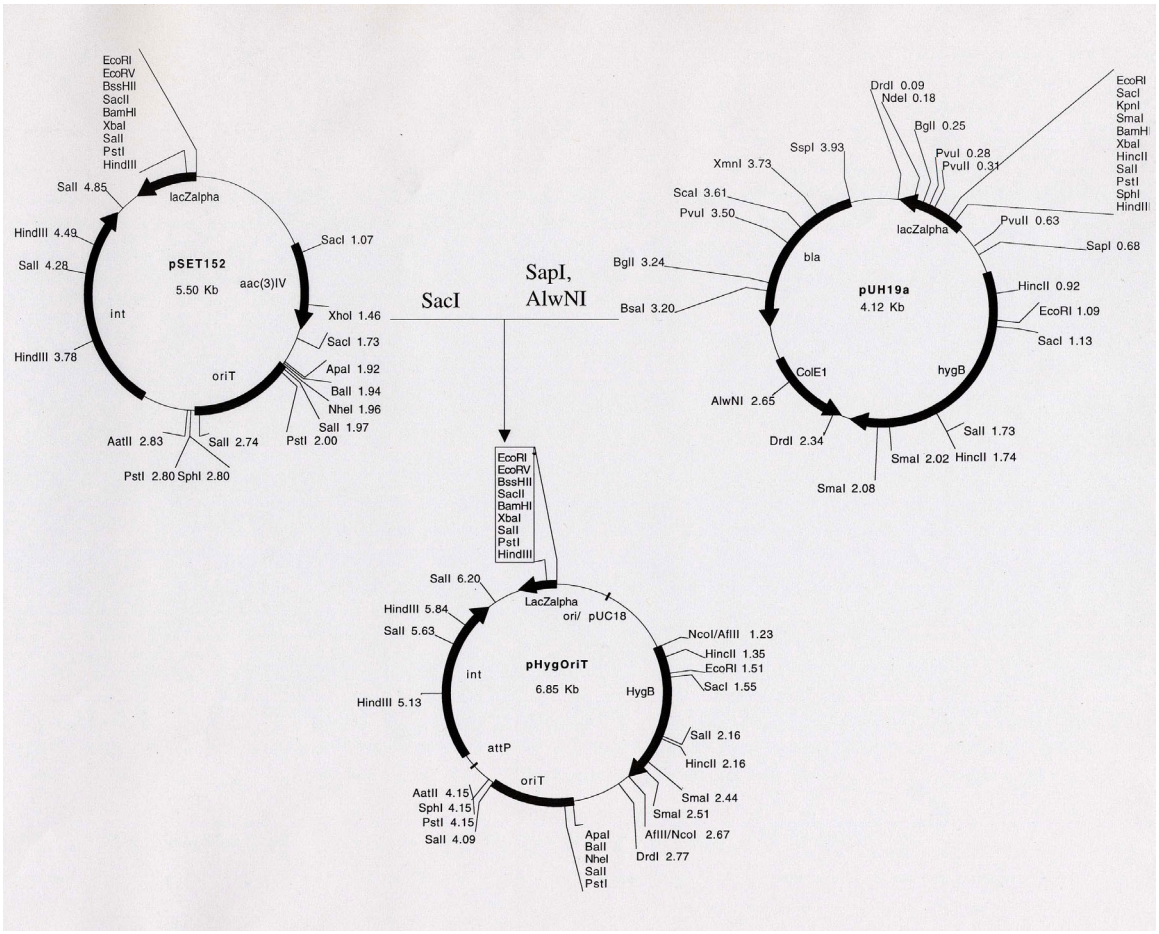


Figure 2.2. Construction of pHyg*oriT*.

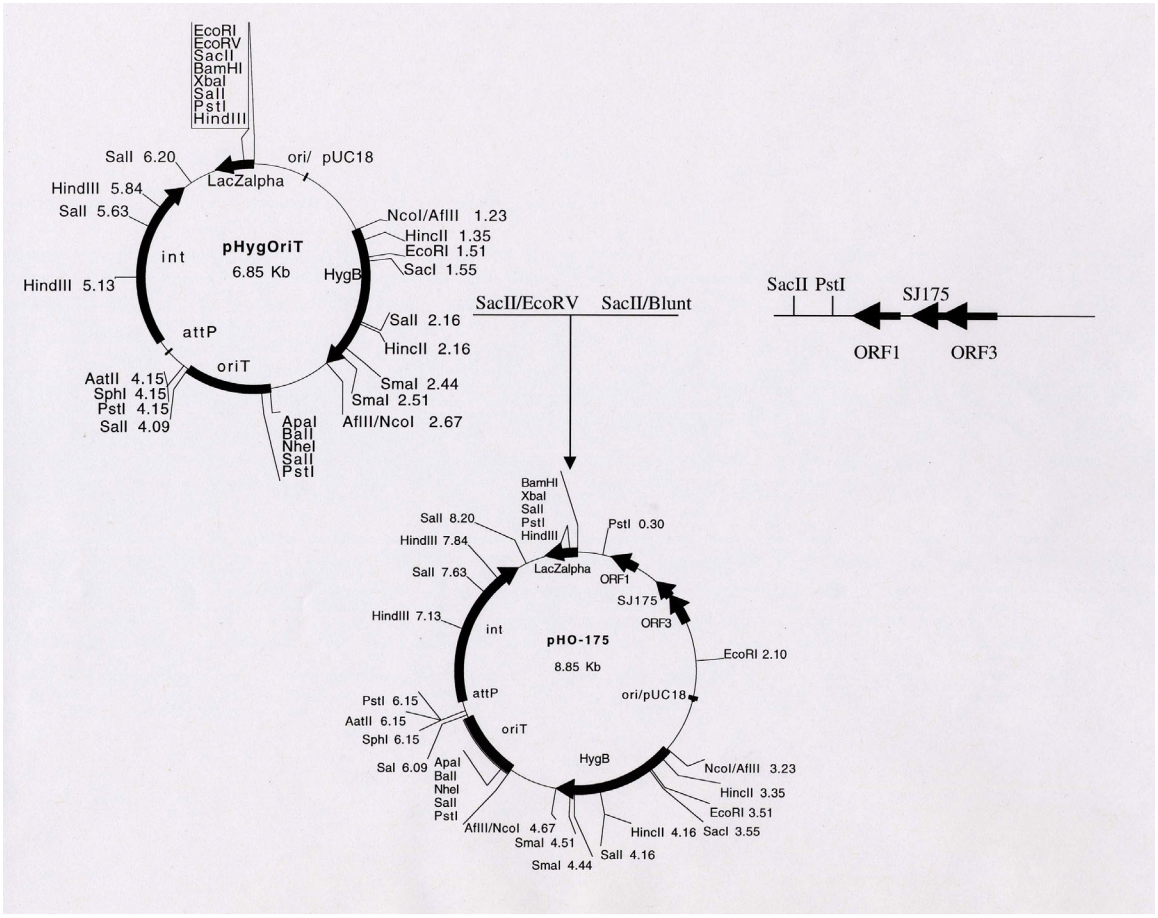


Figure 2.3a. Construction of the Complementation Plasmid for SJ175.

