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MOLECULAR GENETIC ANALYSES OF
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BRENDA SUE PRICE

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MOLECULAR GENETIC ANALYSES OF GLOBAL ANTIBIOTIC REGULATION IN *STREPTOMYCES*

By

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ABSTRACT

MOLECULAR GENETIC ANALYSES OF GLOBAL ANTIBIOTIC REGULATION IN *STREPTOMYCES*

By

Brenda Sue Price

Streptomyces coelicolor is a filamentous soil bacterium that is well known for its ability to produce a vast repertoire of secondary metabolites, most notable of which are the antibiotics. Many of these compounds have become indispensable in human medicine and agriculture. While much is known about the compounds themselves, relatively little is understood about how the organism temporally and spatially regulates the production of antibiotics. A more thorough understanding of antibiotic regulation could reveal ways in which the organisms might be deliberately manipulated to enhance antibiotic production.

This study focuses primarily on the characterization of the *absB* gene in *Streptomyces coelicolor* which globally regulates the production of all four antibiotics produced by that strain. Genetic and molecular approaches were used to determine that the *absB* gene is a ribonuclease III (RNase III) analog. In other bacterial systems, RNase III has been shown to regulate gene expression by modulating the stability of select mRNA transcripts. Also, characterization of the *mia* locus, which is able to inhibit global antibiotic production when cloned in high copy in *S. coelicolor*, is described. Finally, an attempt to elucidate a regulatory pathway for avermectin production in the commercial producer *Streptomyces avermitilis* is presented.

DEDICATION

This dissertation is dedicated to the loving memory of

Dr. Tracy Anne Hammer DVM, Ph.D (1967 - 1996)

I was truly fortunate to have such a wonderful friend and classmate, who also happened to be a remarkable scientist. We shared the trials and tribulations of graduate school, and supported one another through the ebbs and flows of our careers. She inspired me to design innovative approaches to my research, and in that way she has left her mark on this body of work.

In mind and spirit ~ together always

ACKNOWLEDGEMENTS

A student's graduate education is only as good as his or her mentor. Dr. Wendy Champness has given me the tools with which I will continue to grow as a scientist. Her professional and personal attention to the development of her students is a distinction that I will always strive to emulate. I have also had the benefit of a supportive graduate committee: Dr. Michael Bagdasarian, Dr. Lee Kroos, Dr. Barbara Sears, Dr. Loren Snyder. Furthermore, the work discussed in Chapter 4 was possible due to Dr. Kim Stutzman-Engwall's support of the Biotechnology Training Program concept. Thank you also to Dr. Bill Wernau and the rest of the Bioprocess R&D team at Pfizer Inc. for allowing me to experience industry research first-hand.

Many thanks to my classmates in the Microbiology Department, who collectively believed in cooperation rather than competition with each other. Our productivity as a group is a testament to the success of that philosophy.

Of course this work would not have been at all possible without the loving support of my parents and brother. Their pride and enthusiasm for my accomplishments has never waned, even after so many years of having to explain why their daughter/sister is still in school. They share in any and all successes that I may ever have.

Finally, I would like to thank my husband, Hunter, for his loving support during the preparation of this manuscript and throughout our marriage. His commitment to my education helped me though periods of doubt and frustration, and he was the glue that kept me together during the preparation of this dissertation. Together, we can say "We Did It!"

PREFACE

This dissertation is divided into four parts: a general introduction and three chapters presenting the results of the doctoral work. The second chapter is presented in manuscript form and will be submitted to Molecular Microbiology with coauthors Dr. Trifon Adamidis, Kelly Spencer, and Dr. Wendy Champness. Dr. Adamidis' contribution to this paper was the early characterization of the *absB* mutants and the cloning of the *absB* locus to generate pTA108 and pTA128. Ms. Spencer generated the *E. coli* pKS^+ clone pKJ100 and assisted with the detailed restriction mapping of pTA108.

The third chapter describes work done in collaboration with Dr. Perry Riggle and Dr. Gary Brown. Dr. Riggle's contribution to this work was the original isolation and subsequent subcloning of the *mia* sequence, and Dr. Brown carried out further subcloning via PCR amplification. Portions of this work will be submitted for publication with Dr. Brown, Dr. Riggle, and Dr. Champness as co-authors.

The fourth chapter outlines research conducted in the laboratory of Dr. Kim Stutzman-Engwall at Pfizer Inc. (Groton, CT) as fulfillment of requirements for the Biotechnology Training Fellowship Program sponsored by NIH and MSU. The participants undertake additional coursework related to biotechnology as well as a nine month long internship in a biotechnology-related industry. The chapter outlines the objectives of this internship and some preliminary results. Final results, upon completion, will be submitted for publication at a later date, with Dr. Kim Stutzman-Engwall as coauthor.

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ABBREVIATIONS

aa	amino acid
$Abs^{(+/-)}$	synthesis of multiple (+) or no (-) antibiotics
Act	actinorhodin
$Ap^{(r)}$	ampicillin (resistance)
Avm	avermectin
bp(s)	basepair(s)
bsRNase III	ribonuclease III of *Bacillus subtilis*
CDA	calcium-dependent antibiotic
$Hyg^{(r)}$	hygromycin (resistance)
kb	kilobase(s) or 1000 basepairs
kDa	kilodalton(s)
λ	lambda phage
Mbp	megabase(s) or 10^6 basepairs
nt(s)	nucleotide(s)
PKS	polyketide synthase
Red	undecylprodigiosin
RNase III	ribonuclease III of *Escherichia coli*
σ	sigma factor
$Spo^{(+/-)}$	sporulation proficient (+) or deficient (-)
$Thio^{(r)}$	thiostrepton (resistance)
wt	wild-type strain

CHAPTER 1

Introduction and Literature Review

Streptomyces: an Introduction

Streptomycetes (of the family actinomycetes) are Gram-positive soil bacteria that resemble filamentous fungi in their mycelial growth patterns. Developing colonies form a network of branching coenocytic hyphae that penetrate and degrade complex organic material by the secretion of exoenzymes (18). In addition to their complex developmental life cycle, streptomycetes are well-known for their ability to produce a vast array of secondary metabolites, many of which have found uses in medicine or agriculture. In addition to antibiotics, other classes of secondary metabolites include the antiparasitics (i.e. avermectin), antifungals (i.e. polyoxin), β-lactamase inhibitors, (i.e. clavulanic acid), immunosuppressors (i.e. rapamycin), and antitumor compounds (i.e. adriamycin).

The life cycle of the streptomycete begins with a single spore, which under supportive environmental conditions germinates to form a germ tube (for review, see (18)). Extension of the hyphal tips and branching of the hyphae results in the development of a mycelial mat. The colony continues to multiply and radiate outward during the vegetative state. At the point of transition into stationary phase, a signal that is presumably triggered by nutrient depletion initiates the regulatory cascade of differentiation. At this point, both morphological and physiological differentiation are initiated. Aerial mycelia are erected, giving the colony a fuzzy, velvety appearance. A gray spore pigment develops after septation of the aerial mycelia into mature spore chains. Concomitantly the production of secondary metabolites takes place. Partial lysis of the substrate mycelium also occurs, presumably to provide nutrients

for the differentiation processes. This growth-dependent nature of secondary metabolism and morphological development continues to be the subject of intensive study.

Another notable characteristic of the streptomycetes is that the 8 Mbp, 72% G:C chromosome has recently been shown to be a linear molecule (73). This suggests interesting processes for replication, recombination, and partition of the chromosome as growth occurs.

***Streptomyces coelicolor*: a model streptomycete**

S. coelicolor has been the model for studying the processes of secondary metabolism and morphological differentiation in actinomycetes. *S. coelicolor* was the first actinomycete for which a genetic linkage map was developed, aided by methods for protoplast fusion and plasmid-mediated conjugal transfer (47). This ability to genetically manipulate the strain led to further development of plasmid and phage vectors, shuttle vectors, reporter gene fusion vectors, and some transposon mutagenesis schemes as well as methods for their use (48). Recently a physical and genetic map of *S. coelicolor* was compiled (60), and an ordered set of cosmid clones was used to generate a library of wild type chromosomal DNA (96). An adaptation of the genetic map, showing relevant genes discussed in this work, is shown in Figure 1.1.

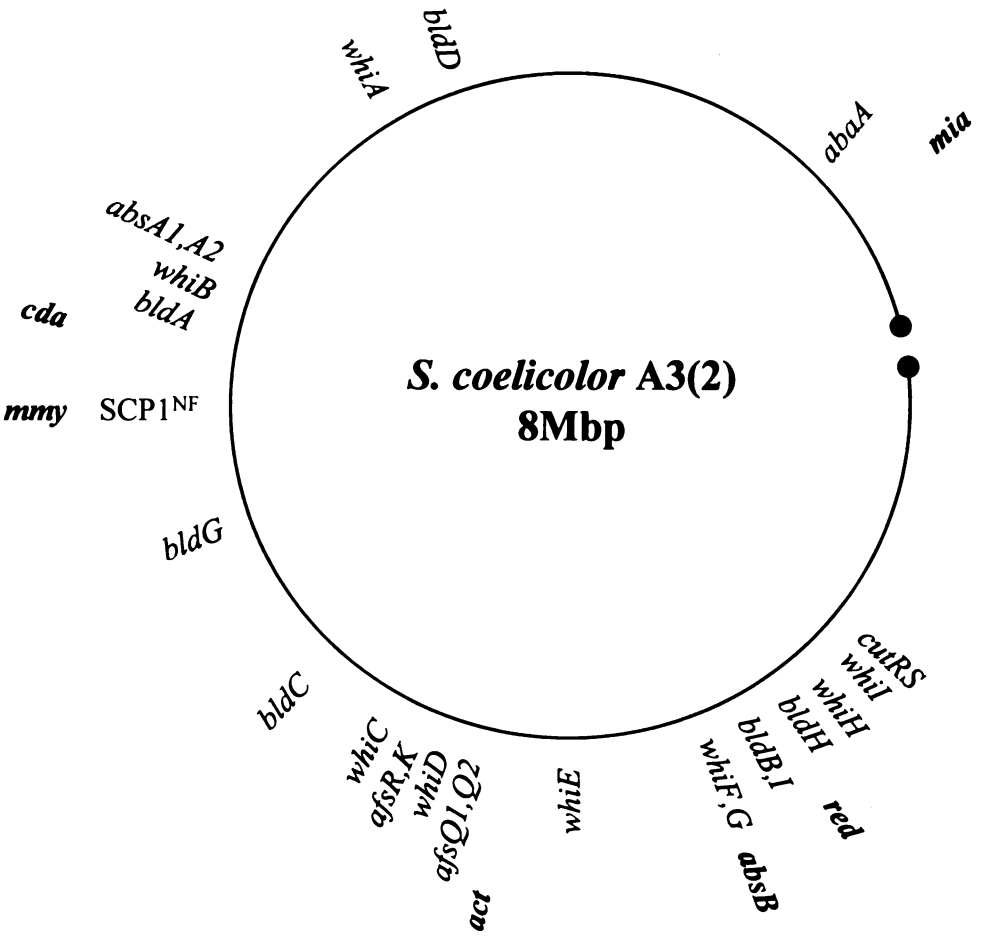


Figure 1.1 A genetic map of *S. coelicolor*. Genes relevant to secondary metabolism are shown. Genes integral to the presented research are highlighted in bold type. Adapted from (49, 60, 96).

S. coelicolor produces four known structurally diverse antibiotics (for review, see (49)). The genes that direct the biosynthesis of each compound are located at distinct locations on the chromosome (Fig. 1.1). Two of these antibiotics are pigmented compounds, and the mature spores of *S. coelicolor* produce a gray pigment; this permits the rapid visual screening for developmental mutants. None of the four antibiotics produced by *S. coelicolor* are commercially useful, thereby relieving any proprietary limitations on the sharing of information between laboratories.

The Antibiotics of *S. coelicolor*: Chemistry and Genetics

Actinorhodin

Actinorhodin (Act) is an intensely blue aromatic polyketide that historically served as the model for the genetic and molecular characterization of antibiotic gene clusters. The Act biosynthetic pathway has been predicted based on pathway intermediates and shunt products found in blocked mutants (6, 23). Actinorhodin results from the condensation of 1 acetate and 7 malonate residues to form a 16-carbon acyl chain (Fig. 1.2) (49). The mechanism of polyketide chain elongation is analogous to that of fatty acid synthesis (98). The polyketide family of compounds are synthesized in prokaryotic and eukaryotic systems and have vast biological activities (50).

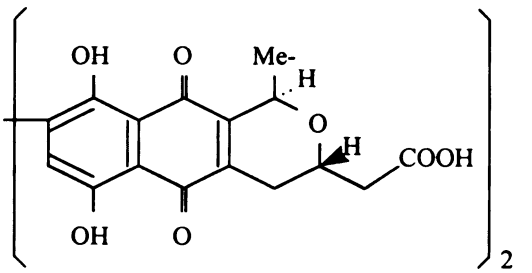


Figure 1.2 Chemical Structure of Actinorhodin

Genetic analysis of the actinorhodin gene cluster began with the isolation of seventy-six mutants blocked in various stages of Act production. Using pairwise combinations of mutants, seven co-synthesis groups were identified (104). Genetic mapping of a representative mutant from each group revealed that the *act* genes were located in a cluster on the *S. coelicolor* chromosome, which later proved to be a recurrent theme among actinomycetes antibiotics (104). The cloning of the entire 25 kb *act* gene cluster allowed a more detailed investigation into antibiotic production and regulation (74). Actinorhodin production requires at least 23 genes expressed as at least 7 transcripts. In addition to biosynthetic enzymes, the *act* cluster contained genes to confer host resistance, export, and regulation. Subsequent studies in other systems have shown that this coordinate expression of relevant antibiotic genes within a cluster has been conserved across species (17).

The *act*II-ORF4 gene located in the middle of the *act* gene cluster has been identified as the positive regulator of Act production (34). *act*II-ORF4$^-$ mutants are completely blocked in Act production and cannot be restored by co-synthesis with any other mutant class (75, 104). When multiple copies of

*act*II-ORF4 are introduced, Act is produced early in exponential growth phase, and at elevated levels, though the growth rate of the culture is unaffected (40). Actinorhodin production, as well as other *S. coelicolor* antibiotics, is dependent on the $tRNA^{Leu}$-encoding *bldA* gene, which recognizes the UUA codon (*bldA* discussed in detail below). Sequence analysis revealed that the actII-ORF4 gene contains one UUA codon, suggesting translational regulation by the *bldA* gene product (34, 70). When the UUA codon is mutated to a silent UUG codon, *bldA* dependence is relieved and actinorhodin is produced in the *bldA* mutant background (34). Multiple copies of *act*II-ORF4 are capable of bypassing the *bldA*$^-$ mutant under some culture conditions, presumably due to imprecise translation (92).

The *act*II-ORF4 gene product shows amino acid homology to other cluster-specific activators, namely RedD (see below) and DnrI, which regulates daunorubicin production in *S. peucetius*. RedD and ActII-ORF4 cannot complement one another, however Act production can be restored in an *act*II-ORF4$^-$ mutant by the introduction of the *dnrI* gene (113). To date amino acid sequence analysis of these three proteins has not revealed a mechanism of action nor are these proteins related to any others in the databases.

Undecylprodigiosin

Undecylprodigiosin (Red) is the major component of a mixture of closely related tripyrolle compounds produced by *S. coelicolor* (120). It has bacteriostatic / bacteriocidal activity against Gram-positive bacteria as well as some antiparasitic activity (13, 45) though is considered too toxic for

therapeutic use. This compound is chemically similar to the prodigiosin produced by *Serratia marcescens* and is believed to be produced from amino acids and polyketides via a similar biosynthetic pathway (33, 128). Under slightly acidic culture conditions, this antibiotic is a deep red pigment; at slightly alkaline pH it appears yellow. Red remains cell associated and is highly nonpolar (120).

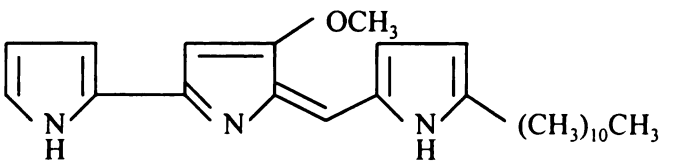


Figure 1.3 Chemical Structure of Undecylprodigiosin

Early genetic studies grouped 37 Red$^-$ mutants into six groups based on co-synthesis with one another (33, 76). Genetic mapping of the representatives from each group (*redA-F*) revealed that the genes for Red biosynthesis were clustered on the chromosome, as had been demonstrated for actinorhodin (105). The Red cluster is located at 5 o'clock on the genetic map, distant from other known antibiotic clusters (Fig. 1.1) (105). The entire gene cluster (37.5 kb) was isolated and characterized (76). To date, eighteen genes have been implicated in the Red biosynthetic pathway (22).

redD was identified as a positive regulator of the Red biosynthetic genes due to several criteria. Earliest characterization showed that *redD*$^-$ mutants could not co-synthesize Red with any other mutant class (32). In a *redD*$^-$ mutant background, transcript levels of biosynthetic genes *redE* and *redBF*

were reduced (84). The introduction of additional copies of the *redD* gene, in either high or low copy vectors, caused an overproduction of Red in both the *S. coelicolor* wild type and *redD*⁻ background (84), and could stimulate Red production in *S. lividans* as well (76). Lastly, RedD protein showed homology to other cluster-specific activators: ActII-ORF4 of *S. coelicolor* (34) and DnrI of *S. peucetius* (113). Transcription analysis over a time course revealed that the expression of *redD* increases dramatically at transition phase and continues into stationary phase, just prior to the onset of Red production (115).

In contrast to the case for *act*II-ORF4, transcription of *redD* is strictly dependent on *bldA* (84, 125). This suggested that there might be an additional regulator of Red that is required by *redD* and is translationally dependent on *bldA*. A search for mutants able to suppress the *bldA* mutation and produce Red, but not Act or aerial mycelia, resulted in the *pwb* (pigmented while bald) mutant class (reviewed in (125)). Genetic mapping placed the suppressor mutation near the right hand end of the Red cluster, approximately 4 to 5 kb downstream of *redD*. Sequence analysis revealed an open reading frame that is expected to be responsible for the Pwb^+ phenotype. Named *redZ*, this gene showed some homology to a response regulator family of proteins, including the global antibiotic regulator *absA2* (see below), yet lacks the functionally significant and conserved phosphorylation regions (43). Deletion of the wild type *redZ* gene results in complete abolition of Red production, while the addition of multiple copies of *redZ* results in copious overproduction of Red (125). Transcript analysis shows that *redZ* is transcribed weakly during exponential growth, and increases at transition and stationary phase.

Mutational analysis indicates that while *redZ* is not dependent on *redD*, *redD* is strictly dependent on *redZ* for transcription. RedZ also appears to negatively autoregulate its own expression. *redD* transcription is *bldA*-dependent, but *redZ* is not. The presence of a UUA codon in *redZ* suggests that it is dependent on *bldA* for translation (125). Presence of multiple copies of *redZ* in a *bldA* mutant restores Red production to the strain, while multicopy *redD* does not. Taken together, these data suggest that *bldA* serves to translationally control *redZ*, which then transcriptionally regulates *redD*. RedD then activates the biosynthetic gene cluster to produce Red (Fig. 1.4) (125).

Figure 1.4 Model for regulation of the undecylprodigiosin gene cluster in *S. coelicolor* (125).

Methylenomycin

Methylenomycin (Mmy) is a structurally simple antibiotic (relative to other known structures) that has antibacterial activity against both Gram-positive and Gram-negative bacteria (44).

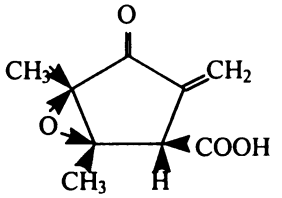


Figure 1.5 Chemical structure of methylenomycin.

Like Act and Red, Mmy is thought to be encoded by a set of genes arranged in a cluster; however the *mmy* gene cluster is carried on the conjugative plasmid SCP1 (129). This linkage was discovered due to the co-transfer of Mmy production and resistance with the fertility properties related to SCP1 (64). SCP1 is a 350kb linear plasmid that can exist autonomously ($SCP1^+$) or integrated within the chromosome (historically termed an NF strain). Integration of SCP1 occurs at the 9 o'clock region of the chromosomal map (Fig. 1.1). The Mmy cluster is thought to be between 17 and 28 kb in length, but has not been as thoroughly characterized as Act or Red. Two genes within the cluster have been characterized; a gene that confers host resistance (*mmr*) and a putative repressor (*mmrR*) (19, 86). Transcription analysis of *mmr* suggests that it is transcribed after biosynthetic genes, and may be induced by methylenomycin or an intermediate precursor (46). *mmrR*, which lies at the leftmost end of the cluster, appears to negatively regulate Mmy, as disruption of the gene results in overproduction of Mmy (19).

Calcium-dependent antibiotic

Calcium-dependent antibiotic (CDA) is the least characterized antibiotic produced by *S. coelicolor*. It requires calcium ions for its antibacterial activity against Gram-positive organisms (68). To date the genes for CDA production have not been cloned or characterized, nor has the structure of the antibiotic been determined. Of fourteen CDA^- mutant strains isolated, none were able to co-synthesize CDA with one another, but all were shown to be closely linked on the chromosome and mapped to the 10 o'clock position of the genetic map (Fig. 1.1) (51).

Pleiotropic Regulation of Antibiotic Production

Several loci have been described which affect the production of more than one antibiotic in *Streptomyces*. In most cases these genes are distinct from the antibiotic clusters on the chromosome. They can be grouped into two broad categories: those that affect antibiotic production and morphological development, and those that appear to affect only antibiotic production.

The *bld* genes: early switches in antibiotic production

Wild type *Streptomyces* colonies on solid medium appear smooth and beige during vegetative growth, but at the onset of stationary growth they become "fuzzy" with the development of aerial mycelium and display pigment due to antibiotic production. The first class of developmental mutants to be identified and characterized never developed the fuzzy appearance of mature colonies (nor the pigmentation) and were thus nicknamed "bald" mutants (82). Since these mutants provided genetic evidence of coupling of antibiotic production and sporulation, they were thought to involve genes required for early switch to secondary metabolism. Nearly all Bld phenotypes are conditional under certain growth conditions - aerial mycelia development can in some cases be restored on particular carbon sources, or when grown next to a wild type strain (15, 82). Antibiotic production is not generally affected by differences in carbon source, which implies multiple levels of control in these mutants. To date, eight major *bld* loci have been identified (A, B, C, D, G, H, I, K) but few have been functionally characterized. Each locus maps to a distinct location on the *S. coelicolor* chromosome (Fig. 1.1).

bldA is the best characterized *bld* locus. *bldA* mutants have been characterized *in S. coelicolor*, as well as *S. lividans* and streptomycin-producer *S. griseus*. Aerial mycelial development is blocked in *bldA* mutants grown on glucose-containing media, but is restored on mannitol or maltose minimal media. In addition, *bldA* mutants are globally blocked in the production of all four antibiotics in *S. coelicolor*, and in *S. griseus* are deficient in streptomycin production (Str^-).

