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David J. Aceti

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Wendy Champness
Major professor

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TRANSCRIPTIONAL REGULATION OF *STREPTOMYCES COELICOLOR* ANTIBIOTIC-SPECIFIC REGULATORY GENES

By

David J. Aceti

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ABSTRACT

TRANSCRIPTIONAL REGULATION OF *STREPTOMYCES COELICOLOR* ANTIBIOTIC-SPECIFIC REGULATORY GENES

By

David J. Aceti

Antibiotics produced by streptomycetes are of great medical value and may play an important role in soil ecology. The regulatory mechanisms governing production of these antibiotics are not well understood. In the model organism *Streptomyces coelicolor*, all four antibiotics produced are affected by mutations in a putative two-component signal transduction system encoded by *absA*1/*absA*2 and by mutations in a putative RNAase encoded by *absB*. S1 nuclease protection assays were used to assess whether these loci control synthesis of the antibiotics actinorhodin and undecylprodigiosin by regulating transcript abundance from biosynthetic and regulatory genes specific for each antibiotic. Strains that were antibiotic-minus due to mutations in *absA* or *absB* were examined. In the *absA* mutant strain, transcripts for the actinorhodin biosynthetic genes *act*VI-ORF1 and *actI*, and for the pathway-specific regulatory gene *act*II-ORF4, were substantially lower in abundance than in the parent strain. The level of transcript for the undecylprodigiosin pathway-specific regulatory gene *redD* was similarly reduced in this mutant. Additionally, a strain that exhibits precocious hyperproduction of antibiotics (Pha phenotype) due to disruption of the *absA* locus contained

elevated levels of the *act*VI-ORF1, *act*II-ORF4 and *redD* transcripts. In the *absB* mutant strain, *act*VI-ORF1, *act*I, *act*II-ORF4, and *redD* transcript levels were also substantially lower than in the parent strain. The simplest explanation for these results is that the *absA* and the *absB* regulatory pathways include transcriptional control of *act*II-ORF4 and *redD*. Studies of transcriptional fusions between the *act*II-ORF4 promoter and the *xylE* reporter gene confirmed the effects of *absB* on *act*II-ORF4 transcription. However, a mutation in *absA* had no apparent effect on the expression of this fusion, suggesting that *absA* and *absB* have distinct regulatory mechanisms. Of seven *bld* mutants tested, none appeared to significantly affect transcription from the *act*II-ORF4::*xylE* fusion. In a separate study, transcriptional reporter gene fusions between the *act*II-ORF4 promoter and the *lux* reporter gene were used in attempts to detect antibiotic synthesis in natural soil. Expression of the fusion was detected during growth in sterile, nutrient-amended soil microcosms; it was concluded, however, that the technique used is unlikely to possess the sensitivity for detection under more natural conditions.

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Abs$^-$	antibiotic synthesis deficient
Act	actinorhodin
ASR	antibiotic-specific regulator
CDA	calcium-dependent antibiotic
CDO	catechol 2,3-dioxygenase
CFU	colony-forming unit
MCS	multiple cloning site
Mmy	methylenomycin
ORF	open reading frame
PCR	polymerase chain reaction
Pha	precocious hyperproduction of antibiotics
Red	undecylprodigiosin
RLU	relative light unit
Sab	suppressor of *abs*
SARP	*Streptomyces* antibiotic regulatory proteins

CHAPTER 1

INTRODUCTION

1.0 Introduction to Streptomycetes

1.1 Streptomycetes are Antibiotic-Producing, Mycelial, Sporulating Bacteria.

By one recent count, the bacterial genus *Streptomyces* includes more than 450 recognized species (7). This number reflects the considerable scientific and economic interest in streptomycetes that has been inspired largely by their ability to produce a tremendous number and variety of antibiotics and other useful pharmaceuticals. Less well known is the status of these complex, mycelial, spore-forming bacteria as a significant research subject in the field of cellular differentiation. A third, somewhat neglected, aspect of streptomycete biology is their role in the environment, where they are ubiquitous and often abundant in soil and sediments.

The genus *Streptomyces*, meaning "chain fungus", was proposed by Selman Waksman in 1943 to encompass that branch of the "aerobic, saprophytic actinomycetes that form catenulate spores" (222). The mycelial morphology of streptomycetes led researchers at that time to classify them variously as fungi, as a form of life intermediate to bacteria and fungi, or as a branch of bacteria separate from the true bacteria. Their classification as true bacteria was established in the 1950's and was based on electron

microscopy showing the absence of a nuclear membrane (96) as well as biochemical characteristics that included a gram positive-type cell wall.

Species classifications within the genus was long based primarily on spore and mycelial morphology and on the synthesis of pigmented compounds during growth on various media. The International *Streptomyces* Project of the 1960's standardized the growth media and the descriptive methods used in characterization (80). Later, the computer-assisted numerical taxonomy approach of Williams *et al.* (233) increased the number of biochemical and morphological characteristics used in classification. Recent analyses based on rRNA and other molecular sequence information confirmed earlier approaches in showing that the streptomycetes are a taxonomically cohesive branch of the eubacteria (7,201). Currently, the genus *Streptomyces* is placed within the Family Streptomycetaceae, Suborder Streptomycineae, Order Actinomycetales, Class Actinobacteria (the high G+C branch of the Gram positive bacteria), the Firmicutes (Gram positive bacteria), and the Domain Bacteria (63,202).

Streptomycetes grow by apical hyphal extension and branching, forming a dense interconnected mat of "vegetative" or "substrate" mycelia on plate media. Chromosomes are distributed through the mycelia with cross walls found between only approximately every tenth chromosome. A single growth cycle on solid media, from spore germination to aerial hyphae bearing mature spores, takes about four days under optimal conditions at 30°C. Spore germination is followed by a period of rapid growth, then a short interval of

near-cessation of growth and macromolecular synthesis, thought to correspond to a conversion from primary to secondary metabolism (164), and another period of substantial mass accumulation as vertically-growing "aerial hyphae" are formed (82). Vertical projection of aerial hyphae is aided in *Streptomyces coelicolor* by a small extracellular protein, SapB, that coats the hyphae and is proposed to break surface tension and/or create a scaffold (230). It is believed that energy and building blocks for the construction of aerial hyphae are derived from the lysis and recycling of vegetative mycelia (51). Little additional mass is accumulated during and after spore formation and maturation (82). Three or more uninucleoid spores per hypha are formed through growth of cell walls between chromosomes in the aerial hyphae. Spore surfaces vary among species and may be smooth, hairy, rugose, spiny, or warty. In liquid media, streptomycetes generally exist as clumps or "pellets" that grow to macroscopic size, new pellets forming from mycelial fragments shed by older pellets. Most species do not form aerial hyphae or sporulate during growth in liquid media; a notable exception is *Streptomyces griseus*.

Streptomycete chromosomes generally consist of 7-8 Mb of DNA (118,136,226). Remarkably, at least some species possess linear chromosomes, a discovery made in the early 1990's that provided the first examples of linear prokaryotic chromosomes other than the small (1 kb) chromosome of *Borrelia burgdorferi* (136,226). Linear plasmids also appear to be characteristic of the streptomycetes (122).

One of the more difficult aspects of *Streptomyces* research is the phenotypic and genetic variability of the organisms. This variability is evident both in terms of phenotypic differences under superficially identical growth conditions and in the occurrence of spontaneous heritable mutations. It is believed to be due in part to the linearity of (at least some) streptomycete chromosomes; telomere-like ends contain repeats that may promote recombination (226). Amplification and deletion of large (sometimes greater than 1 Mb) chromosomal segments are not uncommon and tend to occur at "hotspots"; these are often found at chromosome ends (134,135,194) but are not limited to them (181). Large-scale deletions and amplifications have also been reported in connection with one of the more commonly encountered variants in *Streptomyces coelicolor* cultures, the so-called "scarlet" variant, which is characterized by changes in pigmentation and the loss of resistance to chloramphenicol (68).

Antibiotic production may occur in both plate and liquid culture. Microscopic examination of pigmented antibiotics or of antibiotic regulatory protein/reporter protein fusions in *Streptomyces* colonies on solid media show production only in older vegetative mycelia (46,131,209), and not in aerial hyphae or spores. Antibiotics are almost always produced late in the life cycle as growth rate is decreasing. A common hypothesis to explain this timing proposes that antibiotics are produced to flood the lysing vegetative mycelia, protecting this vulnerable source of nutrients from competing microorganisms until it can be recycled to form aerial hyphae and ultimately

spores (51). Presumably, antibiotics would be useful competitive weapons during earlier growth stages as well; however, synthesis may be too costly in terms of resources during early growth and the antibiotics may be less effective at lower cell density.

Many strains of *Streptomyces* produce more than one antibiotic, and a particular antibiotic may be produced by more than one species. Genes for antibiotic production and resistance appear to have been exchanged frequently in evolutionary history, not only between streptomycetes but among different genera of bacteria and even between bacteria and fungi (88,224).

1.2 Streptomycetes are an Important Component of Soil and Sediment Microfauna.

Actinomycetes are ubiquitous in soils around the world and are sometimes present at concentrations as high as 10^8 per gram of soil (152,221). The genus *Streptomyces* is often the most numerous subclass of actinomycete isolated, typically making up 1-20% of the total bacterial viable count from growth of soil inocula (130). Streptomycetes are easily isolated from lake, river, and ocean sediments as well. It has been argued that their presence in sediments results from the survival of dormant spores washed from terrestrial soil (221); however, recent analysis of 16S rRNA isolated from coastal marsh sediments detected metabolically active streptomycetes and found that they comprised 2-5% of the active microbial community (160).

Despite their abundance, the role of streptomycetes in nature has been little studied.

Growth of streptomycetes in most soil environments is believed to be intermittent; long periods of dormancy alternate with short growth periods induced by the occasional influx of water and nutrients (unlike some soil bacteria, streptomycetes appear to make only limited use of root exudates) (232). An estimated mean generation time in woodland soils of 1.7 days, compared to 1-2 hours in laboratory liquid culture, may reflect intermittent periods of rapid growth instead of slow, constant growth (232). In experiments using soil microcosms seeded with spores of *Streptomyces coelicolor* or *Streptomyces lividans*, a 5-6 day cycle of spore germination, growth, and sporulation was observed, apparently correlating with dehydration of the soil (225).

Their non-fastidious feeding habits and ability to sporulate gives streptomycetes a competitive advantage during extended periods of starvation and dessication, as shown by their increasing numbers as a percentage of total soil bacteria in dry or carbon-poor soils (232). Streptomycete spores are hydrophobic and may be adapted for dispersal by soil arthropods (186).

Growing mycelia prefer moderate temperatures and pH; no true "extremophiles" have been found among the streptomycetes (152). They are strict aerobes but can grow in microaerophilic environments. Obligate heterotrophs, streptomycetes produce a range of degradative enzymes

including cellulases, xylanases, and ligninases that enable them to degrade and use a variety of organic compounds as carbon and energy sources (127). Concentrations of "biodegradative" actinomycetes have been estimated at 10^5 - 10^8 per gram of soil and may play significant roles in the degradation of recalcitrant plant materials, including cellulose and lignin (152). Streptomycetes are thought to have potential as biopesticides (72, 236) and bioremediation agents (196, 237).

To date, no convincing demonstration of antibiotic production by streptomycetes in nature has been published. However, it is generally accepted today that antibiotic production occurs in nature and is not simply a laboratory phenomenon (57). The most compelling evidence is the discovery in the last several decades that streptomycetes devote substantial genetic resources to the synthesis and regulation of antibiotics, often greater than 1% of the total genome. However, this indirect evidence is unsatisfying and tells little about the role of antibiotics in nature. Efforts to obtain more direct evidence have succeeded for certain fungi and bacteria (27, 215) but have failed for streptomycetes. The reason for this disparity is unclear; perhaps streptomycetes produce lower quantities of antibiotics over the course of a life cycle.

What criteria should be set for a rigorous proof of antibiotic synthesis in nature? First, it can be agreed that the use of sterilized soil must be disallowed; although it is relatively easy to recover antibiotics from sterile soil microcosms seeded with streptomycetes (27, 79, 234), such environments

resemble laboratory cultures more than natural soil. Second, concentrations of antibiotic-producers seeded into soil must not greatly exceed concentrations found in natural soil. Third, if biological assays (i.e., inhibition of a sensitive organism by an antibiotic-producing organism) are used, isogenic antibiotic-producing and non-producing strains must be compared so that inhibition by causes other than antibiotics can be ruled out. Finally, is nutrient amendment to soils allowable? Many soil microbes, including streptomycetes, are thought to be essentially dormant except when induced by the occasional influx of nutrients (including water). Therefore, the addition of nutrients that might be normally encountered in natural soil should not be disallowed. Plant matter or compounds such as starch and chitin that could be derived from plant debris, fungal hyphae, or insect carcasses are acceptable.

Several experiments that approach these standards were performed soon after the discovery of antibiotics in the 1950's, when interest in their natural role was at an early peak. In 1952, Gottleib and Simonoff (79) extracted chloramphenicol from soil inoculated with *Streptomyces venezuelae*; this soil had been pre-sterilized but did contain other microbial life in the form of co-inoculated *Bacillus subtilis*. More convincingly, Gregory *et al.* (83) and Krasilnikov (27) recovered traces of substances inhibitory to fungi from non-sterilized, nutrient-amended soil inoculated with streptomycetes. However, the inhibitory substances were not further characterized nor were streptomycete inoculum concentrations reported. In

contrast, Wright *et al.* (27) were able to recover and identify antibiotics produced by various fungi seeded into natural soil but were unsuccessful with *Streptomyces aerofaciens*, *S. griseus*, and *S. venezuelae*.

Important questions in soil microbiology cannot be approached until a method for monitoring streptomycete antibiotic synthesis in soil is found. Given their abundance, it is reasonable to speculate that streptomycetes are a major factor in soil ecology. They may also be involved in suppression of soil-borne plant diseases and biodegradation of xenobiotics. What role do antibiotics play in these phenomena? It is generally believed that, with few exceptions, streptomycete antibiotics are used as weapons against competing microbes. Are they used to protect lysing vegetative mycelia as a nutrient source for the construction of aerial hyphae as proposed by Chater and Merrick (51)? This theory depends on the temporal correllation of antibiotic synthesis and sporulation apparent in laboratory culture but unproven in nature. By what criteria do streptomycetes that produce multiple antibiotics "decide" which to synthesize under various conditions? Is antibiotic synthesis coordinated among closely located colonies and, if so, by what mechanism?

1.3 Synthesis of Pharmaceuticals by Streptomycetes

Streptomycin was first isolated from *Streptomyces griseus* in the laboratory of Selman Waksman; one of the first medically important antibiotics, it was used against tuberculosis beginning in 1944. Over the next

several decades, streptomycetes were intensively isolated from soil and screened for the production of useful antibiotics and other pharmaceuticals. Nearly 7,000 bioactive compounds resulted, including the majority of useful antibiotics (206); these include tetracycline, chloramphenicol, cycloserine, kanamycin, lincomycin, neomycin, nystatin, cycloheximide, chlortetracycline, and vancomycin. Other pharmaceuticals derived from streptomycetes include such immunosuppressants as tacrolimus (FK-506), cyclosporin, and rapamycin, the antitumor drug adriamycin, the antiparasitic ivermectin, and the fungicidal agent polyoxin. Screening strains for new compounds continues to be a significant activity for pharmaceutical companies (206).

Over the last decade, both academic and commercial laboratories have worked towards the development of novel antibiotics through rational recombination of antibiotic biosynthetic genes and *in vivo* synthesis. Antibiotics of the polyketide type are particularly amenable to "mixing and matching" of genes due to the modular nature of gene clusters encoding polyketide synthase (PKS) enzymes. Polyketides are constructed through the action of one or more ketosynthase/acetyltransferase units and acyl carrier proteins, often with numerous auxiliary modifying enzymes. PKS's have been shown to be generally tolerant to modification and substrate alteration (110, 128, 154). Aspects of polyketide synthesis that can be altered by selective deletion or replacement of antibiotic biosynthetic genes include degrees of reduction, chain length, stereochemistry, and post-PKS processing, (39, 110). In addition, feeding of non-natural substrates can alter

the carboxylic acid units used in priming and extension of the chain. Hundreds of variations on bacterially-synthesized polyketide antibiotics have already been produced by engineering of antibiotic biosynthetic pathways; the potential exists for innumerable new bioactive compounds, many of which would be difficult or impossible to synthesize chemically.

In addition to providing antibiotic biosynthetic genes for the creation of recombinant systems, streptomycetes are a source of regulatable promotors and can serve as hosts for the expression of recombinant systems. In a recent example, the Taxol-like polyketide anti-cancer agent epothilone was synthesized using *Streptomyces coelicolor* as a host to express biosynthetic genes cloned from a *Myxobacterium* species (214). Promotors from the actinorhodin gene cluster of *Streptomyces coelicolor* have been commonly used to express recombinant biosynthetic clusters (153, 183). The genetically well-characterized *S. coelicolor* and its close relative *Streptomyces lividans* have often been used as expression hosts (15, 128, 153), taking advantage of well-established large-scale fermentation methods for streptomycetes.

Recently, several vectors that may simplify the construction and expression of recombinant antibiotic biosynthetic clusters have been reported. Actinomycete bacterial artificial chromosomes capable of carrying 100 kb or more of DNA, that replicate in *E. coli*, and that integrate site-specifically into the chromosomes of *S. lividans* and *S. coelicolor* were constructed by Sosio *et al.* (200). Another integrative vector for the

controllable high-level expression of recombinant antibiotic biosynthetic clusters in actinomycetes (using the *actI* promotor of *S. coelicolor*) was reported by Rowe *et al.* (183).

2.0 *Streptomyces* coelicolor A3(2): A Model Organism

2.1 History

As a graduate student at Cambridge University in the early 1950's, David Hopwood chose a *Streptomyces* strain named "A3(2)" as a research subject (96). Streptomycetes appeared to be an interesting intermediate between bacteria and fungi, and strain A3(2) produced a striking blue pigment that promised to be a useful marker for genetic crosses. Hopwood gave this strain the species name *coelicolor*, meaning "sky color". It was later discovered that this name had been previously applied to a different streptomycete and that strain A3(2) properly belonged within the species "*violaceoruber*"; however, the name "*Streptomyces coelicolor* A3(2)" had become established and is still used by most researchers. Taxonomists place *S. coelicolor* strain A3(2) within a group that includes *Streptomyces lividans* 66 and the spiramycin-producer *Streptomyces ambofaciens* (201).

Although research efforts have been scattered over a large number of *Streptomyces* species due to interest in the antibiotics they produce, *S. coelicolor* A3(2) has become a model organism, particularly for genetic

studies. Several properties make it attractive for the study of antibiotic regulation: (1) it produces four antibiotics, each from a distinct chemical class, thus facilitating the search for higher order/global regulators of antibiotics, (2) two of the antibiotics are pigmented and therefore easily assayable, (3) none of the four antibiotics are commercially useful, promoting the free exchange of information, and (4) it is thought to be more stable phenotypically and genotypically than many other streptomycetes.

It is believed that the study of antibiotic regulation in *S. coelicolor* is likely to be widely applicable to other streptomycetes since hybridization studies have shown homologs of many *S. coelicolor* antibiotic regulatory genes in other species (8, 149, 178). In addition, *S. coelicolor* genes cloned into other streptomycetes tend to be temporally regulated, suggesting conservation of regulatory mechanisms (145, 157).

Of several *S. coelicolor* strains commonly used in genetic studies, a strain known as J1501 and its derivatives are probably the most used. J1501 has useful genetic markers and is a good host for plasmids and phage (49). The pedigree of strain J1501 is somewhat convoluted; its derivation from the ancestral A3(2) strain is comprised of a minimum of five steps, resulting in a number of both known and uncharacterized alterations to its genome (118).

2.2 The *S. coelicolor* A3(2) Genome

The *S. coelicolor* chromosome consists of nearly 8 Mb of DNA (118) and has a typical streptomycete G+C composition of approximately 74% (77). Its

size (approximately twice that of the *E. coli* genome) and the tendency of related genes to be located on opposite sides of the genome has led to the suggestion that the *S. coelicolor* genome may have resulted from an ancestral duplication (96). A detailed genetic linkage map of the chromosome has been constructed (180). A physical map, constructed using pulsed field gel electrophoretic analysis of fragments created by *Ase*I and *Dra*I digestion, was reported in 1993 (118). The physical and genetic maps are well correlated. The linearity of the *S. coelicolor* chromosome was revealed in 1993 (136, 226). Chromosome ends contain repeated identical sequences and are covalently bound by proteins (226).

A cosmid library containing nearly the complete genetic complement of *S. coelicolor* has been constructed (180) and used, since 1997, in an effort to sequence the complete *S. coelicolor* genome by 2001. This project, jointly organized at The John Innes Center (Norwich, UK) and the Sanger Center (Cambridge, UK), had completed approximately 90% of the genome as of December 2000. The Sanger Centre *Streptomyces coelicolor* Genome Sequencing Project provides sequence information and annotations to the public at http://www.sanger.ac.uk/Projects/S_coelicolor. Based on data collected thus far, it has been predicted that the *S. coelicolor* genome will contain approximately 7000 genes (96).