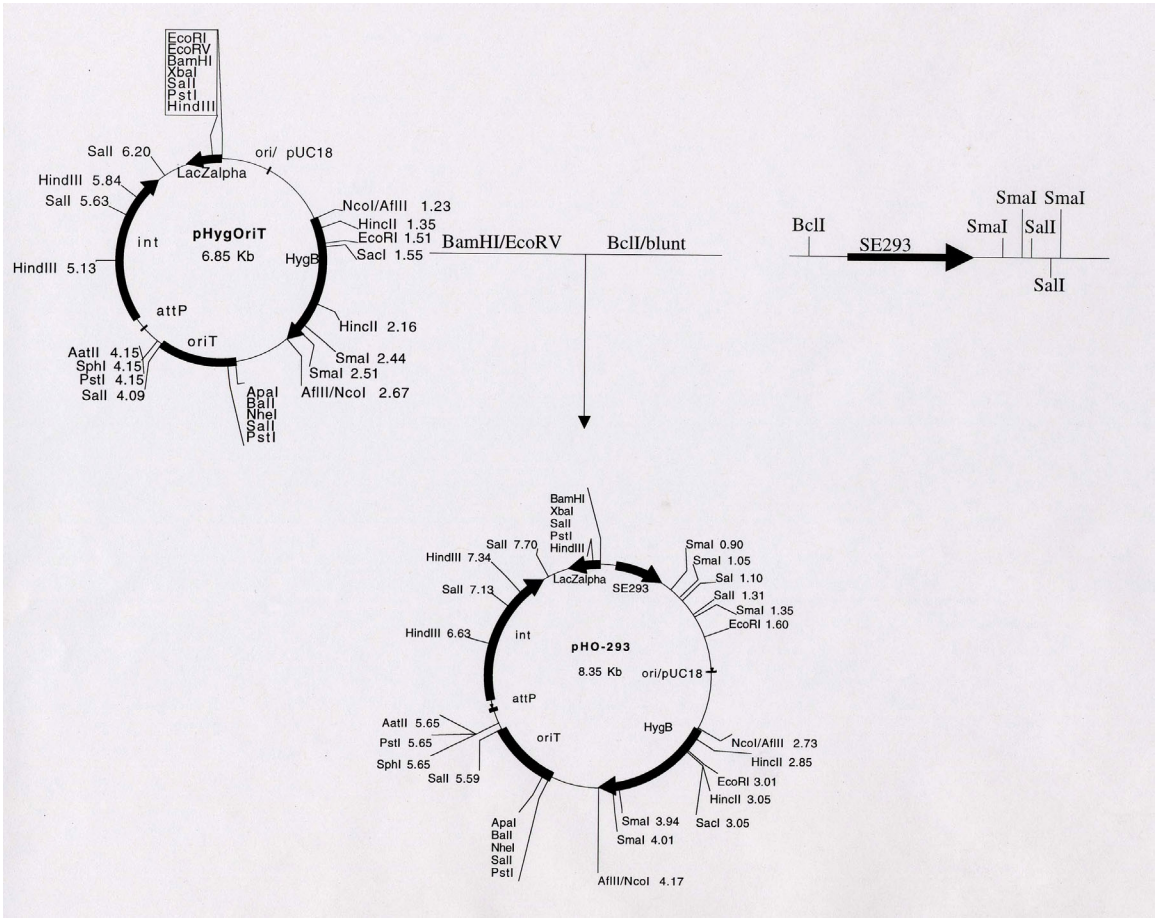


Figure 2.3b. Construction of the Complementation Plasmid for SE293.

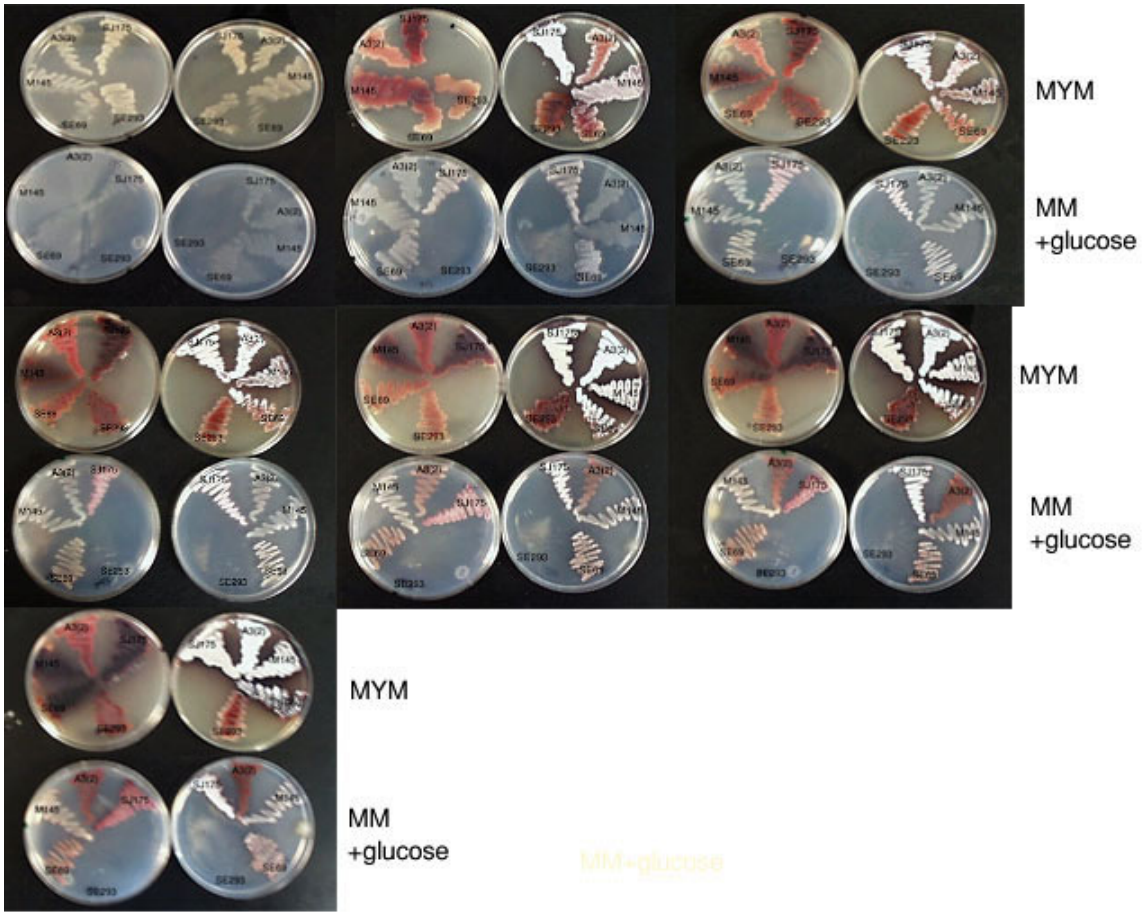


Figure 2.4. Phenotypes of Insertional Mutants. As for each day of growth from 1 to 7 at 30°C. Each day contains a view of the plate from both the top and bottom to allow for visualization of both antibiotic and spore production. Mutants were streaked on both rich carbon source media (MM+glucose) and poor carbon source media (MYM). Order of mutants as viewed from bottom of plate going clockwise: SE293, SE69, M145, A3(2), SJ175.

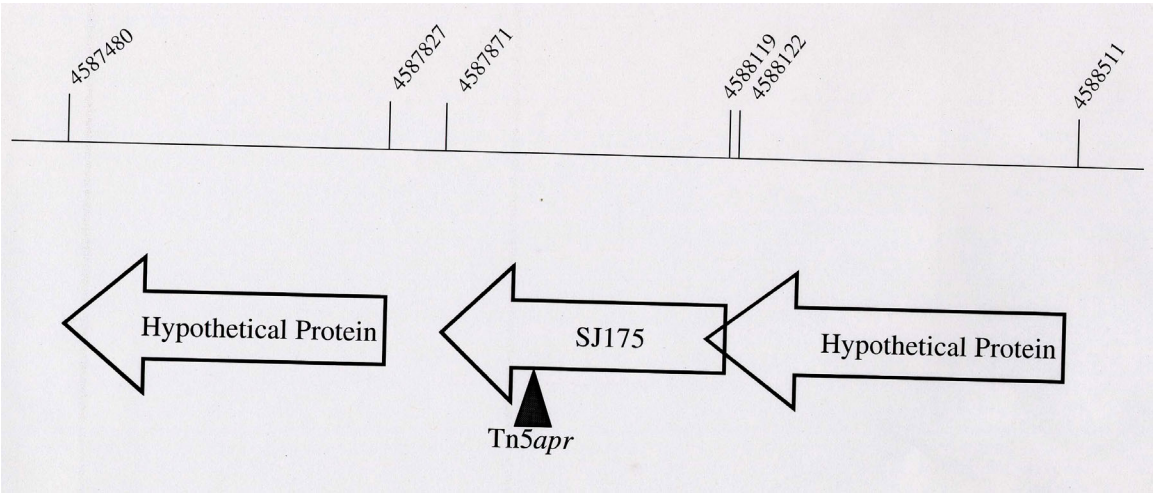


Figure 2.5. Organization of the *S. coelicolor* Genome Region Surrounding SJ175.