The *bldA* gene was mapped to the 10 o'clock position on the *S. coelicolor* map, and was subcloned by complementation of $bldA^-$ mutants (Fig. 1.1) (69). Sequence analysis revealed that the *bldA* gene encodes a tRNA that is charged with leucine and recognizes the UUA codon (69). This codon is extremely rare in the high %G:C DNA of *Streptomyces*. Quite interesting was the fact that the few UUA codons found within known open reading frames were in genes relating to secondary metabolism (70). This brought up the possibility that the *bldA* gene product might function in translational regulation of secondary metabolism. A knockout of the lone *bldA* gene has no apparent effect on vegetative growth, implying that UUA codons are not found in any essential vegetative genes (71). In addition to *act*II-ORF4, *redZ*, and *act*II-ORF2 (Act export) in *S. coelicolor,* UUA codons have been found in known positive regulatory genes for streptomycin (*strR*) in *S. griseus* and bialaphos (*brpA*) in *S. hygroscopicus*, as well as in several antibiotic resistance genes (70). Detailed transcript analysis of the *bldA* showed that the 5' end of the tRNA is processed, and that the processed form increases in concentration in late growth. This

regulation is atypical to most tRNAs, whose abundance generally wanes later in the life cycle (72).

The *absA1/A2* genes

In an attempt to isolate developmental mutants that were completely blocked in the production of all four *S. coelicolor* antibiotics but not in sporulation, UV mutagenesis survivors were visually scored for an Act$^-$ Red$^-$ phenotype, then tested for CDA$^-$ and Mmy$^-$. Of 800,000 mutants screened, thirteen were confirmed to have this phenotype, named "Abs" for antibiotic synthesis deficient (1). Genetic mapping studies concluded that the mutants fell into two classes, *absA* and *absB*, based on their distinct locations at 10 o'clock and 5 o'clock respectively (Fig. 1.1) (14). The mutants were also distinctive in several other respects. The Abs$^-$ phenotype appeared under certain conditions to be conditional; *absA* mutants were slightly leaky for Act on low phosphate medium, while *absB* mutants were slightly leaky for Act and Red on the complex medium R2YE. Additionally, *absA* mutants were found at a much lower frequency (10^{-6}) and generated a significant number of spontaneous suppressors (named *sab*, for suppressors of *absA*).

The *absA* locus was cloned using a low copy plasmid library of wild type chromosomal DNA (1). During the process of transformation, it was observed that spontaneous suppressors were generated at a greater frequency than complementing clones; therefore a scheme that utilized the conjugative properties of the plasmid vector was used. Using this approach, the *absA* locus was cloned and the minimal complementing fragment was sequenced (11).

Database homology searches revealed that the *absA* locus contained a hisitidine kinase protein homologous to the DegS/NarQ,X family of two-component regulatory systems. All four of the original *absA*$^-$ mutations appeared to be localized to this ORF. DNA sequence from further downstream revealed another open reading frame. This gene product showed homology to the corresponding response regulators of the DegU/NarL,P family. Subsequently named *absA1/A2*, these genes were disrupted using an integrative phage. Interestingly, although *absA1* UV mutants were Abs$^-$, the null mutant Δ*absA1/A2* and Δ*absA2* both resulted in hyperproduction of Act and Red. (The parent strain J1501 does not carry the SCP1 plasmid, therefore Mmy was not tested). The antibiotics were also produced earlier in the life cycle, suggesting that the *absA* genes function in negative regulation of antibiotic production, and influence temporal regulation. *absA* genes appear to be conserved among several other streptomycetes (10), though the role of any homologs in antibiotic production is as yet unknown.

The *mia* locus

During an attempt to clone the *absA* locus from a high copy plasmid library, one sequence was independently isolated >40 times (99). This fragment in high copy was able to inhibit the production of at least three of the four antibiotics (named *mia* for multicopy inhibition of antibiotic synthesis), yet sporulation appeared to be unaffected (the Abs$^-$ phenotype). The *mia* locus mapped to the two o'clock region of the chromosome, distinct from any other known antibiotic regulatory gene (Fig. 1.1) (99). A 1.2 kb fragment was

sequenced and subsequently trimmed to a 120 bp region that is sufficient for the Abs$^-$ phenotype (12, 99). This region contains a 20 amino acid ORF, but its codon usage suggests that it may not be expressed. This region also contains a 19bp perfect inverted repeat that could be relevant in the regulation pathway.

The *afsQ1/Q2* system

The *afsQ1* gene from *S. coelicolor* was identified by virtue of its ability to stimulate overproduction of actinorhodin, undecylprodigiosin, and A-factor (a putative cell signal molecule necessary for sporulation) in *S. lividans* when introduced in low copy (56). This clone was able to restore Act production to an *absA* mutant, but not an *absB* mutant. Sequence analysis of the *afsQ1* gene predicted a 225 amino acid protein that revealed homology to the PhoB/OmpR family of response regulators in a two-component signal transduction system. When an aspartate residue, the phosphorylation of which has been shown to be functionally significant in other homologous response regulators, was mutated in the *afsQ1* gene, the protein lost its ability to overstimulate actinorhodin production in *S. lividans*, suggesting that phosphorylation at this residue is involved in this mechanism of antibiotic regulation (91). As protein kinase-response regulators are commonly found in pairs, adjacent DNA was sequenced and the *afsQ2* gene was discovered. The 535 amino acid *afsQ2* sequence is translationally coupled with the *afsQ1* open reading frame, and sequence analysis predicts a histidine kinase gene homologous to the PhoR/EnvZ subfamily. When *afsQ2* was cloned into wild type *S. coelicolor*, no stimulation of actinorhodin was achieved. The *afsQ1/Q2* locus mapped to 7 o'clock on the *S. coelicolor* genetic map near the *act* gene

cluster (Fig. 1.1) (56). Transcript analysis showed that *afsQ1/Q2* message is temporally controlled, occurring just before *act* gene expression. Interestingly, gene disruption of the *afsQ1/Q2* locus resulted in no change in antibiotic production or morphogenesis, indicating that *afsQ1/Q2* is not essential in antibiotic production. Southern hybridization showed that the *afsQ1* and *afsQ2* sequences are conserved across many other actinomycete species.

The *cutRS* system in *S. lividans*

A two-component regulatory system that appears to negatively regulate antibiotic production was isolated from *S. lividans* and was named *cutRS* (16, 121). The histidine kinase *cutS* showed greatest homology to the *afsQ2* of *S. coelicolor*, and the *cutR* gene showed homology to *afsQ1* response regulator (121). Unlike *afsQ1/Q2*, the *cutRS* system does appear to function in normal regulation of antibiotic synthesis by the negatively regulating at least actinorhodin production (16). As evidence, insertional inactivation of the *cutRS* genes resulted in early and increased levels of actinorhodin, which is not normally expressed in *S. lividans*. *cutRS* transcription is growth phase dependent; transcript was detected at the transition to stationary phase prior to actinorhodin biosynthesis (16).

The *afsR/afsK/afsR2* system

In an attempt to characterize a developmental mutant, "*afsB*" (phenotype Act$^-$ Red$^-$ A-factor$^-$ Spo$^+$), a plasmid library of *S. coelicolor* wild type DNA was used to search for a complementing clone. As a result an interesting

bypass clone was isolated, and subsequently named *afsR*. *afsR* mapped to 7 o'clock on the genetic map (Fig. 1.1) (53). A low copy plasmid containing *afsR* introduced into wild type or the *afsB* mutant resulted in overproduction of Act and Red. The *afsR* gene product was determined to be 993 amino acids in length, and portions of the protein were able to stimulate antibiotics to a lesser degree than the entire sequence.

Further experiments determined that the AfsR protein is phosphorylated by a specific kinase, the product of the *afsK* gene. Thus *afsK-afsR* comprise a serine-threonine phosphoprotein signal transduction system in which AfsK phosphorylates AfsR, which then regulates antibiotic production. Sequence analysis determined that the N-terminus of the *afsR* gene is homologous to the cluster-specific activators *act*II-ORF4 and *redD* (52), but it is not yet understood how these proteins interact in the antibiotic regulatory cascade. While *afsR* in multicopy results in an increase in *act*II-ORF4 and *redD* transcription, disruption of the *afsR* gene does not have any deleterious effect on transcript levels (36). *afsR* cannot substitute for *act*II-ORF4 or *redD* in antibiotic production.

An additional gene, separate yet adjacent to *afsR*, was recently reported to also influence antibiotic production (123). Named *afsR2*, this gene encodes a 63 amino acid protein that stimulates Act and Red production when cloned in high copy in *S. lividans*. An analogous gene, *afsS*, has been reported in *S. coelicolor* that is nearly identical in sequence and appears to have the same function (36, 123). The protein has no homologs in the protein databases that might suggest a mode of function. *afsR2* in multicopy stimulates transcription

of the *act*II-ORF4 gene and the biosynthetic gene *act*III; however the AfsR2 protein is not required for the expression of either gene as the null mutant (Δ*afsR2*) is still able to produce Act (123).

The *abaA* gene

The *abaA* locus (named for antibiotic biosynthesis activator) was identified by its ability to stimulate overproduction of actinorhodin when cloned in high copy into wild type *S. coelicolor* and the closely related *S. lividans* (*S. lividans* has the *act* gene cluster intact yet produces no actinorhodin) (35). This locus mapped to the 2 o'clock position on the genetic map, far removed from the actinorhodin gene cluster. Disruption of *abaA* using integrative phage resulted in abolition of actinorhodin production and reduction in Red and CDA levels, yet Mmy levels and sporulation were unaffected (35). The *abaA* gene is not able to bypass a *bldA* mutant, as seen for *act*II-ORF4, which suggests an alternative pathway for actinorhodin activation (92). To date the *abaA* gene is as yet uncharacterized, although hybridization studies have shown that the locus is conserved among other *Streptomyces* species (35).

Regulation of sporulation - a morphological cascade

The *whi* genes

Sporulation in streptomycetes begins with the development of multinucleoid aerial mycelium, which subsequently coil and septate to yield chains of uninucleate spores. At full maturation the spores produce a gray

pigment; thus sporulation mutants were named "whi" mutants for their white appearance. Developmental mutants blocked at various stages of the sporulation process have been identified (Fig. 1.6) and a few have been characterized.

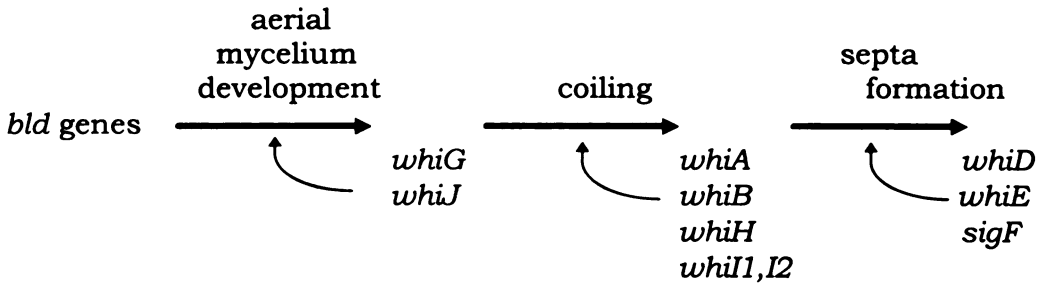


Figure 1.6 Genes involved in spore development.

The *bld* genes control the earliest stages of sporulation with the initiation of aerial mycelium formation. Beyond *bld* genes, morphological differentiation control diverges from antibiotic production. One of the earliest *whi* genes involved in spore development independent of antibiotic production is *whiG* (81). To date the best characterized *whi* gene, *whiG* gene encodes a sigma factor (σ^{whiG}) most similar to σ^{D} of *B. subtilis* and σ^{F} of *S. typhymurium* and *P. aeruginosa* (20). σ^{whiG} is found specifically in actinomycetes that undergo sporulation, and appears to be in limited concentration in the cell (110). Multiple copies of a *B. subtilis* σ^{D}-dependent promoter introduced into wild type *S. coelicolor* resulted in the Whi phenotype, presumably due to sequestration of the *whiG* gene product (20). This result led to a strategy for isolation of two σ^{whiG}-dependent promoters (119). Multiple copies of the *whiG* gene induce sporulation in vegetative mycelium that would otherwise be fated

for lysis (20). *whiG* transcription appears to be constitutive, and is not dependent on any other *whi* gene (58).

Characterization of other *whi* genes has revealed some initial findings. *whiJ* shows homology to the *abaA* gene involved in antibiotic production (106). *whiB* encodes a transcription factor-like peptide (17). *whiI1/I2* is a two component regulatory system most closely homologous to *absA1/A2*, however Δ*whiI2* has no apparent phenotypic affect on *S. coelicolor* (106). *sigF* is a sigma factor gene expressed only during sporulation and is transcriptionally dependent on all *whi* genes (58, 95). Finally, the *whiE* locus appears to encode several genes for formation of the mature spore pigment (131). As further characterization of each *whi* locus is completed, interactions between various *whi* gene products will likely reveal a complex cascade of regulatory control.

The SapA and SapB proteins

Extracellular signal proteins have recently been identified which are involved in aerial mycelium formation. Transcription of *sapA*, which encodes a 13kDa protein found associated with purified mature spores, coincides with aerial mycelium development and is significantly blocked in certain *bld* and *whi* mutants. A SapA-LuxAB fusion protein demonstrated a close spatial correlation between developing aerial mycelium and P*sapA*-driven luciferase expression (42).

The SapB protein has been shown to be required for aerial mycelium formation. All *bld* mutant classes are deficient in SapB production, but *whi* mutants are SapB$^+$ (126). When purified SapB protein was added to *bld*

mutants, aerial mycelium formation was restored, though the inability to produce antibiotics was not (127). Interestingly, when certain *bld* mutant classes were grown near others, aerial mycelium production could be restored to some degree. SapB protein is present in a zone around differentiating bacteria (126).

Further extracellular complementation studies suggested the possible involvement of four or more as yet unidentified intercellular signals in the formation of aerial mycelium (127).

Conclusions

While much work remains to be done to discover the functions of known genes and how they interact, current understanding suggests a complex network of genetic controls of secondary metabolism in *Streptomyces* spp. As more genes involved in antibiotic production and sporulation are identified and characterized, a complex regulatory cascade will undoubtedly emerge to reveal how these organisms sense and adapt to changing environmental conditions. Such information is also likely to be of great value as the streptomycetes continue to be exploited for the production of commercially valuable secondary metabolites.

Ribonuclease III: an Introduction

Chapter 2 of this work characterizes the *absB* gene product of *Streptomyces coelicolor* as a putative Ribonuclease III (RNase III) enzyme. While this is the first known example of an RNase III in an actinomycete, this enzyme has been characterized in other systems.

RNase III is an endoribonuclease that cleaves double-stranded RNA molecules. First identified in *E. coli,* RNase III was shown to cleave perfect duplex RNA *in vitro* (102). In the cell, the most common natural substrates for the enzyme are the duplex regions of stem-loop structures formed by folded RNA molecules. Cleavage by RNase III was first implicated as the initial step in the processing of 16S and 23S rRNA from the 30S precursor transcript (101). Despite this important function, RNase III is not essential for survival of *E. coli.* RNase III cleavage was also shown to be the method of processing of the polycistronic T7 phage early gene transcript into single monocistronic mRNAs (31). This latter function was of considerable interest, as the processing event allowed for translation initiation of each gene and thus directly influenced the expression of the T7 genes. It has been estimated that expression levels of as much as 10% of proteins detected in *E. coli* crude cell extracts are modulated by the RNase III enzyme (39, 117). This ubiquitous nature of RNase III function in the cell has generated much interest in recent years. Additional substrates for RNase III processing in the cell continue to be elucidated, and together they exhibit a complex system of post-transcriptional control of gene expression through the processing of mRNA transcripts.

RNase III and its substrates

The *E. coli* RNase III enzyme is a homodimer composed of two 25 kDa subunits. It was first purified as an endoribonucleolytic activity able to solubilize high molecular weight double stranded RNA *in vitro* (102). The cleavage resulted from two single strand breaks two nucleotides apart, and occurred approximately every 15 bases (10-18 nt range) (101). The resulting fragments had 5' phosphoryl and 3' hydroxyl ends, with no apparent nucleotide specificity at either end (26, 102). Both poly(A)-poly(U) and poly(I)-poly(C) RNA were suitable substrate, suggesting that no particular sequence was required for recognition and cleavage. In addition to perfect duplex RNA, RNase III has since been shown to process double-stranded regions in specific ribosomal and messenger RNAs. These molecules fold to form stem-loop structures, with RNase III cleavage sites located in the stem region. While most RNA molecules have the ability to form stem loops, the vast majority are not recognized by this enzyme (24). Double stranded stems containing RNase III recognition sites often contain unpaired bases, which form internal loops or bulges. The importance of this irregular pairing is questionable as deletions of internal loop regions have shown no effect on RNase III processing in some signals (21).

Elements critical to signal specificity for RNase III processing continue to elude precise definition, but are likely to incorporate sequence and structure-dependent components (21, 66). An attempt to uncover a consensus sequence for RNase III processing was conducted, but the 26bp sequence that resulted was very loosely conserved in only some signals (66). Despite the lack of a well defined consensus, RNase III signal sequences do often show notable symmetry

around the cleavage sites, compatible with the prediction that each cut is made by one of the identical subunits of the enzyme (24). Another common characteristic was the fact that bases immediately adjacent to the cut sites were not at all conserved; this notion was also suggested in the discovery of non-specific terminal nucleotides at cleavage sites (101) and the lack of effect of directed mutations at the cleavage sites (21).

A minimal RNA molecule of a RNase III signal containing only sequence forming the stem-loop was processed by RNase III *in vitro*, indicating that no distant tertiary interactions with external sequences are likely to be required (21, 87). Furthermore, deletion analysis *in vivo* suggested that sequence within the stem loop is required for RNase III processing (25). Taken together, the evidence indicates that the stem-loop structures are both necessary and sufficient for recognition and cleavage by RNase III.

The *rnc* operon

The first RNase III$^-$ mutant, named *rnc105*, resulted from a screen of nitrosoguanidine mutagenesis survivors (61). The RNase III$^-$ mutant demonstrated that the enzyme is not essential for cell survival, however the *rnc105* mutation does generate a somewhat slower growth rate the extent of which is variable depending on culture medium and genetic background (4, 61, 112). The mutation was mapped to the 55-min position on the *E. coli* chromosome (4, 112). This discovery led to the cloning and characterization of the wild type *rnc* gene that encodes RNase III (124). The *rnc* gene was

sequenced, and the deduced peptide sequence predicted a 25 kDa protein consistent with the size of the RNase III monomer (77, 85). A plasmid clone carrying only the *rnc* gene was able to complement the *rnc105* mutant (124).

Genetic analyses showed that *rnc* is the first of three genes in the *rnc* operon (Fig. 1.7) (118). The *era* and *recO* genes are located downstream of the *rnc* gene. The Era protein was characterized as a GTP-binding protein with GTPase activity. The *era* gene is essential for viability in *E. coli*, although the function of the protein is unknown. The *recO* gene is involved in DNA repair and is not essential for the cell. The operon was defined by polar mutations in the *rnc* gene that inhibit the expression of the *era* and *recO* genes, and by polar mutations in *era* that prevent expression of *recO* (118).

Transcript analysis of the *rnc* operon revealed that the expression of the *rnc* operon is autoregulated; processing of a stem-loop structure in the untranslated leader region by RNase III reduces the level of *rnc-era* transcript in the cell (5, 79). A recent detailed analysis of transcription was performed in RNase III$^-$ mutants to avoid complications of autoregulation (79). The most abundant *rnc* transcript was unprocessed at the 5' end and spanned the *rnc* and *era* genes, confirming previous findings (Fig. 1.7) (5, 118). Unexpectedly, the same study revealed that the *rnc* promoter (P*rnc*) can also drive transcription through the *recO* gene to include downstream genes *pdxJ* and *acpS* (78). Previously thought to be independent of the *rnc* operon, *pdxJ* is a nonessential gene involved in pyroxidine biosynthesis, and *acpS* encodes holo-acyl-carrier protein and is essential for cell survival (118). While the *pdxJ-acpS* operon can be transcribed independent of RNase III from the P*pdx*

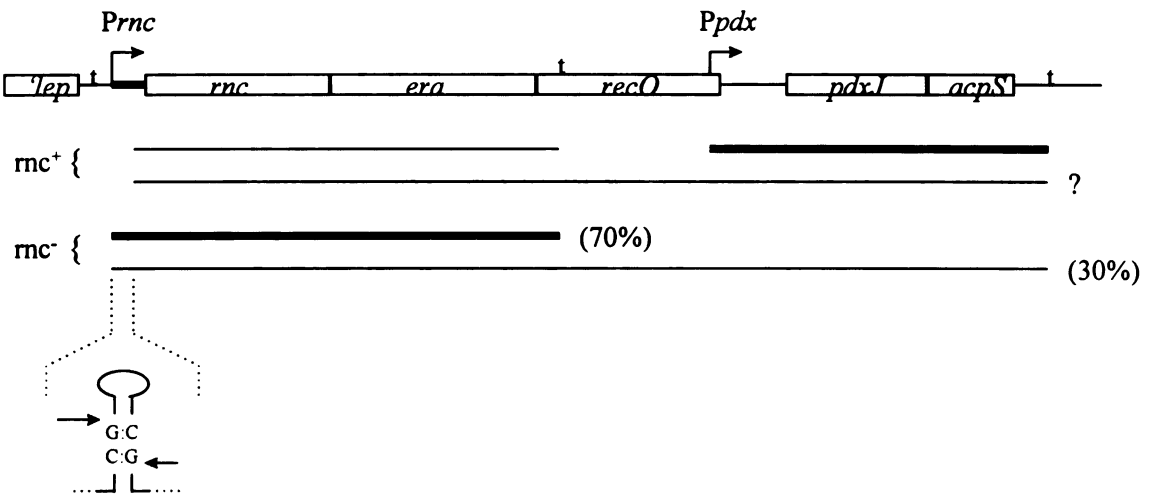


Figure 1.7 The *rnc* operon. The boxes represent genes in the operon as labeled. The promoters of *rnc* (P*rnc*) and *pdxJ* (P*pdx*) operons are noted with arrows, and terminator sites are noted with (t). Transcripts seen in wild type and *rnc*$^-$ mutants are noted; thinner lines indicate reduced RNA levels resulting from negative autoregulation by the enzyme. In the *rnc*$^-$ background, the large transcript is expressed to a lesser degree than the shorter *rnc-era* transcript. The stem loop in the 5' leader region as the site for autoregulation is shown; arrows denote RNase III cleavage sites (see also Fig. 1.10).

promoter, approximately 70% of *pdxJ* transcript seen in RNase III$^-$ cells is driven from the *rnc* promoter (78).

In wild type *E. coli*, the *rnc-era* transcript has been detected by primer-extension analysis, which is processed at the 5' end and is present in low concentrations in the cell due to negative autoregulation (5, 78, 118). The *pdxJ-acpS* transcript is plentiful, confirming that transcription of the *pdxJ* operon from P*pdxJ* is independent of RNase III autoregulation. It may be the case that any larger *rnc-era-recO-pdxJ-acpS* transcript species may be in such low quantities as not to be detectable in wild type strains (78).