One peculiarity of streptomycete genetics is a tendency towards leaderless transcripts (10, 113, 205). A high degree of promotor heterogeneity is also characteristic of streptomycete genes (205), and many

genes are transcribed from more than one promotor (111, 113, 205); this may reflect the need for subtle transcriptional regulation in an organism that undergoes a complex developmental program.

A number of plasmids and transposable elements have been discovered in *S. coelicolor* A3(2). SCP1, a giant (350 kb) linear plasmid, exists in self-replicating form at approximately four copies per chromosome (123) or integrated into the *S. coelicolor* chromosome at 9 o'clock on the genetic map (99, 124). SCP1 is self-transmissable and is responsible for much of the fertility used in genetic linkage mapping in this organism. SCP2 and its more fertile variant SCP2* are 30 kb, low copy-number, self-transmissable plasmids accounting for much of the remaining fertility of *S. coelicolor* strains (21, 195). The genetic element SLP1 is found integrated site-specifically into the chromosome (25). IS117 (formerly known as the 2.6 kb mini-circle) is an insertion sequence integrated at two sites in the chromosome; it can also exist independently as a very low copy number plasmid (143).

2.3 Genetic Techniques

2.3.1 Linkage Mapping. A fairly extensive suite of techniques has been developed for the genetic manipulation of *S. coelicolor*. Conjugal recombination was the basis for early genetic studies and is still useful today. Genetic linkage mapping may be approached using crosses mediated by the self-transmissable plasmids SCP1 and SCP2 (95). Alternatively, crosses accomplished by protoplast fusion result in probable complete diploidy and

produce recombination at very high frequency, but give results that may be more difficult to interpret (14).

2.3.2 Vectors. A number of vectors have been developed for introducing and maintaining DNA in *S. coelicolor*. Many plasmid vectors are based on either the low copy-number (2-3/chromosome) SCP2 (21) or the *S. lividans*-derived moderate copy number (40-300 copies/chromosome) pIJ101 (120). *E. coli/Streptomyces* shuttle vectors have been developed (59). Plasmids with *E. coli* origins of replication (which do not replicate autonomously in streptomycetes) have been adapted for use as suicide vectors (129). A polyethylene glycol (PEG)-assisted transformation protocol for the introduction of plasmids and phage DNA into *S. coelicolor* protoplasts has been in use since 1978 (22). Chromosomal DNA can be introduced by transformation when entrapped in liposomes (144). Plasmids can also be conjugated from *E. coli* to *Streptomyces* (151). The temperate *Streptomyces* bacteriophage ϕC31, with a native genome of 41 kb, has been widely used to integrate DNA into streptomycete chromosomes (31, 129). A number of useful variants of this phage have been developed that carry selective markers, convenient cloning sites, and the ability to integrate either at the *attB* site or (in *attP* strains) by homologous recombination between cloned *S. coelicolor* DNA and the identical chromosomal sequence (31, 182).

2.3.3 Transposon mutagenesis. Transposon mutagenesis of cloned *S. coelicolor* DNA has been accomplished *in vitro* with *mariner* or with a derivative of Tn5 (75). Mutagenesis *in vivo* of the closely related *S. lividans*

has been demonstrated using another Tn5 derivative (220). In each case, insertion seems to have been fairly random.

2.3.4 Generalized transduction. The isolation of generalized transducing phages for *S. coelicolor* and other streptomycetes were reported only very recently (32) though they have been sought for many years. These phages are capable of transducing chromosomal and plasmid DNA between strains of *S. coelicolor* at frequencies comparable to that of the wild-type P22 phage in *Salmonella*. Importantly, they appear to possess broad host ranges among streptomycetes, implying that they may facilitate the analysis and improvement of strains that produce commercially important bioactive compounds but that have resisted genetic manipulation.

2.3.5 Reporter Genes. A number of foreign genes have been used with some success as reporters of transcription in streptomycetes. The *Pseudomonas*-derived *xylE* , encoding catechol 2,3-dioxygenase (CDO), is most widely used (31, 108, 238); others include bacterial *lux* (191, 199), *ampC* (71), *neo* (223), *cat* (19), *melC* (171), *aph* (140), and *gfp* (209). The common reporter gene *lacZ* from *E. coli* is not useful in streptomycetes due to multiple endogenous β-galactosidase activities and poor translation of the *E. coli* gene.

The *xylE* reporter gene bears further discussion due to its popularity and its relevance to the work presented in this dissertation. Its use in streptomycetes was first reported in 1989 by Ingram et al. (108) who constructed a plasmid-borne fusion between *xylE* and an *S. lividans*

galactose-utilization operon promotor, *galP1*. A good correlation between dot blot-quantification of fusion transcripts and *xylE*-encoded CDO activity was demonstrated (however, note that this test does not determine whether *transcription* of the fusion reflects that from the native *galP*1 promotor). In 1994, Paget *et al.* reported a lack of reproducibility from *xylE* fusions (although data was not shown) and touted the use of the *S. glaucescens melC* gene as an alternative (171). Lindley *et al.*, in 1995, reported a discrepancy between results obtained from *strB1*::*xylE* and *strB1*::*aph* fusions in *Streptomyces griseus*, although the fusion at fault was not determined (140). Mogk *et al.* (158), in 1996, fused the reporters *xylE*, *cat* and *bgaB* (beta-galactosidase of *Bacillus stearothermophilus*) to *B. subtilis* heat shock protein promotors *dnaK* and *groE*. Although increased activity was measured from all fusions in response to heat shock, wide variations were observed; again, which if any of these fusions accurately reported transcription rates was not determined. In 1999, Hu *et al.* reported a positive (though rough) correlation between *trpC*::*xylE* fusion expression in *S. coelicolor* and quantitation of the *trpC* transcript itself by S1 nuclease protection (106). Craster *et al.* in 1999 (55) reported sometimes wide variations in CDO activity in replicate *S. coelicolor* cultures expressing *xylE* from amino acid biosynthetic gene promotors; however, the authors were inclined to attribute these variations not to the reporter gene but to small differences in culture inoculum that became amplified during growth. Craster *et al.* also observed a

correlation of fusion-encoded CDO activity from these promotors with the activity of the biosynthetic enzymes themselves (55, 176).

Several cautionary notes regarding *xylE* have been published. Hassett et al. (89) showed that CDO activity is very sensitive to hydrogen peroxide and therefore questioned its reliability, particularly under conditions of oxidative stress. Gonzalez-Ceron *et al.* (78) note that *xylE* in its native state is cotranscribed with *xylT*, a gene encoding a ferredoxin that functions to recycle CDO after each round of catalysis. Several fusion constructs expressing *xylTE* exhibited up to five-fold greater CDO activity as a result of this recycling activity compared to fusions expressing *xylE* alone. The use of *xylTE* promises to increase the sensitivity of this reporter gene assay and may also improve the accuracy of reporting under conditions in which recycling of CDO by endogenous electron-transport proteins is limiting.

In summary, the reputation of *xylE* as a reporter gene is mixed; however, the data at this time does not seem to support its abandonment. All reporter genes (and, indeed, all methods of transcript assay) have limitations as well. The *luxAB* reporter, for example, was shown to affect the activity of a promotor to which it was fused, such that different results were obtained with a *luxAB* fusion compared to fusions with *lacZ*, *cat*, or *galK* (70). Warnings have also been published recently about the reliability of green fluorescent protein (GFP) at higher temperatures and higher concentrations of the protein (163, 193). However, recent successes in the use of GFP in

streptomycetes (86, 131) indicate that it may become the preferred reporter gene for future studies.

2.3.6 Sequence Analysis. Analysis of streptomycete DNA is aided by a computer program called "FRAME" that was devised in the early 1980's by Bibb and co-workers (20). The high G+C content of streptomycete DNA results in a bias towards G's and C's in the third (wobble) position of codons in ORF's, resulting in a typical G+C content of 50%, 70%, and 90% at the first, second, and third positions of the codon, respectively. FRAME identifies ORF's by searching for this pattern.

2.4 Antibiotics Produced by *S. coelicolor* A3(2).

S. coelicolor produces at least four compounds with antibiotic activity; actinorhodin, undecylprodigiosin, methylenomycin A, and calcium-dependent antibiotic. Each is chemically distinct and is produced through the action of a unique set of tightly clustered and contiguous genes. Each cluster includes genes encoding biosynthetic enzymes, antibiotic resistance, and at least one regulatory gene specific to that antibiotic. Antibiotics are produced late in growth under most laboratory conditions, beginning in transition or stationary phases (81, 93, 210). *S. lividans*, which is closely related to *S. coelicolor*, also has the biosynthetic ability to make Act and Red but does not do so under most conditions; induction of the pigmented antibiotics in *S. lividans* through introduction of cloned *S. coelicolor* DNA has proven to be a useful method for identifying genes that may regulate antibiotics.

2.4.1 Actinorhodin or "Act" is an isochromanequinone antibiotic that is blue at alkaline pH and red at neutral or acidic pH. It has weak antibiotic activity against a range of Gram positive bacteria (235). The blue pigment visible in cultures of *S. coelicolor* A3(2) and often in the surrounding media is actually a mixture of actinorhodin and its lactone derivative gamma-actinorhodin; the former (mycelium-bound) form appears to be derivatized to the latter concurrently with export (37).

Act is a member of a class of natural compounds known as polyketides. Varied and widely distributed among organisms, polyketides share a common biosynthetic mechanism in which simple carboxylic acid metabolites are iteratively condensed to form chains of various lengths. Decarboxylative condensation of these metabolites is frequently followed by reduction, giving rise to the keto groups that give the class its name. Many other modifications, including dehydrations, cyclizations, isomerizations, aromatizations, and additional reductions are possible. These potential modifications, as well as the choice of starter and extender carboxylic acid units and final chain length, make possible a great variety of polyketides. The "Polyketide Synthases" (PKS's) that catalyze polyketide construction occur in two classes: Type I, consisting of multiple modules of a core PKS with auxiliary enzymes, each of which is used once in assembly line fashion, and Type II, multienzyme complexes in which individual active sites are used repeatedly.

Act is formed by the condensation of an acetyl-CoA starter unit and seven malonyl-CoA extender units to form an aromatic sixteen-carbon octaketide; two octaketides are dimerized to make Act (28). The Type II multienzyme complex that catalyzes Act synthesis is encoded by a cluster of genes located at 6 o'clock on the *S. coelicolor* genetic map (235) and covering a maximum of 26 kb (146). Early cosynthesis studies grouped the *act* genes into seven categories (*act*I-VII) (184). Sequencing has identified 23 ORF's in the *act* cluster that are transcribed as at least 7 mRNA's (98). The Act biosynthetic pathway and the functions of most of the biosynthetic enzymes have been elucidated through analyses of mutants, chemical intermediates, and shunt products. The core PKS consists of a ketosynthase or "condensing enzyme" which carries the polyketide chain as it is constructed; it is encoded by the *act*I-ORF1 and *act*I-ORF2 genes (67). An acyl-carrier protein, encoded by *act*I-ORF3, carries new building units (67). Other *act* genes encode modifying enzymes including reductases, cyclases, and dehydratases, etc.

Mutants representing the *act*II class did not cosynthesize Act when grown in pairwise combinations with other mutants, suggesting a regulatory function (184). A clone restoring Act synthesis to the *act*II mutant was isolated from a library (146). Introduction into an Act^+ strain in multi-copy resulted in precocious hyperproduction of Act. ActII-ORF4 has been characterized as a positive regulatory protein (65).

Self-resistance to Act is conferred by proteins encoded by *act*II-ORF2 and *act*II-ORF3 that are believed to export Act from the mycelia (37, 65). The product of *act*II-ORF1 is predicted to negatively regulate transcription of these resistance genes (65).

2.4.2 Undecyprodigiosin or "Red" is the major component of a mixture of prodigionine compounds (216) that are red at acidic pH and yellow at alkaline pH. Red possesses antibiotic activity against certain gram-positive bacteria (185). A highly non-polar tripyrrole compound, Red does not visibly diffuse from the mycelia (185) as Act does. Proline is probably a biosynthetic precursor of Red (76); in fact, it has been suggested that Red may be a sink for excess proline, possibly reflecting proline's role as an osmoregulant in some bacteria (121). The Red biosynthetic pathway is well characterized through the analysis of blocked mutants and cosynthesis tests. The products of at least 18 genes (54, 64, 185) clustered within a 35.7 kb segment of DNA (147) and located at 5 o'clock on the genetic map (185) are involved. Much of the cluster has been sequenced and possible functions assigned to a number of ORFs (54, 64). The *red* cluster includes a positive regulatory gene named *redD* that shares extensive sequence homology with *act*II-ORF4 (161).

2.4.3 Calcium-dependent antibiotic or "CDA" is a colorless lipopeptide consisting of an eleven-residue non-ribosomally-synthesized peptide with a hydroxylated fatty acid at the N-terminus (53). In the presence of calcium ions, it apparently acts as a monovalent cation ionophore and is

active against a wide range of Gram-positive bacteria (132). CDA is assayed by placing plugs of growing colonies onto low-calcium nutrient agar with or without added calcium and seeded with a CDA-sensitive bacterial strain (commonly *Staphylococcus aureus*); zones of inhibition are caused by the presence of the antibiotic (132). The *cda* gene cluster, located at 10 o'clock on the genetic map (100), was not cloned until 1998 (53). Identified within the cluster are genes encoding three peptide synthetases with modular structures consistent with the synthesis of an eleven amino acid peptide (53, 187); disruption of one of these genes inactivated CDA synthesis (187). Also identified are a putative regulatory locus (*cdaR*) with homology to *act*II-ORF4 and *redD*, a putative efflux protein, a potential ABC transporter that may be involved in resistance, and predicted amino acid and lipid biosynthetic enzymes (187). In an unexpected development, it was recently discovered that the genes encoding the AbsA1/AbsA2 two-component signal transduction system, a major focus of the research described in this work, are located within the *cda* cluster (187).

2.4.4 Methylenomycin A or "Mmy" is a small (182 Da), colorless, cyclopentanone antibiotic active against both gram-positive and gram-negative bacteria (93). In 1976, it was discovered that the genes coding for Mmy biosynthesis are clustered on the large linear plasmid SCP1 (126, 235) leading to speculation that antibiotic genes might generally be found in clusters borne on plasmids. Clustering has proven to be a general paradigm for antibiotic biosynthetic genes, but to this day the *mmy* genes are the only

confirmed plasmid-borne antibiotic gene cluster. The *mmy* genes cover a maximum of 28 kb (98). Under the growth conditions used by Hobbs *et al.*, Mmy synthesis begins during the transition from exponential to linear growth and peaks as stationary phase is reached (93). Self-resistance to Mmy is believed to occur through the action of a membrane-bound export protein encoded by the *mmr* gene (162). Also located within the cluster is *mmyR* (48, 98), one of the few known negatively acting antibiotic-specific regulators among *Streptomyces* species.

2.5 Antibiotic-Specific Regulatory Genes

The best studied of the *S. coelicolor* antibiotic-specific regulatory genes are *act*II-ORF4 and *redD*. Under most or all conditions, the proteins encoded by these genes appear to be necessary and sufficient to induce transcription of their cognate biosynthetic genes. Strains carrying null mutations of these genes fail to transcribe biosynthetic genes, while the introduction of multiple copies of *act*II-ORF4 and *redD* into *S. coelicolor* results in the production of Act and Red even in exponential phase (65, 210). *act*II-ORF4 and *redD* transcript levels increase dramatically as liquid cultures enter stationary phase, although low levels of transcripts can be detected in the exponential phase (81, 98, 210).

The ActII-ORF4 and RedD proteins share 33-37% amino acid identity (47, 65) with each other and with DnrI, the activator of the duanorubicin biosynthetic cluster in *Streptomyces peucetius* (207). ActII-ORF4 and DnrI

can substitute for each other in activation of antibiotic production (207). It has long been postulated that ActII-ORF4, RedD, and DnrI activate transcription by binding to the promotors of most or all of the biosynthetic and resistance genes. However, none of these proteins contains a traditional helix-turn-helix DNA-binding motif (65, 159, 161, 207). Nevertheless, in 1996 DnrI was shown to bind to *dnr* biosynthetic gene promotors (213). Imperfect inverted repeats with the consensus sequence 5'-TCGAG-3' found in several *dnr* biosynthetic genes promotors were proposed as possible binding targets. In 1997, Wietzorrek and Bibb reported that ActII-ORF4, RedD, DnrI, and a number of other streptomycete antibiotic regulators share a high degree of amino acid sequence identity with OmpR-type regulatory proteins, which contain DNA-binding domains that apparently consist of an alpha-helix and two beta strands (229). The *Streptomyces* antibiotic regulators are predicted to contain this secondary structure as well. Therefore, the *Streptomyces* proteins were proposed to comprise a new class of proteins named SARP's, for *Streptomyces* Antibiotic Regulatory Proteins (229). Potential binding sites consisting of two or three tandem heptameric repeats with the consensus sequence 5'-TCGAGCG/C-3' were also identified in the promotor regions of a number of *act* and *dnr* biosynthetic genes. These sequences are repeated at either 11 or 22 bp intervals and are, therefore, on the same face of the promotor DNA. In some cases, the sequences overlap –35 regions on the face opposite from that of RNA polymerase binding, suggesting that they could promote binding. Recently, ActII-ORF4 was shown to bind to the

sequence 5'-TCGAG-3' in the –35 region of several *act* biosynthetic gene promotors (12).

Additional levels of antibiotic-specific regulation have been identified in the *red* and *dnr* clusters. Each contains a gene encoding a protein with similarity to the response/regulator proteins of two-component signal transduction systems but lacking the phosphorylation site typical of that class. These genes are named *redZ* and *dnrN*. Studies of disruption mutants of *redZ* and *dnrN* indicate that their products activate transcription of *redD* and *dnrI*, respectively (74, 85, 228). Neither the *red* nor *dnr* clusters appear to contain a sensor/kinase-encoding gene, which are often found closely linked to their cognate response-regulators. Recently, yet another apparent regulator of the *dnr* cluster, *dnrO*, has been discovered; this gene encodes a DNA-binding protein that appears to be necessary for *dnrN* transcription (170). In contrast with *red* and *dnr*, there is no evidence for antibiotic-specific regulation in the *act* cluster other than *act*II-ORF4.

3.0 Higher Order Regulation of Antibiotics

3.1 Growth Rate/Nutrient Limitation

Given its practical value, our knowledge of conditions that trigger antibiotic synthesis in streptomycetes is surprisingly limited. One reason for this is the complexity of the relationships involved; it is clear that many

factors can influence the synthesis of a single antibiotic and that individual antibiotics produced by a single strain tend to respond to different stimuli. It is generally believed that a decrease or cessation in growth rate and/or the depletion of specific nutrients triggers synthesis of most antibiotics. Addition of glucose, phosphate, or ammonium has been shown to repress synthesis of many antibiotics (47). Other factors proposed to influence production of specific antibiotics are mycelial density, change in pH, and build-up of excess metabolites or waste products.

Synthesis of Act by *S. coelicolor* can be elicited by nitrogen or phosphate depletion and addition of the same nutrients suppresses Act production (60, 92). However, Act production in continuous cultures is inversely proportional to growth rate regardless whether glucose, ammonium, or phosphate is limiting (116), suggesting that growth rate and not depletion of a specific nutrient is the triggering condition.

Red synthesis appears to be directed by slowing or cessation of growth rate and not the availability of particular nutrients, at least under certain conditions. Takano *et al.* (210) reported that the stationary phase onset of Red synthesis in batch cultures proceeded without significant delay whether carbon, nitrogen, or phosphate was in apparent excess, suggesting that none of these repress and/or inhibit synthesis of Red. In continuous culture, Red production peaks at a doubling time of about 13.9 hours, decreasing at both higher and lower growth rates (116).

Mmy synthesis begins during the transition from exponential to linear growth and concurrently with a rapid drop in medium pH caused by the efflux of pyruvate and alpha-ketoglutarate (93). An artificially induced drop in pH alone can cause transient Mmy synthesis (90); based on this phenomenon, it has been proposed that acid shock reflecting an imbalance in carbon metabolism may trigger synthesis (47).

3.2 *bld* Loci

The existence of genetic regulatory elements common to antibiotic synthesis and sporulation is demonstrated by the existence of *S. coelicolor* mutants affected in both processes. Such mutants are named *bld* (for bald) because they are fail to form the fuzzy aerial mycelia that precede normal sporulation. Each is blocked for the synthesis of at least one antibiotic and generally all four. *bld* mutants are currently divided into 12 distinct types; *A*, *B*, *C*, *D*, *G*, *H*, *I*, *J*, *K*, *L*, *M*, and *N*. Given the existence of a large number of *bld* mutants that are not yet classified, it is likely that this number will increase with time.