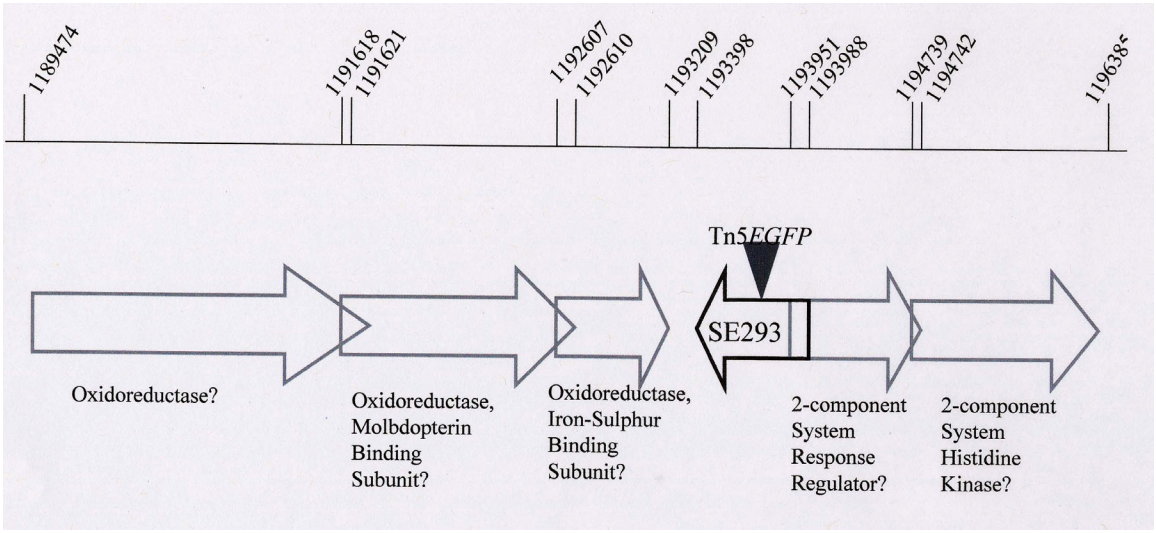


Figure 2.6. Organization of the *S. coelicolor* Genome Region Surrounding SE293.

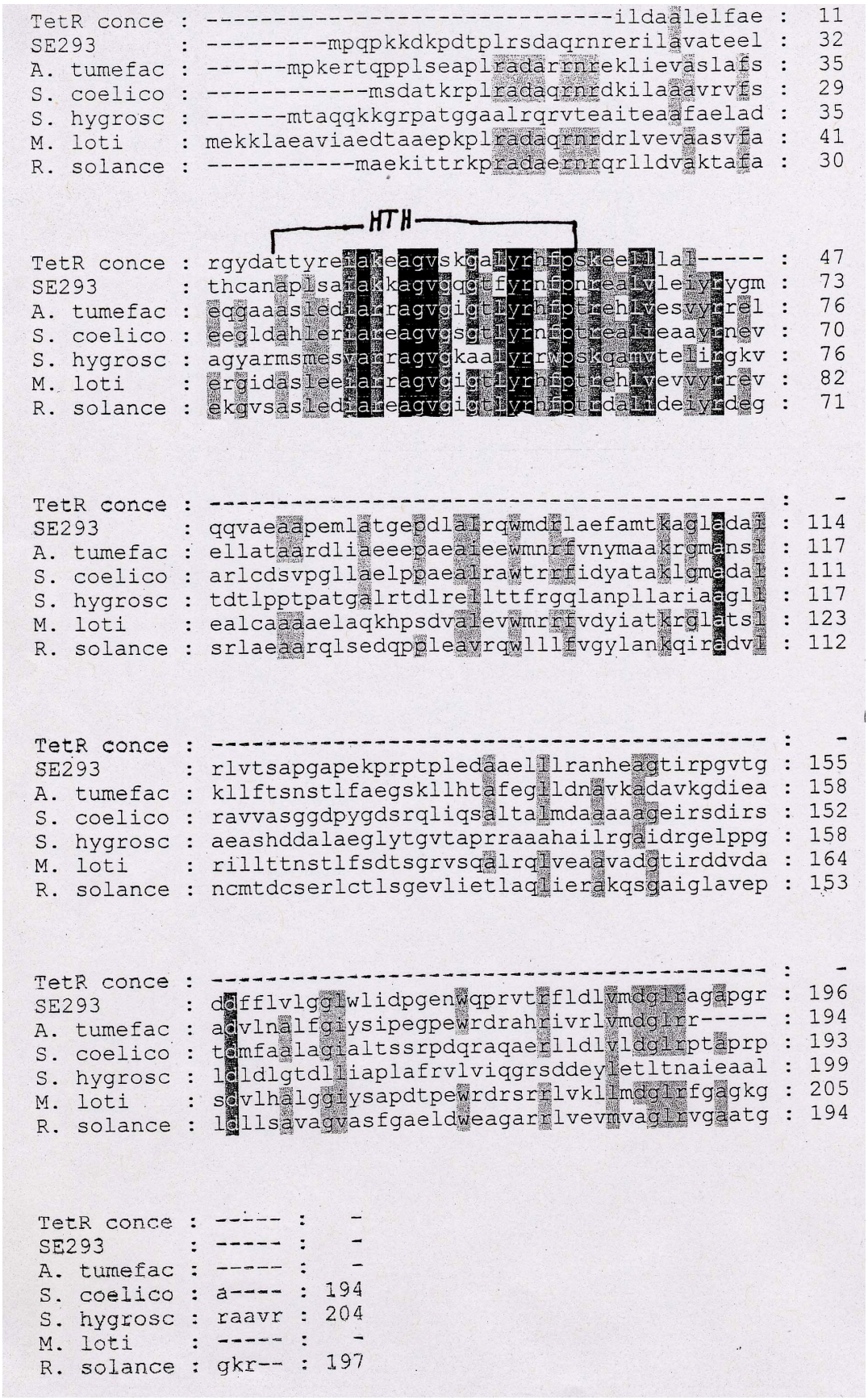

HTH

Figure 2.7. Multiple Alignment of SE293 and Several Other TetR-Like Transcriptional Regulators. Proteins from *Agrobacterium tumefaciens* (Wood 2001), *S. coelicolor* (Redenbach 1996), *Mesorhizobium loti* (Kaneko 2000), and *Ralstonia solanacearum* (Salanoubat 2002) are all putative members of this family. The protein from *S. hygroscopicus* has been determined to be an antibiotic transport regulator protein (Aparicio 1996). The alignment was produced using GeneDoc, which can be found at www.psc.edu/biomed/genedoc/. White letters in black boxes correspond to identities between all proteins, white letters in gray boxes are identical or similar in a majority of the proteins and black letters in gray boxes have some degree of identity or similarity between some of the proteins.