Sequence analyses of the *rnc* wild type and mutant alleles have revealed clues about the functional regions of the enzyme (Table 1.1). The original *rnc105* mutant harbors a missense mutation that changes a glycine residue to an aspartate at position 44 (85). Amino acid sequence comparison among RNase III homologs revealed that this residue lies within a highly conserved region of ten amino acids (Fig. 2.4). This mutation completely abolishes the ability of the enzyme to bind and cleave RNase III substrate. Another interesting mutant, named *rnc70*, contained a missense mutation at position 117 that changes a glutamate to an alanine (55). In this mutant, RNase III is able to bind substrate, but has lost the ability to cleave duplex RNA (24). Additionally, a motif search of the *rnc* gene revealed a double-stranded RNA binding domain (dsRBD) homologous to more than forty other proteins that are known to bind duplex RNA in a myriad of functions (59). This motif has the form $\alpha_1\beta_1\beta_2\beta_3\alpha_2$.

rnc allele	Description	Genotype	rRNA
rnc$^+$	wild type allele	*rnc*$^+$*era*$^+$*recO*$^+$	16,23,5S
*rnc*14::ΔTn10	Tn insert in *rnc*; Tn-promoter-driven expression of *era, recO*	*rnc*$^-$*era*$^+$*recO*$^+$	30S
*rnc*105	Gly^{44}→Asp^{44}; inhibition of binding and cleavage of signals	*rnc*$^-$*era*$^+$*recO*$^+$	30S
*rnc*70	Glu^{117}→Ala^{117}; binds signal but cannot cleave	rnc$^-$era$^+$recO$^+$	30S

Table 1.1 Description of mutant alleles of the *rnc* gene in *E. coli.* Compiled from (24, 55, 85, 118).

RNase III in ribosomal RNA processing

The role of RNase III in the processing of ribosomal RNA has been well established. The *E. coli* chromosome contains seven ribosomal RNA operons, each of which is composed of the genes for the 16S, 23S, 5S, and spacer tRNAs. Each operon is transcribed as one large 7000 nt long transcript, which must be processed to yield individual rRNAs. The role of Ribonuclease III in ribosomal RNA processing came to light when an *E. coli* strain lacking RNase III activity was shown to accumulate a large rRNA species not seen in wild type strain (30, 88). This rRNA was identified as the 30S precursor transcript of the entire ribosomal RNA operon. In vitro experiments showed that this 30S precursor could be processed with the addition of purified RNase III protein. A possible mechanism for RNase III processing was theorized when sequence analysis revealed that the 16S and 23S rRNA sequences are each flanked by

inverted complementary sequences within the spacer RNA. The hybridization of these regions would form a stem-loop structure to effectively "loop out" the 16S and 23S rRNA, and provide a duplex RNA region for a possible RNase III cleavage site (9). Analysis of 16S and 23S rRNA species in wild type and RNase III$^-$ backgrounds proved that RNase III specifically binds to the duplex RNA of each stem and cleaves on both sides, releasing the pre-16S and pre-23S rRNAs as they are transcribed (Fig. 1.9) (38). Other ribonucleases then process the 16S and 23S rRNAs into the mature forms found within the 30S and 50S ribosomal subunits, respectively. *E. coli* cells lacking RNase III do not excise the normal RNA precursors pre-16S and pre-23 from nascent rRNA transcripts. These cells produce instead multiple discreet precursor molecules, such as 17S, 18S, "p23" (different from mature 23S rRNA), 25S and 30S (Fig. 1.8) (38). In the case of pre-16S transcript, other ribonucleases in the RNase III$^-$ cell eventually process the transcript to its mature form, although at a less efficient rate (111). For 23S however, final maturation is never achieved in the absence of RNase III (62). The 23S pre-rRNA accumulates unprocessed in the 50S ribosomal subunits and in polysomes and is still able to function; however it has been speculated that this abnormality may influence translational efficiency and may account in part for the reduced growth rate seen in RNase III$^-$ mutants (109).

The initial cleavage by RNase III is thought to be the rate limiting step in ribosomal RNA processing, after which processing occurs very rapidly (63). 30S rRNA precursors are not detectable in RNase III$^+$ strains, nor are any processing intermediates or linked molecules such as 16S-spacer-23S, or 23S-5S molecules (37).

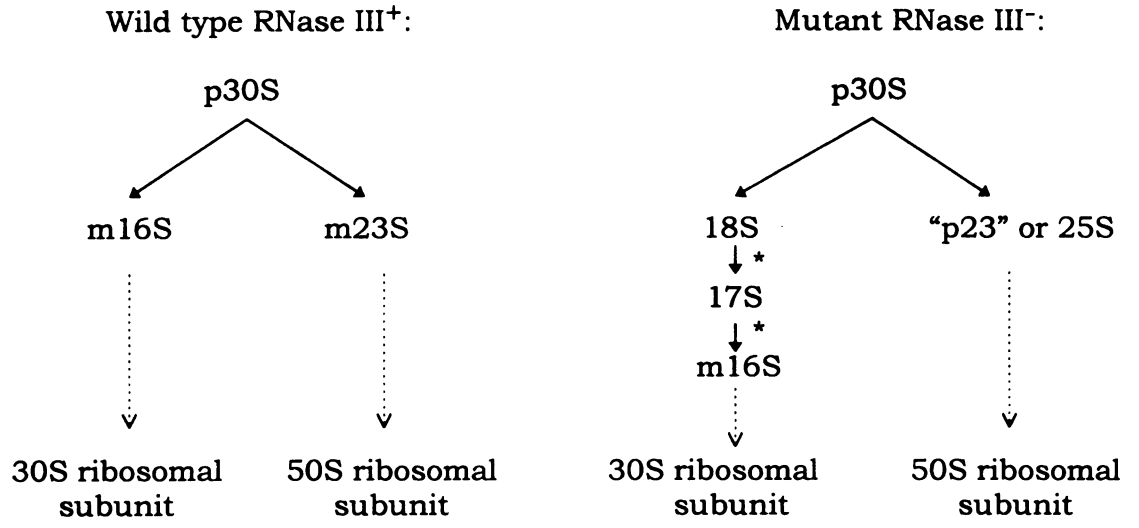
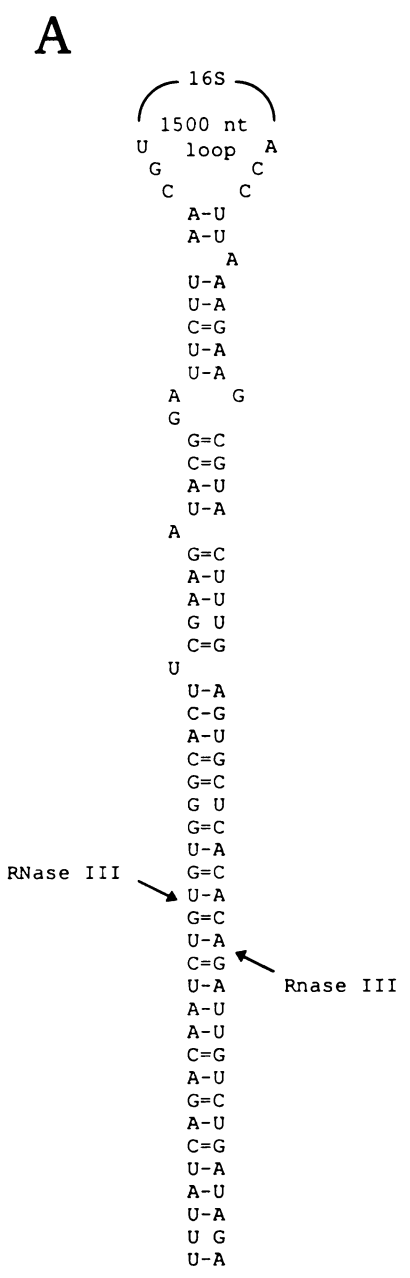


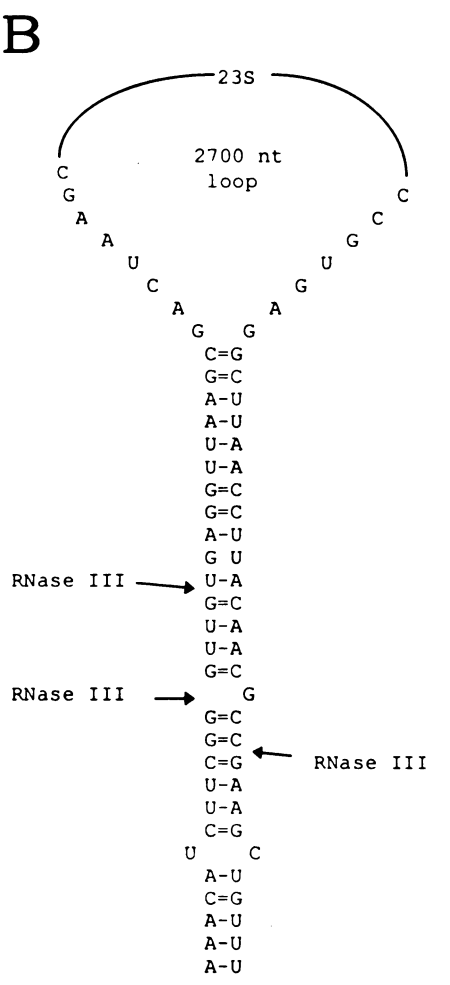
Figure 1.8 Predicted pathways for ribosomal RNA processing with or without RNase III. Steps carried out by other ribonucleases are indicated by (*). "m" indicates mature form, "p" indicates precursor.

Ribosomal RNA operons have been identified in many *Streptomyces* spp. and several have been cloned. Most streptomycete genomes contain six rRNA operons, though *S. venezuela* is reported to have seven (67). The streptomycete rRNA genes are ordered 16S-23S-5S as in other eubacteria, however there are no tRNA-like genes encoded in the spacer regions as seen in other systems (80, 114, 122). Secondary structure analysis determined that the spacer regions surrounding the 16S and 23S genes do contain regions of complementary sequence that are predicted to bind to form duplex regions. Though these regions structurally resemble the 16S and 23S RNase III processing sites reported for *E. coli* (Fig. 1.9), no actual processing site has been defined. There is no obvious sequence similarity between the RNase III processing site of *E. coli* and analogous sequence in *Streptomyces* spp.

Figure 1.9 Processing sites for RNase III in *E. coli* 16S (A) and 23S (B) rRNA flanking sequences (63). RNase III cleavage releases each rRNA molecule to be processed further by other ribonucleases.



16S rRNA
flanking sequences



23S rRNA
flanking sequences

Control of gene expression by RNase III

mRNA decay is one of the principle means by which genes are regulated in prokaryotes. Even under conditions of continuous gene expression, mRNA decay exerts a profound effect on the level of gene expression, having a major impact on the level of protein synthesis (7). The inherent instability of messenger RNA molecules is crucial for rapid genetic response to a changing cellular environment. In *E. coli* mRNA half lives can be as short as 20-30 sec. or as long as 50 min., with average lifetimes between 2-4 min. (93). The rate of decay can be modulated in response to environmental and developmental signals by a few identified ribonucleases. Current research efforts focus on understanding the impact these enzymes have on the genetic control of cellular functions.

RNase III is one of three endoribonucleases known to influence gene expression by modulating mRNA stability and enhancing translation of the gene. A myriad of mechanisms have been described in which the RNase III enzyme processes a mRNA to either up- or down-regulate gene expression. Representative examples of these various mechanisms are given below.

Inhibition of gene expression by 5' leader processing

As previously mentioned, the *rnc* operon is negatively autoregulated by RNase III. The processing of a stem loop structure found in the 5' noncoding region of the *rnc* transcript results in reduced transcript levels and reduced expression of RNase III and Era proteins (Fig. 1.10) (5, 97). RNase III concentration in the cell is limited (0.01% of total protein synthesis) which is

on the same order as other regulatory proteins such as sigma factors (5). Therefore, this autoregulatory mechanism may serve to coordinate RNase III levels in the cell; the presence of a RNase III substrate could titrate the limited amount of protein away from its autoregulatory function, which would ultimately result in increased expression of RNase III (24).

The operon encoding the polynucleotide phosphorylase (PNPase) is also regulated by RNase III in a similar fashion (Fig.1.10) (94, 97, 117). PNPase is a 3'-5' exonuclease that is required for the general degradation of mRNA in cooperation with RNase II. In a RNase III$^-$ mutant strain, the amount of *pnp* transcript and PNPase protein is increased, and the resulting transcript is slightly longer than usual, indicating an absence of processing (94). The mechanism by which RNase III cleavage within the 5' leader reduces half-life of the transcript has not been determined. Processing may expose the transcript to another endonucleolytic enzyme, or an as yet unidentified 5'-3' exonuclease (97). Evidence for further degradative processing comes from the discovery of RNA species containing 5' ends within the coding sequence for both the *rnc* and *pnp* operons (5, 24, 116). These intermediate species have been shown to be RNase III dependent, suggesting that the enzyme initiates further subsequent decay.

Activation of gene expression by 5' leader processing

RNase III processing sites have been characterized in several phage genes. In contrast to the *rnc* or *pnp* operons, cleavage of a RNase III processing site in the 5' noncoding region of the transcript for N gene of λ phage enhances the expression of the N protein (Fig. 1.11) (57). In this case however, transcript

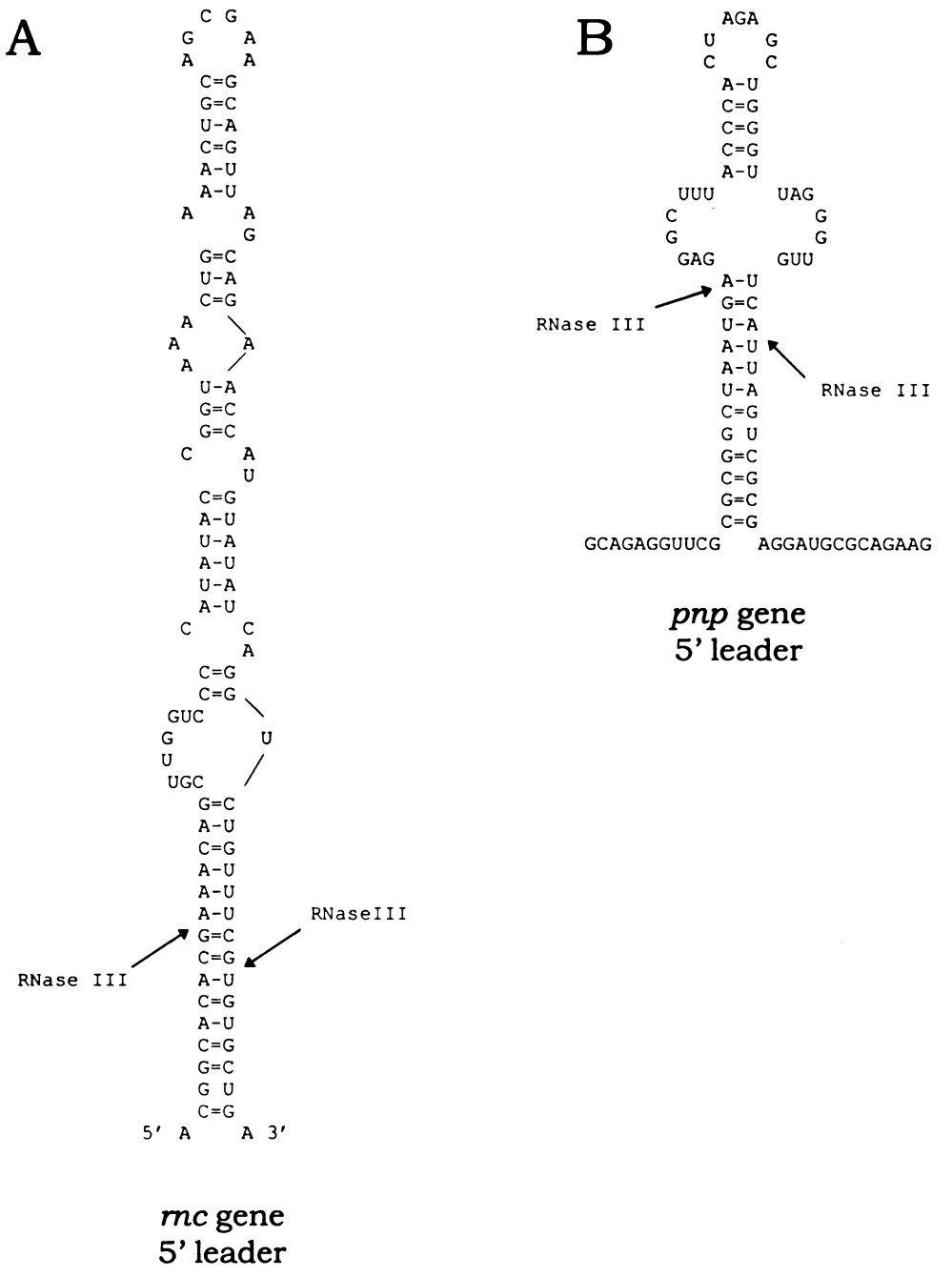
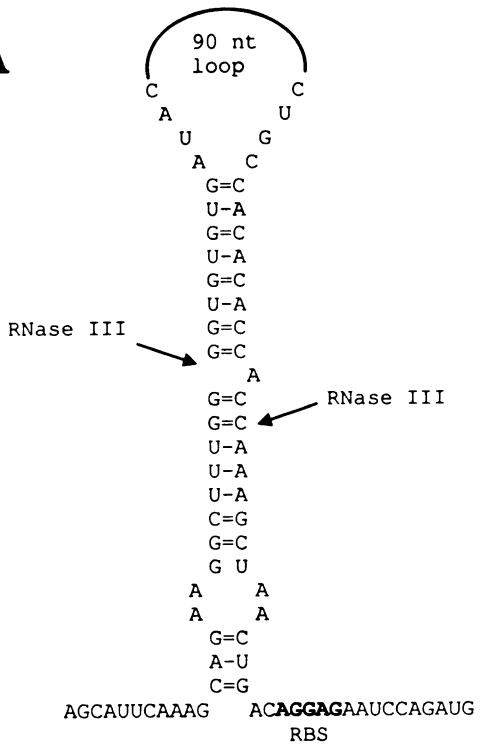


Figure 1.10 Inhibition of gene expression via 5' leader processing. RNase III recognition signals for (A) autoregulation of the *rnc* operon, and (B) polynucleotide phosphorylase operon. In both cases, cleavage by RNase III initiates decay of the transcript by an as yet unidentified mechanism.

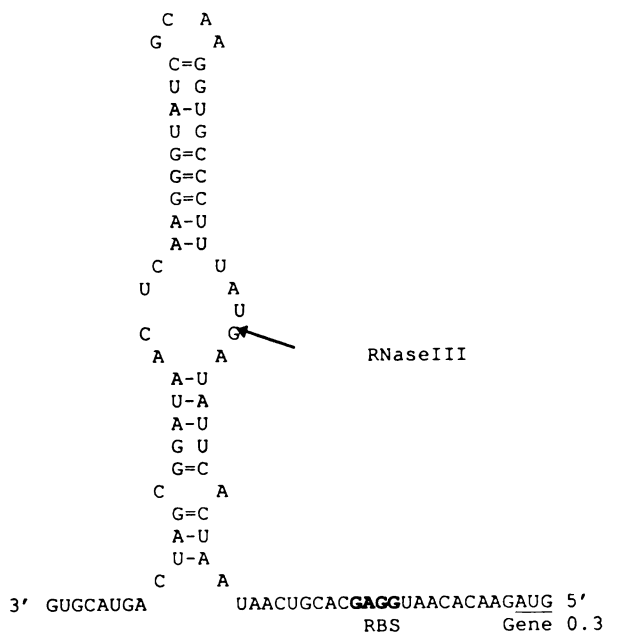
Figure 1.11 Enhancement of gene expression through 5' leader processing. RNase III processing signals for (A) λN gene and the (B) T7 0.3 gene. As a result of processing, the ribosomal binding site (RBS; noted in bold type) is made available for translation initiation.

A



λN gene
5' leader

B



T7 0.3 gene
5' leader

levels of the N gene are not affected by the RNase III processing. Rather, cleavage of the stem-loop signal by RNase III opens up the ribosomal binding site to translation initiation which increases the expression of the N protein (57). N protein, in turn, positively regulates many phage development genes. Therefore, RNase III cleavage opens an entire cascade of genes to permit λ phage development.

The 0.3 gene of T7 phage is activated in a similar manner through the enhancement of translation efficiency. Cleavage occurs at only one side of the stem-loop signal, which releases the ribosomal binding site for translation (Fig. 1.11). In support of this mechanism, the processed transcript has been shown to bind ribosomes better than unprocessed transcript (29, 31).

Inhibition of gene expression by processing within 3' noncoding regions

Examples of RNase III processing of phage genes within the 3' noncoding region demonstrate two different mechanisms by which RNase III processing can inhibit gene expression. The λ*int* protein, which encodes the integrase enzyme, can be expressed from two different promoters, P_L and P_I (for comprehensive review of *int* regulation, see (41)). Transcription from the P_I promoter terminates at terminator T_I and translation occurs normally. However, transcription from the P_L promoter, aided by the antiterminator N protein, proceeds past T_I and transcribes the distal *sib* region (108). *sib* forms an alternative stem loop structure that is recognized and cleaved by RNase III (Fig. 1.12). This processing of the *sib* stem loop leaves a 3' end which is then open to degradation by the 3'-5' exoribonuclease polynucleotide phosphorylase (PNPase). Thus transcription of the *int* gene from the P_L promoter leads to

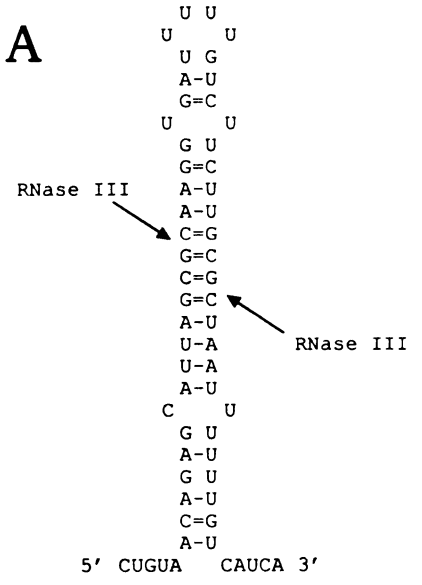
inhibition of gene expression through RNase III cleavage and subsequent degradation of the transcript.

In T7 phage, the expression of early genes 1.1 and 1.2 is also inhibited by RNase III, but via a different mechanism. The 1.1 and 1.2 genes are part of a polycistronic transcriptional unit that is processed within intergenic regions by RNase III. The 1.1 and 1.2 genes are separated from the downstream 1.3 gene by a stem loop designated R5 (Fig. 1.12). Unlike other signals in the T7 transcript, RNase III can cleave the R5 stem loop on both the proximal side (R5a) and the distal side (R5b) (100). Approximately 40% of the 1.1-1.2 transcripts are cut at R5a, and 60% are cut at R5b which results in a longer transcript by an extra 29 nucleotides (107). The shorter R5a transcript expresses the 1.1 and 1.2 genes efficiently, but the longer transcript has a much reduced level of expression for both genes. This inefficient translation of the R5b transcript results from the ability of the extra 29 nucleotides to bind to a complementary region in the 5' end of the 1.1 gene containing the ribosome binding site (107). This pairing blocks translation initiation of 1.1 and 1.2, since they are translationally coupled. Thus the particular cleavage product of the RNase III enzyme determines the translational efficiency of these particular genes.