Many of the *bld* mutants can be induced to form aerial mycelia when grown on poor carbon sources (e.g., mannitol) instead of standard complex or glucose-containing media (43, 156). In some cases, the formation of aerial hyphae can also be induced by growing *bld* colonies next to wild type or certain other *bld* mutants. In fact, most of the *bld* mutants can be placed in a cross-feeding hierarchy, suggesting the presence of a biochemical or

signaling pathway (165, 231). Many *bld* mutants are affected in the regulation of carbon/energy metabolism; for example, *bld A*, *B*, *C*, *D*, *G*, and *H* are deregulated in expression of the galactose-dependent, glucose-sensitive promotor *galP*1. It has been proposed, therefore, that the inability of at least some *bld* mutants to sporulate and produce antibiotics may be due to an inability to sense starvation (175). The BldJ phenotype is apparently due to acidification of growth medium due to an inability to metabolize organic acids (218). Some *bld* mutants (*B*, *C*, and *H*) also differ from parental strains by their inability to modify certain proteins that normally are ADP-ribosylated at approximately the time of differentiation (197).

Only a few conditions are known that allow the recovery of antibiotic synthesis by any *bld* mutants, indicating a division in regulatory and/or biochemical pathways. Growth on low-phosphate media allows synthesis of Red by *bldA* mutants (137), mutants in *bldH* are completely unblocked on a number of non-glucose carbon sources (43), and bldD mutants produce significant quantities of Act on complex media (62).

The best-characterized *bld* mutant is *bldA*. The *bldA* gene encodes a tRNA translating the leucine codon UUA (133). This codon occurs only rarely in the *S. coelicolor* genome and so far the great majority have been found in genes involved in secondary metabolism and sporulation (137). While primary growth is unaffected, *bld*A mutants produce no antibiotics under most conditions and morphological differentiation is severely limited; aerial hyphae are constructed but are not projected vertically (51, 156).

These observations led to the proposal of a novel mechanism in which differentiation would be translationally regulated through the availability of the active $tRNA^{UUA}$ (133, 137, 138). Act and Red synthesis would be subject to this postulated regulatory mechanism, since *act*II-ORF4 and *redZ* transcripts each contain a single UUA codon (65, 85). The hypothetical *bld*A-controlled regulon has been estimated at approximately 100 genes (96). This mechanism is as yet unproven, and apparently conflicting evidence has been published regarding the essential point of temporal availability of active $tRNA^{UUA}$ (81, 139). Alternatively, the presence of UUA codons primarily in transcripts of secondary metabolic genes may simply result from evolutionary drift of more essential vegetative genes from the use of a codon that may be inefficiently translated. A similar mechanism of translational control has been proposed in *Clostridium acetobutylicum*, where a rare codon may be involved in the regulation of the secondary metabolites acetone and butanol (189).

Four other *bld* genes have been cloned. *bldB* (174) and *bldD* (61) encode small, highly charged proteins that are suspected to have regulatory functions. Each possesses a potential DNA binding helix-turn-helix motif and a functionally essential tyrosine as a potential phosphorylation site. Each appears to negatively regulate its own transcription. As cultures near stationary phase, transcription of *bldB* increases. BldD has been shown to bind, and negatively regulate expression from, two developmental sigma factor genes (62). The sequence of *bldG* has a high degree of similarity to *Bacillus subtilis* anti-sigma/anti-anti-sigma factors (23). The *bldK* mutation

occurs in one of 5 ORFs that appear to encode subunits of an ABC-type transporter that is speculated to be an oligopeptide permease (166); therefore, it has been proposed that an oligopeptide signal may begin a cascade leading to sporulation in *S. coelicolor*. A candidate factor has been partially purified from medium conditioned by growth of *S. coelicolor* (165).

3.3 *absA*

Postulating the existence of higher order genetic loci globally regulating the synthesis of antibiotics without affecting sporulation, Champness and co-workers began a search for such loci in the late 1980's. At that time, only the *afsB* locus (discussed later) was known to affect multiple antibiotics (Act and Red, but not CDA or Mmy). Champness and then-graduate student Trifon Adamidis subjected spores of *S. coelicolor* strain J1501 to random mutagenesis by ultraviolet light and N-methyl-N'nitro-N-nitrosoguanidine (NTG). To improve the chances of identifying loci regulating multiple antibiotics, a screen for the simultaneous loss of the two pigmented antibiotics was employed. Of approximately 800,000 survivors of mutagenesis screened in plate culture, eight exhibited the simultaneous loss of Act and Red while retaining normal sporulation (5). Four of the isolates had suffered simultaneous mutations in *act* and *red* biosynthetic genes and were discarded (5). The four remaining isolates (named C505, C542, C554, and C577) were genetically mapped in close proximity to each other at approximately 10 o'clock on the circular map. This location distinguished

them from all known antibiotic-related sites except for the *cda* cluster. Although resolution from the *cda* locus was unsuccessful, sufficient differences in phenotype with any known mutations in the *cda* region prompted the classification of these mutations as a new locus to be named *absA*, for antibiotic synthesis deficient.

Each *absA* mutant strain was tightly Act-minus and Red-minus on all media tested and deficient in CDA and Mmy synthesis as well. Introduction of *act*II-ORF4 or *redD* in multi-copy restored Act and Red synthesis, respectively (4, 42), demonstrating that the mutants remained metabolically capable of producing the antibiotics. Strain C505 was chosen for a comparison of growth rate and mycelial accumulation and found comparable to the parental strain (5).

To clone the wild type *absA* gene, a library of parental strain J1501 chromosomal DNA was constructed using the low copy-number plasmid vector pIJ922 (26). Initial attempts at mutant complementation through transformation of the library into *absA* mutant strain C542 were complicated by two interesting characteristics of the mutant strain: first, protoplasts of C542 were found to be less transformable (by more than an order of magnitude) than the parental type; second, apparent reversion of the *absA* strains to a pigment-producing phenotype occurred at high frequency (approximately 0.1%), making identification of complemented mutants difficult. The apparent revertants also recovered the transformation efficiency of the parental type, making them more numerous on

transformation plates. Therefore, the DNA library was conjugated into strain C542 using the mobilizing ability of pIJ922. Eight clones from the library restored production of Act, Red, and CDA to all four mutant strains (26). Mmy was also restored in the two representative transconjugants tested. A hybridization experiment showed that six of the clones shared some DNA sequence. A 1.95 kb subcloned fragment complemented all four *absA* mutant strains when a single copy was introduced by integration of a recombinant phage containing the fragment. Phage released from lysogens and used to infect the parental strain created an antibiotic-minus phenotype at a high frequency. When used to probe the *S. coelicolor* chromosomal DNA cosmid library, the 1.95 kb fragment hybridized to a clone corresponding to the 10 o'clock location of the genetic map previously determined for the *absA* locus. These experiments demonstrated the identity of the cloned fragment with the *absA* locus.

Sequencing of the 1.95-kb fragment revealed two incomplete ORFs, divergently oriented with approximately 130 intervening nucleotides (26). The larger ORF included the region previously shown by marker exchange to contain the *absA* mutations; this ORF was named *absA*1. Sequencing of a downstream fragment revealed the remainder of the *absA*1 sequence, for a total length of 1715 nt. The deduced amino acid sequence of AbsA1 showed significant similarity to the histidine protein kinases of bacterial two-component signal transduction systems, especially the Deg/Uhp subgroups. Typical motifs of this class were present, including the conserved sequence

surrounding and including a histidine likely to be a site of autophosphorylation, and N-terminal hydrophobic regions proposed to span the cell membrane.

Just downstream of *absA*1 and oriented in the same direction was another ORF that would be cotranscribed with *absA1*. This ORF consisted of 668 nt that showed 39% end-to-end identity with the response regulators DegU and NarP. Named *absA2*, the ORF contained typical conserved motifs, notably a putatively phosphorylated aspartyl residue and a C-terminus with a helix-turn-helix DNA-binding motif.

Paul Brian, a post-doctoral researcher in the Champness laboratory, constructed several deletion mutants in the *absA*1/*absA*2 system (26). In the first, all conserved regions of *absA*1 were deleted, probably resulting in a polar effect on *absA*2 transcription. This strain, named C420, exhibited a phenotype essentially opposite to the original *absA* mutants; i.e., precocious hyperproduction of Act and Red. Both Act and Red were produced 6-12 hours earlier than in the parental type and quantities of the antibiotics were five-fold and eight-fold greater, respectively, after 75 hours of growth. This phenotype was given the name "Pha" for Precocious hyperproduction of antibiotics. Colony morphology was also affected in C420; the colony surface was crenulated, sporulation sparse, and transformation efficiency significantly decreased. Another disruption mutant of *absA*1, strain C430, probably did not have a polar effect on *absA*2 but resulted in the same phenotype. Experiments monitoring transcription from the *actI* biosynthetic

gene promotor using a transcriptional fusion to the *xylE* reporter gene demonstrated detectable promotor activity 6-12 hours earlier and a significantly higher peak of activity in C420 compared to the parental strain (26). Mutants deleted in the proposed transmitter domain (including both kinase and phosphatase activities) of *absA*1 exhibited the Pha phenotype, as did mutants deleted in proposed essential regions of *absA*2 (10). From these results, it was concluded that *absA* negatively regulates antibiotics, directly or indirectly.

Phosphorylated AbsA2 is the negatively regulating form of the response regulator protein. This was demonstrated through the creation of strains with mutations in the proposed phosphorylation sites of AbsA2 (D54) and AbsA1 (H202), both of which exhibited the Pha phenotype. In addition to their effects on the Act and Red antibiotics, precocious hyperproduction of CDA was noted in these mutants as well (10).

Analysis of absA transcription revealed that *absA*1 and *absA*2 are cotranscribed (10). This transcript is leaderless (transcription and translation beginning at the same nucleotide), a not uncommon phenomenon in streptomycetes. Transcription is positively autoregulated (apparently by the phosphorylated form of AbsA2) and growth phase regulated, with transcript abundance rising by approximately five-fold between 18 hours and 30 hours in liquid cultures (10).

Based on these results, a model for AbsA function has been proposed. In this model, low concentrations of AbsA1 and AbsA2 are present (as

indicated by modest transcript levels) during early growth, when the organism is probably not competent to synthesize antibiotics. Responding to an increase in the concentration of an unknown signal during the transition phase, AbsA1 is converted to kinase mode and phosphorylates AbsA2, resulting in repression of antibiotic synthesis. Simultaneously, AbsA2~P positively regulates transcription from its own promotor, resulting in an approximately 5-fold increase in *absA1*/*absA2* transcripts (10). Later, signal depletion or degradation results in derepression of antibiotic synthesis through conversion of AbsA1 to the phosphatase form and the resulting dephosphorylation of AbsA2.

Marker rescue experiments showed that the four original *absA** (antibiotic deficient) isolates contained mutations in *absA1* (26). Two of the mutants were characterized at the sequence level (9). Strain C577 carries a substitution, L253R, within a region proposed to be involved in aspartylphosphatase activity. Strain C542 carries two closely placed substitutions, I360L and R365Q, in a region involved in nucleotide binding; it is not known whether both mutations are required for the antibiotic minus affect. It is now thought that these mutations lock AbsA1 into a kinase dominant form; in the case of C577 this would occur through inactivation of phosphatase activity, in C542 through the creation of a signal-independent kinase.

The AbsA system apparently functions to delay/decrease antibiotic synthesis. Why is this important to the cell? One speculation is based on

the sparse sporulation of knockout (Pha phenotype) mutants; early expression or overexpression of antibiotics may inhibit sporulation (8).

As noted previously, *absA* mutants are characterized by the frequent occurrence of spontaneous apparent revertants. Although antibiotic-producing apparent revertants appear occasionally in other non-producing mutants, they are much less common than in *absA* mutants and most are of a known type resulting from a spontaneous chromosomal deletion (68). Those arising from *absA* strains can be isolated and stably cultured (5, 26). A number of such mutant strains have been characterized at the sequence level for the information they might reveal about the mechanism of *absA*1/A2 action. In all cases, they were found to be not true revertants but second-site suppressive mutations; these were named *sab* mutants (for suppressors of *abs*). *sab* mutants occur in two forms: SabI, resembling the parental strain, and SabII, which overproduce Act and Red for a Pha-like phenotype. The *absA* mutation in strain C542 (located in the nucleotide binding region of *absA*1) was suppressed to a SabI phenotype by the substitution G252V in the *absA*1 aspartyl phosphatase region. Of two Pha-like *sab*II strains in the C542 background, one was associated with a S171W substitution occurring in the DNA-binding region of *absA*2, the second with a nonsense codon in the middle of *absA*1 with a possible polar effect on *absA*2. The L253R substitution in the proposed aspartyl phosphatase region of *absA*1 strain C577 was suppressed to a SabI phenotype by the substitution V29A in an

alpha-helical region of *absA*2, and to a SabII phenotype by a deletion including much of *absA*1, all of *absA*2, and additional downstream sequence.

The *absA1/absA2* locus has recently been localized to within the *cda* cluster (187), near one end between the *cdaR* regulatory gene and the bulk of the biosynthetic genes. This discovery raises interesting evolutionary questions; for example, did the *absA* system evolve with the *cda* cluster and only later become a global regulator of antibiotics? If so, does *absA* control CDA synthesis to a greater degree or in a different manner than the other antibiotics?

3.4 *absB*

The mutant screen that led to the discovery of the *absA* mutants also produced a "leaky" mutant that synthesized small amounts of pigment on the complex "R5" medium used in the screen (3). A second mutant hunt was undertaken to isolate more representatives of this type, resulting in five additional isolates from a screen of 120,000 survivors of UV light- and NTG-treated spores. This frequency is consistent with loss-of-function mutations. The six isolates (named C120, C170, C175, C246, C252, and C576) were genetically mapped to an apparent single locus at 5 o'clock, which was named "*absB*". Strain C120 was tested for Act and Red synthesis on seven types of minimal or complex media and produced none on media other than R5. Multiple copies of *act*II-ORF4 or *redD* bypass the mutation, showing that the strain is metabolically able to make Act and Red (3, 4). *absB* mutants

also produce much less CDA and Mmy than the parent. Strain C120 was not impaired in growth rate, although sporulation was somewhat less than normal on thin R5 plates. Strains C175 and C252 formed smaller than normal colonies on some media and their antibiotic-minus phenotype was more severe than C120's (178). In contrast with *absA** strains, the occurrence of spontaneous suppressing mutants in *absB* strains are rare.

Trifon Adamidis isolated a clone from the parental J1501 chromosomal DNA library that restored synthesis of all four antibiotics to the *absB* mutants (178). Brenda Price, also a graduate student at that time, continued characterization of the cloned fragment. The original fragment was reduced to a 1.4 kb sequence that was still capable of complementing each of the *absB* mutants. Sequencing revealed an ORF encoding a putative 276 amino acid protein with 41% amino acid sequence identity to *E. coli* RNAase III (encoded by the *rnc* gene) as well as homology to RNAase III's from other organisms. The *E. coli* RNAase III is a double-stranded endo-RNAase that processes rRNAs and many mRNAs. Although it is not essential for growth, RNAase III-minus *E. coli* strains grow slower than normal. Processing of transcripts by RNAase III results in either upregulation or downregulation of as many as 10% of *E. coli* proteins. The *E. coli* RNAase III contains a dsRNA binding domain and other conserved residues that are also predicted for the AbsB protein. The target sequence in *E. coli* is difficult to define, but often includes a stem-loop with an ~20 bp double-helical region. The *S. coelicolor*

RNAase III was shown to process 30S rRNA, as does the *E. coli* enzyme (178).

The native *S. coelicolor absB* gene was amplified from J1501 chromosomal DNA by PCR (178). The native gene was able to complement *absB* mutants as either a high- or low copy-number clone. *absB* was mapped by hybridization to a position on the physical map that corresponded to the 5 o'clock position previously determined by genetic mapping. The genetic structure of the *absB* region suggests that it may be cotranscribed with two upstream ORF's, one predicted to encode a 50S ribosomal protein, the other of unknown function. Homologs of *absB* have been detected in other streptomycetes by hybridization.

The *absB* gene was sequenced in three mutant strains: C120, C175, and C252 (178). In strain C120, the gene contained a mutation that would replace a Leu residue with a Pro, probably disrupting an alpha-helix. In strain C175, a mutation results in a nonsense codon at residue 8. In strain C252, a nucleotide duplication of nucleotides 505 to 511 would result in a frameshift mutation.

Disruption of the *absB* gene resulted in essentially the same phenotype as did the original point mutations, i.e., creation of a fairly tight Abs^- phenotype but with the more severe antibiotic deficiency typical of strains C175 and C252 (178). If the putative AbsB RNAase III functions as a regulator of antibiotics, it would appear to be a positive regulator. Alternatively, AbsB may not be a true regulator of antibiotics, but more simply

a processing enzyme that confers stability upon transcripts from the antibiotic-specific regulatory genes or higher order regulators.

3.5 *afs* Loci and Autoregulator Signaling Factors

The best studied of a group of small, diffusible, signaling molecules produced by streptomycetes is "A-factor", a γ-butyrolactone autoregulatory molecule produced by *Streptomyces griseus* that is necessary for both streptomycin synthesis and sporulation in that organism. A-factor is similar in structure and proposed function to the homoserine lactones (HSL's) found in various gram-negative bacteria. HSL's are bound with high affinity and specificity by receptor proteins and are believed to function as quorum sensors in some bacteria (101, 102). Based on the observation that A-factor is produced mainly during transition and stationary phases, Bibb (17) has proposed that it acts not as a quorum sensor but in response to physiological or environmental signal(s). In contrast, Neumann *et al.* (164) found evidence for a programmed developmental cycle in which the presence of low levels of A-factor during exponential growth were necessary to commit a colony to later differentiation; this program appeared to be independent of nutritional state.

S. coelicolor A3(2) produces at least four compounds structurally very similar to A-factor (17, 211). These compounds are capable of cross-stimulating *S. griseus* but, under conditions studied thus far, are not required for antibiotic synthesis or sporulation in *S. coelicolor* (87). However, one of

these (named SCB1) causes precocious production of Act and Red when added exogenously (47, 117).

Several *S. coelicolor* genetic loci affect synthesis of both antibiotics and A-factor-like compounds; these are named *afs* (for A-factor synthesis). A long-standing mystery regarding the identity of one of these loci, *afsB*, was recently solved. First reported in 1983, *afsB* mutants are defective in Act, Red, and SCB1 (6, 87, 103); CDA and Mmy are unaffected (3), as are all other observed characteristics. The effects on Act and Red are apparently mediated through significant decreases in transcription of *act*II-ORF4 and *redD*; interestingly, *red*Z transcription is unaffected (6, 105). Exogenous addition of SCB1 does not restore Act and Red (6). The *afsB* mutation is now known to be located in an RNA polymerase sigma factor, σ^{HrdB} (6) (see later discussion of sigma factors).

Another *afs* locus was discovered when a cloned fragment of *S. coelicolor* DNA, in multi-copy, restored Act, Red, and A-factor-like compounds to an *afsB* mutant strain. It was later discovered that the fragment did not correspond to the *afsB* locus (103, 203). Two ORFs were identified on this fragment and were named *afsR* (69, 103, 203) and *afsS* (150, 219). *afsR* alone, in multi-copy, can suppress the AfsB$^-$ phenotype and stimulate precocious hyperproduction of Act and Red in AfsB$^+$ *S. coelicolor* with corresponding increases in *act*II-ORF4 and *redD* transcription. *afsS* alone, in multi-copy, can also stimulate Act and Red; its effect on Act synthesis is moderated through *act*II-ORF4 transcription. *afs*R encodes a

993-residue protein with a potential DNA-binding helix-turn-helix motif, ATP-binding consensus sequences, and an N-terminus with significant sequence similarity to both *act*II-ORF4 and *redD* (104). *Afs*S encodes a 63-residue protein of unknown function (150, 219). The AfsR protein is phosphorylated by a membrane-bound kinase named AfsK (94, 149) which belongs to a class of serine/threonine kinases generally associated with eukaryotic two-component signal transduction systems. Deletion of *afsR* results in the absence or reduction of Act, Red, and CDA synthesis under certain nutritional conditions, while Mmy is unaffected (69). Phosphate concentration is a key nutritional factor in this conditional phenotype, leading to speculation that AfsR may play a role in a cascade that regulates antibiotics in response to phosphate depletion (69). Interestingly, *act*II-ORF4 and *redD* transcript abundance were not affected by disruption of *afsR*, but transcripts of the *act* biosynthetic gene *act*III were severely reduced (69). However, despite this and the sequence similarity between *afs*R and the antibiotic-specific regulators, *afsR* cannot substitute for *act*II-ORF4 and *redD* (69). When introduced into *absA* and *absB* mutant strains in multi-copy, a DNA fragment containing both *afsR* and *afsS* restores Act and Red synthesis in a medium-dependent manner but does not restore CDA or Mmy (44).

Two recently discovered *S. coelicolor* genes encode proteins with homology (35% identity) to *S. griseus* A-factor receptor proteins (169). These proteins, CprA and CprB, are predicted to contain DNA-binding helix-turn-helix motifs. Although they share 91% amino acid sequence identity,

CprA and CprB have quite different effects on antibiotic production. CprA appears to be a positive regulator of Act and Red, as disruption of *cprA* severely reduced and significantly delayed synthesis of those antibiotics, while introduction of several plasmid-borne copies into the parental strain resulted in overproduction. CprB appears to be a negative regulator of Act, as disruption of the encoding gene resulted in Act overproduction while additional copies reduced Act synthesis (Red was unaffected) (169).