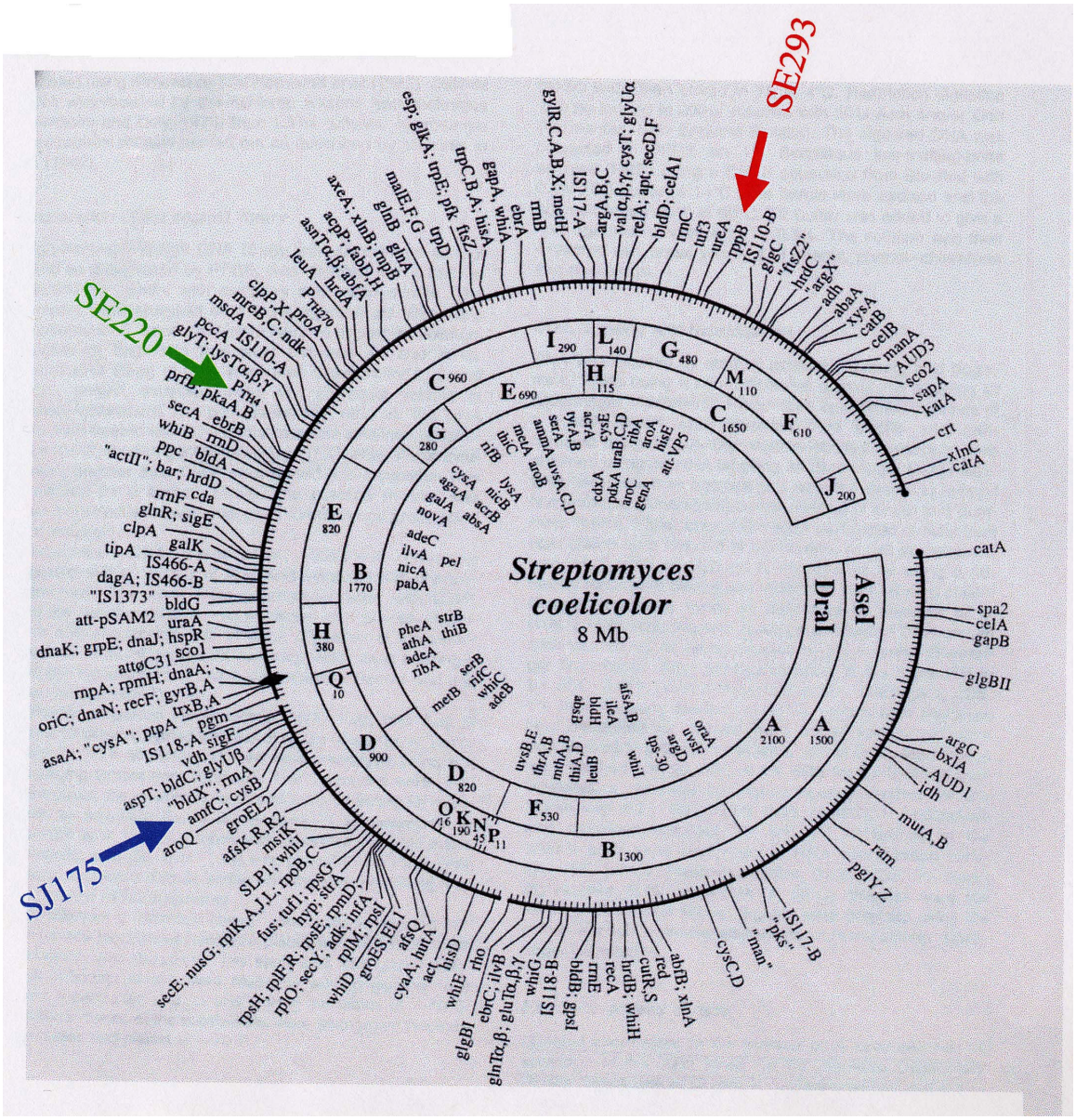


Figure 2.8. Locations of Tn5 Insertions on the *S. coelicolor* Genome.

Table 2.1. Transformation of Tn5 Insertion Libraries into *S. coelicolor*.

Tn5 DERIVATIVE USED	RECIPIENT	APR^R	SPC^S	MUTANTS
Tn5*apr*	A3(2)	276	11	11
Tn5*EGFP*	M145	8096	158	35
Tn5*xylE*	M145	5216	151	14

Table 2.2. Complementation of Tn5 Insertion Mutants with pHO-175 and pHO-293.

S. coelicolor Recipient	Complementation Plasmid	Conjugation Efficiency	Spontaneous Resistance	Phenotype
M145	pHO	5×10^{-5}	$<10^{-7}$	slight decrease in spores
M145	pHO-175	1.5×10^{-4}	$<10^{-7}$	slight decrease in spores
M145	pHO-293	3×10^{-4}	$<10^{-7}$	slight decrease in spores
A3(2)	pHO	8 x 10-5	$<10^{-7}$	wild type
A3(2)	pHO-175	2×10^{-4}	$<10^{-7}$	wild type
A3(2)	pHO-293	6×10^{-4}	$<10^{-7}$	wild type
SJ175	pHO	6.4×10^{-8}	$<10^{-8}$	over-sporulation
SJ175	pHO-175	8×10^{-7}	$<10^{-8}$	wild type
SE293	pHO	2×10^{-6}	$<10^{-8}$	*bld*
SE293	pHO-293	1.2×10^{-5}	$<10^{-8}$	*bld*

CHAPTER 3

IMPROVEMENTS IN CONJUGAL TRANSFER FOR AN ATTEMPT TO ISOLATE INSERTIONAL MUTANTS IN *STREPTOMYCES VENEZUELAE*

Abstract

We attempted to obtain mutants in *Streptomyces venezuelae* by the conjugation of an insertional library of *S. coelicolor* mutagenized *in vitro* with Tn*5*. All attempts to recover ex-conjugants in these experiments were unsuccessful. However, we were able to determine that ex-conjugant frequencies in tri-parental matings were similar to frequencies obtained in di-parental matings. Also, methylation-deficient *E. coli* strains are not needed for high frequency conjugation into *S. venezuelae*.

Introduction

Streptomyces are filamentous, multi-cellular soil bacteria that undergo a complex cycle of differentiation that starts with the sensing of extracellular signals released upon starvation. Coincident with this sporulation is the production of antibiotics and other secondary metabolites. Isolation of genes involved in these processes has focused on *Streptomyces coelicolor*, which has become the model organism for the actinomycetes.

However, there are other species, which offer many benefits over *S. coelicolor*. One such species *is S. venezuelae*. *S. venezuelae* is able to grow and differentiate much more quickly than *S. coelicolor* on both agar plates and in liquid media. Its spores

germinate synchronously, unlike spores of *S. coelicolor*, which allows for transcriptional analyses to be performed with a much higher sensitivity. Also, *S. venezuelae* is able to sporulate in liquid media, while *S. coelicolor* can only sporulate on agar plates (Glazebrook 1990). Unfortunately, not much is known about how sporulation and antibiotic production occur in *S. venezuelae* as it is difficult to transform protoplasts of this species.

A system for the conjugal transfer of DNA from *E. coli* to *Streptomyces* has been developed (Mazodier 1989). This system has been shown to work for a number of *Streptomyces* species (Bierman 1992; Flett 1997; Giebelhaus 1996; Smokvina 1990), including *S. venezuelae* at a much higher efficiency than can be achieved with protoplast transformation (Green personal communication). Any plasmid can be mobilized from *E. coli* into *Streptomyces* by the cloning of an origin of transfer (*oriT)* onto the plasmid (Mazodier 1989). Although it has been shown that tri-parental matings are possible and that some *Streptomyces* species are able to accept DNA transferred from a methylation-proficient strain of *E. coli*, this has not previously been shown for *S. venezuelae*. We have shown here that both of these methods are possible for *S. venezuelae*.