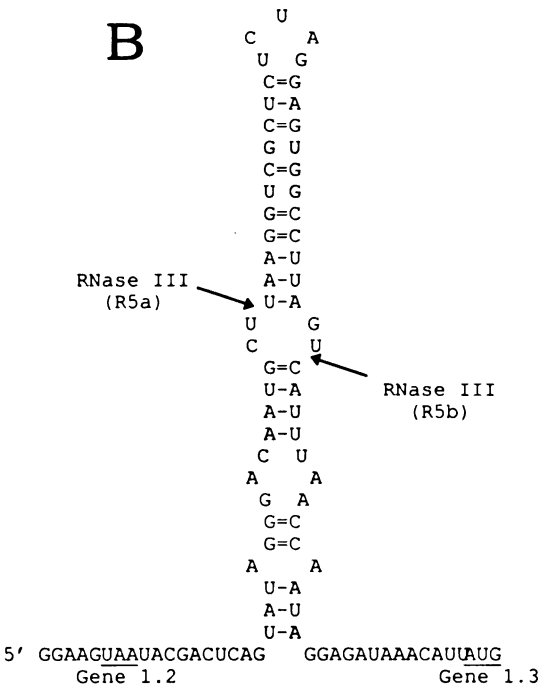
Enhancing gene expression by processing within the 3' noncoding region

The earliest example of mRNA processing by RNase III described the processing of intergenic regions of the T7 early gene polycistronic message. The resulting monocistronic messages are very stable, with half-lives up to 20 min. While the typical RNase III cleavage results in a double strand break,

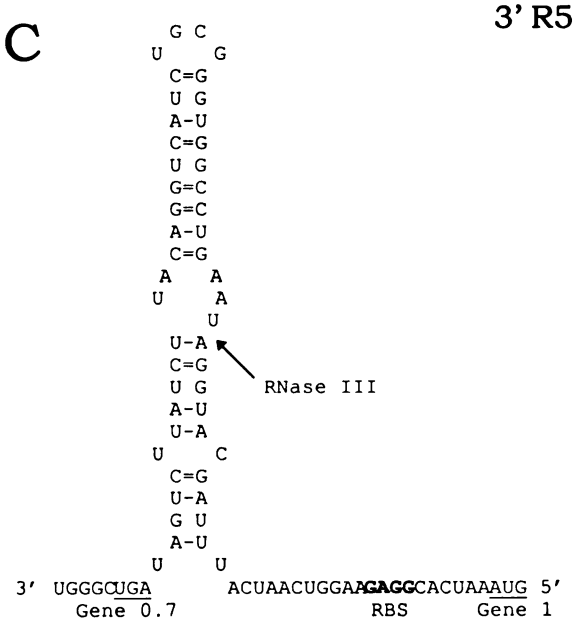
Figure 1.12 RNase III processing within the 3' noncoding region. (A) The *sib* processing site of λ*int* that forms upon transcription from the P_L promoter. Processing results in reduced mRNA stability due to 3'-5' exonucleolytic degradation. (B) The R5 processing site of T7 1.1 and 1.2 genes. Cleavage at the R5b site (as opposed to the R5a site) allows binding of the 29+ nts to the 5' leader region of gene 1.1 resulting in impaired translation initiation. (C) RNase III processing enhances the stability of the T7 0.7 gene by leaving a stable hairpin structure at the 3' end of the mRNA. This hairpin structure, and possibly the presence of the RNase III enzyme itself, blocks exonucleolytic degradation of the transcript.



λ_{int} - sib
3' noncoding region



T7 1.1, 1,2 genes
3' R5 cleavage site



T7 0.7 gene
3' noncoding region

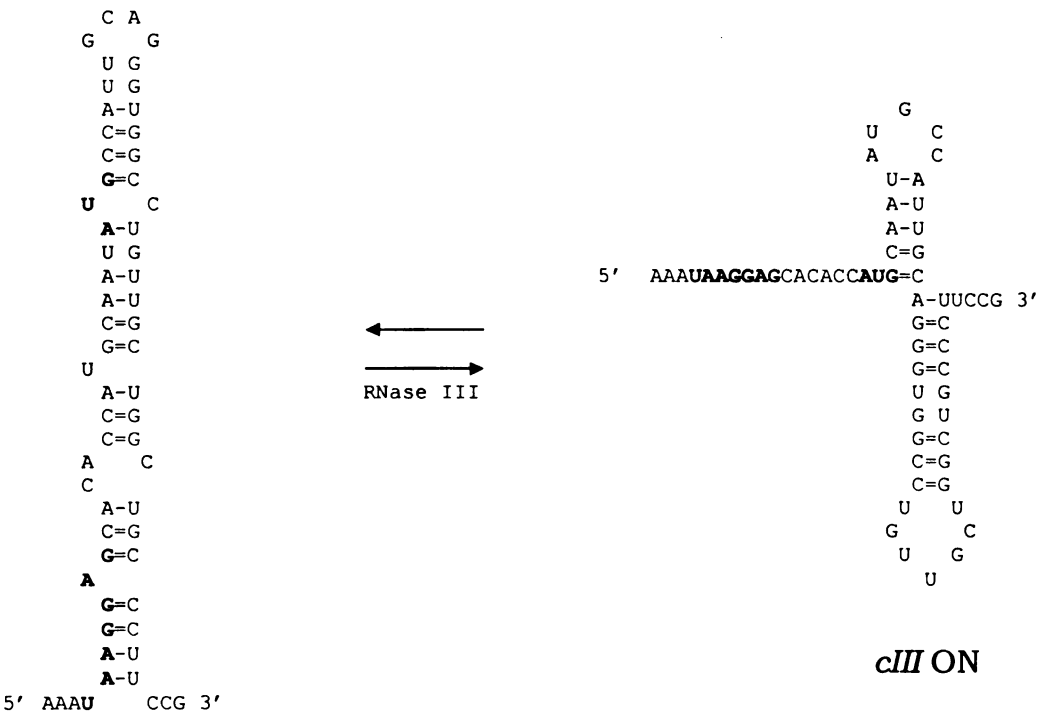
most of the T7 early genes are cleaved by a single stranded break at the distal side of the stem-loop region (29, 107). The RNase III signal for T7 0.7 gene is shown in Figure 1.12 as an example. RNase III cleavage leaves a hairpin structure at the 3' end of the transcript that has been proposed to impart stability by blocking the message from 3'-5' exonucleolytic digestion (28). Additionally it is possible that the RNase III may remain bound to the resulting stem loop and may itself block exonucleolytic degradation.

Control of gene expression without processing

The expression of the *cIII* gene of phage λ is enhanced by the presence of RNase III, yet the transcript is not cleaved at all (3). Rather, RNase III appears to stabilize one favorable conformation by binding at the 5' end of the transcript to open up the ribosomal binding site thereby aiding in translation initiation (Fig. 1.13). In the absence of RNase III, translation is inhibited and most transcripts are found in the obstructive conformation "*cIII* OFF" (2, 3).

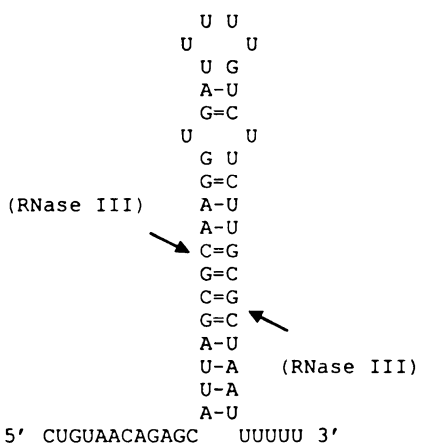
As discussed previously, expression of the λ*int* gene is inhibited by RNase III when it is transcribed from the P_L promoter through cleavage of the *sib* site and subsequent exonucleolytic degradation. During later stages of λ development, *int* is transcribed from the P_I promoter and terminates at the Rho-independent terminator, T_I (Fig. 1.13). RNase III does not process the T_I stem loop, which is thought to protect the transcript from PNPase, the same exoribonuclease that degrades the P_L-*int-sib* transcript (41). However, in an RNase III^- mutant, *int* expression is reduced from the P_I-*int*-T_I transcript. Two possible explanations exist for this dependence on RNase III for optimal expression. First, RNase III negatively regulates PNPase, so that in an

Figure 1.13 Enhancement of gene expression without transcript processing. (A) Two conformations of the *cIII* gene. In the presence of RNase III the ribosomal binding site and the start codon (boldface) are not obscured by secondary structure. (B) The T_I terminator formed when λ*int* is transcribed from the P_I promoter. (RNase III) signifies RNase III cleavage sites *in vitro* under excess enzyme concentrations.



cIII OFF

cIII ON



T_I terminator of λ*int*

RNase III$^-$ mutant, increased PNPase could accelerate degradation of the transcript. Alternatively, the RNase III may be able to bind to the T_I terminator without cutting it, thereby protecting it from PNPase attack. This possibility is supported by the fact that RNase III, in excess concentrations, is capable of cleaving T_I *in vitro*, indicating that binding to T_I is possible (108). While neither model can be favored over the other, it is clear that RNase III is intimately involved in the regulation of the *int* gene under a variety of conditions.

The role of RNase III in antisense RNA control

A typical example of antisense control involves a gene that is transcribed from both the sense and antisense strands. Commonly the antisense RNA inhibits translation of the sense RNA by forming a duplex at the ribosomal binding site. In several cases, RNase III has been shown to participate in this control through cleavage of the sense:antisense duplex. One example in *E. coli* involves the RepA protein which is required for the replication of the R1 plasmid. An antisense RNA, *copA*, inhibits R1 replication by binding to *copT*, the leader region of the *repA* transcript (8). This duplex is cleaved by RNase III, which results in a marked decrease in *repA* expression compared to an RNase III$^-$ mutant control. It has yet to be determined whether the reduced expression is due to inefficient translation or mRNA decay.

In λ phage, the *c*II gene is involved in the delicate balance between lysogenic and lytic development. One level of control involves the antisense RNA *OOP*, which is complementary to the 3' end of the *c*II coding region (65). Formation of the duplex leads to RNase III cleavage and prevents complete

translation of the cI gene. In a RNase III$^-$ mutant, the *OOP* RNA has no effect on cI expression, indicating that it is the cleavage event, and not the binding of the antisense RNA, that affects the expression level of the protein. Thus RNase III processing directly inactivates cI expression by a process that is dependent on the presence of the *OOP* antisense RNA (65).

Ribonuclease III in Other Systems

Recently, several RNase III homologs have been reported in other bacteria and in the yeast *Schizosacchromyces pombe.* While only a few homologs have been functionally characterized, it appears as though RNase III has multiple functions in other systems as seen in *E. coli.*

Bacillus subtilis RNase III (bsRNase III)

Transcription analysis of the *B. subtilis* phage SP82 demonstrated that *in vivo* transcripts of the early genes were considerably shorter than those transcribed *in vitro.* This suggested the possibility that the transcripts were being post-transcriptionally processed. Studies in uninfected strains proved that the processing function was produced by the host and not the phage (27). Protein extracts were purified for this processing function and the resulting protein was characterized as a RNase III homolog (83, 89, 90). The *B. subtilis* RNase III (bsRNase III) proved similar to RNase III of *E. coli* in many respects. It was double strand RNA specific, and processed poly(I)-poly(C) and ribosomal RNA. However, there were notable differences. The bsRNase III appeared to be

a monomer 27-29 kDa in size. The bsRNase III was not able to cleave poly(A)-poly(U) RNA *in vitro*. Comparative studies of signal recognition revealed that the *E. coli* RNase III could not process the SP82 phage transcript, but the bsRNase III was able to cleave the T7 1.1 gene signal at the same site as RNase III (83). Neither enzyme was able to process the rRNA signal of the other. The bsRNase III gene was cloned and sequenced, and showed 36 % homology to the *E. coli* enzyme. Residues and regions that had proved to be functionally significant in *E. coli* through mutational analysis (see Table 1) were strictly conserved in the bsRNase III homolog.

RNase III in *Coxiella burnetii* (cbRNase III)

Although *Coxiella* does not produce a capsule as part of its pathogenic life cycle, a genomic fragment was isolated which caused capsule synthesis when cloned into *E. coli* resulting in a mucoid phenotype. In an effort to identify genes that may suppress capsule synthesis in *C. burnetii*, a plasmid clone was isolated that suppressed the mucoid phenotype in the *E. coli* mutant (132). Sequence analysis of the cloned insert revealed homologous genes of the *rnc-era-recO* operon of *E. coli*. Sequence identities between the genes of *E. coli* and *C. burnetii* were *rnc* (49.3%), *era* (51.2%), and *recO* (37.4%), indicating a high degree of conservation across the entire operon. When the *E. coli rnc* gene was expressed in high copy in the mucoid mutant, the mucoid phenotype was suppressed, indicating that the *rnc* gene is involved in capsule synthesis. The mucoid phenotype was not suppressed when either the *Coxiella rnc* or the

E. coli rnc were expressed in low copy. Deletion analysis showed that *era* and *recO* were not involved in the suppressive function (132).

Additional functional comparisons showed that the cbRNase III was capable of complementing *E. coli rnc*$^-$ mutants. Also, cbRNase III was able to stimulate λN gene expression, as seen for *E. coli* RNase III.

An RNase III homolog in *Schizosacchromyces pombe*

A gene with homology to RNase III of *E. coli* was found to block conjugation and sporulation when overexpressed in *S. pombe* (130). The gene, named *pac1*, had no effect on vegetative growth in high copy, yet inhibited the expression of certain genes related to mating and meiosis. Sequence analysis revealed that the *pac1* gene was 363 amino acids in length, and 230 amino acids of the C-terminus showed homology to RNase III of *E. coli* (54). Unlike *rnc* in *E. coli*, *pac1* is essential for survival of the yeast. The Pac1 protein was isolated and was able to degrade duplex RNA in vitro. Site directed mutagenesis revealed that the analogous residues found to be important to *E. coli* RNase III function, the glycine^{44} of *rnc105* and glutamate^{117} of *rnc70* (Table 1.1) were also required for function in Pac1 mating inhibition (103).

Other RNase III Homologs

Database searches revealed putative RNase III homologs in *Mycobacterium tuberculosis*, *Haemophilus influenzae*, and *Mycoplasma genitalium*. These genes are the result of total genome sequencing, and to date no functional analysis of these genes has been reported.

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CHAPTER 2

Genetic and Molecular Characterization of *absB*, a Ribonuclease III Analog that Globally Regulates Antibiotic Synthesis in *Streptomyces coelicolor*

Abstract

Streptomyces coelicolor absB mutants are developmentally blocked in the production of all four of the antibiotics produced by that strain, yet undergo normal morphological differentiation. To uncover mechanisms involved in global antibiotic regulation, the *absB* locus was cloned using a low copy plasmid library of wild type chromosomal DNA. Two plasmid clones, pTA108 and pTA128, were identified by their ability to restore global antibiotic production specifically to the *absB* mutants. The *absB* locus was delimited to a 1.0 kb region shared by each clone. DNA sequence analysis within this region revealed one open reading frame homologous to the Ribonuclease III (RNase III) enzyme. The *E. coli* RNase III, the most functionally characterized, is a double-strand RNA specific endoribonuclease which functions in the processing of an rRNA precursor and has been shown to regulate gene expression by modulating stability of select mRNA molecules. Sequence analysis of *absB* mutant alleles revealed mutations within the RNase III gene that would predict a truncated or functionally debilitated enzyme. Gene disruption mutants generated by homologous recombination using an internal *absB* fragment also exhibited the Abs^- phenotype. Southern hybridization of other streptomycetes demonstrates that the *absB* gene is highly conserved across species. Preliminary evidence that rRNA processing may be affected in one $absB^-$ mutant suggests that AbsB may share functional similarity with RNase III of *E. coli* and other homologs.

Introduction

The streptomycetes are Gram-positive coenocytic soil organisms that undergo true cellular differentiation in a multicellular developmental life cycle (for review, see (15)). This life cycle begins with the germination of spores which then extend filamentous vegetative (substrate) mycelia that grow on and into the agar medium. In response to unknown stimuli, certain hyphae differentiate and grow into the air as aerial mycelia, which give the colony a fuzzy white appearance. The aerial hyphae then septate and develop into mature haploid spores with a characteristic gray pigment. Portions of the substrate mycelium are lysed and provide nutrients to the developing aerial hyphae.

The production of secondary metabolites is temporally coupled to the morphological differentiation of aerial hyphae (16). While most widely known for the variety of antimicrobial compounds they produce, streptomycetes also produce various antiviral, antitumor, antiparasitic, and immunosuppresive compounds which have found uses in human medicine and agriculture (for review, see (28)). This fact has made *Streptomyces* invaluable to the pharmaceutical industry. Currently, however, the commercial processes of strain improvement for the development of overproducers is devoid of a comprehensive understanding of the regulatory controls of secondary metabolite production. A better understanding of the regulation of antibiotic production could increase the efficiency of antibiotic isolation and commercial production (7).

Streptomyces coelicolor, the best characterized and most genetically manipulatable species, continues to be the model species of choice for the

study of secondary metabolism regulation for many reasons. A complete physical and genetic map has been defined, and a cosmid library of the entire *S. coelicolor* genome is available (33, 49). A broad range of plasmid and phage vectors has been developed, as well as reliable procedures for their use (27). *S. coelicolor* produces four known antibiotics; actinorhodin (Act), undecylprodigiosin (Red), methylenomycin (Mmy) and calcium-dependent antibiotic (CDA). Two antibiotics, Act and Red, are pigmented compounds which facilitates the visual identification of developmental mutants.

The majority of studies of secondary metabolites in *Streptomyces* have focused on the large gene clusters which encode the compound's biosynthetic enzymes, host-resistance genes, and cluster-specific regulators. By comparison, little is as yet understood about the mechanisms of regulation of secondary metabolism by these bacteria at the onset of stationary phase. Clues about the mechanisms involved in the switch to antibiotic synthesis have begun to surface, due to studies on a few defined loci. Evidence for concerted regulation of secondary metabolic pathways in *S. coelicolor* comes from the isolation of "bald" mutants, which are inhibited in aerial mycelium development as well as antibiotic synthesis (42). A second family of developmental mutants, named *whi*, are blocked in various stages of spore formation, resulting in white coloration indicative of immature spore chains, while antibiotic production appears unaffected (17). In an effort to identify global regulation pathways specific to antibiotic production and independent of sporulation, our laboratory has isolated a third class of developmental mutants which are named *abs* for antibiotic synthesis deficient. These mutants are blocked in the production of

all four antibiotics in *S. coelicolor*, yet sporulate normally. Two distinct loci have been identified, *absA* and *absB* (13).

Characterization of the *absA* locus revealed a two component signal transduction system that consists of a histidine kinase (*absA1*) and a response regulator (*absA2*)(11). Current studies seek to determine the mechanism by which these proteins function in the antibiotic regulation cascade. Interestingly, the *absA* null mutant results in copious overproduction of Act and Red, indicating that *absA* functions as a global negative regulator of antibiotic production at some level. This result underscores the potential that a greater understanding of antibiotic regulation might have on production levels of antibiotic.

Current models of antibiotic regulation mechanisms focus on transcription initiation (*absA1/A2* (11), *afsQ1/Q2* (29), *afsK/R* (40)) and translation (*bldA,* (37)) as points of control of gene expression. In this report we provide evidence for a new level of antibiotic gene regulation. We will show that the *absB* locus encodes an analog of Ribonuclease III, which has been shown in other systems to regulate gene expression by modulating mRNA stability. RNase III was first shown to initiate the processing of the 30S ribosomal RNA precursor into the 16S and 23S components (10, 58). Interestingly, RNase III is not essential for growth, as this processing still takes place by a redundant mechanism (24). More recent studies have focused on the ability of RNase III to process certain mRNA transcripts and either up- or down-regulate gene expression as the result of double-stranded RNA processing by this enzyme (18). The possible involvement of a Ribonuclease III enzyme in antibiotic production will be discussed.

Experimental Procedures

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Tables 2.1 and 2.2. *Streptomyces* cultures were propagated on R2YE agar or in YEME broth (27) unless otherwise indicated. Thiostrepton (10μg/ml final conc.) or hygromycin (100μg/ml final conc.) were added as needed. Conditions for growth of cultures and preparation of spore stocks were as described in (27). *E. coil* cultures were grown on LB medium (51) supplemented with ampicillin (100μg/ml final conc.) as required to maintain plasmids.

Genetic techniques and DNA manipulations. *Streptomyces* protoplasts were generated and transformed as described previously (27). Transformants were regenerated on R2YE and thiostrepton was applied (10μg/ml final concentration) at 18-20h. High copy Streptomyces plasmid DNA was isolated either manually (8) or by using QIAgen Midi Plasmid Columns (QIAgen Inc., USA). Low copy plasmid (pIJ922) derivatives were isolated using the procedure for SCP2* derivatives (27). Chromosomal DNA used for PCR and Southern hybridizations was isolated as described in (27). *E. coli* cultures were transformed as described in (51). *E. coli* plasmid DNA was isolated by the alkaline lysis procedure (51) or by QIAprep spin columns (QIAgen, Inc.) when used in automated sequencing. All *E. coli* plasmids were passed through *dam*$^-$ *dcm*$^-$ *E. coli* strains ET12567 (39) or DM-1 (Gibco BRL) to generate unmethylated DNA suitable for introduction into *Streptomyces*.