A fragment of *S. coelicolor* DNA that stimulated Act, Red, and A-factor synthesis when introduced into *S. lividans* led to the discovery of the gene *afsQ*1 (109). This gene is homologous to the response/regulator components of two-component signal transduction systems. The corresponding histidine protein kinase was subsequently discovered downstream and named *afsQ*2. Disruption of both genes in *S. coelicolor* had no noticeable affect on antibiotic synthesis, therefore this system may be non-essential for antibiotic production or may operate under conditions that have not yet been defined. The ability of an *afsQ*1-carrying low copy-number plasmid to suppress an *S. coelicolor absA** mutation (109) tends to support the latter conclusion.

An *S. coelicolor* gene that may be involved in an autoregulator signaling pathway distinct from those discussed above has been discovered. This gene, known as *spaA*, is homologous to the RspA protein of *E. coli* (192). RspA has been implicated in a starvation-sensing pathway; it is proposed to affect induction of the stationary-phase sigma factor σ^S by degrading or

synthesizing homoserine lactones (107). Colonies of *spaA*-disrupted *S. coelicolor* strains exhibited delayed and decreased Act and Red production when growing on a poor carbon source and at low colony density. At high colony density, Act was overproduced. This phenotype was not rescued by the presence of *spaA*$^+$ colonies next to *spaA*$^-$ mutants, suggesting an intracellular signaling pathway. Its effect on antibiotic production and its proposed intracellular role points toward involvement of *spaA* in a pathway distinct from that of A-factor.

3.6 (p)ppGpp

Guanosine tetraphosphate [(p)ppGpp] has been proposed as a possible signal in intracellular nutrient-limitation detection pathways. In *E. coli*, (p)ppGpp is an important signal molecule for the regulation of growth rate-related gene expression. Depletion of amino acids in *E. coli* cultures triggers the "stringent response", which is initiated as uncharged codon-specific tRNAs are bound by a ribosomal protein (168) encoded by *relC*. This leads to activation of a (p)ppGpp synthetase encoded by the *relA* gene. Carbon/energy depletion can also result in (p)ppGpp synthesis through the action of another synthetase encoded by *spoT*. Ultimately, a reduction in RNA synthesis and an increase in the expression of amino acid biosynthetic genes occurs in response to the (p)ppGpp signal. A number of antibiotic-producing streptomycetes (67) exhibit a spike in (p)ppGpp concentration near the end of exponential growth in amino acid-limited media (204). The

temporal correlation between (p)ppGpp synthesis and the onset of production of many antibiotics has led to suggestions that (p)ppGpp may serve as an intracellular signal for antibiotic synthesis, at least under some nutritional conditions (204, 210).

S. coelicolor homologs of the *E. coli relA* and *relC* genes have been cloned (41, 148, 167, 208). A *relA* null mutant of *S. coelicolor* strain M600 produced no detectable (p)ppGpp and made neither Act nor Red when grown under nitrogen-limiting conditions; CDA synthesis was unaffected (40). Failure to produce Act and Red was reflected in severe reductions in *act*II-ORF4 and *redD* transcripts (40, 91). In the case of *act*II-ORF4, this effect was linked directly to (p)ppGpp levels through the use of a cloned, inducible copy of *relA* introduced into a Δ*relA* mutant (91, 208). Induction of the *relA* construct during late exponential growth led to a spike in (p)ppGpp, followed by increased transcription from *act*II-ORF4 and an *act* biosynthetic gene, and Act production (however, this response was not obtained in mid-exponential phase, indicating the necessity of other factors). Although low levels of *redD* and *red* biosynthetic gene transcription and Red antibiotic were obtained in response to modest concentrations of (p)ppGpp, higher (p)ppGpp concentrations failed to elicit significant responses, suggesting the necessity of another factor or factors. Growth in phosphate-limited conditions allowed Act and Red synthesis by a *relA* mutant, a result consistent with the theory that *relA* is involved in sensing nitrogen limitation instead of starvation conditions in general (41). In continuous culture, *relA* proved to be important

for optimal production of Act and Red under glucose-limited conditions as well (116).

A second gene with similarity to *relA* and *spoT* was recently discovered in the *S. coelicolor* genome (208); named *rshA*, its role is uncertain at this time since deletion of the gene has no apparent effect. A *relC* deletion mutant did not produce (p)ppGpp, Act, or Red (168). These results strongly imply that (p)ppGpp plays a role in triggering the synthesis of Act and Red, at least under certain nutritional conditions.

3.7 RNA Polymerase Sigma Factors

One method by which organisms can regulate gene expression is by altering the specificity of RNA polymerase through its exchangeable sigma subunit. *S. coelicolor* possesses at least nine unique sigma factors. This number is likely to increase as additional described transcribing activities and sigma-homologous DNA sequences are classified; it was recently predicted that the final count will be greater than twenty (96). Of the current nine, two have been linked to general vegetative gene expression (30, 114, 227), two with sporulation (50, 177), one with extracytoplasmic functions (142, 173), and one with response to oxidative stress (115, 172). Of these, four have also been linked in some way to antibiotic synthesis.

The *S. coelicolor* sigma factor genes *hrdA*, *hrdB*, *hrdC*, and *hrdD* were identified through sequence similarity to a conserved sequence in the principal sigma factor RpoD of *E. coli* and *B. subtilis* (212) and *M. xanthus*

(34) (*hrd* stands for homolog of rpoD). The sigma factor product of the gene *hrdD*, σ^{HrdD}, is the major *in vitro* transcribing activity of the antibiotic-specific regulatory genes *act*II-ORF4 and *redD* (73). *hrdD* is transcribed in liquid-grown cultures (34) and translated into a protein of Mr =46 kDa (73). σ^{HrdD} is controlled by σ^{E} (142), which itself has recently been found to be controlled by a putative two-component signal transduction system which may respond to signals from the cell envelope (173). However, disruption of *hrdD* results in no obvious defects in antibiotic synthesis or any other function, placing in doubt a role for σ^{HrdD} for *in vivo* antibiotic synthesis (35).

σ^{HrdB} (Mr=66 kDa) is believed to be essential based on the failure of attempts to isolate *hrdB*-disrupted mutants and may be a "housekeeping" sigma factor equivalent to *E. coli* σ^{70} (30). Somewhat higher σ^{HrdB} – dependent transcriptional activity is detected in exponential phase compared to stationary phase (114). *In vitro*, σ^{HrdB} recognizes a ribosomal RNA promotor (114), several other promotors that are likely to be expressed in exponential phase (30, 36), and supports the synthesis of minor quantities of *act*II-ORF4 and *redD* transcripts (73). It is now quite certain that σ^{HrdB} is the major (and perhaps only) sigma factor supporting transcription of the antibiotic-specific regulators *act*II-ORF4 and *redD in vivo*. As discussed earlier, a mutation in σ^{HrdB} was recently found to be the cause of the Act^{-}, Red^{-}, "AfsB" phenotype. Transcription of *act*II-ORF4 and *redD* are strongly reduced in the presence of this mutation, a G243D substitution located in a highly conserved region of unassigned function. How could a mutation in

σ^{HrdB} have such narrow consequences? Aigle *et al.* (6) have hypothesized, based on the location of the mutation, that it may result in ineffective binding to either a regulatory factor or to RNA polymerase. It is easily imagined how the former case could lead to highly specific effects. Aigle *et al.* have proposed that, in the latter case, the mutant σ^{HrdB} might bind to RNA polymerase well enough to be sufficiently effective during exponential growth when present in high concentrations, but at low concentrations during transition phase it is ineffective. It is not known whether σ^{HrdB} has any role in regulating antibiotic synthesis.

Another sigma factor known as σ^{52} (Mr = 52 kDa) (36, 114) and a putative sigma factor with M_r = 31 kDa (114) each recognizes one of two identified promotors of the *act*III biosynthetic gene *in vitro*. Each has higher activity in stationary than in exponential phase (114).

3.8 Other Potential Regulators

The *cutR*/*cutS* two-component signal transduction system was discovered fortuitously within a cloned fragment of *S. lividans* DNA (217) that negatively regulates Act and Red production in *S. coelicolor* when in high copy-number (45). Disruption results in antibiotic overproduction. Little else is known of this system.

abaA was isolated as a fragment of *S. coelicolor* DNA causing Act synthesis when introduced into *S. lividans* in a high copy-number plasmid (66). This fragment maps to a different location from the *abs* and *afs* genes,

at approximately 2 o'clock. Two ORFs (*orfA* and *orfB*) on the fragment were shown to be responsible for the phenotype; *orfA* shows similarity to the transmitter region of two-component systems, while *orfB* did not show similarity to any known genes. Disruption of *orfB* gene in *S. coelicolor* results in the complete loss of Act and significant reductions in Red and CDA synthesis without affecting Mmy or sporulation.

The *mia* (for multi-copy inhibition of antibiotics) sequence was discovered fortuitously by Adamidis and Champness on an *S. coelicolor* DNA fragment that inhibited production of Act, Red, and CDA when introduced in copy numbers greater than 2-3 (42). As little as 90 bp of sequence from the fragment (which includes a portion consistent with the formation of a large stem loop) is capable of causing this effect (9). The 90 bp sequence includes the likely promotor region and beginning of an ORF; the antibiotic-inhibitory effect of *mia* is therefore likely to result from titration of a DNA-binding protein. The *mia* promotor appears to transcribe an operon, tentatively named *absC1* (188). The N-terminus of AbsC1 aligns with two-component histidine kinases, the C-terminus with protein phosphatases.

The recently discovered ClpP ATP-dependent proteases (ClpP1 and ClpP2) and their putative regulators ClpX and ClpC (56) may also regulate antibiotic production. Overproduction of ClpX accelerates Act production in *S. coelicolor* and induces Act production in *S. lividans*. Proteases of this family affect stationary phase or differentiation phenomena in *E. coli* and *B. subtilis* through degradation of specific proteins. Degradation of competing

primary phase regulatory proteins may be an important function of these proteases since slow rates of cell growth and division at the time of differentiation prevents dilution of such regulators.

3.9 Overview of Antibiotic Synthesis Regulation in *S. coelicolor.*

It is becoming clear that streptomycetes possess complex and finely tuned genetic systems that control antibiotic production in response to many environmental and internal signals. Not only are the onset and shutdown of antibiotic synthesis controlled, it appears that quantities are modulated as well and that each of the four antibiotics produced by *Streptomyces coelicolor* is (to some degree) individually controlled.

The genetic circuitry governing metabolic and morphological differentiation in *S. coelicolor* is often pictured as a regulatory tree with *bld* genes at the trunk; mutations at these loci generally result in a global blockage of antibiotics and arrest sporulation at its earliest steps. The *bld* loci that have been cloned and sequenced are predicted to encode proteins with a wide variety of functions including extracellular signaling, signal transduction, and both transcriptional and postranscriptional regulation. Environmental and internal signals feeding into this regulatory level are likely to include carbon and/or energy starvation and, through an extracellular oligopeptide signal, colony density, growth rate, or coordination of a group response. It is likely that some of the *bld* loci will be found to encode

"housekeeping" functions that are necessary for differentiation but do not truly regulate it.

At the next level, the regulatory tree forms branches consisting of master regulators dedicated to sporulation (*whi* genes) or antibiotics. Genetic elements at this level may be controlled to some degree by *bld* genes. Master regulators of antibiotics include some that globally influence the four antibiotics (*absA*, *absB*, and possibly the *mia/absC1* locus) and others that affect two or more antibiotics (*afsK/afsR*, *afsS*, *afsQ2/Q1*, *cutR/cutS*, *cprA*, *cprB*, *spaA*. *relC*, and *relA*). Some signals that are likely to feed into regulation at this level are Scb1 and other gamma-butyrolactones (possibly communicating growth rate or colony density) and nitrogen, phosphate, and carbon starvation. Signal transduction is accomplished by phosphorelay in many of these systems. Other conditions that may be monitored (as suggested by some evidence or precedent) are levels of trace elements, pH, temperature, and waste products; some of these are likely to be detected directly, others monitored through a general mechanism such as growth rate.

At the final level of regulation are antibiotic-specific regulators. It is very likely that many of the higher order regulatory pathways target these antibiotic specific regulators, while others influence antibiotic biosynthetic genes directly.

4.0 This Work

A primary goal of researchers studying streptomycetes is to understand, at the molecular genetic level, the regulation of secondary metabolism and cellular differentiation in these organisms. In addition to valuable basic knowledge, an enormous practical value is associated with the potential for efficient, knowledge-based manipulation of the expression of antibiotics and other pharmaceuticals produced by streptomycetes.

A significant body of knowledge has been amassed and continues to accumulate regarding antibiotic biosynthetic genes and their protein products. A number of confirmed or putative "higher order" regulators of antibiotics have been discovered; in most cases, the mechanism by which they affect antibiotic production is poorly understood. The majority of the work described in this dissertation seeks to define unknown aspects of several of these regulatory pathways; specifically, potential transcriptional regulation of several *S. coelicolor* antibiotic-specific regulatory and biosynthetic genes by the *absA*1/*absA*2, *absB*, and various *bld* loci were investigated. Transcription was monitored in two ways: by S1 nuclease protection transcript assays and by transcriptional fusions between promotors of interest and the reporter gene *xylE*. The use of two methods has allowed comparison of the techniques as well as exploitation of the strengths of each.

A second facet of this work is the investigation of techniques to monitor antibiotic synthesis by streptomycetes growing in soil, their natural

environment. A number of researchers have tried and failed to detect antibiotic synthesis by streptomycetes in soil. A new approach was made possible by the relatively recent development of reporter genes encoding enzymes that catalyze light-producing reactions. Several approaches were investigated towards the goal of detecting transcription of *lux* reporter gene-fused *S. coelicolor* antibiotic biosynthesis-related genes in soil microcosms.

CHAPTER 2

TRANSCRIPTIONAL REGULATION OF *Streptomyces coelicolor* PATHWAY-SPECIFIC ANTIBIOTIC REGULATORS BY THE *absA* AND *absB* LOCI

PART A

Aceti, D. J. and W. C. Champness. 1998. Transcriptional regulation of *Streptomyces coelicolor* Pathway-Specific Antibiotic Regulators by the *absA* and *absB* Loci. **180**:3100-3106.

Note: Higher quality reproductions of the figures on pages 60, 61, and 62 can be found on pages 133-135.

p. 3100

Aceti, D. J. and W. C. Champness. 1998. Transcriptional regulation of *Streptomyces coelicolor* Pathway-Specific Antibiotic Regulators by the *absA* and *absB* Loci. **180**:3100-3106.

p. 3101

Aceti, D. J. and W. C. Champness. 1998. Transcriptional regulation of *Streptomyces coelicolor* Pathway-Specific Antibiotic Regulators by the *absA* and *absB* Loci. **180**:3100-3106.

p. 3102

Aceti, D. J. and W. C. Champness. 1998. Transcriptional regulation of *Streptomyces coelicolor* Pathway-Specific Antibiotic Regulators by the *absA* and *absB* Loci. **180**:3100-3106.

p. 3103

Aceti, D. J. and W. C. Champness. 1998. Transcriptional regulation of *Streptomyces coelicolor* Pathway-Specific Antibiotic Regulators by the *absA* and *absB* Loci. **180**:3100-3106.

p. 3104

Aceti, D. J. and W. C. Champness. 1998. Transcriptional regulation of *Streptomyces coelicolor* Pathway-Specific Antibiotic Regulators by the *absA* and *absB* Loci. **180**:3100-3106.

p. 3105

Aceti, D. J. and W. C. Champness. 1998. Transcriptional regulation of *Streptomyces coelicolor* Pathway-Specific Antibiotic Regulators by the *absA* and *absB* Loci. **180**:3100-3106.

p. 3106

Aceti, D. J. and W. C. Champness. 1998. Transcriptional regulation of *Streptomyces coelicolor* Pathway-Specific Antibiotic Regulators by the *absA* and *absB* Loci. **180**:3100-3106.

PART B

ADDITIONAL RESULTS AND DISCUSSION

Transcripts of an *act* biosynthetic gene are more severely reduced than *act*II-ORF4 transcripts in the *abs* mutants. Figures 2 and 4 (above) show that *act*II-ORF4 and *redD* transcripts are present at higher levels in the *abs* mutant strains than might be expected based on the absence of detectable antibiotic production in those strains. Substantial levels of *redD* transcript were again detected in the *absA-542* mutant in recent experiments of Ryding and Champness as well (187). Therefore, it was of interest to determine whether antibiotic biosynthetic gene transcript levels were similarly disproportionate to antibiotic synthesis. The comparison presented in Table 1, below (which incorporates experiments presented in Part A of this work as well as additional, unpublished data), indicates that transcripts of the *act*VI-ORF1 biosynthetic gene are reduced substantially more than are *act*II-ORF4 transcripts in both *abs* mutants. The degree of reduction in biosynthetic gene transcripts may, in some cases at least, explain the actinorhodin-deficient phenotypes of these mutants (a similar comparison of the effects of *abs* mutations on *red* regulatory and biosynthetic genes could not be made due to the lack of a well-characterized *red* biosynthetic gene). However, it should be noted that Act production is not observed even in cases where substantial quantities of actVI-ORF1 transcripts are present (i.e., Figure 3 and Table 1

Timecourse 3). Possible implications of this phenomenon are discussed in Chapter 4 of this work, "Conclusions and Prospects".

Table 1. Comparison of reductions in *act*II-ORF4 and *act*VI-ORFI transcript levels in *abs* mutants.

RNA Time-course[a]	Fold transcript reduction in C542 (*absA**) relative to J1501, for each assay performed		Fold transcript reduction in C120 (*absB*$^-$) relative to J1501, for each assay performed	
	*act*II-ORF4	*act*VI-ORF1	*act*II-ORF4	*act*VI-ORF1
#1	6, 5[b]	>50	2, 2	12, 25
#2	4, 4	11	4, 4	18
#3	3[c]	4, 6[d]	12[c]	13[d], 48

[a]Total RNA was isolated as described in Materials and Methods from 48, 54, and 60 hour-old minimal mannitol medium plate cultures (Timecourse #1), from 39, 48, 54, and 60 hour-old PGA medium plate cultures (Timecourse #2), and from 39, 48, 54, and 65 hour-old PGA plate cultures (Timecourse #3).
[b]Transcripts were quantitated as described in Material and Methods. Data deemed unreliable due to low signal-to-noise ratio is not presented.
[c]Derived from experiment shown in Figure 2, this work.
[d]Derived from experiment shown in Figure 3, this work.

Do the *abs* genes influence the timing of onset of transcription from *act* and *red* genes? Clearly, the *abs* genes influence *act*II-ORF4, *redD*, and *act*VI-ORF1 transcript abundance. Less clear is whether the *timing* of antibiotic regulatory and biosynthetic gene transcriptional onset is affected by the *abs* genes. The experiment shown in Figure 4 (above) suggests that the timing of *redD* transcriptional onset is not (or is not greatly) altered in the *abs*

mutants; the first appearance of substantial quantities of *redD* transcripts occurs at the 48-hour timepoint in both of the mutants and in J1501. Similarly, an increase in *act*II-ORF4 transcript levels was detected at the 48-hour timepoint in strains J1501, C542, and C120 in Figure 6 (below). In each of these experiments, however, temporal shifts of less than nine hours could have occurred without detection.

The above data suggest that the *abs* genes do not (or do not greatly) affect the timing of transcriptional onset of *act*II-ORF4 and *redD*. Additional data bearing on this question, derived from reporter gene assays presented in the following chapter, as well as the work of Brian *et al.* (26), Anderson *et al.* (10), and Ryding *et al.* (187), are discussed in Chapter 4 of this work.

Identity of a secondary band in *act*VI-ORF1 assays. In assays of *act*VI-ORF1 transcripts performed in this work (e.g., Figure 3, above), minor protected fragments corresponding to a length of approximately 170 bp were observed in addition to those resulting from the predicted 191 nucleotide transcript. These minor bands were unexplained at the time of publication of that figure. Data published since that time by Arias *et al.* (12) suggests a possible explanation. Footprinting studies identified a secondary ActII-ORF4 binding site within the *act*VI-ORF1 gene that, if transcription were initiated at this position, would result in a protected fragment of approximately the size observed. Furthermore, a potential GTG start codon is located in the same area,16 bp downstream from the primary transcription initiation site.

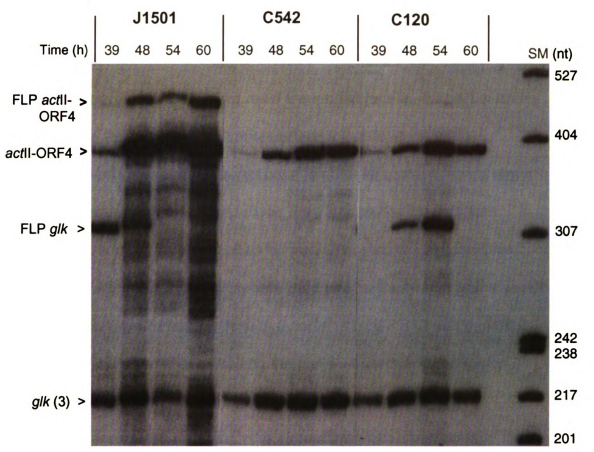


Figure 6. Expression of *act*II-ORF4 mRNA in J1501 (*abs*+), C542 (*absA*") and C120 (*absB*-). RNA was isolated from PGA plate-grown cultures at the times indicated and used in S1 nuclease protection assays. The size markers (SM) and probes for *act*II-ORF4 and *glk* were as described in Figure 1. "FLP *act*II-ORF4" indicates the position of full-length probe; transcriptional readthrough from the upstream promotor for *act*II-ORF3 likely contributes to the signal as well.