Another recent advance in *Streptomyces* biology has been the development of a convenient system of generating transposon insertions *in vitro* (Gehring 2000). These mutated libraries can then be introduced into *Streptomyces* on suicide vectors to generate transposon insertion mutants. The aim of this study was to generate mutations in genes involved in morphogenesis in *S. venezuelae* by introduction of a chromosomal library of *S. coelicolor* fragments, mutated by transposon insertion, via conjugation from *E. coli*.

Although all attempts to isolate insertional mutants in this manner were unsuccessful, we were able to show that conjugal transfer of mutagenized libraries will be more efficient into *S. venezuelae* than into *S. coelicolor* as the ability to use a methylation-proficient *E. coli* donor eliminates a transformation step, increasing the likelihood of obtaining a representative library.

Results

Use of tri-parental mating from a methylation proficient host in S. venezuelae. Although tri-parental matings (Hopwood 2000) and matings from methylation proficient *E. coli* hosts (Mazodier 1989) have been shown to be efficient methods of DNA transfer into *Streptomyces*, use of these methods has not been reported for *S. venezuelae*. Matings were performed to mobilize pSET 152 using both bi-parental and tri-parental matings as described in the materials and methods. The frequencies of the two methods are comparable and at least one order of magnitude above the background of spontaneous resistance (figure 3.1). This suggested to us that tri-parental mating could be used to mobilize a transposon-mutagenized library into *S. venezuelae* from a methylation proficient *E. coli* host. It was important to establish this, as attempting to transform a library into a strain already containing a plasmid would mean increased selection pressures, and result in a decrease in the chance of obtaining a representative library.

These experiments also established that *S. venezuelae* was able to receive DNA from a methylation-proficient *E. coli* donor, as the XL-10 gold cells used in the tri-parental matings do methylate DNA. As seen in table 3.1, exconjugants were recovered

in these experiments well above background. Again, this was important to establish as methylation-deficient strains of *E. coli* are harder to transform and result in a much lower transformation efficiency. Also, it would be necessary to perform two transformations of the library, first into a methylation-proficient strain, followed by plasmid purification, and a second transformation into the methylation-deficient strain. This would also result in a reduction of the library, further increasing the likelihood that library would not be representative.

Attempt to construct insertional mutants. Plasmid libraries of *S. coelicolor* were mutagenized with Tn*5*-hyg *in vitro* as described in the materials and methods. The *in vitro* reaction mixture was then transformed into *E. coli* with selection for hygromycin (hyg) and apramycin (apr) resistance genes, conveyed by the transposon and vector respectively. This passage into *E. coli* repaired the single-strand gaps generated by transposition of Tn*5* (Goryshin 1998). The insertional library was then conjugated into *S. venezuelae* as described in the materials and methods.

Selection for hyg^R would yield two kinds of ex-conjugants, as the vector does not contain a *Streptomyces* origin of replication. Those generated by a single cross-over and thereby integration of the whole plasmid vector, and those generated by homologous recombination of the genomic DNA, resulting in a marker-replacement event. These two outcomes are easily distinguished since ex-conjugants generated by a single cross-over would also be apr^R (plasmid-borne resistance gene) while those that were generated by a double cross-over will be apr^S. We would only be interested in pursuing those mutants

created by homologous recombination as they resulted in clean marker replacement. Any mutant phenotypes generated by plasmid integration could have been caused by downstream effects rather than gene disruption. Despite extensive manipulation of the experimental procedures, no hyg^R ex-conjugants were ever recovered from these experiments (figure 3.2), suggesting that not enough homology exists between *S. coelicolor* and *S. venezuelae* to allow for a significant number of homologous recombination events. Conjugation experiments were performed with a pSET152 derivative, pSET-hyg, to ensure that conjugal transfer was possible into *S. venezuelae* when selecting hyg^R. As seen in figure 3.2, ex-conjugants were recovered from these experiments, indicating that the inability to recover ex-conjugants from the library mating experiments was not due to the method of selection.

Discussion

Although we were unsuccessful in isolating insertional mutants in *S. venezuelae* from the conjugal transfer of a library of *S. coelicolor* DNA, mutagenized *in vitro* with Tn*5*, we were able to show that tri-parental matings are an efficient method of DNA transfer into this species. We were also able to show that *S. venezuelae* does not restrict methylated DNA as is seen with some other *Streptomyces* species (Kieser 1991). It was of crucial importance to establish these two facts, as both contribute to the ability to utilize a representative library to generate mutations. The ability to use these methods in *S. venezuelae* reduces the number of transformations necessary, as well as reduces the amount of selection that any single *E. coli* donor must endure. This should make the

conjugation of in vitro mutagenized libraries much more efficient than has been observed with *S. coelicolor* (Gehring 2000), which does restrict methylated DNA.

We have also shown that hyg^R is a suitable marker for use in conjugation experiments in *S. venezuelae*. Therefore, the most likely reason we were unable to isolate ex-conjugants in our experiments was a lack of enough homology between *S. coelicolor* and *S. venezuelae* to facilitate the amount of recombination events necessary to recover ex-conjugants. This suggests that it would be possible to perform a genome-wide search for genes involved in morphology and antibiotic production in *S. venezuelae* if a library of *S. venezuelae* DNA was mutagenized and subsequently mated into wild type strains.

Intergeneric conjugation of *S. venezuelae* not only allows for transfer of DNA into a previously unmanipulable species, but also greatly reduces the background of spontaneous mutations previously seen in this kind of experiment. This would result in more mutants being recovered whose phenotype had been caused by transposon insertion and not a spontaneous event. In similar experiments performed in *S. coelicolor,* where transformation was used to deliver the insertional library, only approximately 1 in 4 of the mutant phenotypes observed was shown to be linked to the transposon insertion (Gehring 2000). *S. venezuelae* offers many advantages over *S. coelicolor*, which has been the model organism for the streptomycetes. However it has been impossible to perform genome-wide screens for genes involved in differentiation in this species due to the lack of the necessary technology for molecular manipulations. It should now be possible to identify these genes and begin to characterize their functions.

Materials and Methods

Strains and culture conditions. S. venezuelae strain used was 10712. Growth was on MYM plates, MYM supplemented with 10mM $MgCl_2$ or MM with 1% glucose (Hopwood 2000). As indicated 50 μg/ml of hygromycin B and 50 μg/ml apramycin sulfate (Sigma) were added to growth media. All growth was at 30°C.

E. coli strains used were Top 10 and XL-10 gold ultracompetent cells (Stratagene) and ET 12567 (*dam*$^-$, *dcm*$^-$) and all manipulations were as described (Maniatis 1989).

Construction of Tn5 transposon derivative. To construct Tn*5*-hyg, the 1.53kb fragment resulting from digestion of pUH19A with *Pvu*II and *Blp*I was ligated into pMOD that had been digested with *Sma*I and *Hinc*II (figure 3.3). This resulted in plasmid pLQ9, which was used directly for the *in vitro* transposon reactions.

Construction of a plasmid library of M145. A library of M145 genomic fragments in pSpec*oriT* was kindly provided by Gehring, et. al. and was constructed as previously described (Gehring 2000).

Construction of pSET-hyg. pUH19a was digested with *Sap*I and *Alw*NI to obtain the 1.97 kb fragment containing the hyg^R gene. This fragment was blunted with Klenow and

ligated into pSET 152 that had been cut with *Bam*HI and blunted with Klenow (figure 3.3). This resulted in the plasmid pSET-hyg.

In vitro transposition reactions. Transposition reactions were carried out with the EZ::TN Tranpsosase kit from Epicentre as described by the manufacturer.