Strain	Genotype	Reference
Streptomyces Strains		
S. coelicolor A3(2) J1501	*hisA1 uraA1 strA1* SCP1$^-$ SCP2$^-$ pgl$^-$	(27)
M124	*cysD18 proA1 argA1* SCP1$^-$ SCP2$^-$	(27)
M145	Prototroph, SCP1$^-$, SCP2$^-$	(27)
C120	*absB120 hisA1 uraA1 strA1* SCP1$^-$ SCP2$^-$ pgl$^-$	(3)
C170	*absB170 hisA1 uraA1 strA1* SCP1$^-$ SCP2$^-$ pgl$^-$	(3)
C175	*absB175 hisA1 uraA1 strA1* SCP1$^-$ SCP2$^-$ pgl$^-$	(3)
C120-310	*absB120*::pBK310 *hisA1 uraA1 strA1* SCP1$^-$ SCP2$^-$ pgl$^-$	This study
C542	*absA542 hisA1 uraA1 strA1* SCP1$^-$ SCP2$^-$ pgl$^-$	(4)
C301	*bldA*301 *hisA1 uraA1 strA1* SCP1$^-$ SCP2$^-$ pgl$^-$	(14)
C112	*bldB112 hisA1 uraA1 strA1* SCP1$^-$ SCP2$^-$ pgl$^-$	(14)
C109	*bldH109 hisA1 uraA1 strA1* SCP1$^-$ SCP2$^-$ pgl$^-$	(14)
S. albus		
S. ambofaciens		
S. avermitilis		
S. cinnamonium		
S. griseus		
S. halstedii JM8		
S. lincolnensis		
S. lividans TK64		
***E. coli* Strains**		
DH5α		Gibco BRL
ET12567	dam- dcm-	(39)
DM-1	dam- dcm-	Gibco BRL

Table 2.1 Strains used in this study

Plasmid	Construction / Distinguishing Characteristics	Reference
***Streptomyces* Plasmids**		
pTA108	pIJ922 + 11.0kb *Sau3A* fragment	(2)
pIJ922	low copy SCP2* derivative	(27)
pBK600	1.4kb *BglII* fragment from pBK412 in pIJ922	This study
pBK601	1.0kb *BglII* fragment from pBK651 in pIJ922/*BamHI*	This study
pBK602	1.0kb *BglII* fragment from pBK652 in pIJ922/*BamHI*	This study
pBK603	1.0kb *BglII* fragment from pBK653 in pIJ922/*BamHI*	This study
pIJ702	high copy pIJ101 derivative, Thio^r	(27)
pBK650	1.4kb *BglII* fragment from pBK412 in pIJ702	This study
pBK651	1.0kb PCR-generated *BglII* fragment (Primers A and D) from J1501in pIJ702/*BglII*	This study
pBK652	1.0kb PCR-generated *BglII* fragment (Primers A and D) from C120 in pIJ702/*BglII*	This study
pBK653	1.0kb PCR-generated *BglII* fragment (Primers A and D) from C175 in pIJ702/*BglII*	This study
***E. coli* plasmids**		
pKS$^+$Bluescript, pSK$^+$Bluescript	Amp^r, *lacZ* insertional inactivation *KpnI-SacI* MCS (pKS$^+$) or *SacI-KpnI* MCS (pSK$^+$)	Stratagene
pKJ100	8.3 kb BglII fragment from pTA108 in pKS$^+$	This study
pBK200	3.3 kb *PstI* fragment from pTA108 in pKS$^+$	This study
pBK210	2.6 kb *PstI* fragment from pTA108 in pKS$^+$	This study
pBK212	1.4 kb *PstI-ApaI* fragment from pBK210 in pKS$^+$	This study
pBK213	1.2 kb *ApaI-PstI* fragment from pBK210 in pKS$^+$	This study
pBK802	1.0 kb *BglII* fragment from pBK651 in pSK$^+$	This study
pBK803	1.0 kb *BglII* fragment from pBK652 (*absB*120) in pSK$^+$	This study
pBK804	1.0 kb *BglII* fragment from pBK653 (*absB*175) in pSK$^+$	This study
pIJ963	pIJ2925 derivative with Hyg^r	(38)
pBK300	3.3 kb *BamHI/KpnI* fragment from pBK200 in pIJ963	This study
pBK310	2.6 kb *BamHI/KpnI* fragment from pBK210 in pIJ963	This study
pBK312	1.4 kb *BamHI/KpnI* fragment from pBK212 in pIJ963	This study
pBK313	1.2 kb *BamHI/KpnI* fragment from pBK213 in pIJ963	This study
pBK314	460 bp PCR-generated *BglII* fragment (Primers B and IF) from J1501 in pIJ963	This study
pIJ2925	pUC19 derivative with modified multiple cloning site	(30)
pBK412	1.4kb BamHI/KpnI fragment from pBK212 in pIJ2925	This study

Table 2.2 Plasmids used in this study

For PCR amplification, Deep Vent polymerase was used (New England Biolabs) and reactions were carried out essentially as recommended by the manufacturer, with the addition of glycerol to 10% final concentration per reaction. Cycling conditions were as follows: hold at 95°C 5 min; denature 96°C/45 sec, anneal 70°C/45 sec, extend 72°C/1 min for 35 cycles. Primer sequences (heterologous tails containing *BglII* sites underlined) were:

Primer A - *5'*CCAGATCTGCAGCACATCGCGTGCCCG*3'*;

Primer B - *5'*GCAGATCTCGGCCTCGTCGTCACGGACACG*3'*;

Primer IF - *5'*GCAGATCTGCCGTACGAGACGCCTCCGACG*3'*;

Primer D - *5'*CGAGATCTCG ACCTCTACCTCGGGCAACTCGGG3'.

Cloning the absB *locus.* The isolation of the *absB*-encoding DNA was carried out using a low copy pIJ922 plasmid library (2). The library contained 10-30 kb fragments of *Sau3A* partially-digested J1501 chromosomal DNA (11). The cloning scheme took advantage of the self-transmissible nature of pIJ922. M124 protoplasts were transformed with the pIJ922 plasmid library. $Thio^r$ resistant transformants were replicated onto lawns of the *absB* mutant C120 on R2YE medium. After sporulation, the mating plates were replicated onto medium selective for C120 recipients and nonpermissive for the M124 host strain (i.e. glucose minimal medium containing uracil, histidine, thiostrepton and streptomycin). The transconjugants were visually screened for Act^+ Red^+ phenotype, and subsequently tested for CDA^+ and Mmy^+ (the Abs^+ phenotype) (2). Assays for actinorhodin, undecylprodigiosin, methylenomycin and calcium-dependent antibiotic have been described previously (4).

Physical mapping of the absB *locus.* The *absB* locus was localized on the *S. coelicolor* physical map (33, 49) as follows. *AseI*-digested J1501 chromosomal DNA was separated by pulsed-field gel electrophoresis and blotted on nitrocellulose membrane (51). The 3.3kb *PstI* fragment from *absB* complementing clone pTA108 was labeled with ATPα^{32}P for hybridization to the membrane. High stringency conditions were used (65°C; 2xSSC, 0.5xSSC washes), and the annealed probe was detected by film exposure for 24 h. at -70°C.

Functional analysis of the absB *locus through recombinational rescue and complementation.* Subfragments of the 11.0 kb insert of pTA108 were cloned into the *E. coli* vector pKS$^+$Bluescript (Stratagene). In recombinational rescue experiments, fragments were then ligated into the *E. coli* vector pIJ963 (38), which carries a Hyg^r gene suitable for selection in *Streptomyces*. Unmethylated pIJ963 derivatives were used to transform *absB* mutant strains. Hyg^r recombinants were isolated (indicating the occurrence of a single crossover event via homologous recombination within the insert) and scored for an Abs$^+$ phenotype 3-4 days after transformation. In initial complementation studies, pTA108 fragments were cloned into pIJ2925, which has *BglII* sites flanking the multiple cloning site. Following passage through ET12567, clones were digested with *BglII* to yield unmethylated fragments which were ligated into both the high copy pIJ702 and the low copy pIJ922 (27). In subsequent experiments, the PCR generated *absB* gene was amplified using primers with heterologous *BglII* sites designed at the ends for cloning directly into pIJ702. In

either case, ligation mixtures were used to transform *absB* mutant C120, and Thio^r transformants were scored for the Abs$^+$ phenotype. Complementing clones from Abs$^+$ transformants were isolated and the constructs confirmed by restriction mapping and Southern hybridization. Complementing clones were then used to retransform C120 and to transform other *absB* mutant strains as well as wild type J1501. PCR-generated *absB* mutant alleles were similarly cloned and introduced back into their respective *absB* mutant backgrounds as a negative control for complementation.

DNA sequencing and analysis. Automated sequencing of the *absB* locus from wild type and *absB* mutant strains was performed at the Iowa State University and the Michigan State University sequencing facilities. Nested deletion clones from pBK210 and PCR-generated fragments were used as sequencing templates. PCR primers A and D (Figure 2.3) were used to generate templates of the *absB* gene from wild type and mutant strains; these were then functionally characterized by complementation (see above) and then cloned into pSK$^+$Bluescript for sequencing using standard universal and reverse primers and Primer B (Figure 2.3). All sequencing templates were purified using QIAprep Spin Columns and were resuspended in water to a concentration ≥200 ng/μl. When necessary, 10% glycerol or 10% DMSO was added to the cycling reactions to aid sequencing through regions of complex secondary structure. Sequence data was compiled and analyzed using the Wisconsin Package Version 9, GCG, Madison, WI.

Identifying absB *homologs in other streptomycetes.* Chromosomal DNA was isolated from various *Streptomyces* strains (27) and digested with *BglII, BamHI* or *PstI.* Normalized amounts of digested DNA (50μg/lane) were electrophoresed on a 0.7% agarose gel. Southern blotting was performed as described previously (51). A 1.0kb *PstI* fragment from *E. coli* clone pBK802 containing the wild type *absB* gene was gel purified (QIAquick) and labeled with Dig-UTP. Probe preparation, non-radioactive hybridization and colormetric detection was performed using the Genius Kit (Boeringer Mannheim) under high stringency conditions according to manufacturer's instructions.

Identification of 30S precursor rRNA. RNA samples isolated over a 65 hour timecourse from J1501, C120 (*absB*), and C120-310 (*absB*C120::pBK310) were kindly provided by D. Aceti (1). 50 μg of each RNA sample was run on 1% agarose at 25V for 16 h. The gel was stained with ethidium bromide and photographed.

Results

Cloning the *absB* locus

Six *absB* mutants had been isolated previously, and genetic analysis suggested that a single mutant locus was responsible for the Abs$^-$ (Act$^-$Red$^-$ Mmy$^-$CDA$^-$) phenotype of the strains (3). To isolate the wild-type *absB* locus, a low copy plasmid library of *Sau3A* partially-digested J1501 chromosomal DNA was employed (see Experimental Procedures). Two clones were isolated, pTA108 and pTA128, which were capable of restoring the production of all four antibiotics to the *absB* mutants. In contrast no Act, Red, or CDA production resulted when the plasmids were transformed into other developmental mutants such as C542 (*absA*), C301 (*bldA*), C112 (*bldB*), and C109 (*bldH*) (4, 14). Preliminary restriction mapping and Southern hybridization indicated that the inserts in pTA108 (12.0 kb) and pTA128 (13.0 kb) shared at least two internal *PstI* fragments (2). Therefore, we hypothesized that the two plasmid clones carried the *absB* locus, and that its location was within the shared region of DNA.

Detailed Restriction Analysis of the pTA108 complementing clone.

Preliminary restriction data had previously been generated (2), but more detailed information was necessary to facilitate subcloning into various plasmid vectors. An 8.3 kb *BglII* fragment containing nearly the entire insert from pTA108 was subcloned into pKS$^+$Bluescript and named pKJ100. A detailed restriction map was generated using this clone as well as pTA108 itself (Figure

2.1). The insert within pTA108 was actually shown to be an 11.0 kb fragment, and not 12.0 kb as initially estimated (2).

Physical mapping of the *absB* locus

Recently a physical map of ordered *AseI* and *DraI* fragments from the *S. coelicolor* A3(2) chromosome has been reported and correlated with the genetic map (33). To determine the location of the *absB* locus, a 3.3 kb *PstI* fragment common to both complementing clones pTA108 and pTA128 was radioactively labeled and hybridized to *AseI*-digested chromosomal DNA of *S. coelicolor* that had been separated by pulsed-field gel electrophoresis. *AseI* digestion generates 17 fragments from chromosomal DNA, designated A - Q. Upon exposure to film, the probe hybridized to fragment B, which is located at 5 o'clock relative to the genetic map (Figure 1.1). This result was consistent with previous genetic mapping results, which localized the *absB* mutation to the same region, between the *cysD* and *mthB* auxotrophic markers (2, 3).

Localization of the *absB* locus within a 1.4 kb fragment

To delimit the putative *absB* locus within the 11.0 kb insert of complementing clone pTA108, the shared *PstI* fragments (3.3 kb and 2.6 kb in Figure 2.2) were independently isolated and tested for the ability to restore the wild type phenotype (Abs^+) to the *absB* mutants. Each was ligated into pKS^+Bluescript to assist in further cloning and restriction mapping to yield pBK200 and pBK210, respectively. From these vectors, *PstI* fragments were

Figure 2.1 A detailed restriction map of pTA108. *absB* complementing clone pTA108 (constructed by T. Adamidis, (2)) containing the 11.0 kb fragment from *Sau3A* partially-digested *S. coelicolor* J1501 chromosomal DNA. Vector DNA (pIJ922 (27)) is indicated by arrows. Approximate fragment sizes (in kilobases) are noted.

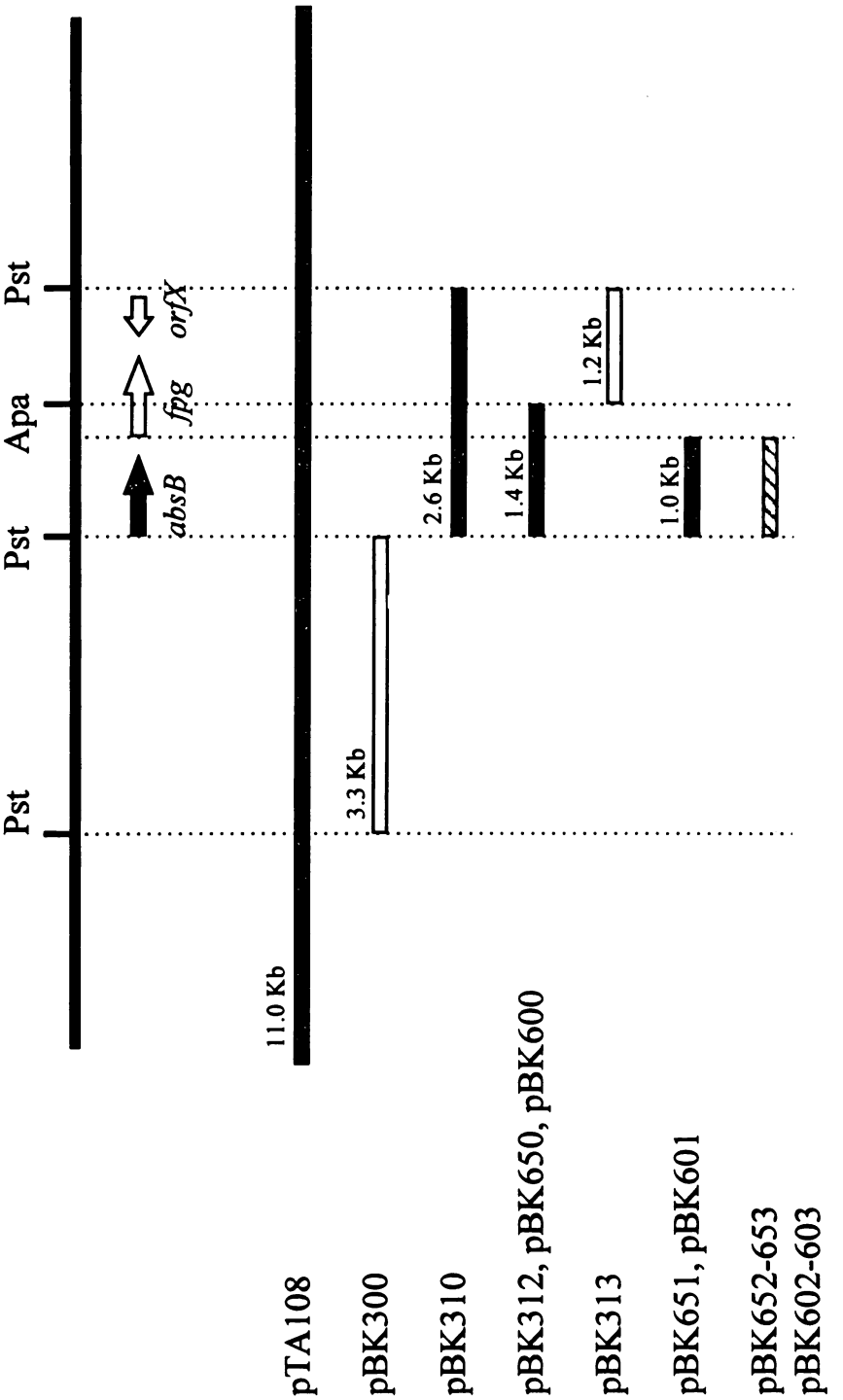
EcoRI
BamHI / Sau3A
BglII
SphI
PstI
EcoRI
BamHI
SacI
PstI
ApaI
SphI
PstI
KpnI
BglII
Sau3A / BamHI
BglII
pIJ922
pIJ922
0.6
0.6
0.23
1.0
0.55
1.05
1.1
0.6
1.4
0.46
0.74
1.26
1.8
0.4
1.0 kb

then cloned into pIJ963, an *E. coli* plasmid carrying the Hyg^r cassette suitable for selection in *Streptomyces* (38) to yield pBK300 and pBK310. Since pIJ963 cannot replicate in *Streptomyces*, Hyg^r colonies arise following homologous recombination within the cloned insert. Hyg^r colonies of the *absB* mutant C120 are expected to display the Abs^+ phenotype only if the cloned insert contains at least one end of the *absB* gene and includes the region spanning the *absB* mutation. Using such an approach, homologous recombination between the *absB* mutant locus and a clone containing the entire *absB* gene would result in phenotypic rescue of nearly 100% of recombinants, while a clone containing only a portion of the *absB* gene might result in a mixture of Abs^+ and Abs^- recombinants with the proportion dependent on the location of the crossover.

When pBK300 was introduced into *absB* mutant C120, all Hyg^r recombinants remained Abs^-. However 100% (n=21) of the Hyg^r pBK310 recombinants were Abs^+ (Figure 2.2). This result suggested that the region of DNA responsible for rescuing the *absB* mutation was located within the 2.6 kb *PstI* subfragment from pTA108. Transformation of either pBK300 or pBK310 into the Abs^+ parent strain J1501 had no effect on antibiotic production by that strain.

A convenient *ApaI* restriction site located within the 2.6 kb fragment was used to further delimit the *absB* locus. The 1.4 kb and 1.2 kb *PstI-ApaI* fragments from pBK210 were subcloned into pKS^+Bluescript to yield pBK212

Figure 2.2 Defining the *absB* gene. Wild type J1501 DNA fragments from pTA108 used in cloning schemes outlined in the text are diagrammed. Those fragments capable of restoring the wild type Abs$^+$ phenotype to the *absB* mutants are shaded. Plasmid clones used in rescue experiments (pIJ963 derivatives) are designated pBK3xx, while complementation clone derivatives from pIJ922 and pIJ702 are named pBK60x and pBK65x, respectively. The *absB* mutant alleles cloned into pIJ922 and pIJ702 that were unable to complement the *absB* mutants are designated by a hatched box. Open reading frames determined by sequence analysis are shown as arrows. Relevant restriction sites are noted.


Pst
Pst
Apa
Pst
absB
fpg
orfX
11.0 Kb
3.3 Kb
2.6 Kb
1.4 Kb
1.2 Kb
1.0 Kb
pTA108
pBK300
pBK310
pBK312, pBK650, pBK600
pBK313
pBK651, pBK601
pBK652-653
pBK602-603

and pBK213 respectively. Each insert was recovered as a *KpnI-BamHI* fragment and ligated into pIJ963 to yield pBK312 (1.4 kb) and pBK313 (1.2 kb) (Figure 2.2). Following transformation of C120, none of the resulting recombinants from pBK313 were Abs^+, yet all of the pBK312 recombinants were Abs^+ (n=47). These results suggested that the *absB* gene was contained entirely within the 1.4 kb *PstI-ApaI* fragment.

To determine whether the 1.4 kb *PstI-ApaI* fragment had the ability to complement the *absB* mutations *in trans*, the autonomously replicating *Streptomyces* plasmids pIJ702 (high copy) and pIJ922 (single copy) were used (27). The 1.4kb *PstI-ApaI* fragment was cloned into unmethylated pIJ2925, an *E. coli* plasmid with *BglII* sites flanking the multiple cloning site (30). This allowed the fragment to be recovered as a *BglII* fragment suitable for cloning into both pIJ702 and pIJ922. The ligation mixture was transformed into C120, and several $Thio^r$ Abs^+ transformants were obtained. Plasmid was recovered from both Abs^+ and Abs^- transformants and the presence of the 1.4 kb fragment was determined by restriction digestion and Southern hybridization; the 1.4 kb fragment was found in only the Abs^+ clones (n=6). Plasmid DNA extracted from Abs^+ colonies was then used to retransform C120 with the result that 100% of transformants were Abs^+. Hence both the low copy pIJ922 clone (named pBK600) and the high copy pIJ702 clone (pBK650) were able to complement the *absB* mutant C120 *in trans*. Transformation of two other *absB* mutants, C170 and C175, with pBK600 and pBK650 also resulted in 100% Abs^+ transformants. This result established that the 1.4 kb

PstI-ApaI fragment carried all information necessary and sufficient for the restoration of the Abs^+ phenotype to the *absB* mutants.

DNA sequence analysis of the *absB* locus

One strand of the 2.6 kb *PstI* fragment was sequenced. DNA sequence analysis of the region revealed three open reading frames using CODON PREFERENCE (Figure 2.2). The 3' end of the 2.6 kb fragment contained a 306 bp ORF, designated *orfX*, that showed no homology to any sequences in the protein databases. A second open reading frame spanning the *ApaI* site showed homology (57.5-31.6%) to several formamidopyrimidine DNA glycosylase (*fpg*) genes in the databases. The Fpg protein is a DNA repair enzyme that excises the imidizole ring-opened form of N7-methylguanine from damaged DNA (9, 26, 46, 57). We have similarly designated this ORF *fpg*.

A third gene was found to be entirely within the 1.4 kb *PstI-ApaI* fragment (Fig. 2.3). Because this fragment was able to rescue and complement *absB* mutants, the third gene presented a strong candidate for the *absB* gene. This 276 amino acid open reading frame showed a high degree of identity to Ribonuclease III, which had been shown in *E. coli* to regulate gene expression by modulating mRNA stability. Six other RNase III homologs were also found in the databases; amino acid alignments of these homologs are shown in Figure 2.4. All of the functionally significant residues defined for the *E. coli* RNase III homolog are strictly conserved in the *Streptomyces* sequence, as is the double-stranded RNA binding domain.

Secondary structure analysis of the 3' end of the putative RNase III gene as well as the 3' noncoding region using RNAFOLD revealed two large stem loops (Figure 2.3). One stem loop lies just within the coding region of the putative RNase III gene, and the other, a 21 bp perfect inverted repeat with a 6 bp loop, lies within the noncoding region. This structure may function as a transcriptional terminator, and/or as an autoregulatory site (see discussion).

Identification of the *absB* gene

Because the 1.4 kb fragment used in rescue and complementation studies also contained the 5' end of the *fpg* gene, we sought to isolate the RNase III ORF and repeat the complementation experiments to confirm that the putative RNase III gene was responsible for complementation of *absB*. PCR primers A and D (Figure 2.3) were used to generated a 1046 bp fragment containing the RNase III ORF and the noncoding regions on either side of the gene from wild type J1501. The *BglII* digested fragment was ligated with high copy plasmid pIJ702. The ligation mixture was used to transform C120 and several Abs^+ colonies were recovered. Plasmid was isolated from several transformants and the constructs were confirmed by restriction analysis. A representative plasmid was named pBK651. Upon retransformation into C120, pBK651 gave 100% Abs^+ colonies (n=264). Subsequent transformation into *absB* mutants C175 and C170 gave similar results. The *BglII* fragment was recovered from pBK651 and cloned into low copy pIJ922 and used to transform each of the *absB* mutants. The resulting clone, pIJ601, was also able to restore the Abs^+ phenotype.