APPENDIX

Complementation of the *absB*⁻ mutation with the cloned *absB* gene partially restores *act*II-ORF4 and *redD* transcript levels. As part of an effort to isolate the genetic locus represented by the *absB* mutation, I assayed *act*II-ORF4 and *redD* transcripts in a putative complemented strain of the *absB*⁻ mutant C120. Brenda Price, then a graduate student, had isolated a candidate 2.6 kb sequence from an *S. coelicolor* genomic DNA library (178). After cloning this fragment into the *Streptomyces* suicide vector pIJ963, Brenda transformed the resulting construct (pBK310) into the *absB*⁻ strain C120. Transformants exhibiting the pIJ963-encoded hygromycin resistance contained pBK310 integrated into the chromosome by homologous recombination through the cloned fragment. Several Hyg^R colonies were obtained; these produced Act and Red at levels similar to the parental strain J1501 (178).

To determine whether restoration of *act*II-ORF4 and *redD* transcription had occurred coincident with antibiotic synthesis, I assayed total RNA isolated from cultures of strains J1501, C120, and C120/pBK310. The C120/pBK310 plate cultures from which RNA was obtained began producing visible quantities of Act at approximately 39 hours compared to 48 hours in J1501 cultures. Both J1501 and C120/pBK310 cultures had produced copious quantities of Red by 39 hours. S1 nuclease protection assays were used to determine *act*II-ORF4 and *redD* transcript abundance (Figure 7).

Figure 7. Complementation of *absB*⁻ mutants by a cloned DNA fragment. RNA was isolated from PGA plate cultures at the times indicated and used in S1 nuclease protection assays. The size markers (SM) and probes for *act*II-ORF4, *redD*, and glk were as described in Figures 1 and 4. "FLP" indicates full-length probe.

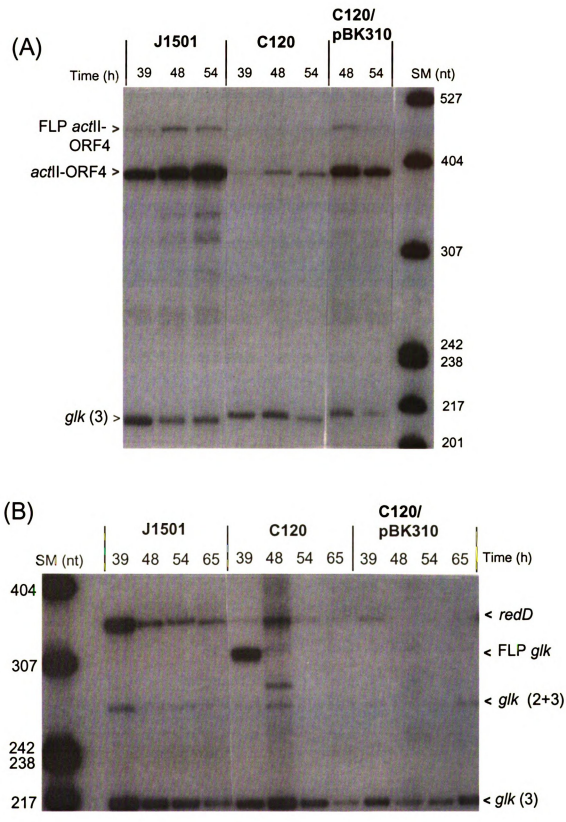

(A)
J1501
C120
C120/
pBK310
Time (h)
39
48
54
39
48
54
48
54
SM (nt)
527
404
307
242
238
217
201
FLP actII- >
ORF4
actII-ORF4 >
glk (3) >
(B)
J1501
C120
C120/
pBK310
SM (nt)
39
48
54
65
39
48
54
65
39
48
54
65
Time (h)
404
307
242
238
217
< redD
< FLP glk
< glk (2+3)
< glk (3)

Figure 7

The quantity of *act*II-ORF4 transcript in strain C120/pBK310 was nearly that of the parental J1501 (Figure 7A). Restoration of *redD* transcript levels, however, was less complete (Figure 7B); following adjustment for the glucose kinase internal control transcript, *redD* was found to be 2.5-fold higher in C120/pBK310 than in C120 but still 5-fold lower than in J1501. This apparently incomplete restoration of *redD* transcription may be an artifact caused by assaying at timepoints when transcription was dropping rapidly, resulting in magnification of small differences in growth phase. As described by Price *et al.* (178), an 828 nucleotide open reading frame located within the 2.6 kb fragment of pBK310 was determined to be the *absB* gene.

CHAPTER 3

TRANSCRIPTIONAL REGULATION OF THE *act*II-ORF4 PROMOTOR: REPORTER GENE FUSION STUDIES

ABSTRACT

Transcriptional fusions between the promotor of the *act*II-ORF4 antibiotic regulatory gene of *Streptomyces coelicolor* and the *xylE* reporter gene were created in a low-copy plasmid and in an integrative phage. These fusions were used to investigate the possible effects on *act*II-ORF4 transcription of mutations in the proposed master regulators *absA*, *absB*, and several *bld* genes. When introduced into the parental strain J1501, expression patterns reflected those observed in previous direct assays of *act*II-ORF4 transcripts, suggesting normal regulation of the promotor. Transcript levels in *absB* mutant cultures were about 50% less than in the parental strain when averaged over a timecourse, a result compatible with prior direct transcript assays. In contrast, transcription was not altered significantly in the *absA* mutant; this result contrasts with prior S1 nuclease protection experiments showing a 3 to 6-fold reduction in *act*II-ORF4 transcription in the absA mutant. Several hypotheses are proposed to explain this apparent conflict. In addition, transcription was not altered significantly in *bldA*, *bldB*, *bldD*, *bldG*, *bldH*, *bldI*, or *bldA95* mutant strains.

INTRODUCTION

Prior to investigating the possible roles of *absA* and *absB* in regulating antibiotic-specific regulatory gene transcription as described in the previous section (through S1 nuclease protection assays), an effort was made to address these questions using reporter gene fusions. The reporter gene approach possessed several potential advantages: (1) A number of reporter genes have previously been used with success in streptomycetes, (2) Once constructed, fusions are often easily and quickly introduced into a large number of backgrounds, and (3) The relative ease of reporter gene assays may allow the survey of many timepoints with many assay repetitions, potentially resulting in more precise data.

The catechol 2,3-dioxygenase (CDO)-encoding *xylE* gene derived from *Pseudomonas putida* was chosen for these studies due to its superior characterization as the most commonly used reporter gene in *Streptomyces* research. Despite its popularity, certain potential pitfalls exist in its use (as is the case with any reporter gene, or indeed with any method of transcript quantification); these were discussed in more detail in the Introduction to this work.

Transcriptional fusions between the *act*II-ORF4 promotor and *xylE* were constructed in a low-copy replicative plasmid and in an integrative phage. These were introduced into the J1501 parental type and derivative *absA*, *absB*, and *bld* mutant strains. Preliminary results, in which CDO activity was

assayed visually (uncorrected for changes in cell mass), suggested abnormal expression of the fusions; in particular, an apparent lag of greater than 20 hours between transcription from *act*II-ORF4 and from an *act* biosynthetic gene suggested constitutive expression of the former. Therefore, the reporter gene approach was initially abandoned in favor of S1 nuclease protection assays. However, later reassessment of the data led to additional experiments that are presented in this chapter. These experiments were improved over those performed earlier by spectrophotometric determination of CDO activity with normalization for changes in cell mass. They were designed to extend the study of transcriptional regulation of *act*II-ORF4 to a number of *bld* strains, and to test the viability of the *xylE* reporter gene method through comparison with S1 nuclease protection assay data.

MATERIALS AND METHODS

Bacterial strains. *S. coelicolor* strains used were: J1501 (*hisA*1, *uraA*1, *strA*1, *pgl*)(49), C542 (*hisA*1, *uraA*1, *strA*1, *pgl*, *absA*1-542) (5), C120 (*hisA*1, *uraA*1, *strA*1, *pgl*, *absB*-120) (3), J1501/KC900 (31), and *bld* strains (1) C103 (*bldA301*), C186 (*bldB186*) , C112 (*bldB112*), C181 (*bldH181*), C536 (*bldG536*), C109 (*bldH109*), C249 (*bldI249*), J774 (*bldD53*) (156), and A95 (2),

Growth conditions. Cultures used for reporter gene studies were grown by spreading spore suspensions ($1x10^5$ cfu/plate) onto cellophane discs placed on SpMR agar media containing 6.25 μg/mL thiostrepton.

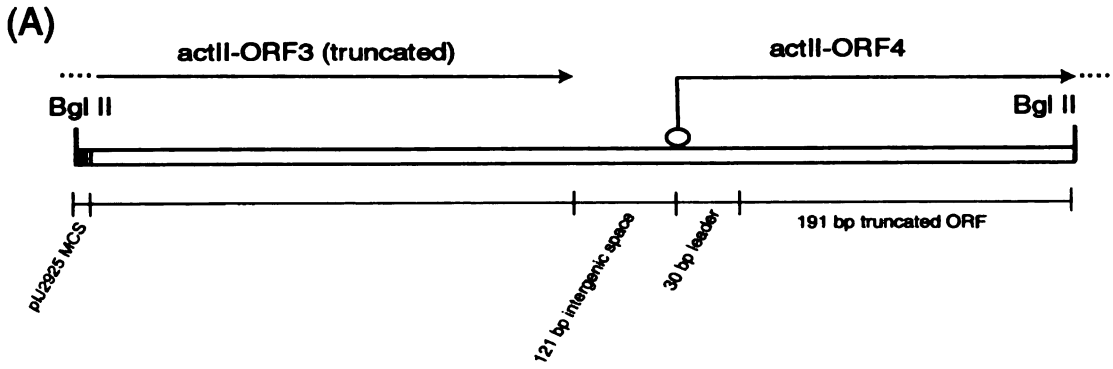
Enzyme assays. Cell extracts were prepared and catechol dioxygenase activity was determined spectrophotometrically essentially as described by Ingram *et al.* (108), except that cell material scraped from plates and suspended in 1 mL sample buffer was substituted for liquid cultures. Where activity was visually determined, plates were sprayed with 0.5 M catechol solution and incubated for 1 hour at 30°C before color development was assigned. Protein concentration was determined by the method of Bradford (24) using reagent purchased from Sigma with bovine serum albumin as the standard.

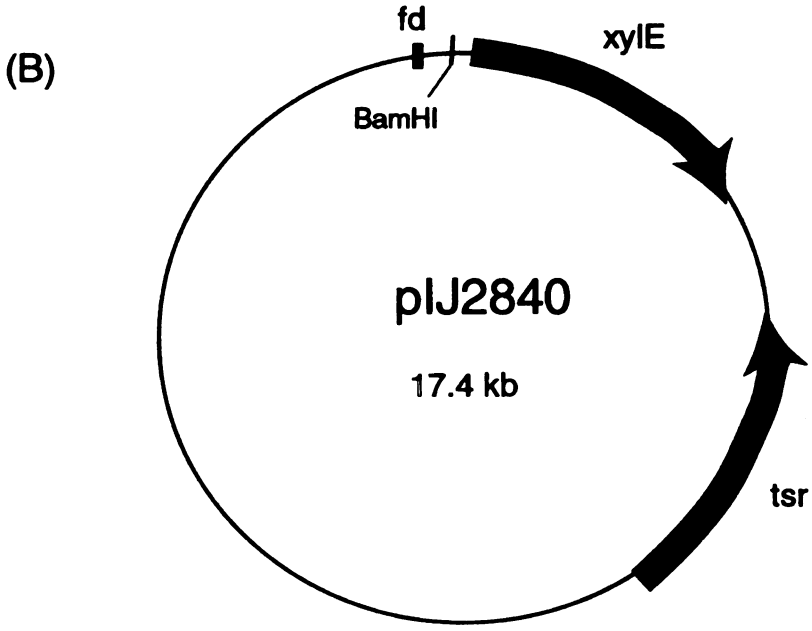
Note: Images in this dissertation are presented in color.

RESULTS

Construction of low-copy and single-copy *act*II-ORF4::*xylE* transcriptional fusions. Two types of *act*II-ORF4::*xylE* transcriptional fusion were created. A low-copy (1-3/chromosome) replicating plasmid construct was made by ligating a fragment of *S. coelicolor* DNA containing the *act*II-ORF4 promotor (Figure 8A) into the plasmid pIJ2840 (18), which carries a promotorless *xylE* gene (Figure 8B). This plasmid construct, named pDA4, had the advantage of ease of construction and introduction into *Streptomyces* hosts. Next, a construct that would be maintained at

Figure 8. Construction of the *act*II-ORF4::*xylE* fusion vectors pDA4 and DA1. A 1.6 kb *S. coelicolor* DNA fragment that included the *act*II-ORF4 promotor region (A) was ligated into the vectors depicted (B and C) to produce transcriptional reporter gene fusions. The promotor fragment was prepared by subcloning from pAT107, an *act*II-ORF4-containing clone isolated by T. Adamidis (3), into the pUC18 derivative pIJ2925 (112) and re-isolating it with *Bgl*II ends to obtain the fragment depicted ("pIJ2925 MCS" is the multiple cloning site sequence of that vector). The promotor fragment was ligated into the *Bam*HI site of pIJ2840 (B) to produce the *xylE* fusion construct pDA4. The phage construct DA1 was produced by ligating the promotor fragment into phage KC859 (C) that had been digested with *BamHI* and *BglII*. Both vectors contain the *tsr* thiostrepton resistance gene as a selective marker. "*fd*" is the major transcriptional terminator of coliphage fd. "t_o" is the terminator of coliphage lambda.





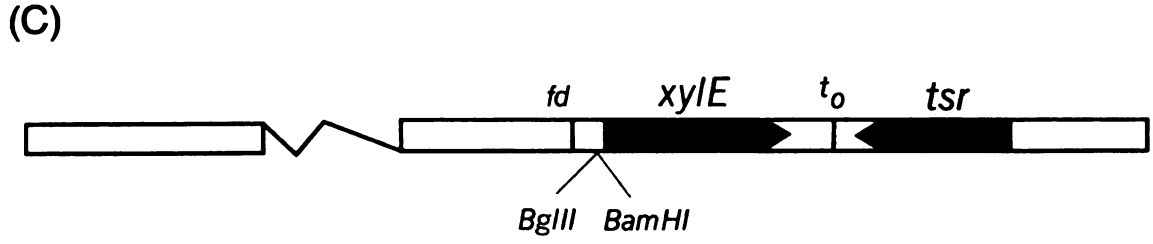


Figure 8 KC859 (39 kb)

single-copy in the *S. coelicolor* chromosome was made by ligating the *act*II-ORF4 promotor fragment into KC859 (31), a derivative of the ϕC31 actinophage with a promotorless *xylE* gene (Figure 8C). The resulting phage, DA1, integrated at single copy into the chromosomal *attB* site when transfected into S. *coelicolor*. Several attempts to create a third vector by which the promotorless *xylE* could be integrated into the native *act*II-ORF4 gene were unsuccessful.

The *act*II-ORF4::*xylE* fusions are subject to regulation in S. coelicolor. Before using the *act*II-ORF4::*xylE* fusions to investigate the effects of mutant backgrounds on transcriptional activity, it was necessary to establish expression patterns in the parental strain. Therefore, pDA4 and DA1 were introduced into *S. coelicolor* strain J1501 by transformation and transfection/integration, respectively. It was desired to compare the timing of *act*II-ORF4 expression with that from an *act* biosynthetic gene (i.e., a gene known to be regulated by *act*II-ORF4). The actinorhodin biosynthetic gene chosen for comparison in this study was *actI*, which consists of three ORF's encoding the central β-ketoacyl-synthase/acyl carrier/acyl transferase activities of the actinorhodin polyketide synthase (67). Therefore, a transcriptional *actI*::*xylE* fusion was created using KC900 (31), a bacteriophage ϕC31 derivative supplied by Keith Chater (John Innes Institute, U.K.). In this *attP*⁻ phage, an internal fragment of the *actI* gene sequence cloned upstream of a promotorless *xylE* allows homologous

recombination into the *S. coelicolor* chromosome upon transfection, fusing *xylE* to the native *actI* promotor. Such a fusion was created in strain J1501.

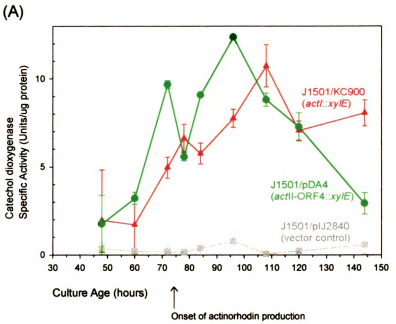
Expression patterns from the *act*II-ORF4 and *actI* fusions were expected to correspond generally to those observed by S1 nuclease protection assays for *act*II-ORF4 and *actV*I-ORF1, respectively, in Chapter 2. Additional precedent was provided by the studies of Gramajo *et al.* (81), who monitored transcripts of *act*II-ORF4 and the *act* biosynthetic genes *act*III and *actV*I-ORF1 in liquid cultures by S1 nuclease protection assays. Gramajo *et al.* captured early timepoints in the growth cycle, showing that *act*II-ORF4 is transcribed at low rates from the earliest assayable timepoints in exponential phase but increases dramatically as a culture nears and passes through transition phase, then decreases in stationary phase. Transcription from *act*III and *actV*I-ORF1 was detectable 1 to 2 hours after the onset of major *act*II-ORF4 transcription and was followed almost immediately by visible Act production. In summary, it was expected that as cultures aged, rising levels of regulatory gene transcripts would be followed closely by biosynthetic gene transcripts, itself followed by Act production.

Differences in the growth media used in these studies should be noted here due to their effects on the timing of transition to secondary metabolism. Gramajo *et al.* reported visible Act production after approximately 14 hours of incubation in stirred casamino acid-supplemented liquid minimal medium cultures. In Chapter 2 of this work, RNA was isolated from cultures grown on Peptone Glucose Agar plate medium; Act was visibly produced by J1501

(Abs+) cultures beginning at approximately 42 hours. In these two studies, act genes were strongly expressed shortly before the appearance of Act. A relatively rich solid medium, SpMR, was used for the reporter gene studies reported in this chapter since attempts to use PGA medium were unsuccessful due to poor growth (possibly stemming from variation in medium components). Act was visibly produced on SpMR after approximately 73 hours of growth, and it was expected that actII-ORF4 expression in the current experiments would occur shortly before visible Act formation, as it did in the previous experiments. It was unclear whether the longer lag times between events (i.e. transcription from *act*II-ORF4, *act* biosynthetic genes, and Act production) would also result from a lengthened growth cycle.

Fusion strains were plated at equal densities on selective agar medium and CDO specific activity monitored over time (Figure 9). Transcription patterns from the *act*II-ORF4::*xylE* fusions generally correlate with those expected based on the experiments presented in Chapter 2 of this work (Figures 1 and 2) and on the data of Gramajo *et al.* (81). The time span between major *act*II-ORF4 expression and the onset of *actI* expression appears to be between 0 and 24 hours or 0 and 12 hours based on the data shown in Figures 9B and 9D, respectively. Therefore, this span may not be significantly greater than the 1-2 hours noted by Gramajo *et al.* or it may be considerably longer.

Figure 9. Expression from *act*II-ORF4::*xylE* and *act*I::*xylE* transcriptional fusions in *S. coelicolor* strain J1501. Cultures were grown and assayed as describe in Materials and Methods. Strains J1501/KC859 and J1501/pIJ2840 carry the parental (promotorless *xylE*) vectors as negative controls. Graphs A and B represent replicate experiments, as do graphs C and D, but with an additional timepoint (at 36 hours) in graphs B and D. A single experiment using concurrently-grown cultures resulted in the data shown in graphs A and C; data was divided into two graphs for readability, with the J1501::KC900 timecourse used in both. Graphs B and D also result from a single experiment with similar treatment of data. With the exception of 36 hour timepoints, each data point represents the mean of three determinations of catechol dioxygenase specific activity in a single cell extract; the former represent triplicate assays of each of two extracts. Error bars indicate standard deviation. A unit of catechol dioxygenase activity was defined as $\Delta OD_{375} x 10^6$/sec. The onset of actinorhodin synthesis was determined visually as the first detectable appearance of red pigment that became blue upon addition of sodium hydroxide solution.