Conjugal transfer into S. venezuelae. *S. venezuelae* 10712 cells were conjugated with *E. coli* strains ET 12567, with pUB 307 (Flett 1997), and Top 10 , with the insertional library, as described (Hopwood 2000) or XL-10 gold, with pSET152 or pSET-hyg, except: 50 μl of each *E. coli* parent were used instead of 100 μl for tri-parental matings, MYM with 10 mM MgCl2 plates were used instead of MS agar and *S. venezuelae* spores were heat shocked in 0.05 M TES, pH 7.0 instead of 2X YT broth. Selection was by flooding with 0.5mg nalidixic acid and 2 mg hygromycin in 2 ml water (for the library) or 0.5mg nalidixic acid and 1 mg apramycin in 2 ml water (for pSET 152) after 18 hours incubation at 30^{o}C.

References

1. Bierman, M, Logan, R., O'Brien, K, Seno, E.T., Nagaraja Rao, R., and Schoner, B.E. (1992). "Plasmid cloning vectors for the conjugal transfer of DNA from *Escherichia coli* to *Streptomyces* spp." *Gene* 116: 43-49.

2. Flett, F., Mersinias, V., and Smith, C.P. (1997). "High efficiency intergeneric conjugal transfer of plasmid DNA from *Escherichia col*i to methyl DNA-restricting stretpmycetes." *FEMS Microbiol. Lett*. 155: 223-229.

3. Gehring, A., Nodwell, J.R., Beverley, S.M., and Losick, R. (2000). "Genomewide insertional mutagenesis in *Streptomyces coelicolor* reveals additional genes involved in morphological differentiation." *Proc. Natl. Acad. Sci. USA* 97: 9642-9647.

4. Giebelhaus, L.A., Frost, L, Lanka, E., Gormley, E.P, Davies, J.E., and Leskiw, B. (1996). "The tra2 core of the IncPα plasmid RP4 is required for intergeneric mating between *Escherichia coli* and *Streptomyces lividans*." *J. Bacteriol*. 178: 6378-6381.

5. Glazebrook, M.A., Doull, J.L., Stuttard, C., and Vining, L.C. (1990). "Sporulation of *Streptomyces venezuelae* in submerged cultures." *J. Gen. Microbiol*. 136: 581-588.

6. Goryshin, I.Y., and Reznikoff, W.S. (1998). "Tn5 *in vitro* transposition." *J. Biol. Chem*. 273: 7367-7374.

7. Hopwood, D.A., Kieser, T., Bibb, M.J., Buttner, M.J., and Chater, K.F. (2000). Practical *Streptomyces* genetics. The John Innes Foundation. Norwich, England.

8. Kieser, T., and Hopwood, D.A. (1991). "Genetic manipulation of *Streptomyces*: integrating vectors and gene replacement." *Methods Enzymol*. 204: 430-458.

9. Maniatis, T., Sambrook, J., and Fritsch, E.F. (1989). Molecular cloning: a laboratory manual, second edition. Cold Spring Harbor Laboratory Press. New York.

10. Mazodier, P., Petter, R., and Thompson, C. (1989). "Intergeneric conjugation between *Escherichia coli* and *Streptomyces* species." *J. Bacteriol*. 171: 3583-3585.

11. Smokvina, T., Mazodier, P., Boccard, F., Thompson, C.J., and Guerineau, M. (1990). "Construction of a series of pSAM2-based integrative vectors for us in actinomycetes." *Gene* 94: 53-59.

Figure 3.1. Frequency of Conjugation Between *E. coli* and *S. venezuelae.*

Cross #	*E. coli Donor*	Conc. Of Donor Cells (cfu)	*Streptomyces* Recipient	Conc. of Recipient Spores (cfu)	Spontaneous Resistance Frequency (cfu/cfu)	Exconjugant Frequency (cfu exconj/cfu recipient)
1a	**ET 12567** (pUB307/pSET152)	1×10^{8}	*S. venezuelae*	1×10^{8}	$< 10^{-8}$	3×10^{-5}
2a	**ET 12567**(pUB307)	3×10^{7}				
	XL-1 Blue(pSET152)	3.2×10^{6}	*S. venezuelae*	5.58×10^{8}	$< 10^{-8}$	4.06×10^{-7}
2b	**ET 12567**(pUB307)	1.97×10^{7}				
	XL-1 Blue(pSET152)	2.41×10^{6}	*S. venezuelae*	8.33×10^{8}	$< 10^{-8}$	4.82×10^{-7}
2c	**ET 12567**(pUB307)	2.92×10^{6}				
	XL-1 Blue(pSET152)	3.17×10^{6}	*S. venezuelae*	8.33×10^{8}	$< 10^{-8}$	1.34×10^{-6}
3a	**ET 12567**(pUB307)	3×10^{7}	*S. venezuelae*	5.58×10^{8}	$< 10^{-8}$	$< 10^{-8}$
3b	**XL-1 Blue**(pSET152)	3.2×10^{6}	*S. venezuelae*	5.58×10^{8}	$< 10^{-8}$	$< 10^{-8}$

* pSET152 has an oriT and apr^{R} genes, inserts into att sites in *S. venezuelae* genome

* ET 12567 contains the pUB307 plasmid that confers *tra2* gene functions

* When 2 *E. coli* strains are listed in one experiment, tri-parental mating was performed with ET 12567 supplying conjugation genes and the other parent supplying the plasmid to be transferred

* Exconjugant frequency expressed as the number of exconjugants/recipeint spore

Figure 3.2. Attempts at Conjugal Transfer of an Insertional Library.

Cross #	*E. coli* Donor	Conc. of Donor Cells (cfu)	*Streptomyces* Recipient	Conc. Of Recipient Spores (cfu)	Frequency of Spontaneous Resistance (cfu/cfu)	Frequency of Conjugation (cfu ex-conj/cfu recipient)
1a	**ET12567**(pUB307) **XL-10**(pSET-hyg)	1×10^{8} 1.25×10^{8}	*S. venezuelae*	1.5×10^{9}	$<10^{-9}$	9×10^{-8}
2a	**ET12567**(pUB307) **Top 10**(library)	2.2×10^{8} 1.5×10^{9}	*S. venezuelae*	1×10^{9}	$<10^{-9}$	$<10^{-9}$
2b	**ET12567**(pUB307) **Top 10**(library)	2.2×10^{7} 1.5×10^{8}	*S. venezuelae*	1×10^{9}	$<10^{-9}$	$<10^{-9}$
2c	**ET12567**(pUB307) **Top 10**(library)	2.2×10^{8} 1.5×10^{9}	*S. venezuelae*	1×10^{8}	$<10^{-8}$	$<10^{-8}$