Upon observation of complementation of the *absB* mutants, the PCR-generated fragment in pBK651 was recovered and cloned into pSK+Bluescript to yield pBK802. DNA sequencing of the insert, using vector priming sites, was performed in both directions three times to directly correlate the phenotypic results with the sequence data. DNA sequence obtained from pBK802 was identical to preliminary sequence data obtained from the 2.6kb *PstI* fragment, indicating that the PCR product ligated into complementation clones was identical to the fragment carried on pTA108 and encoded the RNase III-like sequence.

Sequencing the *absB* mutant alleles

Two *absB* mutants, C120 and C175, were chosen for detailed sequence analysis. Primers A and D (Figure 2.3) were used to amplify the mutant alleles from *absB* mutant strains. Each reaction generated a fragment the same size as the J1501 1.0 kb fragment, indicating that no major deletions of the locus were responsible for the Abs^- phenotype. PCR fragments were cloned into pIJ702 for complementation testing. Unlike the wild type gene, neither mutant allele was able to complement the *absB* mutant phenotype (C120, n=137; C175, n=88). These clones were named pBK652 (*absB*120) and pBK653 (*absB*175). To correlate DNA sequence with phenotypic results, the insert from each non-complementing plasmid was cloned into pSK^+Bluescript for sequencing. The inserts were sequenced in both directions at least two times. Sequence comparisons showed that each mutant contained a mutation that could predictably disrupt the production or function of an RNase

Figure 2.3 Sequence of the *absB* locus. Nucleotide and protein sequence of the *absB* gene and the 5' end of the *fpg* gene is shown. PCR primer annealing sites are highlighted in bold text. Putative ribosomal binding sites (rbs?) and possible GTG start sites for AbsB and Fpg are underlined. The point mutations identified in *absB* mutants C120 and C175 are noted, as are the resulting amino acid changes. The highly conserved 10 amino acid region common to all Ribonuclease III homologs is underlined. The double-stranded RNA binding domain as defined in *E. coli* is underlined. Stem-loop structures predicted by RNAFOLD are marked with (>><<).

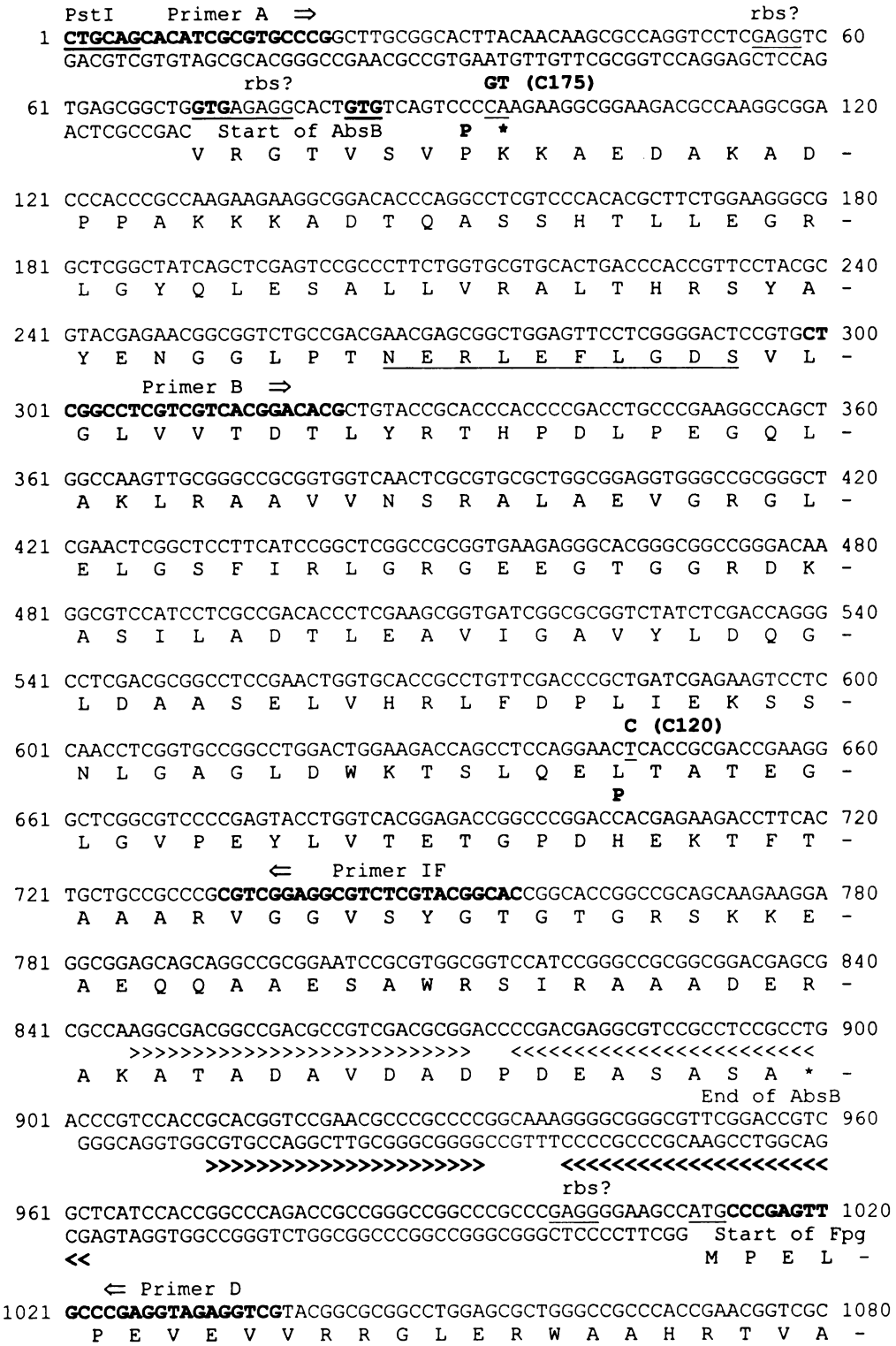
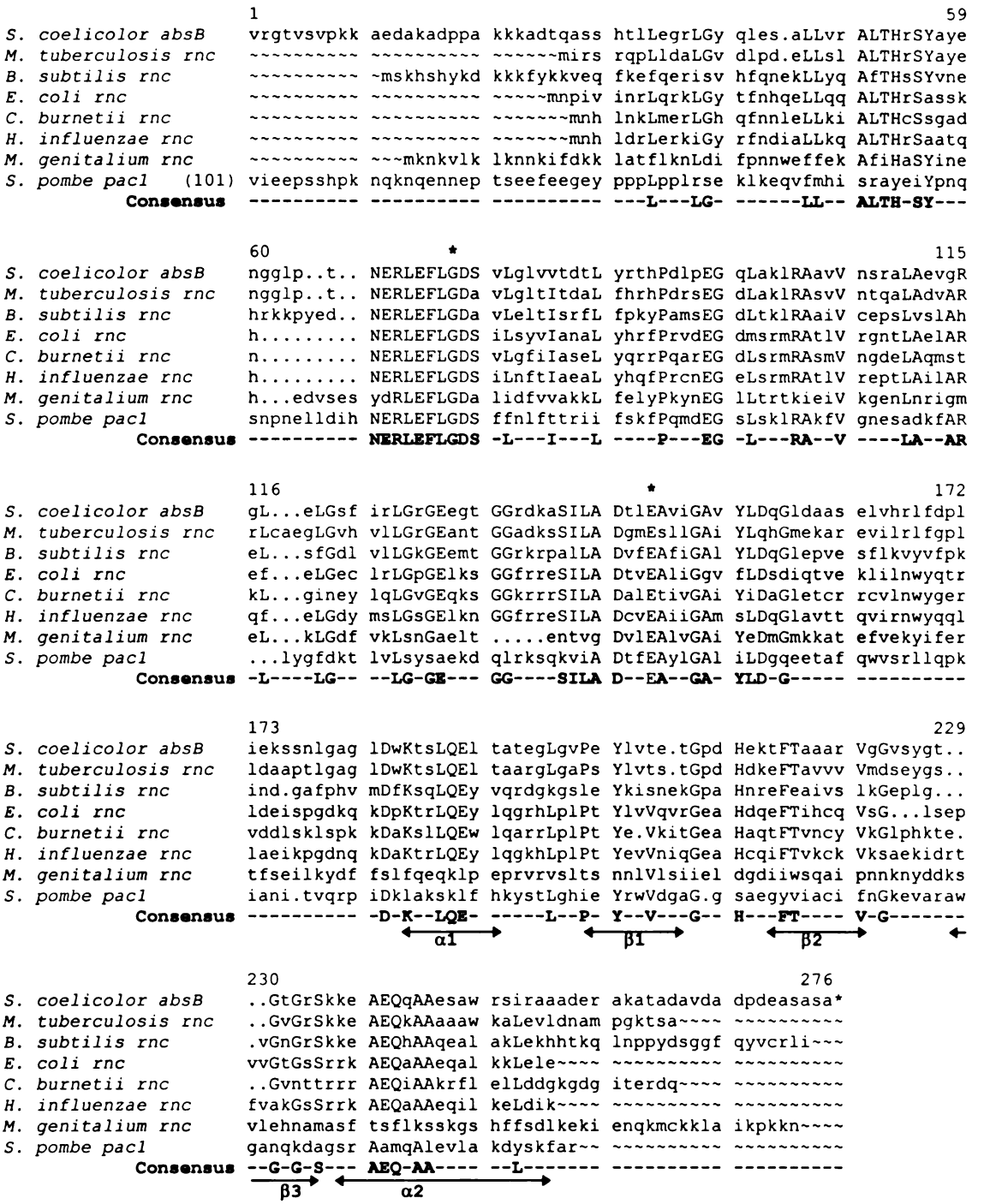

PstI
Primer A ⇒
rbs?
rbs?
GT (C175)
Start of AbsB
P
*
Primer B ⇒
⇐ Primer IF
C (C120)
P
End of AbsB
rbs?
Start of Fpg
⇐ Primer D

Figure 2.4 Amino acid alignment of *absB* and Ribonuclease III homologs. Homologs are listed in the order of highest identity to *absB* (*Mycobacterium tuberculosis* 62.2%, *Bacillus subtilis* 40.9%, *Escherichia coli* 40.9%, *Coxiella burnetii* 39.7%, *Haemophilus influenzae* 38.6%, *Mycoplasma genitalium* 35.0%, *S. pombe* 27.3%). Amino acid numbering is based on the *absB* sequence (first 100 amino acids of *S. pombe pac*1are not shown). Compiled using PILEUP of the U. Wisconsin GCG Group Package, V.9. The consensus is noted for amino acids conserved in ≥ 5 homologs. Residues shown to be important for function in E. coli are shown with (*). The double-stranded RNA binding domain (dsRBD) as defined for *E. coli* (α_1-β_1-β_2-β_3-α_2; (32)) is noted, where α-helices and β-sheets are highlighted with arrows.


1 59
S. coelicolor absB vrgtvsvpkk aedakadppa kkkadtqass htlLegrLGy qles.aLLvr ALTHrSYaye
M. tuberculosis rnc ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~mirs rqpLldaLGv dlpd.eLLsl ALTHrSYaye
B. subtilis rnc ~~~~~~~~~~ ~mskhshykd kkkfykkveq fkefqerisv hfqnekLLyq AfTHsSYvne
E. coli rnc ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~mnpiv inrLqrkLGy tfnhqeLLqq ALTHrSassk
C. burnetii rnc ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~mnh lnkLmerLGh qfnnleLLki ALTHcSsgad
H. influenzae rnc ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~mnh ldrLerkiGy rfndiaLLkq ALTHrSaatq
M. genitalium rnc ~~~~~~~~~~ ~~~mknkvlk lknnkifdkk latflknLdi fpnnweffek AfiHaSYine
S. pombe pac1 (101) vieepsshpk nqknqennep tseefeegey pppLpplrse klkeqvfmhi srayeiYpnq
Consensus ---------- ---------- ---------- ---L---LG- ------LL-- ALTH-SY---
60 115
S. coelicolor absB ngglp..t.. NERLEFLGDS vLglvvtdtL yrthPdlpEG qLaklRAavV nsraLAevgR
M. tuberculosis rnc ngglp..t.. NERLEFLGDa vLgltItdaL fhrhPdrsEG dLaklRAsvV ntqaLAdvAR
B. subtilis rnc hrkkpyed.. NERLEFLGDa vLeltIsrfL fpkyPamsEG dLtklRAaiV cepsLvslAh
E. coli rnc h..... NERLEFLGDS iLsyvIanaL yhrfPrvdEG dmsrmRAtlV rgntLAelAR
C. burnetii rnc n..... NERLEFLGDS vLgfiIaseL yqrrPqarEG dLsrmRAsmV ngdeLAqmst
H. influenzae rnc h..... NERLEFLGDS iLnftIaeaL yhqfPrcnEG eLsrmRAtlV reptLAilAR
M. genitalium rnc h...edvses ydRLEFLGDa lidfvvakkL felyPkynEG lLtrtkieiV kgenLnrigm
S. pombe pac1 snpnelldih NERLEFLGDS ffnlfttrii fskfPqmdEG sLsklRAkfV gnesadkfAR
Consensus ---------- NERLEFLGDS -L---I---L ----P---EG -L---RA--V ----LA--AR
116 172
S. coelicolor absB gL...eLGsf irLGrGEegt GGrdkaSILA DtlEAviGAv YLDqGldaas elvhrlfdpl
M. tuberculosis rnc rLcaegLGvh vlLGrGEant GGadksSILA DgmEsllGAi YLqhGmekar evilrlfgpl
B. subtilis rnc eL...sfGdl vlLGkGEemt GGrkrpalLA DvfEAfiGAl YLDqGlepve sflkvyvfpk
E. coli rnc ef...eLGec lrLGpGElks GGfrreSILA DtvEAliGgv fLDsdiqtve klilnwyqtr
C. burnetii rnc kL...giney lqLGvGEqks GGkrrrSILA DalEtivGAi YiDaGletcr rcvlnwyger
H. influenzae rnc qf...eLGdy msLGsGElkn GGfrreSILA DcvEAiiGAm sLDqGlavtt qvirnwyqql
M. genitalium rnc eL...kLGdf vkLsnGaelt .....entvg DvlEAlvGAi YeDmGmkkat efvekyifer
S. pombe pac1 ...lygfdkt lvLsysaekd qlrksqkviA DtfEAylGAl iLDgqeetaf qwvsrllqpk
Consensus -L----LG-- --LG-GE--- GG----SILA D--EA--GA- YLD-G----- ----------
173 229
S. coelicolor absB iekssnlgag lDwKtsLQEl tategLgvPe Ylvte.tGpd HektFTaaar VgGvsygt..
M. tuberculosis rnc ldaaptlgag lDwKtsLQEl taargLgaPs Ylvts.tGpd HdkeFTavvv Vmdseygs..
B. subtilis rnc ind.gafphv mDfKsqLQEy vqrdgkgsle YkisnekGpa HnreFeaivs lkGeplg...
E. coli rnc ldeispgdkq kDpKtrLQEy lqgrhLplPt YlvVqvrGea HdqeFTihcq VsG...lsep
C. burnetii rnc vddlsklspk kDaKslLQEw lqarrLplPt Ye.VkitGea HaqtFTvncy VkGlphkte.
H. influenzae rnc laeikpgdnq kDaKtrLQEy lqgkhLplPt YevVniqGea HcqiFTvkck Vksaekidrt
M. genitalium rnc tfseilkydf fslfqeqklp eprvrvslts nnlVlsiiel dgdiiwsqai pnnknyddks
S. pombe pac1 iani.tvqrp iDklaksklf hkystLghie YrwVdgaG.g saegyviaci fnGkevaraw
Consensus ---------- -D-K--LQE- -----L--P- Y--V---G-- H---FT---- V-G-------
α1 β1 β2
230 276
S. coelicolor absB ..GtGrSkke AEQqAAesaw rsiraaader akatadavda dpdeasasa*
M. tuberculosis rnc ..GvGrSkke AEQkAAaaaw kaLevldnam pgktsa~~~~ ~~~~~~~~~~
B. subtilis rnc .vGnGrSkke AEQhAAqeal akLekhhtkq lnppydsggf qyvcrli~~~
E. coli rnc vvGtGsSrrk AEQaAAeqal kkLele~~~~ ~~~~~~~~~~ ~~~~~~~~~~
C. burnetii rnc ..Gvnttrrr AEQiAAkrfl elLddgkgdg iterdq~~~~ ~~~~~~~~~~
H. influenzae rnc fvakGsSrrk AEQaAAeqil keLdik~~~~ ~~~~~~~~~~ ~~~~~~~~~~
M. genitalium rnc vlehnamasf tsflksskgs hffsdlkeki enqkmckkla ikpkkn~~~~
S. pombe pac1 ganqkdagsr AamqAlevla kdyskfar~~ ~~~~~~~~~~ ~~~~~~~~~~
Consensus --G-G-S--- AEQ-AA---- --L------- ---------- ----------
β3 α2

III-like protein. C120 contained a C→T transition that changes Leu^{172} to a proline residue. This amino acid lies within the first alpha helix of the double-stranded RNA binding domain (dsRBD) (32). The presence of a proline residue, which is reputed to disrupt the structural integrity of an α-helix (19), could structurally distort the dsRBD and inactivate the enzyme's ability to bind to its target. The C175 allele revealed a two base pair change, the second of which results in a nonsense mutation at Lys^{8} to yield a severely truncated protein.

absB is conserved in other streptomycetes

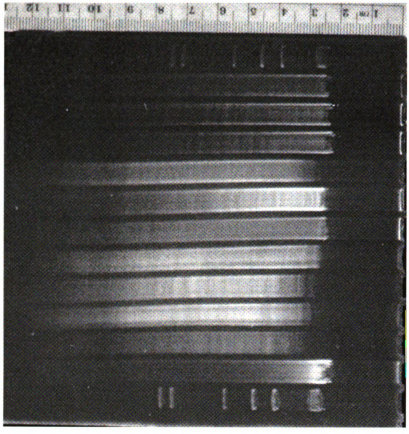
To determine if the *absB* gene is conserved across species, a 1.0 kb *PstI* fragment from sequencing clone pBK802 containing the entire *absB* ORF was used to probe the chromosomal DNA of various streptomycetes (Figure 2.5). A *PstI* digest of prototrophic strain *S. coelicolor* M600, as well as *PstI* and *BglII* digests of the parent strain J1501 were used as positive controls and bands were visible at 2.6 kb and 8.1 kb respectively, as expected from the pTA108 restriction map (Fig. 2.1). In other streptomycete species, a single *BamHI* fragment hybridized to the probe under high stringency conditions. Fragments from *S. albus* (2.4 kb), *S. ambofaciens* 2035 (6.5 kb), *S. avermitilis* (9.1 kb), *S. cinnamonium* (8.8 kb), *S. griseus* (2.7 kb), *S. halstedii* JM8 (2.7 kb), and *S. linclonensis* (1.6 kb) were detected. The *BamHI* digest of *S. lividans* was incomplete, with the *absB* probe hybridizing to the single high molecular weight band. The hybridization conditions used (0.5x SSC, 0.1% SDS at 65°C) predictably exclude probe binding when less than 90% homology exists,

according to the manufacturer. Since the identity between the *absB* gene and the actinomycete *Mycobacterium tuberculosis rnc* gene is 62.2%, it is not surprising that like genes of the streptomycetes would have greater degrees of homology. This data, in conjunction with computer-assisted homology searches, indicates that the putative Ribonuclease III gene is highly conserved across a wide variety of bacteria and lower eukaryotes.

Preliminary evidence for the 30S rRNA precursor in an *absB* mutant strain

One distinguishing characteristic of an *E. coli* RNase III$^-$ mutant is the presence of the 30S precursor rRNA in total RNA preparations (22). This precursor accumulates only in RNase III$^-$ mutants, due to the inefficient processing in the absence of the enzyme. To determine if an rRNA processing deficiency is present in *absB* mutants, RNA isolated at various time points from J1501, C120, and the Abs$^+$ pBK310 recombinant C120-310 was run on a 1% agarose gel. At each time point, an additional rRNA band can be seen in the *absB* mutant, but not in either the wild type or recombinant strain (Fig. 2.6). The migration of the fragment is consistent with 30S rRNA, though its identity has not been confirmed. This result provides preliminary evidence that the putative *Streptomyces* RNase III analog has retained the rRNA processing function defined in other RNase III enzymes and that this function is inhibited in the *absB* mutant strain C120.

Figure 2.5 Hybridization of the *absB* gene to other *Streptomyces* species. A 1.0 kb *PstI* fragment from pBK802 was used to probe digested chromosomal DNA from eight streptomycetes. Digested DNA was run on a 0.7% agarose gel (left) and Southern blotted onto nylon membrane. Probe hybridization was detected using non-radioactive Genius Kit (right). Molecular weight marker (*HindIII*-digested λ DNA) fragment sizes are noted.



M600 / PstI

J1501 / PstI

J1501 / BglII

S. albus / BamHI

S. ambofaciens / BamHI

S. avermitilis / BamHI

S. cinnamonensis / BamHI

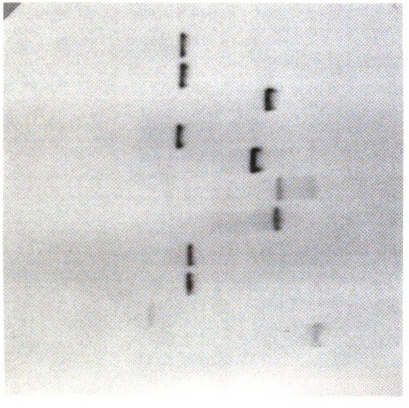
S. girseus / BamHI

S. halstedii / BamHI

S. lincolnensis / BamHI

S. lividans / BamHI

24.1
9.4
6.6
4.4
2.3
2.0



M600 / PstI

J1501 / PstI

J1501 / BglII

S. albus / BamHI

S. ambofaciens / BamHI

S. avermitilis / BamHI

S. cinnamonensis / BamHI

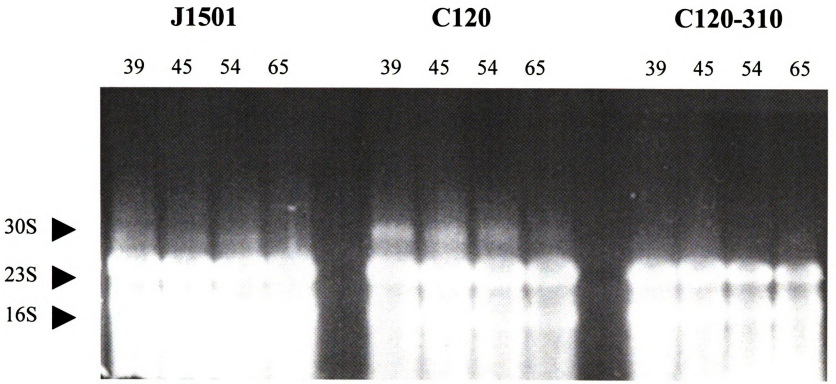
S. girseus / BamHI

S. halstedii / BamHI

S. lincolnensis / BamHI

S. lividans / BamHI

Figure 2.6 Ribosomal RNA processing in the *absB* mutant. Normalized concentrations of RNA from wild type strain J1501, *absB* mutant C120, and C120 rescued to the wild type phenotype with pBK310 (C120-310) were isolated from four time points and run on a 0.7% agarose gel at low voltage for 16 hours, then stained with ethidium bromide. 23S and 16S rRNAs are noted, as well as the putative 30S rRNA that is seen in the C120 (*absB*$^-$) mutant.