(A)
Catechol dioxygenase
Specific Activity (Units/ug protein)
J1501/KC900
(actI::xylE)
J1501/pDA4
(actII-ORF4::xylE)
J1501/pIJ2840
(vector control)
Culture Age (hours)
Onset of actinorhodin production

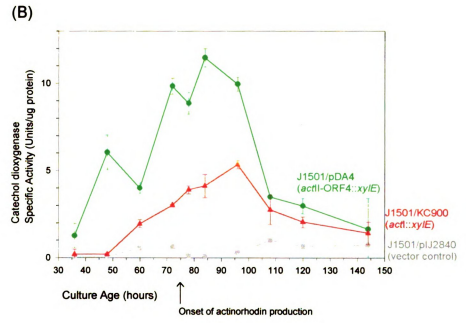
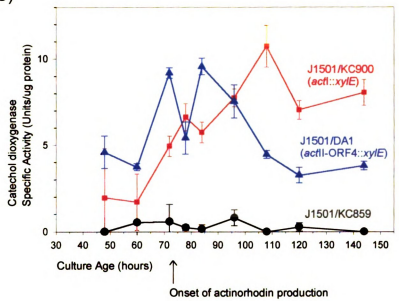

(B)
Catechol dioxygenase
Specific Activity (Units/ug protein)
J1501/pDA4
(actII-ORF4::xylE)
J1501/KC900
(actI::xylE)
J1501/pIJ2840
(vector control)
Culture Age (hours)
Onset of actinorhodin production

Figure 9A.

(C)


Catechol dioxygenase
Specific Activity (Units/ug protein)
0
5
10
30
40
50
60
70
80
90
100
110
120
130
140
150
Culture Age (hours)
J1501/KC900
(actI::xylE)
J1501/DA1
(actII-ORF4::xylE)
J1501/KC859
Onset of actinorhodin production

(D)

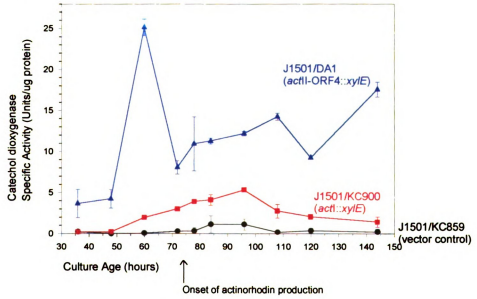

Catechol dioxygenase
Specific Activity (Units/ug protein)
0
5
10
15
20
25
30
40
50
60
70
80
90
100
110
120
130
140
150
Culture Age (hours)
J1501/DA1
(actII-ORF4::xylE)
J1501/KC900
(actI::xylE)
J1501/KC859
(vector control)
Onset of actinorhodin production

Figure 9B.

Expression from *act*II-ORF4::*xylE* fusions in *absA* and *absB* mutant backgrounds. The fusion constructs pDA4 and DA1 were introduced into the *absA* mutant strain C542 and the *absB* mutant strain C120. In Figure 10, transcription from *act*II-ORF4 in those strains and in strain J1501 are compared. A general reduction in expression is noticeable in the *absB*$^-$ strain; in experiments A, B, C, and D, respectively, expression in C120 was 56%, 35%, 31% and 62% of that in J1501 for an overall mean value of 46% (percentages were determined from the mean of all data points for C120 and for J1501 in each timecourse). In the *absA*1-542 strain, however, differences are substantially less; values in C542 were 68%, 86%, 78%, and 155% of those in J1501 for experiments A, B, C, and D, respectively, for a combined mean of 97%.

Figure 10. Expression from *actII*-ORF4 fusions in *absA*⁻ and *absB*⁻ strains. Experiments were done essentially as described in Figure 9. Graphs A and B represent replicate experiments with the exception of the 36 hour timepoint, as do graphs C and D. Actinorhodin was visible at the time indicated in the J1501 cultures; no pigment was visible in the C542 and C120 cultures over the timecourses.

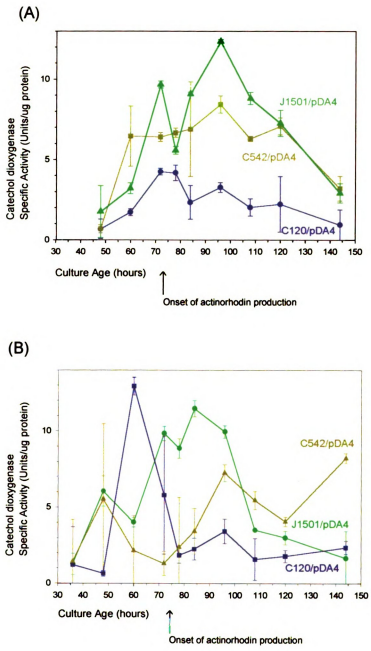
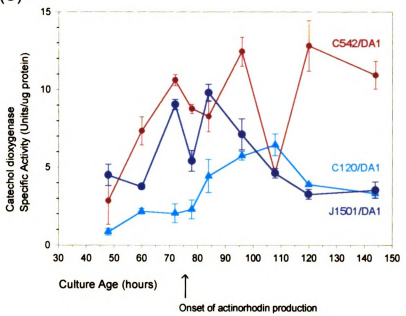

(A)
Catechol dioxygenase Specific Activity (Units/ug protein)
J1501/pDA4
C542/pDA4
C120/pDA4
Culture Age (hours)
Onset of actinorhodin production
(B)
Catechol dioxygenase Specific Activity (Units/ug protein)
C542/pDA4
J1501/pDA4
C120/pDA4
Culture Age (hours)
Onset of actinorhodin production

Figure 10A.

(C)



(D)

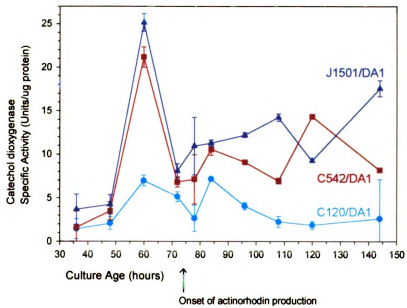


Figure 10B

Expression of *act*II-ORF4::*xylE* fusions in *bld* mutant backgrounds. The *act*II-ORF4::*xylE* fusion constructs were introduced into a number of *bld* mutant strains; these are characterized by blocks in both sporulation and (generally) in antibiotic synthesis. It is important to note that two of the *bld* strains tested are exceptions to this antibiotic-minus rule; strain J774 (*bldD53*) made substantial quantities of Act and strain C181 (*bldH181*) made slight quantities (none was detected in the bldH109 strain) as is common for these strains (4, 43). Therefore, it was expected that these strains would express actII-ORF4 to some degree. In addition, it was predictable that the *bldA* mutation would not affect expression from the fusion since that mutation is known to affect *act*II-ORF4 expression at the level of translation (65).

The phage construct DA1 was introduced into strains C301 (*bldA301*), C112 (*bldB112*), J774 (*bldD53*), C536 (*bldG536*), C109 (*bldH109*), C181 (*bldH181*), C249 (*bldI249*), and A95 (no *bld* locus assigned). Each strain was also lysogenized with the parent phage KC859 to determine background levels of expression. Plate cultures were assayed visually for catechol dioxygenase activity at 44 hours and at 60 hours post-inoculation. All *bld* DA1 lysogens, J1501/DA1, and J1501/KC900 were strongly positive at both timepoints, while all KC859 lysogens were negative (data not shown). These results suggest that the lack of Act synthesis in the Act-minus *bld* mutants is not due to a lack of *act*II-ORF4 transcription. The pDA4 construct was transformed into *bld* strains C103 (*bldA301*), C112 (*bldB112*), C186

(*bldB186*), C536 (*bldG536*), C109 (*bldH109*), C181 (*bldH181*), and C249 (*bldI249*). The experiment shown in Figure 11 compares expression from the *act*II-ORF4 promotor in these *bld* strains, in J1501, and from the *act*I promotor in strain J1501/KC900. It is important to note that, in contrast with the experiments shown in Figures 9 and 10, catechol dioxygenase activity was assayed visually in this experiment and was not corrected for changes in cell mass. The expression pattern in each of the *bld* mutant backgrounds tested generally resembled that of the parental strain; therefore, these results again suggest that *act*II-ORF4 transcription is not affected by these *bld* mutations. This conclusion must be considered with the caveat that the *act*II-ORF4 promotor in this fusion may not respond normally to all regulatory mechanisms (see Discussion)

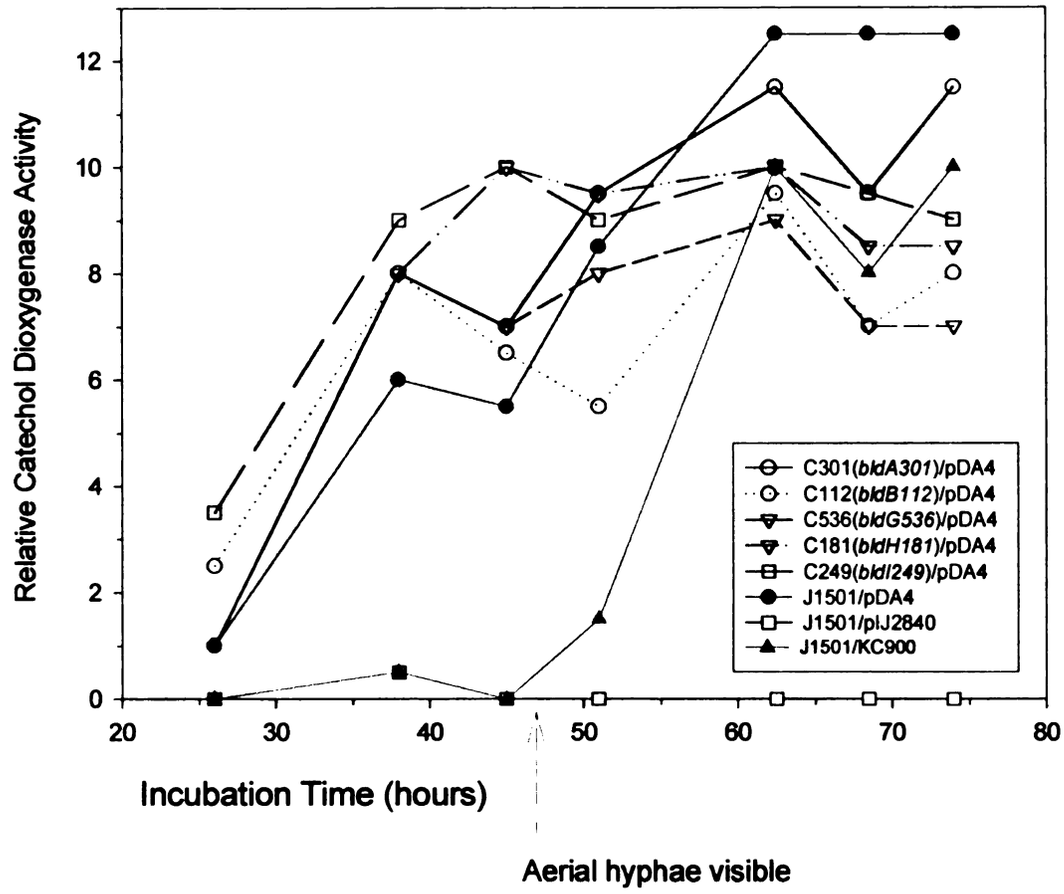


Figure 11. Expression of plasmid-borne *act*II-ORF4::*xylE* transcriptional fusions in *bld* mutants. Spores of each pDA4-carrying strain, J1501/KC900 (*act*I::*xylE*), and J1501/pIJ2840 (promotorless *xylE*) were streaked onto R5 medium containing thiostrepton. At each timepoint, fresh plates incubated at 30°C for the time indicated were sprayed with catechol and assayed visually for catechol dioxygenase activity, which was scored on a relative scale. Values represent the mean of two experiments. Strains carrying alternate alleles of *bldB* and *bldH* not shown in the figure (*bldB186* and *bldH109*) gave similar results to those shown and are not included in the figure.

DISCUSSION

Summary:

-Transcriptional patterns from the *act*II-ORF4::*xylE* fusions generally conformed to what is known about the *act*II-ORF4 promotor, indicating that the fusions are responsive to at least some regulatory functions.

-Transcription from the *act*II-ORF4::*xylE* fusion was significantly reduced in the *absB* mutant strain.

-Transcription from the *act*II-ORF4::*xylE* fusion was not significantly affected by the *absA1-542* mutation or the *bld* mutations tested.

-Several hypotheses are proposed to explain these results.

Based on the following observations, the *act*II-ORF4 promotor appears to be subject to regulation in both the plasmid-borne and the chromosomally-integrated reporter gene constructs; (1) Expression from the promotor varies significantly over the cultures' life cycle instead of exhibiting an essentially constant rate as would be expected of an unregulated promotor, (2) These variations in expression generally correspond to expected patterns relative to culture age, *act*I transcription, and actinorhodin production, (3) The time lag between *act*II-ORF4 and *actI* expression is not inconsistent with independent data.

Transcription from *act*II-ORF4 is reduced approximately 2-fold in the *absB*⁻ background, a result that conforms with the 2- to 12-fold reductions

observed by S1 nuclease protection assays (Chapter 2). The *absB*-encoded RNAase is predicted to function by cleaving a transcript or transcripts, thereby affecting message stability. The target transcript(s) are unknown, those from antibiotic-specific regulatory genes such as *act*II-ORF4 being one possibility (increased transcript stability would be predicted in this case, since mutational inactivation of *absB* results in lower levels of detectable *act*II-ORF4 transcript). However, the following observations suggest that *act*II-ORF4 is not a target. First, approximately two thirds of the 3-prime end of *act*II-ORF4 is deleted in these fusions and replaced with *xylE* , yet *absB* continues to affect transcript abundance; this implies that the 3-prime region of the *act*II-ORF4 transcript is not the target. Second, if the transcript were cleaved at the 5-prime end, one would expect to see evidence of a longer (albeit less stable) transcript in the *absB*$^-$ background; there is no evidence of this in the S1 nuclease protection assays presented in Chapter 2, Figure 2.

Expression was affected very little, if at all, in the *absA1-542* mutant; this contrasts with the three to six-fold decreased expression observed by S1 nuclease protection assays in Chapter 2. Although no satisfying explanation for this phenomenon has been found, several possibilities are considered below.

Given the variability in the data, it must be considered that this result could be an artifact. Additional repetitions of the experiments presented here would be helpful in settling this issue, as would investigation of fusion expression in other *absA* mutants such as the Pha type. However, this

explanation seems unlikely, since in none of the four timecourses presented was there a reduction in expression approaching that seen in S1 nuclease protection assays.

A second possibility is that the regulatory locus targeted by *absA* (and possibly *bld* genes) could be missing from the fusion construct, while those for *absB* (and possibly other regulators) are present. Since greater than 200 bp of sequence downstream and about 1400 bp upstream of the transcriptional start site are included in the construct, the *absA* target would have to be a novel *cis*-acting locus distant from the *act*II-ORF4 promotor region. This seems unlikely, partly due to the distance required. While regulatory mechanisms operating at such a distance are not unknown (58), they are presumably rare. In addition, an *act*II-ORF4 promotor unresponsive to regulation by *absA* would be expected to exhibit not normal levels of expression but *hyperexpression* resembling that in an *absA*-knockout Pha- type mutant. This was not observed.

Thirdly, it is possible that the reporter gene constructs are not faithfully reporting transcription. This explanation also seems unlikely, since the *act*II-ORF4::xylE construct appears to be regulated by *absB* and exhibits an expression pattern in J1501 consistent with regulation.

The variability of signals within and between experiments in this study is a concern. One of the likely causes for this variability is that cell material was not consistently harvested from within lawns of confluent growth on plates; this is desirable since mycelia at the middle and at the edges of

growth often develop at different rates. Nevertheless, overall mean activity was fairly consistent between replicate experiments for each strain, with the exception of strain J1501/KC900 (*act*I::*xylE*). This strain exhibited approximately 50% less activity in the replicate experiment (Figure 9, graphs B and D) than in the first trial (Figure 9, graphs A and C). The reason for this difference is not obvious. On the possibility that a fraction of the mycelia in the culture used in the replicate experiment had lost the ability to express the fusion, inocula used in that experiment were plated for isolated colonies and assayed visually for CDO activity; no significant differences between colonies were seen. A second possibility (which was not tested) is that the spore suspension used as inoculum was not accurately titered; although it was ensured that equivalent amounts of cell material were used in CDO assays (through normalization of signals for cell extract protein content), differences in growth density could affect antibiotic expression.

Relatively few inferences can be drawn from results involving *bld* mutants since there is no independent data regarding the affect of the *bld* mutations on *act*II-ORF4 transcription. Therefore, the simplest explanation for lack of response of *act*II-ORF4 expression to the presence of *bld* mutations is that Act production does not occur through regulation of *act*II-ORF4 transcription, instead targeting a downstream function such as biosynthetic gene expression.

APPENDIX

ASSESSMENT OF *LUX* REPORTER GENE FUSIONS FOR MONITORING *S. COELICOLOR* ANTIBIOTIC SYNTHESIS IN SOIL

ABSTRACT

Antibiotic production by streptomycetes in nature has never been conclusively demonstrated. A new approach to this question, in which transcriptional activity of streptomycete antibiotic regulatory or biosynthetic genes would be monitored through fusions to *lux* reporter genes, was investigated. A fusion between the *S. coelicolor act*II-ORF4 promotor and the *V. harveyi luxAB* genes was created in a plasmid vector and introduced into *S. coelicolor*. Expression from this strain was detected during growth in sterile, nutrient-amended soil microcosms. Also, several light-detecting instruments were compared for their abilities to detect expression of a *lux*-expressing bacterium mixed into soil; an ATP Photometer proved to be the most sensitive of the three instruments tested. Finally, a technique for the rapid extraction and microscopic localization of mycelia from soil was tested for potential use in monitoring *lux* expression by microscopic imaging of individual soil-grown mycelia or colonies. Based on this work, it was concluded that further advances in reporter gene technology or detection

methods may be required before this approach can be used successfully to monitor antibiotic synthesis in natural soil.

INTRODUCTION

Streptomycetes produce the majority of medically useful antibiotics. However, no convincing demonstration of antibiotic synthesis by streptomycetes in nature has been reported. Researchers attempting to investigate this matter have taken two major approaches. The more direct of these is extraction and detection (13) of the antibiotic itself from a soil microcosm seeded with the producing strain and incubated under appropriate conditions. The failure of this approach is most likely due to the small quantities of antibiotics produced, degradation by other microbes, and sequestering of antibiotic compounds by soil colloids. The more indirect "bioassay" approach monitors variation in the population of a co-inoculated organism sensitive to the antibiotic produced by the streptomycete. In this type of study, researchers have often failed to exclude possible interactions between producer and indicator strains that are unrelated to antibiotic synthesis.

Developments in reporter gene technology have suggested a new approach to this problem. A reporter gene fused to an antibiotic biosynthetic or regulatory gene promotor could allow detection of expression, by

established and sensitive methods, of gene activity reflecting antibiotic synthesis. Reporter genes encoding enzymes that catalyze light-producing reactions are particularly attractive for soil *in situ* assays; light can be detected with great sensitivity and speed, with minimal disturbance of cultures, and is not bound by soil as either antibiotics themselves or chemical reporter gene products may be. The *luxAB* genes derived from *Vibrio harveyi* are in common use as reporters. They encode a heterodimeric luciferase that catalyzes the following reaction, where RCHO represents a long-chain aldehyde substrate that can be supplied exogenously (commonly as *n*-decanal) and cellular $FMNH_2$ provides reducing potential;

$$FMNH_2 + RCHO + O_2 \rightarrow FMN + RCOOH + H_2O + light$$

Fusions involving *lux* genes have been used successfully for soil *in situ* studies; for example, King *et al.* (125) reported detection of *lux* activity from a pseudomonad in soil slurries. The *lux* reporter has also been used to study streptomycetes in plate culture (191).

For this work, it was proposed to construct fusions driven by *S. coelicolor* actinorhodin regulatory and biosynthetic gene promotors. Detection of transcription from these promotors in mycelia growing under conditions as "natural" as possible in soil microcosms could provide convincing evidence for the production of antibiotics as well as a tool for further soil ecology

studies. Two somewhat different approaches to detection of light emission were considered. In one, "bulk" light emission from a sample would be collected and reported as a single cumulative numerical result. Several instruments capable of this type of assay were available, including an ATP Photometer, a Photonics System or "Photon Camera", and a Luminometer. In addition, a "Fiber Optic System" was specifically designed and constructed for light detection *in situ* by Prof. Mark Worden of the MSU Dept. of Chemical Engineering; this system amplifies and quantifies light transmitted through a fiber optic cable, the tip of which can be inserted into a soil or other sample.

The second approach, undoubtedly less sensitive but potentially more informative, would use microscopic imaging to discriminate between light emission from different parts of a mycelium or colony. Localization of *lux*-based light emission to individual bacterial cells has been demonstrated by (among others) Cai and Wolk (38), who showed heterocyst-specific expression of genes related to nitrogen uptake in *Anabaena* . This was achieved using a Hamamatsu Photonics System or "Photon Camera", an instrument capable of detecting the very small number of photons generated by single *lux*-expressing cells in the field of a light microscope. Patterns of *lux*-based light emission collected by the instrument can be superimposed onto visual microscope images of the organism to localize expression. Using a similar instrument, Schauer *et al.* (191) have demonstrated spatial and temporal patterns of *lux* fusion expression within streptomycete colonies. An additional challenge was faced in the current study; that of extracting mycelia

from soil and completing the imaging process within a short period of time so that any signals detected could be attributed to expression during soil growth and not to disturbance of the culture.