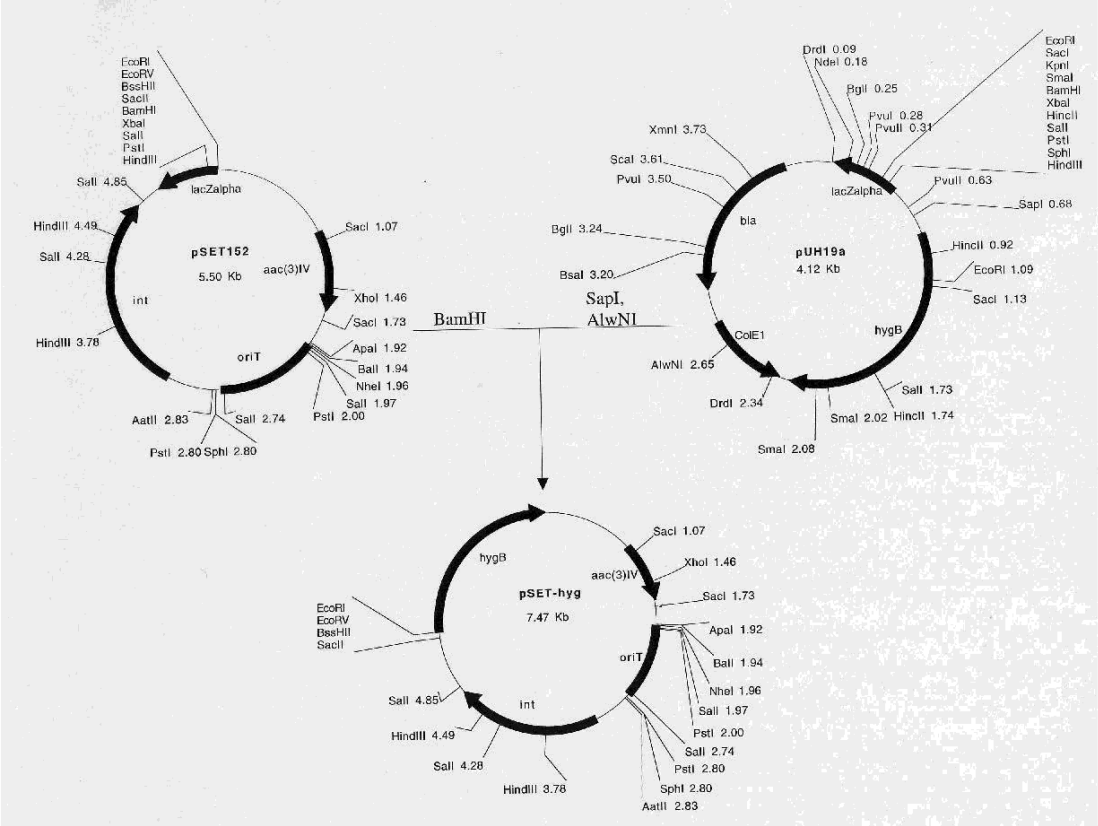


Figure 3.3. Construction of pSET-hyg.

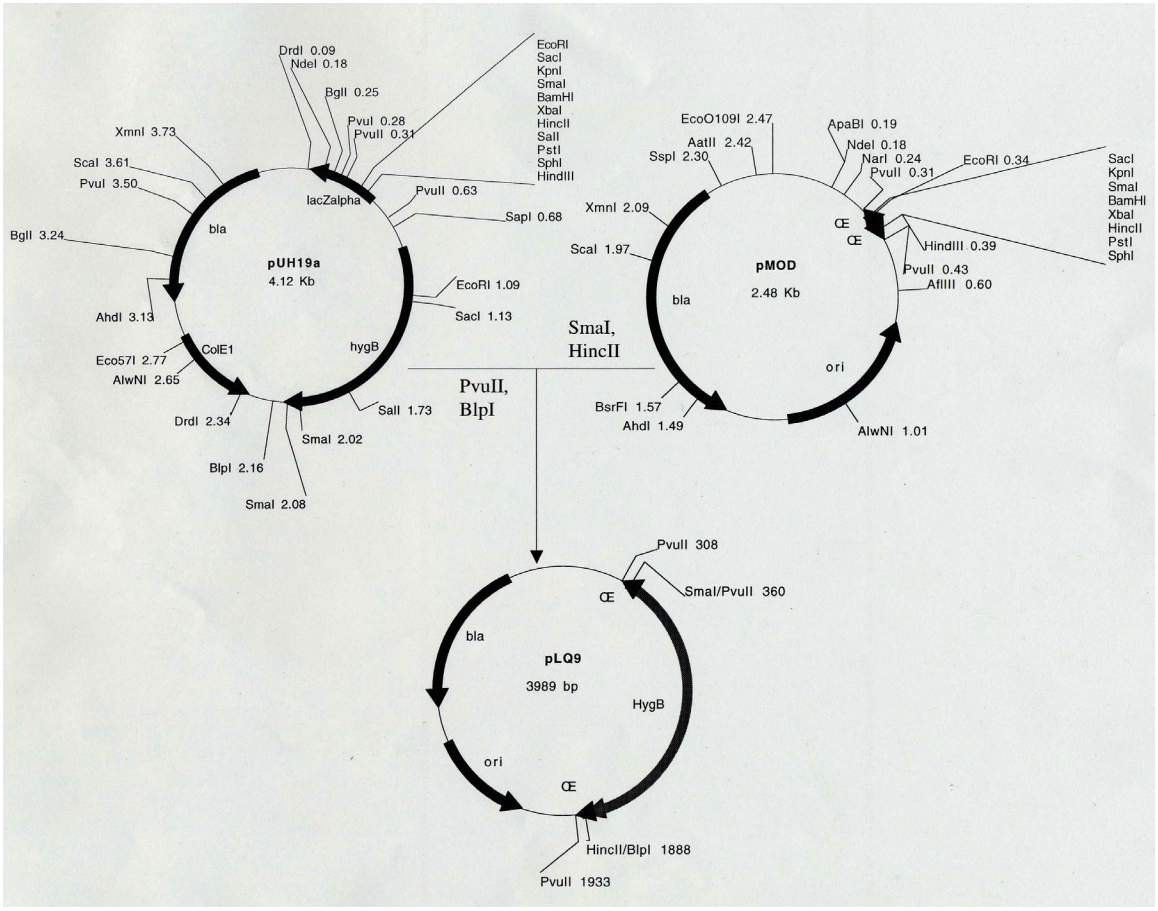


Figure 3.4. Construction of Tn5-hyg.

CHAPTER 4

DISCUSSION AND CONCLUSIONS

Despite the interesting biology of *Streptomyces* morphological differentiation and antibiotic production, little is known about how these pathways function. In other organisms, transposon mutagenesis has been useful in performing genome-wide screens for genes involved in particular processes. However, until recently, this has not been possible for *Streptomyces*. This is the result of 2 main problems. First, in order to get the transposon into *Streptomyces*, it is necessary to protoplast the cells. This process is mutagenic and results in a high background of spontaneous mutations. Second, it is necessary to remove the transposon donor after infection to prevent multiple, deleterious hops of the transposon. Initial studies with transposons in *Streptomyces* attempted to use temperature sensitive delivery vectors as had been done for other bacterial genera (Muth 1989). However, curing of these vectors requires elevated temperatures, which are also mutagenic in *Streptomyces*, adding to this background of spontaneous mutation. This results in a background of mutation that is so high that it becomes impractical to try to find the real insertion mutants.

Recent advances by Gehring, et. al. have led to the development of a system for transposition of Tn*5 in vitro* in *Streptomyces* (Gehring 2000), which decreases this background of spontaneous mutation, as it is no longer necessary to heat shock the cells to cure them of the delivery vector. However, it is still necessary to protoplast these cells

to introduce the transposon and therefore, it is imperative to establish linkage between transposon insertions and the observed mutant phenotypes. In the past, linkage has been established by Southern analysis, complementation analysis and sequencing. However, these methods are time and cost prohibitive when dealing with the large number of false positives resulting from protoplasting. Advances in DNA mediated transformation of *Streptomyces* (Oh 1997) have allowed for linkage verification via co-transformation of mutant phenotypes and markers contained within the transposon.