J1501
C120
C120-310
39 45 54 65
39 45 54 65
39 45 54 65
30S
23S
16S

Discussion

In the study we have presented evidence that the *S. coelicolor absB* gene, which is involved in the global regulation of antibiotic synthesis, encodes an analogue of Ribonuclease III. Through recombinational rescue and complementation experiments we have shown that three NTG-induced *absB* mutants can be restored to the wild type Abs^+ phenotype by the introduction of the putative RNase III gene. Sequence analysis of two mutant alleles shows that each *absB* mutant harbors a mutation in the putative RNase III gene. One mutation is predicted to prematurely halt translation, and the second would likely alter the secondary structure of the dsRBD. The *absB* gene is highly conserved across species of *Streptomyces*. Finally, preliminary evidence suggests that *absB* mutant C120 is deficient in ribosomal RNA processing as seen for *E. coli* and *B. subtilis* RNase III^- strains, and that this processing ability appears to be restored in the rescued clone C120-310.

Ribonuclease III is a double-stranded RNA specific endoribonuclease that has been best characterized in *E. coli*. RNase III was first identified by its ability to degrade long duplex RNA and to initiate the processing of the 30S rRNA precursor into the mature 16S and 23S subunits (22, 44). The 30S rRNA precursor is only seen in strains devoid of RNase III activity, as the processing occurs very rapidly in wild type strains (22, 45). Surprisingly, *rnc*, the gene that encodes RNase III in *E. coli*, is not essential for cell growth; the mature 16S rRNA is formed, albeit less efficiently, by a redundant mechanism in rnc^- strains (35, 53). In a mutant lacking RNase III, mature 23S rRNA never forms,

but the 50S ribosomes containing immature pre-23S rRNA are competent for translation (36, 52). *E. coli rnc*$^-$ mutants have been shown to grow at a somewhat slower growth rate than *rnc*$^+$ strains. The effect of the mutation on growth rate is variable depending on culture medium and genetic background (5, 34, 54).

A more recent focus of interest is the ability of RNase III to control the regulation of gene expression by processing specific mRNA transcripts. It has been estimated that the abundance of as many as 10% of *E. coli* proteins detectable by gel analysis are either under- or overproduced in the well-characterized *rnc105* mutant (25, 56). Through endonucleolytic cleavage of stem-loop structures within the 5' or 3' noncoding regions of select mRNAs, RNase III is able to up- or down-regulate the expression of the gene(s) post-transcriptionally. In phage λ, RNase III has been shown to cleave a signal stem-loop in the leader RNA of the N protein transcript and in the 3' end of the *int* gene transcript. However, in λN this processing *enhances* λN gene expression by opening up the Shine-Delgarno sequence to translation initiation (31); in contrast, processing at the 3' end of the λ*int* gene *inhibits* gene expression by exposing the transcript to exonucleolytic degradation (48). The T7 phage early and late gene transcripts have RNase III processing signals in intercistronic regions; their specific cleavage yields mature mRNAs that have increased translational activity and stability (20-22). Yet the messages of the *E. coli* polynucleotide phosphorylase, and the *rnc* transcript itself are destabilized upon RNase III processing and are thus negatively regulated by this enzyme (6, 55).

To date, many RNase III processing signals have been identified in *E. coli* and other systems, yet the specific structural and sequence determinants required for RNase III-dependent cleavage have eluded definition. Although stem-loop structures are found in almost all RNA molecules, the vast majority are not processed by RNase III (18).

It has yet to be determined whether RNase III in *Streptomyces coelicolor* actively regulates antibiotic production by specific mRNA processing, or whether the antibiotic production defect of the *absB* mutants is the result of a less direct biological influence. The ability of the *absB* mutants to sporulate normally indicates the lack of any gross disruption in normal cellular differentiation. We have not seen any discernible growth rate difference between the *absB* mutants and the wild type J1501, however more quantitative growth rate experiments under varying culture conditions would determine whether RNase III mutation has any appreciable affect on growth as is seen in the *E. coli* system.

If RNase III does specifically regulate the stability of antibiotic-specific mRNAs, then the targets for such an enzyme would be interesting to study. We have several candidates for potential RNase III targets. One of the most intriguing is the *mia* sequence. *mia*, named for multicopy inhibition of antibiotic production, is defined by a 120 bp fragment that when cloned in a high copy vector and introduced into wild type *S. coelicolor* produces the Abs$^-$ phenotype (50). DNA sequence analysis of this fragment predicts a small open reading frame that is unlikely to be translated, but does contain a 19bp perfect inverted repeat, with a ten basepair loop. Preliminary transcription analysis using an inducible pTipA promoter indicates that the region must be

transcribed to produce the phenotype (12). It is tempting to speculate that an RNA stem-loop structure such a *mia*, expressed in high copy, might sequester the AbsB protein and interfere with its intended function in antibiotic regulation.

We have not excluded the possibility of a direct interaction between AbsB and the activators for antibiotic gene clusters. S1 nuclease protection assays have shown that in the *absB* mutant C120 there are notable decreases in the levels of transcripts encoding the actinorhodin gene cluster activator, *act*II-ORF4, and the undecylprodigiosin activator, *redD*, transcripts (1), which could represent instability of those transcripts in the absence of AbsB. Both *act*II-ORF4 and *redD* have been shown to bypass the *absB* mutants when introduced in high copy. One could speculate that AbsB might be needed for stabilization of these transcripts, perhaps to reach a certain threshold after which antibiotic production can occur. Both of these gene transcripts have substantial stem-loop structures reported in noncoding regions (23, 43). A 25bp perfect inverted repeat has been reported within the 3' noncoding region between *act*II-ORF4 and *act*III; *in vitro* studies of this sequence and purified AbsB could determine if any interaction exists.

The presence of a 22bp perfect inverted repeat in the 3' noncoding region of *absB* poses the possibility that this region could function as an autoregulatory site. In *E. coli*, RNase III processes a signal in its own transcript to negatively autoregulate its own expression (6, 41). While the autoregulatory signal for *E. coli rnc* is located in the 5' end of the operon, in other RNase III-regulated genes the processing signals have been located in the 3' end of transcripts. Northern and Western analysis of the *absB* gene product in wild

type and *absB* mutant strain C120 could discern whether the gene is autoregulated.

Preliminary functional characterization of the putative RNase III in *Streptomyces coelicolor* comes from analysis of preparations of ribosomal RNA from the wild type J1501 and *absB* mutant strains. A large RNA corresponding in size with the 30S rRNA precursor can be seen in the *absB* mutant C120, but not in the wild type J1501 or in C120(*absB*) recombinationally rescued to the Abs^+ phenotype with pBK310 (C120-310). This result suggests that the *absB* gene has retained the function of 30S precursor rRNA processing in addition to any possible function in antibiotic production. Upon isolation of the AbsB protein, this presumed 30S ribosomal RNA processing can be specifically investigated *in vitro*.

If future experiments show the direct involvement between the putative RNase III and antibiotic-specific transcripts, this would represent the first evidence of regulation of antibiotic production through mRNA stability. To date many types of genes in *E. coli*, coliphage, and *B. subtilis* and its phage have been shown to be processed by RNase III, though no streptomycete genes related to antibiotic production have been shown to be post-transcriptionally regulated in this way.

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CHAPTER 3

Characterization of the *mia* Locus, a Multicopy Inhibitor of Global Antibiotic Production in *Streptomyces coelicolor*

Abstract

The goal of this collaborative project is to determine the mechanism by which an antibiotic regulatory locus, named *mia*, causes multicopy inhibition of global antibiotic synthesis. This genetic element inhibits the synthesis of at least three of the four antibiotics produced by *Streptomyces coelicolor* when cloned into a high copy vector and introduced into that strain; this phenotype has been designated Abs^-. Emphasis has been placed on determining whether *mia* sequesters a regulatory element, exhibits a gene dosage effect, or expresses a regulatory RNA that is exerting an effect on the global regulation of these antibiotics. Elucidating this mechanism could contribute to a better understanding of the regulation of secondary metabolites in *Streptomyces*.

Introduction

Antibiotic production by *Streptomyces* spp. has been the subject of intensive investigation for several decades. Driven by commercial and academic interest, a wealth of information about the mechanisms by which this genus synthesizes a vast arsenal of antibiotics and other secondary metabolites has been documented. With this knowledge, researchers strive to understand and possibly manipulate the genetics of antibiotic producing strains to enhance yield and perhaps develop new antibiotic derivatives through genetic engineering (11).

Efforts to commercially exploit antibiotic production by *Streptomyces* has provided an abundance of information about growth and nutritional conditions required for optimal antibiotic production. Trial and error mutagenesis screenings have yielded strains that produce antibiotics in hundreds or even thousands times greater quantities than the original soil isolates. Despite this, production strains still regulate the production of antibiotics to occur late in the life cycle at the onset of stationary phase. The mechanisms by which streptomycetes regulate antibiotic production remain a relative mystery, but understanding them may provide a new approach to the rational design of overproducing strains (10).

Most of the current understanding of secondary metabolism regulation comes from the study of developmental mutants in *Streptomyces coelicolor* which are blocked at various stages of antibiotic production pathway. One class of mutants thought to represent the very earliest stages of secondary metabolism are the "bald" mutants. These mutants are blocked in the

production of all four *S. coelicolor* antibiotics and in morphological differentiation (sporulation) (13). Under certain growth conditions morphological differentiation in these mutants can sometimes be restored, however antibiotic production is not typically affected. This suggested that there might be another level of control that affects antibiotic production exclusively.

The work by the Champness laboratory has focused mainly on a class of developmental mutants named *abs* for "antibiotic synthesis deficient". These mutants are able to sporulate, but are not able to produce antibiotics (9). A screen of 800,000 mutagenesis survivors led to the identification of two distinct *abs* mutant types, *absA* and *absB* (1, 2). These mutations were physically and genetically mapped to two distinct loci on the chromosome (Fig. 1.1). Each locus has been cloned and elucidation of the mechanisms by which they regulate antibiotic synthesis is currently being conducted ((4); see Chapter 2).

While the *absB* mutants were isolated at a frequency of 10^{-4}, the *absA* mutants appeared to be relatively rare because they were found at a frequency of 5×10^{-6} (2). This rarity raised the possibility that the *absA* mutant alleles could be dominant. Thus one attempt to clone the *absA* locus involved constructing a *BglII* library of *absA* mutant DNA, introducing it into an Abs^+ recipient, and screening for the Abs^- phenotype. Although no $absA^-$ clones resulted from this approach, a 2 kb *BglII* fragment was isolated (> 40 independent times) that conferred the Abs^- phenotype in *S. coelicolor* when cloned into the high copy vector pIJ702 (15). When this 2 kb fragment was cloned into the low copy vector pIJ922, no Abs^- clones resulted. A homologous fragment was also isolated from wild type *S. coelicolor* which exhibited the Abs^-

phenotype in high copy, thus the absA$^-$ background was irrelevant to the phenotypic effect. The element was therefore named *mia* for multicopy inhibition of antibiotic synthesis. This fragment was physically and genetically mapped to the 2 o'clock position on the genome, distinct from both the *absA* and *absB* loci (Fig. 1.1). To insure that all elements responsible for the Abs$^-$ phenotype were contained within the 2 kb fragment, *mia* was cloned into a high copy vector with terminators flanking the cloning site. This construct also conveyed an Abs$^-$ phenotype to wild type J1501. Therefore no read-through vector transcription is required for the phenotypic effect of the 2 kb *mia* fragment.

The Abs$^-$ phenotype conferred by *mia* was not a strain-specific effect. The *mia* fragment in multicopy induced the Abs$^-$ phenotype in each of four *S. coelicolor* strains tested. The *mia* fragment was also put into a high copy transmissible vector and mated into the host, and the phenotype was shown to be the same regardless of the method by which *mia* was introduced. To determine whether antibiotic production in other *Streptomyces* species could be similarly affected, *mia* was introduced into *S. peucetius*. This species also produces multiple antibiotics, including daunorubicin, which has an activator (*dnrI*) that is homologous to the *act*II$^-$ORF4 activator of the *S. coelicolor* antibiotic actinorhodin. With *mia* in high copy, *S. peucetius* also demonstrated an Abs$^-$ phenotype. The results of a *Staphylococcus aureus* killing assay suggested that in addition to daunorubicin, at least one other polyether antibiotic was inhibited as well. Therefore, the global regulatory mechanism of *mia* may be a common phenomenon across different streptomycetes.

Delimiting the *mia* locus

The 2 kb *BglII* fragment was reduced to a 1.2 kb *BglII-SacI* fragment which also gave rise to the Abs$^-$ phenotype in high copy. This DNA fragment was sequenced (Lark Technologies) and the sequence analyzed using Wisconsin Package Version 9, GCG, Madison, WI to construct further directed deletions. The 1.2 kb *mia* sequence contained a 819 nucleotide partial open reading frame (ORFY) truncated at the 3' end (Fig. 3.1, 3.2). However this ORF was shown to be unessential for the phenotype after a deletion clone that contained 367 bp of *mia* upstream of ORFY and a clone in which this ORF was disrupted by an out-of-frame deletion each still conveyed the Abs$^-$ phenotype.

Plasmid clones containing the 367 bp *Sau3A* fragment upstream of the ORF and the 242 bp *Sau3A-SmaI* fragment each generated the Abs$^-$ phenotype in high copy, while the 125 bp *SmaI-Sau3A* did not give the Abs$^-$ phenotype (Fig. 3.1). The results of these cloning experiments defined the minimal sequence shown to give the Abs$^-$ phenotype as the 242 bp *BglII-SmaI* fragment. Sequence analysis of this region revealed several features. A small 18 or 20 amino acid ORF was predicted using CODONPREFERENCE, but the codon usage is such that it is probably not translated. Also the fragment contained the 3' end of another gene, designated ORFA. This truncated gene showed a high degree of homology to α-galactosidase in the protein databases (6). Finally, structural analysis revealed a 48 bp region containing a 19 bp perfect inverted repeat. This stem loop structure was positioned 17 bp downstream of the ORFA stop codon and may possibly function as a rho-independent terminator for the ORFA gene although this has not been demonstrated..

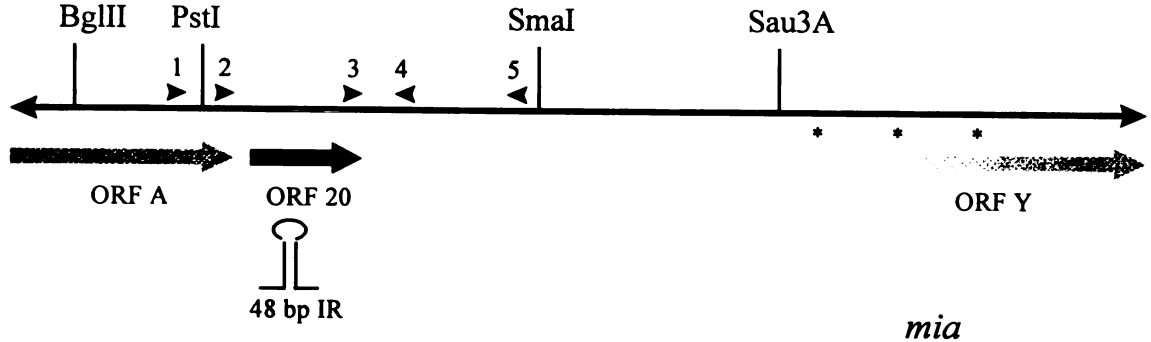
Figure 3.1 Features of the *mia* locus. Relevant restriction sites within the *mia* locus are noted relative to predicted open reading frames (arrows). ORFY contains three possible GTG start sites (*). The relative location of the 48 bp perfect inverted repeat region is shown. Fragments cloned into high copy vector pIJ702 to evaluate the *mia* phenotype (Abs^-) are shown. The first three clones were generated by restriction digestion (P. Riggle), and the lower four were generated by PCR amplification using primers 1-5 (G. Brown). Primer sites are noted with arrowheads

(➤). Preliminary promotor activity analysis is noted:

[a] Promoter activity evaluated in *xylE* vector; promoter activity in the leftward orientation only.

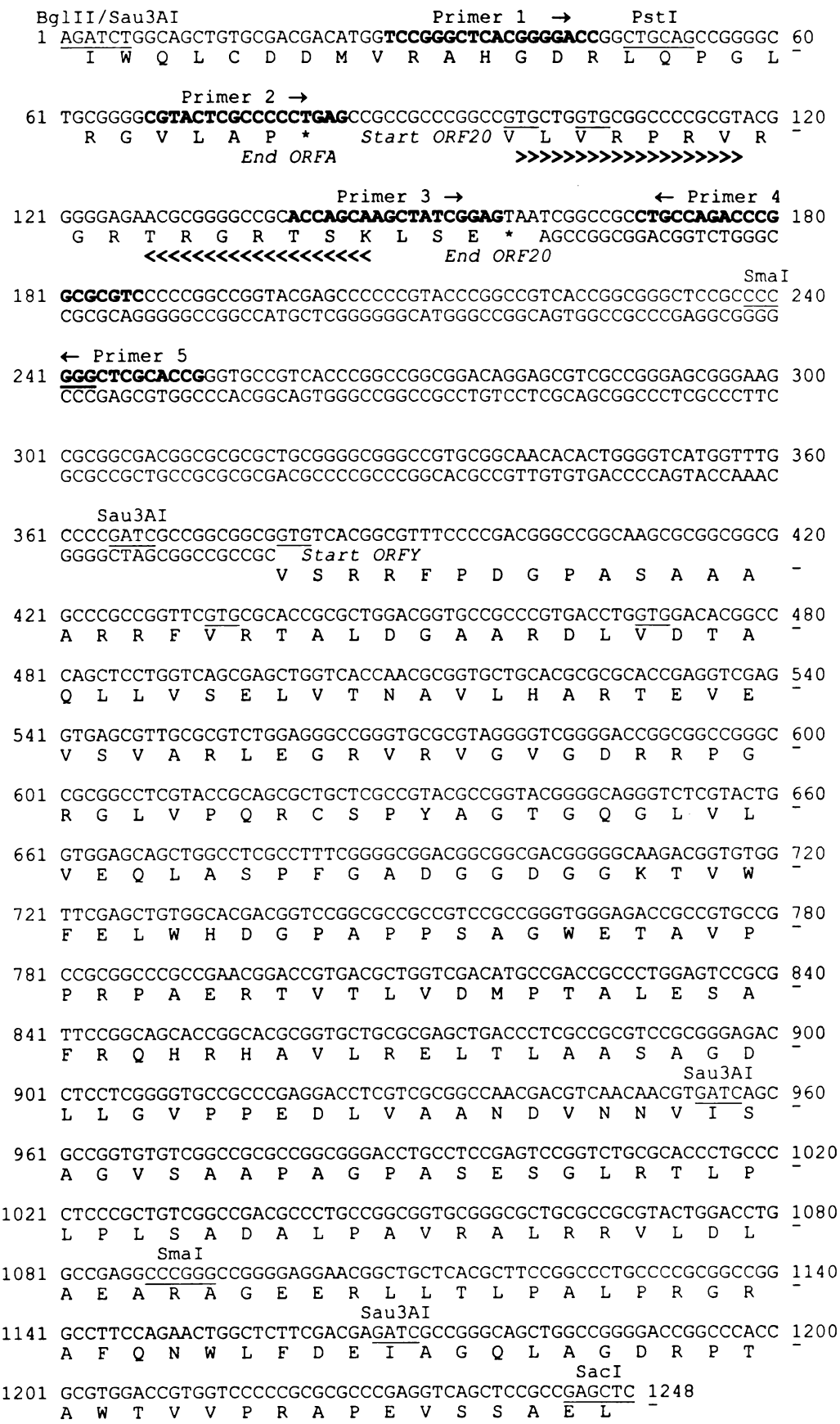
[b] Promoter activity evaluated in *xylE* vector; orientation unknown.

[c] Promoter activity evaluated in promoterless Neo^r vector, orientation unknown.



fragment cloned in pIJ702	*mia* phenotype	promotor activity
367 bp Bgl/Sau	+	+[a]
242 bp Bgl/Sma	+	+[b]
125 bp Sma/Sau	–	–[b]
PCR 1/5	+	ND
PCR 1/4	+	ND
PCR 2/4	+	+[c]
PCR 3/5	–	ND

Figure 3.2 Nucleotide and amino acid sequence of the *mia* locus (5, 16). The *BglII* - *SacI* region including the minimal *mia* sequence is shown. Primer sequences used in cloning are highlighted in boldface. Start sites for ORF20 and ORFY are underlined. The location of the 19 bp perfect inverted repeats are denoted (>> <<).


BglII/Sau3AI
Primer 1 →
PstI
Primer 2 →
End ORFA
Start ORF20
Primer 3 →
← Primer 4
End ORF20
SmaI
← Primer 5
Sau3AI
Start ORFY
Sau3AI
SmaI
Sau3AI
SacI

In an attempt to further delimit the *mia* locus, PCR generated fragments were cloned into pIJ702 (15). The smallest fragment able to convey the Abs$^-$ phenotype in multicopy was amplified from primers 2 and 4, to yield a 120 bp insert (Fig. 3.1). This fragment retained the ORF20 and the 48 bp inverted repeat region, but eliminated all but the last four amino acids of ORFA.

Features of the mia locus

Promoter probing of the *mia* locus.

The 367bp *BamHI-Sau3A mia* fragment was cloned into *xylE* reporter vector pXE4 in each direction to determine whether the fragment contained any promoter activity. The fragment showed promoter activity by producing yellow pigment in the presence of catechol only when cloned in the leftward orientation (see Fig. 3.1). Thus there appears to be a promoter directing transcription away from ORF20 and ORFY. Because this assay was qualitative, the possibility of promoter activity directing transcription towards the ORFs cannot be ruled out. Alternatively, the ORFY promoter may be contained between the *Sau3A* site and any of the three possible GTG start sites (between 15-104 bps).

The 242 bp and 125 bp fragments were also tested for promoter activity (Fig. 3.1). The 242 bp fragment was shown to contain promoter activity when cloned in front of the promoterless *xylE* gene, while the 125 bp fragment did not. In this preliminary experiment the orientation of the fragments was not determined (16).

Additionally, the 120 bp PCR-generated fragment (2/4) was cloned into a plasmid vector, pIJ487, in front of a promoterless neomycin resistance gene. This fragment was able to drive the expression of the Neo^r gene to permit growth on the antibiotic, but the orientation of the fragment has not yet been determined (Fig. 3.1; (5)).

While more detailed characterization of promoter activity is required, these preliminary experiments indicate that the small *mia* fragment is a complex sequence.

Secondary structure of the *mia* sequence.

Secondary structure analysis of the small 120bp minimal *mia* sequence reveals a complex predicted structure centered around the 19 bp inverted repeat. The RNAFOLD (Wisconsin Package Version 9, GCG, Madison, WI) predicted secondary structure is pictured in Figure 3.3.