MATERIALS AND METHODS

Bacterial Strains, Vectors, and Growth Conditions. *S. coelicolor* strains J1501 (49) and M600 (119) used in this work were grown in liquid YEME medium (97) supplemented with thiostrepton (10 μg/mL, final concentration) for selection where appropriate. *R. meliloti* strains 1021 (155) and CV1 (179) were grown in TY medium (16) containing 200 μg/mL kanamycin. The *lux* reporter gene constructs used were pHI342 (190, 198) and pDA7 (this work).

Luciferase Assays. All assays were performed with instruments set for maximum sensitivity (i.e., maximum digital gain and, where applicable, widest possible aperture) and represent 60 second cumulative signal collections unless otherwise stated. Signal-to-noise ratio was calculated by dividing peak signal by the root mean square of noise. The luciferase substrate *n*-decanal was used as an emulsion prepared the day of use by 5 minutes sonication of a mixture of *n*-decanal (Sigma Chemical Co., St. Louis, MO) at a concentration of 0.1% v/v in deionized water containing 20 mg/mL fatty-acid free Bovine Serum Albumin (Sigma). For comparison of luciferase activity from *S. coelicolor* M600/pDA7 and *R. meliloti* strain CV1, 0.2 mL

samples of cultures diluted 1:5 with fresh medium were mixed with 10 μl decanal solution and, after a 5 second delay, a 10 second cumulative assay was initiated. Three replicate assays of each culture were done. Dry weights were determined (four replications each culture) by pelleting cells from 1 mL culture by centrifugation, washing pellets with distilled water, transferring to pre-baked and pre-weighed alumininum weigh boats, and drying for 40 hours at 42°C before re-weighing.

Soil and soil microcosm preparation. Agricultural CAPAC soil from Kellogg Biological Station was collected and stored at 4°C in sealed plastic bags until use. Prior to inoculation, soil was spread in a thin layer and air-dried for several days followed by sieving to eliminate clumps. Soil amended with starch and chitin was prepared by mixing powdered soluble starch (Sigma) and powdered crab shell chitin (Sigma) each to 1% w/w and sterilizing by autoclaving twice for 30 minutes each. Glucose-amended soil was prepared by mixing 200 μl 1M glucose solution into 1g soil. Soil microcosms consisted of 0.1 g of sterile, nutrient-amended soil placed in 1.5 mL (8x50 mm) polypropylene luminometer assay tubes (Turner Designs) that had been disinfected by soaking for 24 hours in 70% ethanol. *S. coelicolor* spore inocula were prepared as previously described (97) and stored at –20°C until use; spores were pelleted by centrifugation and resuspended in sterile deionized water before using as inocula.

Note: Images in this dissertation are presented in color.

RESULTS

Test of the "buried slide" method for extraction of soil-grown *Streptomyces* for microscopic imaging. One approach considered for this study was to attempt observation of *lux* fusion expression from individual *Streptomyces* mycelia or colonies extracted from soil using a light microscope linked to a Hamamatsu Photonics System (Hamamatsu Photonics K. K., Hamamatsu City, Japan). This approach would require rapid extraction of mycelia from soil, location by microscope, and imaging for light emission; a time limit of several minutes for this process would ensure that any luciferase activity observed resulted from transcription during soil growth and not as a result of disturbance. Therefore, the "buried slide" or "Cholodny" method (52) for extraction of soil-grown bacteria for microscopy was attempted. Sterile microscope cover slips were partially buried in samples of sterilized soil that had been inoculated with spores of the prototrophic *S. coelicolor* strain M600. After 4 days incubation, cover slips were withdrawn from the soil cultures. A light microscope was used to search for mycelia clinging to the cover slip. Mycelia could be located within several minutes of extraction, and both undifferentiated vegetative mycelial and differentiated spore chains were observed (Figure 12). Therefore, this technique appears to be suitable for the desired purpose.

Figure 12. Soil-grown *S. coelicolor* mycelia on a "buried slide". A 50 gram sample of sterilized agricultural soil was inoculated with approximately $1x10^8$ spores of *S. coelicolor* strain M600 suspended in 5 mL sterile deionized water. After partially inserting sterile microscope cover slips into the soil, it was incubated loosely covered at 30°C for 4 days. Cover slips were then withdrawn, cleaned on one side, then suspended (using a ring of petroleum jelly around the edge of the cover slip) "dirty" side down over a microscope slide. Both differentiated spore chains (photographs A and C) and vegetative mycelia (B) are visible. Photographs are at 1000x magnification. Photography by Prof. Frank Dazzo.

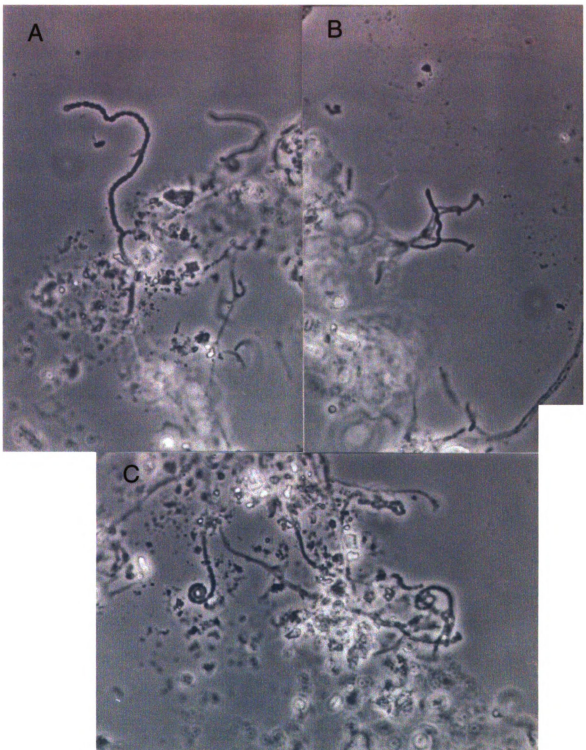


Figure 12.

Detection limits of three light-detecting instruments for *lux*-based light emission from bacteria in liquid or mixed into soil (in collaboration with Daniel Ragatz, Leann Matta, Mark Worden, and Frans Debruijn). To determine whether available light-detecting instruments were capable of detecting "bulk" (cumulative from all sources in the sample) *lux* expression from bacterial concentrations likely to be found in soil, the detection limits of three instruments for a *lux*-expressing bacterium were tested. The instruments tested were; i) An "ATP Photometer" (Lab-Line Instruments, Melrose Park, IL), ii) A Hamamatsu Photonics System or "Photon Camera" and, iii) A "Fiber Optic System" designed and constructed by Prof. Mark Worden (MSU Dept of Chemical Engineering). *Rhizobium meliloti* strain CV1, which carries an uncharacterized *lux*-bearing Tn5 insertion (179), was used as a test strain. Strain CV1 was grown to OD_{600} = 1.0 in rich liquid medium; the culture was then serially diluted with fresh medium and the dilutions mixed into soil. Bacterial concentration was determined by plate counts of the liquid culture.

The detection limit for liquid dilutions of bacterial culture and for soil/liquid culture mixtures were tested for each instrument. Samples consisting of 10 mL diluted liquid culture or 4 g soil mixed with 0.4 mL diluted liquid culture were placed into glass "scintillation" vials (2.5 cm diameter). Addition of 0.2 mL *n*-decanal solution, mixing, and 5 minutes room temperature incubation were followed by immediate assay in one of the

instruments. For ATP Photometer assays, vials were placed into the instrument's light-tight chamber and light emission from the sample assayed through a window located directly below the vial. For Photon Camera assays, vials were placed uncovered into a light-tight box and a video camera located above the vial activated to collect light emission; the signal distribution displayed on a video monitor was converted to a numerical value. For assays using the Fiber Optic System, the fiber optic probe was inserted 14-16 mm into the sample and vials were placed in a light-tight box; light absorbed by the tip of the fiber optic cable was transmitted to the photomultiplier/detector and reported as a numerical value. For each instrument, cumulative signal from 60 second assays were reported.

First, the ability of the instruments to detect light emission from cells in liquid was tested. Concentrations as low as $1x10^4$ cfu (colony-forming units) per mL were detectable with the ATP Photometer (Table 2; signals considered significant are in bold type). In contrast, the lowest concentrations reliably detected by the Photon Camera or Fiber Optic System were $1x10^7$ cfu/mL. Thus, under these conditions, the ATP Photometer was approximately 1000-fold more sensitive than either the Photon Camera or Fiber Optic System.

Table 2. Light emission from liquid dilutions of *lux*-bearing *R. meliloti* strain CV1 cells as detected by three instruments.

CONC. (CFU/ML) of *R. MELILOTI* STRAIN CV1	LIGHT DETECTED, FOR EACH INSTRUMENT		
	ATP PHOTOMETER	PHOTON CAMERA	FIBER OPTIC SYSTEM
0[a]	1[b]	90	831
1×10^3	0	ND	ND
1×10^4	**162**	160	475
1×10^5	**2320**	163	644
1×10^6	**30010**	577	981
1×10^7	ND[c]	**40013**	**9188**
1×10^8	ND	ND	**21512**

[a] *R. meliloti* strain 1021, the *lux*-minus parent of CV1, was used here as a control at 2×10^9 cfu/mL.
[b] Each value is in Relative Light Units specific to the instrument and is the mean two independent experiments, each consisting of three replicate assays.
[c] Not Determined.

In soil/liquid culture mixtures, the lowest concentration detectable by the ATP Photometer was 1×10^6 cfu/g soil (Table 3). This concentration is well within the range of actinomycete populations found in some soils; therefore, the *lux* fusion approach was not rejected due to this experiment. Comparison of detection limits in liquid with those in liquid/soil mixtures indicated that 100-fold greater bacterial concentrations are required for detection in soil due to light blockage by soil particles. Significant signals were not obtained using the Photon Camera and Fiber Optic System, which thus do not appear to possess the sensitivity required for this purpose.

Table 3. Light emission from soil dilutions of *lux*-bearing *R. meliloti* strain CV1 cells as detected by three instruments.

CONC. (CFU/GRAM SOIL) OF *R. MELILOTI* STRAIN CV1	LIGHT DETECTED (RLU)		
	ATP PHOTOMETER	PHOTON CAMERA	FIBER OPTIC SYSTEM
0[a]	3[b]	104	432
1 X 10^4	3	ND	ND
1 X 10^5	4	ND	ND
1 X 10^6	**181**	79	ND
1 X 10^7	**968**	195	191
1 X 10^8	ND[c]	548	ND

[a] *R. meliloti* strain 1021, the *lux*-minus parental strain of CV1, was used here as a control at $2x10^8$ cfu/g soil.
[b]Values are in Relative Light Units (RLU) that are specific to each instrument. Each value is the mean of two independent experiments each consisting of three replicate assays.
[c]Not Determined

Development of an *S. coelicolor* strain expressing a *lux* fusion. An *S. coelicolor* strain that strongly expressed a *lux* fusion was required for preliminary tests of detection in soil cultures. One candidate, provided by Alan Schauer (U. Texas-Austin), was a strain of S. *coelicolor* J1501 carrying the low-copy plasmid (1-3 copies/chromosome) pHI342. This plasmid carries *V. harveyi luxAB* transcribed from the *S. coelicolor* sporulation-related promotor *sapA* (84, 191); an uncharacterized mutation results in earlier-than-normal expression of the fusion. As an alternative, I constructed (Figure 13) a transcriptional fusion between *V. harveyi luxAB* and the promotor of the *S. coelicolor* actinorhodin-specific positive regulatory gene (*act*II-ORF4) in the

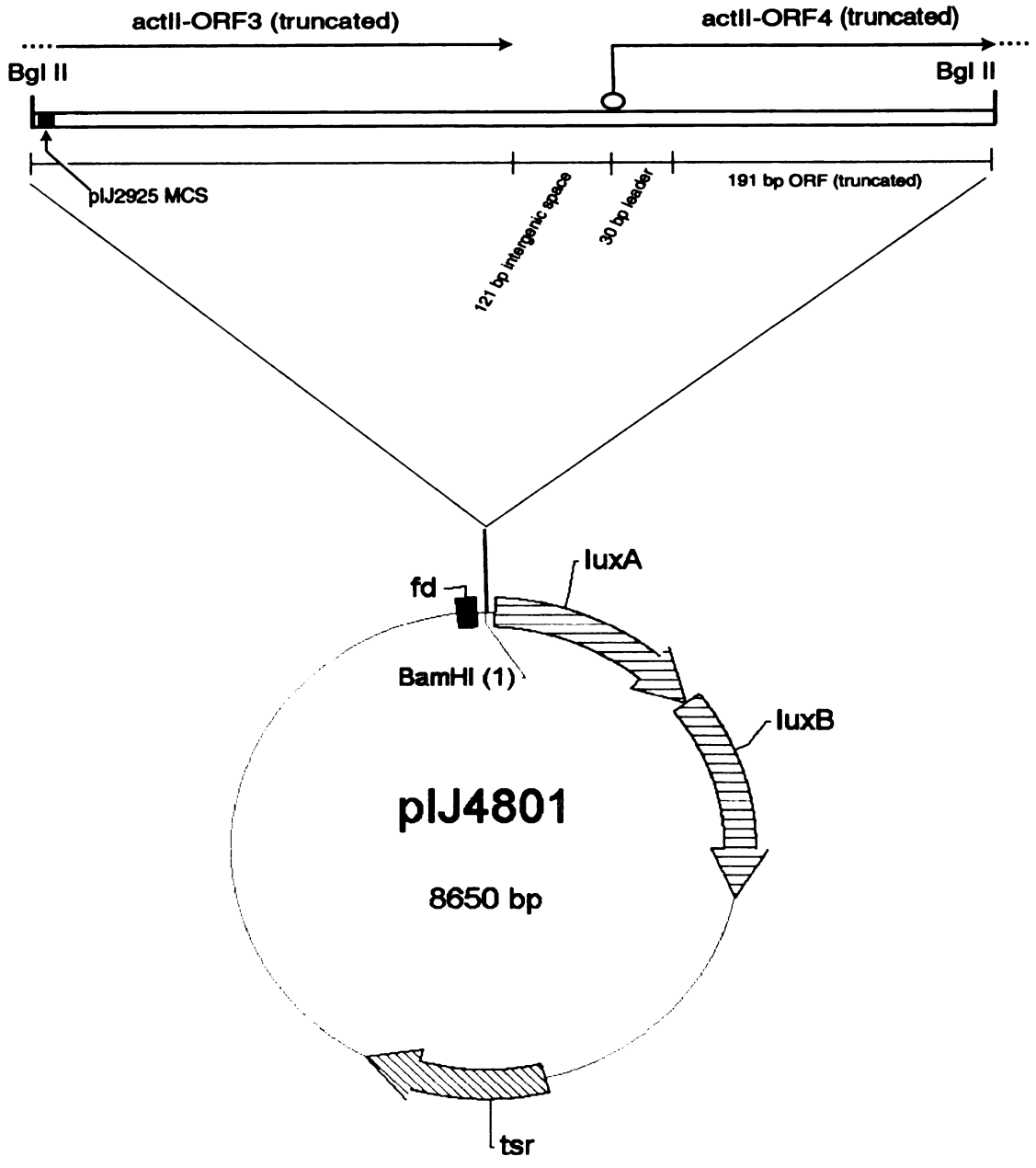


Figure 13. Construction of pDA7. A 1.6 kb *Bgl*II fragment containing the *S. coelicolor act*II-ORF4 promotor (previously described in Chapter 2) was ligated into the *Bam*HI site of pIJ4801 in the orientation shown so that transcription of the *V. harveyi lux*AB genes were driven by that promotor. "*fd*" is the major transcriptional terminator of coliphage fd. "*tsr*" is the thiostrepton resistance gene.

moderate copy-number vector (30-300/chromosome) pIJ4801 (29). This struct, pDA7, was transformed into the prototrophic *S. coelicolor* strain M600.

Expression from strains J1501/pHI342 and M600/pDA7 grown in complex liquid medium were compared. Assays were performed using a Turner Designs Luminometer (Model TD-20e, Turner Designs, Inc., Sunnyvale, CA), an instrument similar to the ATP Photometer used in earlier experiments. Light emission from J1501/pHI342 was easily detectable, peaking at 34 RLU compared to background values of 0-1 RLU (Figure 14). However, expression from strain M600/pDA7 peaked at 1954 RLU/sec, approximately 60-fold higher; therefore, strain M600/pDA7 was used in further experiments. *S. coelicolor* strain M600/pDA7 was also compared to *R. meliloti* strain CV1. Cultures grown in rich liquid media to OD_{600} = 1.0 were assayed by luminometer. In this experiment, signals were normalized to dry cell weight since optical density measurements of *Rhizobium* and *Streptomyces* are not comparable (luciferase and dry weight measurements were performed as described in Materials and Methods). Approximately 5-fold greater light emission was detected from *S. coelicolor* M600/pDA7 ($1.8x10^4$ RLU/sec/mg dry weight) compared to *R. meliloti* ($3.7x10^3$ RLU/sec/mg dry weight). However, normalizing for *lux* fusion copy number per chromosome (approximately 100-fold higher in the streptomycete) results in an approximately 20-fold greater expression level/mg dry weight for *R. meliloti* CV1.

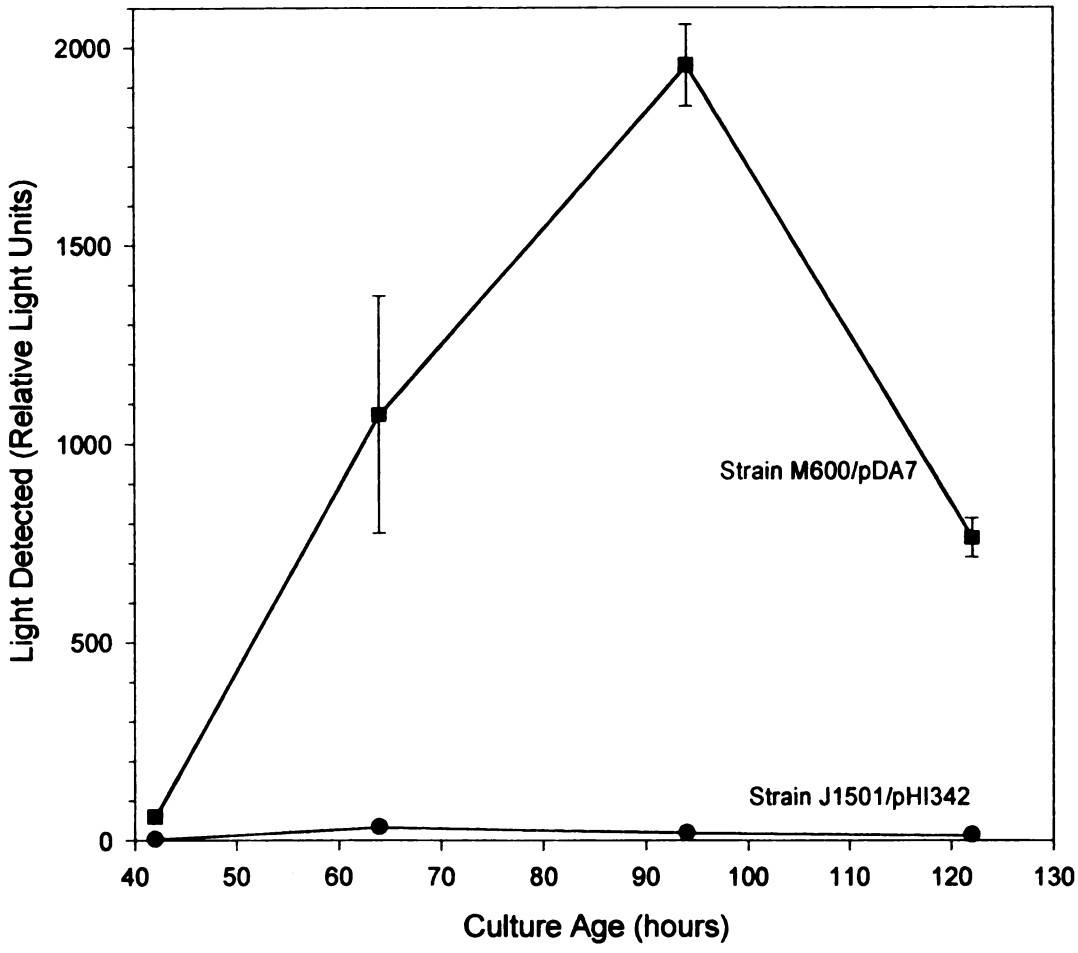


Figure 14. Timecourse of luciferase expression from *S. coelicolor* strains M600/pDA7 and J1501/pHI342 in liquid culture. Cultures were grown in liquid YEME medium. At intervals, four replicate samples were removed from each culture and assayed. Cell densities were comparable between cultures as determined by plate count. Each value is the mean of four assays of a single culture. Error bars indicate standard deviation from the mean.