We utilized the Tn*5apr* transposon originally created by Gehring, et. al. (Gehring 2000), in addition to 2 new variations of Tn*5*, which we constructed, Tn*5xylE* and Tn*5EGFP*. These transposons can generate transcriptional fusions to promoters within the *Streptomyces* chromosome, enabling us to examine the transcriptional regulation of these genes, both temporally and spacially. A transcriptional fusion to the *xylE* gene, which encodes a catechol deoxygenase, would lead to formation of a yellow pigmented oxidation product upon exposure to catechol, which is colorless (Ingram 1989). A fusion to the *EGFP* gene would result in production of a protein that fluoresces green upon exposure to UV light (Chalfie 1994; Sun 1999). If a transcriptional fusion is generated between these reporter genes and promoters within the *Streptomyces* genome, then these gene products will be expressed at the same time and/or in the same location as the gene product naturally transcribed by that promoter. This is easily observed by the conversion of catechol, as indicated by a yellow pigmentation on colonies sprayed with catechol for *xylE*, or green fluorescence for *EGFP*.

We report here a genome-wide screen for genes involved in differentiation and antibiotic production using the newly developed system of Tn*5 in vitro* mutagenesis. We have isolated 5 genes involved in sporulation and antibiotic production, 2 of which are new genes that have not been previously described. We have established linkage between the 5 mutants and transposon insertion via co-transformation of the observed mutant phenotypes with the apramycin resistance gene marker encoded within Tn*5*. We then sequenced out from the transposon into the surrounding DNA to determine which gene had received the insertion. This has so far been possible for 4 of the 5 mutants. Of these 4 mutants, one was a re-isolation of a previously known gene and one was the result of a chromosomal rearrangement. However, 2 of the mutants were clean insertions into genes of previously uncharacterized function. For these 2 mutants, complementation experiments were performed by introducing a wild-type copy of the disrupted gene into the mutants via conjugation. These 2 novel mutants were analyzed further.

Mutant SJ175 was caused by an insertion into a gene predicted to be an integral membrane protein based on regions of hydrophobic residues within the translated protein. The protein displayed no homology to any known proteins in the database upon multiple searches with several different matrices. A mutation in this gene resulted in a de-regulation of sporulation and antibiotic production, as this mutant produced both spores and the pigments associated with antibiotic production a full day earlier than wild-type on both rich and poor carbon source media. This mutant produced a larger amount of both spores and pigments compared to wild-type as well. This would suggest that this gene may be involved in repressing these pathways until the appropriate time. This mutant

was also complemented by introduction of a wild type copy of the gene region determined to cause the mutant phenotype by co-transformation. Upon receipt of the complementation plasmid, these cells resumed wild type levels of sporulation. This further supports that the insertion did cause the observed phenotype, and that the mutation is recessive.

Mutant SE293 was the result of an insertion into a gene predicted to be a transcriptional regulator of the tetR-family based on homology to known members of this family (Beck 1982). The predicted protein also possessed the conserved tetR domain, the helix-turn-helix DNA binding motif of this family of proteins, as well as the surrounding area. This mutant was *bld* on poor carbon source media, which is unusual for *bld* mutants, which are usually complemented on this media. It was also defective in antibiotic production as indicated by the lack of production of the blue pigment associated with the antibiotic actinorhodin. The mutant failed to grow on minimal media plates containing the rich carbon source glucose, indicating that it may be auxotrophic as well. The protein would seem to be involved in regulating the transcription of genes involved in initiation of the sporulation process. It was not rescued by introduction of a single copy of the gene into a different chromosomal region, indicating that this mutation is dominant. Perhaps this protein must act in *cis* to direct transcription of the neighboring genes.

Characterization of these genes will further contribute to what is known about the pathways of differentiation and antibiotic production in *Streptomyces*. Some key experiments to begin with would involve confirming the predicted functions of the

protein products of these genes. S1 nuclease mapping could be used to determine if the product of SE293 does regulate the transcription of several nearby genes. Membrane analysis could be undertaken to determine if SJ175 really is a membrane-bound protein. Plus, it would be of interest to see where SE293 fits into the *bld* complementation cascade described by Willey, et. al. (Willey 1991) as the only other known *bld* mutants to not be rescued by growth on poor carbon sources, *bldB*and *bldN*, do not fit into this cascade. It is also crucial to continue efforts to obtain sequence data for SE69 to establish whether this mutation will identify a new gene that can be characterized.

We also report an attempt to utilize a library of *S. coelicolor*, mutated by Tn*5* insertion *in vitro*, to generate mutants in the more ideal strain for genetic analysis of *Streptomyces, S. venezuelae*, via conjugation from *Escherichia coli*. However, all attempts were unsuccessful, possibly because not enough homology exists between the two species to facilitate the homologous recombination events necessary to recover ex-conjugants. It would be worthwhile to pursue generation of transposon mutants in *S. venezuelae* by making a genomic library of *S. venezuelae* DNA to mutagenize *in vitro* with Tn*5*. This would allow for easier molecular characterization of genes involved in morphogenesis and antibiotic production as this species is much more amenable to molecular techniques currently in use in other organisms (Glazebrook 1990).

Streptomyces are of valuable scientific interest for 2 very unique biochemical pathways that they contain. The ability to study the process of differentiation in a prokaryotic organism offers many advantages over trying to study the process in eukaryotic organisms. However, very few prokaryotes undergo this process.

Streptomyces does. As prokaryotes are haploid, generation of mutation is much easier to accomplish and maintain. Also, methods for introduction of DNA into prokaryotes are highly refined and easier to accomplish than introduction of DNA constructs into eukaryotes.

Perhaps even more importantly, *Streptomyces* produce over 70% of the known natural product antibiotics currently used in human and veterinary medicine. Understanding how these pathways function is important not only because it could lead to increased production of the naturally produced antibiotics, but also because it could lead to manipulation of these pathways to generate novel antibiotics. Many antibiotics are synthesized by complex operons of proteins that each performs a particular chemistry, many of which are impossible to duplicate *in vitro*. If we could shift different parts of these pathways together, we could generate novel drugs that would be impossible to create in any other way. In this age of increased resistance to currently prescribed antibiotics by deadly pathogens, finding new drugs has become imperative.

References

1. Beck, C.F., Mutzel, R., Barbe, J., and Muller, W. (1982). "A multifunctional gene (*tetR*) controls the Tn10-encoded tetracycline resistance." *J. Bacteriol*. 150: 633-642.

2. Gehring, A., Nodwell, J.R., Beverley, S.M., and Losick, R. (2000). "Genomewide insertional mutagenesis in *Streptomyces coelicolor* reveal additional

genes involved in morphological differentiation." *Proc. Natl. Acad. Sci. USA* 97: 9642-9647.

3. Glazebrook, M.A., Doull, J.L., Stuttard, C., and Vining, L.C. (1990). "Sporulation of *Streptomyces venezuelae* in submerged cultures." *J. Gen. Microbiol.* 136: 581-588.

4. Ingram, C., Brawner, M., Youngman, P., and Westpheling, J. (1989). "*xylE* functions as an efficient reporter gene in *Streptomyces* spp.: use for the study of *galP1*, a catabolite-controlled promoter." *J. Bacteriol.* 171: 6617-6624.

5. Muth, G., Nussbaume, B., Wohlleben, W., and Puhler, A. (1989). "A vector system with temperature sensitive replication for gene disruption and mutational cloning in *Streptomyces*." *Mol. Gen. Genet*. 219: 341-348.

6. Oh, S.H., and Chater, K.F. (1997). "Denaturation of circular or linear DNA facilitates targeted integrative transformation of *Streptomyces coelicolor* A3(2): possible relevance to other organisms." *J. Bacteriol*. 179: 122-127.

7. Sun, J., Keleman, G.H., Fernandez-Abalos, J.M., and Bibb, M.J. (1999). "Green fluorescent protein as a reporter for spatial and temporal gene expression in *Streptomyces coelicolor* A3(2)." *Microbiol*. 145: 2221-2227.

8. Willey, J., Santamaria, R., Guijarro, J., Geislich, M., and Losick, R. (1991). "Extracellular complementation of a developmental mutation implicates a small protein in aerial mycelium formation by *S. coelicolor*." Cell 65: 641.