Possible mechanisms for *mia*

The phenomenon of multicopy inhibition of the antibiotic synthesis by the *mia* locus suggested three possible mechanisms. First, the *mia* locus could encode a gene whose product, when overexpressed on a multicopy vector, exerts an inhibitory effect. Examples of a similar outcome include the *spoOF* gene product of *Bacillus subtilis* which was shown to inhibit sporulation when cloned in multicopy (12), and *nif* gene expression in *Klebsiella pneumoniae* was shown to be inhibited by the overexpression of the

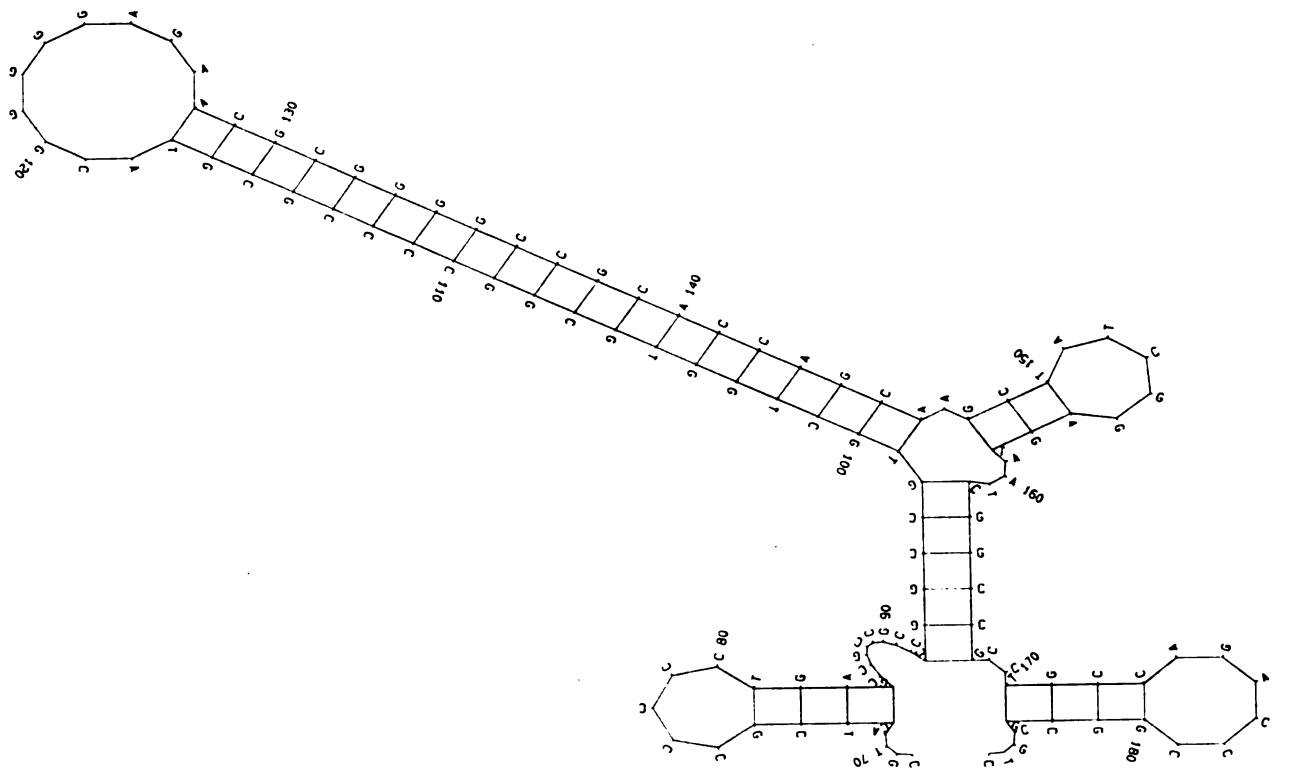


Figure 3.3 Secondary structure prediction of the 120 bp minimal mia sequence. RNAFOLD of the Wisconsin Package Version 9, GCG, Madison, WI was used to compute the structure, which has a ΔG of -57.3.

NifL protein from a multicopy clone (7). However, results to date suggest that this is not a likely mechanism for *mia*. The ORF20 located within the minimal *mia* sequence contains several rare codons that are not likely to be translated in *Streptomyces*. In a second possible mechanism, the *mia* DNA sequence could contain a binding site which, in high copy, titrates out a positive regulatory factor, as was shown for *spoVG* of *B. subtilis* (3) and for the nitrogen fixation genes *nifH* and *nifU* of *K. pneumoniae* (8). This mechanism also allowed for the isolation of the σ^{WhiG} - dependent promoters in *S. coelicolor* by titrating out the σ^{WhiG} RNA polymerase holoenzyme when cloned in high copy to give a "white" phenotype (20). Preliminary evidence suggests however that transcription of the *mia* locus is required for the Abs$^-$ phenotype. When the *mia* sequence was cloned downstream of the thiostrepton-inducible pTipA promoter, the Abs$^-$ phenotype was seen only in the presence of thiostrepton, which induces transcription of the locus (6). This would suggest that a transcript is required for the *mia* effect. In a third possible mechanism for *mia*, the inhibition could be the result of an overexpressed regulatory RNA. Through multicopy inhibition, the *micF* gene of *Escherichia coli* was shown to produce an antisense RNA that bound to the translational start region of the *ompF* mRNA to inhibit its translation. This system, in wild-type *E. coli*, is thought to represent a translational control mechanism for osmoregulation (14). A similar antisense RNA mechanism was discovered for IS10 of transposon Tn10, through multicopy inhibition (19). A regulatory RNA has been isolated from *S. fradiae* which activates the production of actinorhodin when cloned in high copy in *S. lividans* (18). Although the *S. lividans* genome does encode the actinorhodin gene cluster, Act is not normally produced by that strain. This

132 nt RNA has been predicted to function as an antisense RNA. An analogous gene, encoding an 86 nt transcript, has been isolated from *S. lividans* and similarly induces Act production in high copy in that strain (17). While the phenomenon of multicopy inhibition has proven to be a useful tool in the isolation of novel genes, an understanding of the mechanism could lead to the discovery of new regulatory pathways. Furthermore, the fact that this antibiotic inhibition by *mia* is global, affecting distantly located gene clusters, makes the mechanism of *mia* all the more intriguing.

Future directions for the characterization of *mia*

Future experiments will focus on deciphering which of the above mechanisms is integral to *mia*. To discern whether a gene product (either an RNA or a protein) is needed for the *mia* effect, further characterization of the fragment downstream of the pTipA inducible promoter will be performed. If the minimal *mia* fragment, cloned downstream of the chromosomally-integrated pTipA, is able to induce the Abs$^-$ phenotype in one orientation only, then either a regulatory RNA structure or the ORF20 product could be required. However, the ability of the fragment to induce the Abs$^-$ phenotype in both orientations would suggest involvement of the stem loop, as the structure would not be affected by the orientation of the cloned insert. Additionally, site-directed mutagenesis could eliminate the ORF20 while maintaining the structural integrity of the stem loop formed by the 19 bp inverted repeat, since a single base change within the loop region ($A^{125} \rightarrow T^{125}$, Fig. 3.2) would create a stop

codon within ORF20 while maintaining the stem loop structure. If this sequence is still capable of conveying the Abs- phenotype, then the stem loop structure is more likely to be the crucial element involved in the *mia* effect.

If the results of the above experiments suggest the involvement of the stem loop structure in the Abs$^-$ phenotype, then the possibility of interaction between the *absB* gene product and *mia* will be investigated. The *absB* gene, when mutated, results in global blockage of antibiotic production. The *absB* gene encodes a putative Ribonuclease III homolog (see Chapter 2). RNase III, as it has been characterized in *E. coli* and *B. subtilis*, is an endoribonuclease that cleaves duplex RNA. It functions in the processing of rRNA precursor into the 16S and 23S subunits, and also processes certain mRNA transcripts to regulate gene expression by modulating mRNA stability. The substrate for this enzyme *in vivo* is the stem region of particular stem loop structures. While most RNA molecules form stem loops, only a small fraction are recognized by RNase III. Yet the specific features required for enzyme recognition have eluded definition. It appears that sequence and structural components are important.

It is tempting to speculate that the *mia* stem loop could be a substrate for the AbsB protein. If AbsB is required for antibiotic regulation, the *mia* stem loop in high copy might titrate out the protein resulting in the Abs$^-$ phenotype. Several experiments could determine if *mia* and the AbsB protein interact. Cloning the *absB* gene in multicopy into the intergrated pTipA-*mia* construct may determine whether the *mia* stem loop sequesters the AbsB protein. If *mia* titrates limited concentrations of AbsB in the cell, then overexpression of the AbsB protein in the *mia* clones might relieve the *mia* effect. Also, in the *absB* mutant C120, rRNA processing appears to be adversely affected. As is seen for

RNase III mutants in other systems, the 30S rRNA precursor molecule is seen in the *absB* mutant indicative of inefficient rRNA processing. While additional experiments need to precisely define this rRNA processing deficiency, it would be of interest to determine whether rRNA processing is affected in wild type *S. coelicolor* carrying *mia* in high copy. If *mia* titrates the AbsB protein from a presumed function in antibiotic production, it might also sequester AbsB from rRNA processing function.

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CHAPTER 4

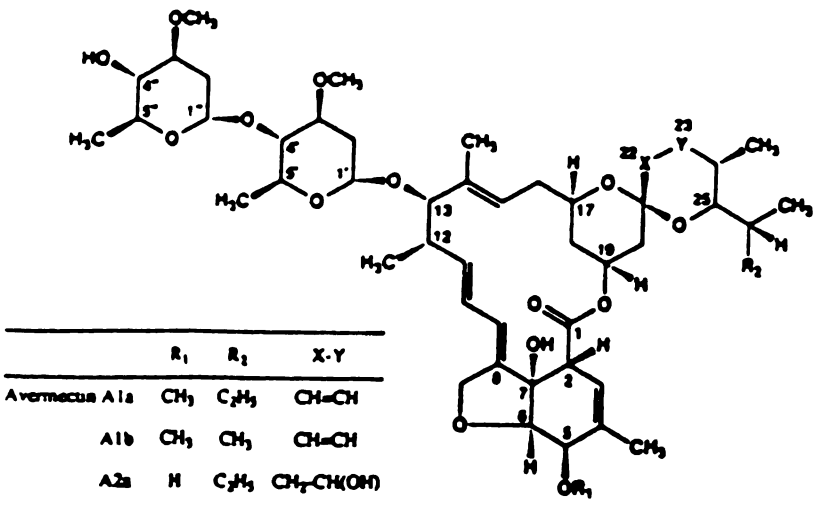
The Regulation of Doramectin Production in *Streptomyces avermitilis*: A Biotechnology Training Program Internship

INTRODUCTION

The Biotechnology Training Program at Michigan State University enrolls students who have a career interest in biotechnology within a wide range of fields. The program, partially funded by the National Institutes of Health, offers students the opportunity to explore the specific problems of biotechnology that have current industrial relevance. To do this, students take biotechnology-related coursework and participate in a biotechnological research internship for two semesters. This internship is arranged by the student, his/her mentor, and an industry researcher. Research performed during the internship is preferably directly-related to the student's thesis program, though not a necessity.

This manuscript describes research conducted as a requirement for the Biotechnology Training Program in the laboratory of Dr. Kim Stutzman-Engwall at Pfizer Inc, in Groton CT. Pfizer Inc. is one of the worlds leading pharmaceutical companies in human and animal health. Dr. Stutzman-Engwall's research focuses on the genetics of secondary metabolites produced by *Streptomyces avermitilis* that are used in the animal health industry.

S. avermitilis was shown in 1979 to produce a family of eight closely related compounds with anthelmintic properties called the avermectins (Fig. 4.1) (1). Each compound differs in composition at positions C5, C22-23, and C26 according to the following designations: "A" or "B" denote -CH3 or -H at R1



	R_1	R_2	X-Y
Avermectin A1a	CH_3	C_2H_5	CH=CH
A1b	CH_3	CH_3	CH=CH
A2a	H	C_2H_5	CH_2-CH(OH)
A2b	H	CH_3	CH_2-CH(OH)
B1a	CH_3	C_2H_5	CH=CH
B1b	CH_3	CH_3	CH=CH
B2a	H	C_2H_5	CH_2-CH(OH)
B2b	H	CH_3	CH_2-CH(OH)
Ivermectin B1a	H	C_2H_5	CH_2-CH_2
B1b	H	CH_3	CH_2-CH_2

Figure 4.1 The general structure of the avermectin family. Variations in the structure of each compound is noted. Adapted from (3).

respectively; "1" or "2" denote -CH=CH- or -CH2-CHOH at X-Y; and "a" or "b" denote -CH2CH3 or -CH3 at R2. They also undergo a glycosylation step that adds the disaccharide oleandrose to the C13 position. Characterization of these compounds has revealed a broad spectrum of activity against various nematode and arthropod parasites, thus increasing the commercial importance of these compounds.

Doramectin, commercially marketed as Dectomax™, is one derivative of the avermectins that has exhibited expansive market growth since its U.S. launch in June, 1996. Currently marketed in an injectable form for the eradication of parasites in cattle, Dectomax™ sales in the first quarter of 1997 increased 125% to $27 million over fourth quarter 1996 earnings. According to the Pfizer 1996 Annual Report, the projected market for this product is $1.6 billion dollars worldwide. Currently under regulatory review are injectable forms for sheep and swine, and topical ("pourable") applications for cattle. The introduction of these products will insure Dectomax's position as the keystone product for the Animal Health Care Division earnings.

The avermectins (Avm) are produced by a type I polyketide synthase (PKS) which is a large multifunctional protein complex and is thought to function analogously to type I fatty acid synthase (FAS) (2). The avermectins are produced by the stepwise addition and condensation of 12 acyl units (5 proprionates and 7 acetates) to a starter unit of isobutyl or 5-methyl butyryl, followed by cyclization of the 16 membered ring (4).

The gene cluster for Avm biosynthesis is 95kb in length, and has a highly organized structure (Fig. 4.2) (4). Each of the two PKS subunits contain 6 modules; these modules specify the condensation and modification of each acyl unit addition. Flanking the PKS subunits are the genes for the glycosylation of the avermectins, and the C5 O-methyl transferase.

While the biosynthesis of the avermectins has been the subject of intensive investigation, relatively little is known about how the PKS gene cluster is regulated in *S. avermitilis*. Like other streptomycete secondary metabolites, the production of avermectin is regulated to the onset of stationary phase. In 1993, MacNeil et. al. published a paper in which they made a host of gene cluster replacements throughout the avermectin locus to functionally map the region (5). Three of the clones containing deletions to the left of the PKS gene completely inhibited avermectin production and were not able to be complemented by the addition of any avermectin intermediates. They concluded that this region might contain a pleiotropic regulator for avermectin production. The minimum deletion giving this phenotype was 7.8 kb. In 1995, Ikeda et.al. published a similar finding, in which a 10 kb deletion upstream of the PKS (overlapping the 7.8 kb fragment of (5)) was not able to be complemented (3). They noted that mutants with this deletion could not methylate or glycosylate avermectin substrates even though these genes were intact in the clone. They therefore concluded that a positive regulator may reside here. Thus two researchers independently identified an 8 kb region

upstream of the PKS cluster as a putative regulatory region based on the fact that clones with deletions in this region had no PKS, OMT, ring formation, or glycosylation activity and could not be rescued by the addition of any intermediate substrates. This region was named *aveR*.

Project Goals

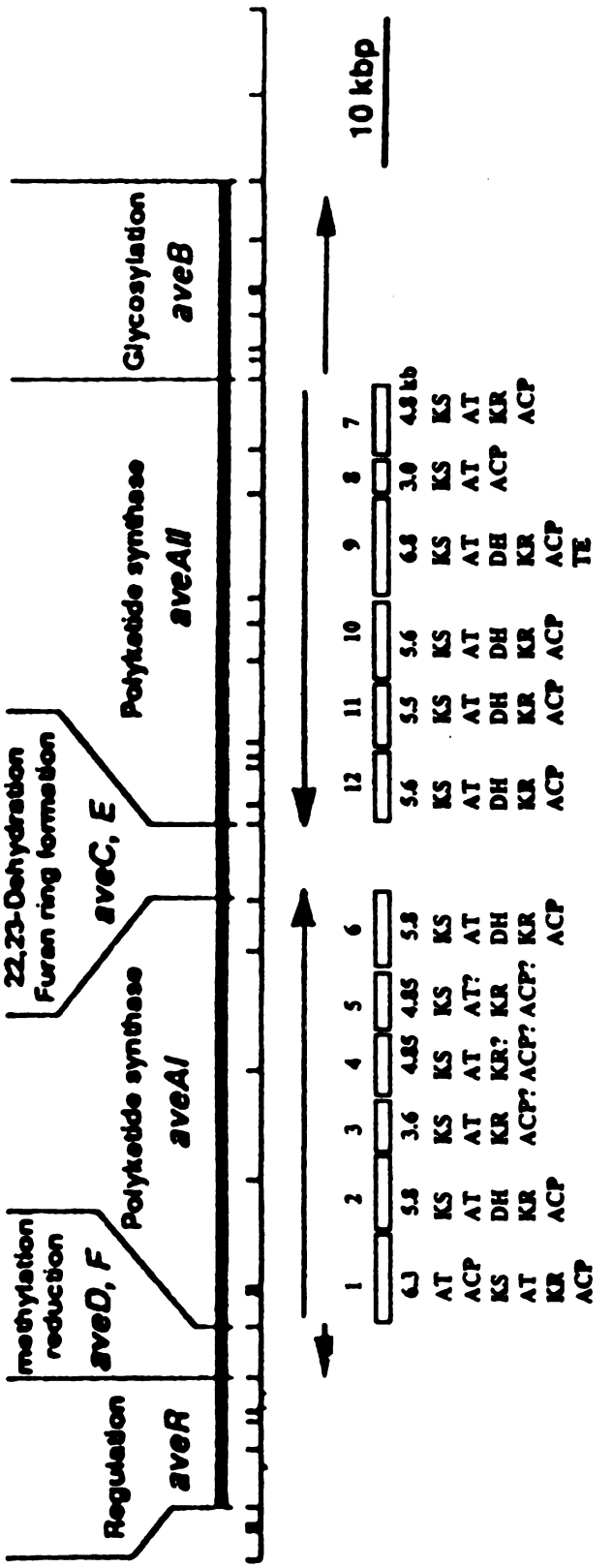
Characterization of the *aveR* region.

In an attempt to identify any regulatory elements of PKS, the *aveR* region was studied further. A multi-pronged approach was designed to study PKS regulation during the internship. The first strategy was to construct and sequence various plasmid clones containing subfragments of the *aveR* region. Sequence analysis of the *aveR* DNA would identify any putative regulatory genes for targeting in subsequent deletion clone construction.

In lieu of DNA sequence data, detailed restriction mapping of the *aveR* region revealed potential sites for the construction of deletion subclones. Gene replacement with the erythromycin cassette, *ermE* was planned to generate various knockout mutants within this region.

One final project involved developing a tool for studying any perturbations of PKS regulation. The avermectins, unlike Act and Red for

Figure 4.2 Physical map of the gene cluster for avermectin biosynthesis in *Streptomyces avermitilis*. Arrows denote transcripts of genes needed for avermectin biosynthesis: *aveA1* and *aveA2* encode PKS. Each PKS transcript contains six modules (boxes) and each module encodes functions as noted (AT, acyltransferase; ACP, acyl carrier protein; KS, β-ketosynthase; KR, β-ketoreductase; DH, dehydratase; TE, terminal thioesterasease). Adapted from (3,5)


Regulation
aveR
methylation
reduction
aveD, F
Polyketide synthase
aveAI
22,23-Dehydration
Furan ring formation
aveC, E
Polyketide synthase
aveAII
Glycosylation
aveB
10 kbp
1 2 3 4 5 6 12 11 10 9 8 7
6.3 5.8 3.6 4.85 4.85 5.8 5.6 5.5 5.6 6.8 3.0 4.8 kb
AT KS KS KS KS KS KS KS KS KS KS KS
ACP AT AT AT AT? AT AT AT AT AT AT AT
KS DH KR KR? KR DH DH DH DH DH ACP KR
AT KR ACP? ACP? ACP? KR KR KR KR KR ACP
KR ACP ACP ACP ACP ACP ACP ACP ACP TE
ACP

S. coelicolor, are not pigmented compounds. Assays for the presence of the avermectins require a three week long fermentation followed by several intensive days of product isolation, and finally, characterization by HPLC. In an attempt to simplify a method for determining PKS expression, a method for generating a *xylE* fusion to the PKS was designed.

Results and Discussion

Of the above approaches, the sequencing project proved to be the most successful. Five plasmid subclones containing various fragments of the *aveR* region were submitted for sequencing, carrying a total of 11.5 kb of insert DNA. Results were compiled with previously determined sequence, to yield 12.9 kb of continuous DNA sequence inclusive of the *aveR* region. This DNA sequence was analyzed for putative open reading frames using the Wisconsin Package, v. 9, GCG Group. Seven open reading frames were predicted, as shown in Figure 4.3. (Details of the sequence have been withheld pending publication of the final results.) When compared to deletion clones reported previously (3, 5), the Reg1 ORF probably lies outside the boundary of the PKS locus, as deletions in this region were reported to have no effect on Avm production. Therefore the six remaining ORFs had potential involvement in Avm production. Computer-assisted homology searches were carried out for each ORF to attempt to gain

Figure 4.3 Schematic diagram of the *aveR* region. Open reading frames predicted by CODONPREFERENCE are indicated with arrows. Previously characterized genes are shown as hatched arrows and are labeled: PKS, polyketide synthase; OMT, O-methyl transferase; KR ketoreductase.

1kb

clues about their possible functions. Four of the seven genes showed homology to proteins in the protein databases that were reportedly involved in secondary metabolism in other systems. The contribution of each of these genes to Avm production is under current investigation. The remaining three ORFs had no homologs in the databases.

***xylE* - PKS reporter fusion**

To generate a *xylE*-PKS fusion strain, a *xylE* cassette and the PKS Module 1 were sequenced. This provided the information needed to design an in frame insertion of the *xylE* cassette within the PKS transcript. One caveat to this project was the fact that recombination within the PKS region is extremely low, presumably a necessity given the extensive homologies between each module in the PKS locus (Fig. 4.2). This fact made insertion of the *xylE* cassette within this coding region technically more difficult. Though not completed during the internship, this project has been continued by another researcher. The construction of this reporter gene fusion would simplify the assay for PKS expression from a costly four week procedure down to five days required for growth of the strain.

Conclusions

The goal of this internship was to experience scientific research from a market-driven viewpoint. The opportunities to participate in strategy sessions, quarterly report meetings, and budget planning sessions afforded me that opportunity.

In addition, I was given the opportunity to apply my knowledge of secondary metabolite regulation in *Streptomyces* to a commercial strain that generates millions of dollars each year in company revenues. While the preliminary results of this work are awaiting completion to be submitted for publication, it will be interesting to see if perturbations of any of the genes discovered will affect avermectin production levels, or have any effect on the final product.

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