The limits of detection of *S. coelicolor* M600/pDA7 in liquid culture and in liquid culture/soil mixtures were determined (experiments were performed essentially as previously done with *R. meliloti*). Significant signals were detected by luminometer at concentrations as low as $5x10^3$ cfu/mL in liquid culture and $5x10^4$ cfu/gram of soil/liquid mixture (Table 4; values deemed significantly above background are in bold type). Since detectable concentrations in soil were well within the range of streptomycete populations found in nature, these results did not rule out the *lux* reporter gene approach.

Table 4. Detection of light emission from *S. coelicolor* strain M600/pDA7 in liquid and in liquid/soil mixtures.

CFU/mL[a] Or CFU/g Soil[b]	Signal (RLU)[d] In Liquid	Signal (RLU) In Soil
0[c]	0.1	0.00
5×10^2	0.1	0.03
5×10^3	**1.7**	0.10
5×10^4	**11.2**	**0.34**
5×10^5	**109.0**	**2.50**
5×10^6	**957.0**	**26.80**

[a]Cultures grown in liquid YEME medium were serially diluted with fresh medium.
[b]Prepared by mixing liquid medium dilutions into soil.
[c]Uninoculated medium or soil.
[d]Relative Light Units. Each value is the mean of four assays of a single culture.

Detection of *lux* fusion expression from *S. coelicolor* M600/pDA7 during soil growth. *S. coelicolor* strain M600/pDA7 was grown in sterile soil amended with either glucose or a starch/chitin mixture. Soils were inoculated

with $1x10^9$ spores per gram of soil and incubated for 48 hours in humidified conditions. Significant signals were obtained from soil microcosms with either glucose or starch/chitin (Figure 15). No signal was detected when humidity was not maintained (data not shown).

Stronger signals were attained through several modifications to the assay procedure. Combining *n*-decanal solution and phosphate-buffered saline before addition to soil (instead of adding these sequentially as done previously) increased light emission, presumably through more homogeneous distribution of *n*-decanal. More vigorous mixing after addition of *n*-decanal solution, additional mixing before subsequent assays of the same sample, and determination of the time of optimal emission following addition of *n*-decanal (approximately 4 minutes) also resulted in stronger signals. These modifications were combined in the timecourse experiment shown in Figure 16 in which the peak signal is nearly 20-fold higher than in the previous experiment. A signal-to-noise ratio of 188 was determined for this signal.

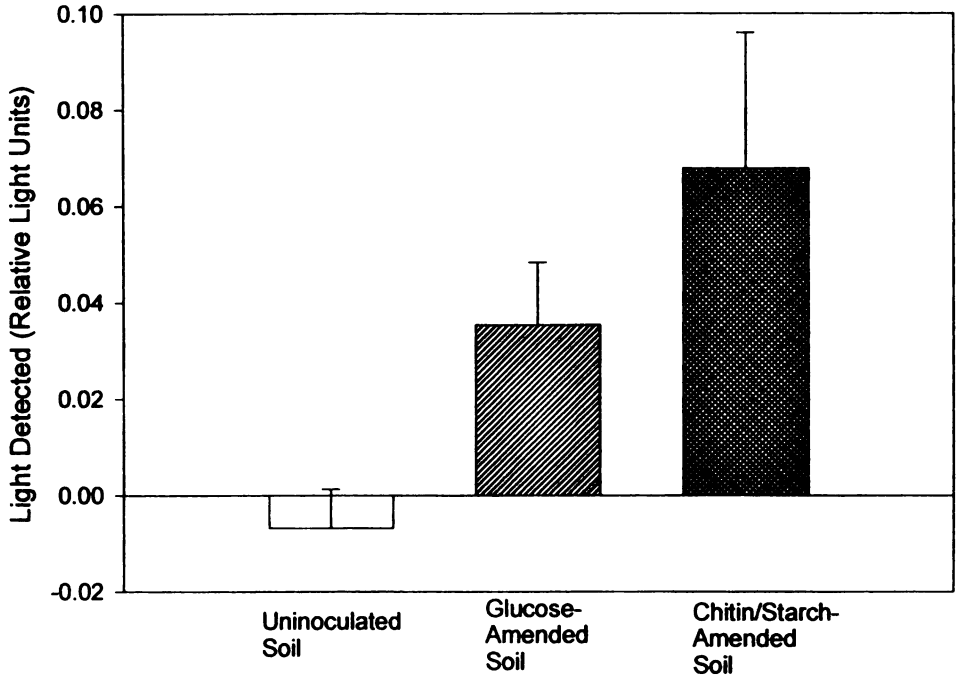


Figure 15. Detection of *lux* fusion expression from *S. coelicolor* M600/pDA7 growing in sterilized, nutrient-amended soil microcosms. Soil microcosms consisted of 8x50 mm luminometer assay tubes containing 0.1 g sterilized soil amended with glucose or with a starch/chitin mixture. Each microcosm was inoculated by adding $1x10^8$ spores of *S. coelicolor* M600/pDA7 suspended in 20 µl water and mixing, followed by incubation at 30°C in a humidified chamber for 48 hours. Humidity was provided by a layer of water at the bottom of the sealed container containing the microcosms. Luciferase assays were initiated by adding 10 µl 0.1% *n*-decanal solution and 100 µl Phosphate-Buffered Saline (the latter providing additional liquid volume to promote homogenous mixing of the *n*-decanal into soil) and mixing vigorously. After 1.5 min incubation at room temperature, light emission was assayed by luminometer. Each point represents the mean of assays of 6 microcosms. Error bars indicate standard deviation from the mean.

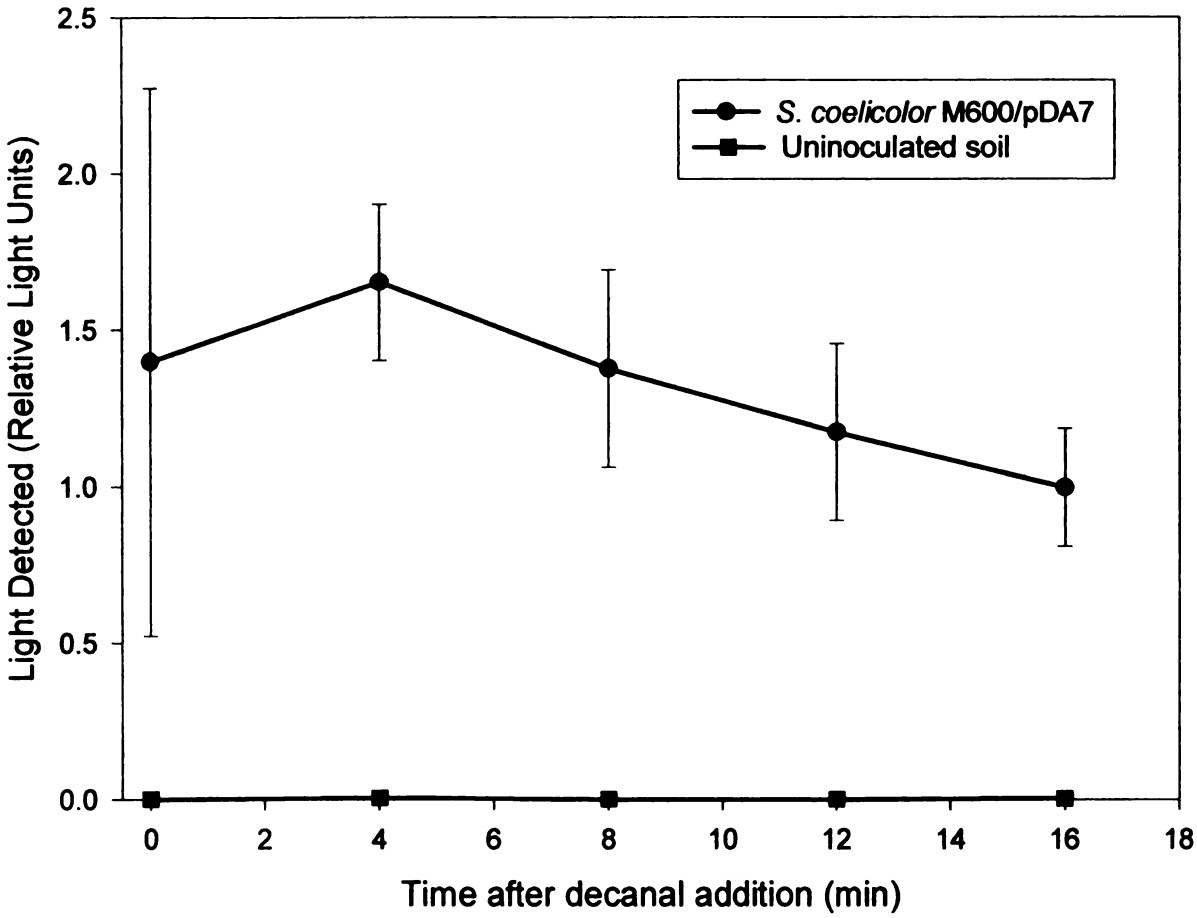


Figure 16. Timecourse of light emission from *S. coelicolor* M600/pDA7 following addition of *n*-decanal. Microcosms were incubated and assays performed essentially as described in Figure 15, but with the alterations described in the text. Values are the mean of assays of 3 microcosms. Error bars indicate standard deviation.

DISCUSSION

Significant steps were taken towards the development of a *lux*-based system for detecting *S. coelicolor* gene expression in soil. A rigorous demonstration of *in situ* antibiotic synthesis would require additional steps, most importantly; (i) The development of a *lux* fusion that would be stably maintained without selection (i.e., chromosomally-integrated) and demonstrated to be subject to normal regulation, (ii) The use of natural, unsterilized soil in microcosms, and (iii) Inoculum concentrations limited to streptomycete concentrations found in nature. Each of these steps would necessarily result in decreased signal strength compared to that from the microcosms described in this study. Extrapolation from current data suggests that, at best, a marginally detectable signal will result. Therefore, it is likely that significant improvements in methodology giving increased signal strength or detection sensitivity or both will be necessary for the *lux* fusion approach to succeed.

It was shown that the time required for extraction and visualization of mycelia from soil is not a barrier to the microscopic visualization approach. *S. coelicolor* mycelia were extracted from soil and located microscopically within several minutes of disturbance of the soil microcosm. Exposure to the luciferase substrate *n*-decanal could be accomplished concurrently with location by microscope by placing a drop of the volatile liquid into the

chamber created by the microscope slide, cover slip, and petroleum jelly; mycelia would be quickly exposed to vapors. Cai and Wolk (38) successfully monitored *lux* expression from individual cells of *Anabaena* strain PCC 7120 by this approach; however, a very strong light-emitting strain was required for detection. It is unknown whether any *S. coelicolor* antibiotic genes would be expressed to the same degree as the *Anabaena* nitrogen uptake-related genes studied by Cai and Wolk, particularly under relatively nutrient-poor soil conditions. Improvements to the Photon Camera (most obviously, the addition of a close-up lense to the video camera) would increase sensitivity. However, it must be concluded that the microscopic imaging approach, while appealing, is less likely to succeed than assays of bulk light emission from soil samples due to sensitivity limitations. Other reporter genes or reporter gene versions might be considered as a solution to this problem, e.g., genetically engineered versions of *lux* or *gfp* with enhanced signal strength. Recently, confocal laser scanning microscopy has been used to detect expression in individual mycelia of single-copy chromosomal fusions between enhanced *gfp* and *redD* (209), and *lat* and *ccaR* (antibiotic biosynthetic and regulatory genes, respectively in *S. clavuligeris* (86, 131).

Of three light-detecting instruments tested in "bulk" soil assays, the Lab-Line ATP Photometer was the most sensitive. Detection by the Fiber Optic System or Photon Camera required bacterial concentrations several orders of magnitude higher. The Fiber Optic System potentially allows fast, location-targeted, and convenient assays, but its sensitivity may be limited by

the small area sampled by the tip of the fiber optic cable. As with microscopic imaging, the addition of a close-up lense to the Photon Camera would improve its sensitivity for bulk soil assays.

The *S. coelicolor* strain M600/pDA7 constructed in this study expressed lux at approximately 60-fold higher levels than did *S. coelicolor* carrying a low-copy *sapA*::*lux* fusion; this factor roughly correlates with fusion copy number, suggesting similar promotor strengths. *S. coelicolor* M600/pDA7 was also compared to *R. meliloti* CV1, which carries a single-copy *lux* fusion. Expression from the *Rhizobium* strain was approximately 20-fold higher per mg cell dry weight when adjusted for fusion copy number. The reason for this difference is unknown; obvious possibilities lie in the strength of the respective promotors and in physiological differences between the species (the latter perhaps resulting in reduced $FMNH_2$, O_2, or *n*-decanal substrate availability in the streptomycete). Since promotor strength is probably the easiest factor to manipulate, the identification of an antibiotic-related promotor with greater transcriptional activity than *act*II-ORF4 might be a good first step to signal improvement.

Given this comparison of the *Streptomyces* and *Rhizobium* strains, it may seem surprising that minimum detectable concentrations in liquid were very similar ($5x10^3$ cfu/mL and $1x10^4$ cfu/mL, respectively) and favored the streptomycete in soil/liquid culture mixtures ($5x10^4$ cfu/gram and $1x10^6$ cfu/gram, respectively). The explanation undoubtedly lies in the differing properties of a "colony-forming unit" for each organism. Streptomycetes grow

in liquid culture as pellets of intermeshed mycelia that may reach considerable cell mass, and each pellet is likely to appear as a single colony in a plate count. In contrast, a colony-forming unit of *Rhizobium* will consist of one or a few cells. Therefore, at comparable cfu/mL or cfu/gram concentrations, it is likely that much more streptomycete cell mass was assayed for luciferase activity.

What can be inferred about the detectability of soil-grown *Streptomyces* from the values obtained by mixing liquid-grown culture with soil? When inoculated into sterilized soil containing appropriate nutrients and moisture, most spores will germinate and grow to form branching networks of mycelia (141); however, these will undoubtedly consist of significantly less mass than those grown in rich liquid media. Moreover, in unsterilized soil many spores remain dormant due to unspecified interactions with other soil microbes (141). Therefore, it can be expected that detection of mycelia grown in natural soil will require populations several orders of magnitude higher than the $5x10^4$ cfu/gram detected when a liquid-grown culture was mixed into soil.

Expression from *S. coelicolor* M600/pDA7 was easily detected after spores were inoculated at $1x10^9$ cfu/gram soil in sterile, nutrient-amended soil microcosms that were then incubated for several days. Method improvements resulted in increased light emission with a signal-to-noise ration of 188.

S. coelicolor M600/pDA7 is unsuited for attempts to detect expression under natural conditions because the *lux* fusion is carried on a multi-copy

plasmid. It is unlikely that this plasmid will be stably maintained during soil growth in the absence of selection. Therefore, a strain expressing a single-copy, chromosomally-integrated fusion construct would be highly preferable. Based on available data, would expression from such a fusion be detectable? An approximately 100-fold decrease in fusion copy number would likely result in a corresponding decrease in light emission. Given the signal-to-noise ratio of 188 achieved in the experiment depicted in Figure 16, it seems probable that a marginally detectable signal would result. Greater signal strength might be achieved by using an *act* biosynthetic gene promotor, at least some of which are likely to be expressed to a greater degree than the *act*II-ORF4 promotor (in addition, the use of a biosynthetic gene promotor would have the benefit of more directly reporting antibiotic synthesis). Furthermore, it is likely that assay sensitivity can be further increased by experimenting with such factors as *n*-decanal concentration and method of addition, nutrient amendment, and by determining the time period during incubation of a soil microcosm during which fusions are optimally expressed. However, these improvements must be balanced against requirements for "natural" conditions. First, inocula must be reduced approximately 100-fold to reflect streptomycete concentrations found in natural soil. Actinomycetes have been reported at up to $1x10^8$ cfu/gram of natural soil (152, 221) and streptomycetes often make up 10% of the total actinomycetes recovered from soil (130); therefore, a concentration of $1x10^7$ cfu/ gram soil would seem to be the maximum allowable. Since this number corresponds to *recoverable*

concentrations of streptomycetes, it would be permissable to inoculate with higher concentrations if (11) predation and competition lower recoverable numbers. However, it has been found that a high percentage (approximately 90%) of spores inoculated into unsterilized soil do not germinate (141). The combination of these factors will likely result in a signal reduction of approximately 1000-fold. Considering both factors that will likely increase and decrease signal strength, in balance it appears unlikely that antibiotic synthesis in soil can be monitored using *luxAB* without additional improvements in expression or detection.

As mentioned above, rather impressive results have been published recently in the detection of gene expression through fusions with the *gfp* reporter gene. Although cultures used in those studies were grown in rich laboratory medium, nevertheless the use of enhanced versions of *gfp* would appear to be a promising approach to *in situ* detection.

CHAPTER 4

CONCLUSIONS AND PROSPECTS

A primary goal of this work is to determine whether the *absA* and *absB* loci affect Act and Red synthesis through transcriptional control of the antibiotic-specific regulators (ASRs) in the *act* and *red* gene clusters. It is hoped that this knowledge will help to define the pathways by which the *abs* loci globally influence *S. coelicolor* antibiotic biosynthesis. In the following discussion, two models are considered in light of the data generated in this study and the findings of other researchers. In one, ASR (i.e., *act*II-ORF4 and *redD*) transcripts are, in fact, targeted by the *abs* loci (note that control of either transcription rate or transcript stability is possible in this model). In the alternative "downstream target" model, a process downstream of ASR transcription/transcript stability (for example, biosynthetic gene transcription) is targeted. It should be noted that control of transcript abundance does not necessarily imply regulation; the possibility exists that *absB*, in particular, performs a "housekeeping" and not a true regulatory function.

Do the *abs* loci control *act*II-ORF4 and *redD* transcript abundance? It has been shown through S1 nuclease protection assays that mutations in *absA* and *absB* result in altered levels of *act*II-ORF4 and *redD* transcripts. Compared to an absA+ strain, plate cultures of an *absA** mutant (in which *absA1* is believed to be locked into phosphorylation mode) showed 3 to 6-fold less *act*II-ORF4 transcripts, 7-fold less *redD* transcripts, and between 4-

fold and greater than 50-fold less *act*VI-ORF1 biosynthetic gene transcripts. Disruption of *absA2*, in contrast, resulted in approximately 2-fold increases in each of these transcripts. An *absB*⁻ mutation resulted in 2 to 12-fold less *act*II-ORF4 transcripts and 7 to 13-fold less *redD* transcripts. It should be noted here that the *absB-120* mutation is now known to only partially inactivate *absB* activity; thus, different results might be obtained if other mutations that completely inactivate *absB* (resulting in a tight, *absA**-like antibiotic-minus phenotype) are studied.

The simplest interpretation of the results described above is that the *abs* genes control ASR transcript abundance which, in turn, affects biosynthetic gene expression. In contrast, a "downstream target" model must incorporate a speculative mechanism to account for the observed decrease in ASR transcripts. One such possibility is a positive feedback loop in which Act, Red, or their precursors serve to stimulate ASR transcription; in an *absB*⁻ mutant, for example, reduced levels of antibiotic precursors resulting from decreased biosynthetic gene expression might feed back to reduce *act*II-ORF4 and *redD* transcription. A possible precedent for such a feedback mechanism can be found in the tylosin pathway of *Streptomyces fradiae* (33). Production of the core polyketide component of tylosin is severely reduced when a deoxyhexose component, also synthesized by *tyl* gene-encoded enzymes, is unavailable for addition to the polyketide. A positive feedback mechanism involving expression of the regulator TylR (analogous to ActII-ORF4 and RedD) has been suggested to account for this effect. In addition

to a feedback mechanism, it is not difficult to imagine the existence of other indirect effects of the *abs* genes on ASR transcription, given the pleiotropic effects of the *abs* mutations.

An ASR target is also favored by the discovery that introduction of *act*II-ORF4 and *redD* in high copy-number overcomes the antibiotic-minus effects of both *absA* and *absB* mutations (3, 4, 42). The most straightforward interpretation of this result is that deficiencies in ASR transcripts have been repaired by the introduction of the high-copy genes. However, this is not a particularly strong argument since it is quite possible that deficiencies "downstream" might also be overcome by overexpression of ASRs.

Both of these models must account for two apparent discrepancies in the S1 nuclease protection data: (a) The disproportionate effects of the *abs* mutations on ASR transcripts and on biosynthetic gene transcripts, and (b) The disproportionate effects on biosynthetic gene transcripts and on antibiotic production. The first discrepancy is illustrated in Table 1, which shows (in three separate cultures) mean decreases in *act*II-ORF4 transcripts of 4-fold in an *absA** mutant and 6-fold in an *absB* mutant, compared to mean reductions of approximately 22-fold for *act*VI-ORF1 in each of the mutants. This phenomenon is easily accounted for by the "downstream target" model, since ASR transcripts would be affected indirectly, if at all, by alteration of downstream activities. In the "ASR target" model, however, *abs* mutations influence biosynthetic gene transcription through levels of ASR transcripts. In a simple system where *act*II-ORF4 and *redD* transcripts are limiting factors

in Act and Red synthesis, alterations in levels of those transcripts should translate to approximately proportional changes in biosynthetic gene transcription. On the other hand, if ASR transcripts are not a limiting factor, alterations should result in *less* acute changes in downstream processes, contrary to what is observed.

Mechanisms that could account for this phenomenon and that fit into the "ASR target" model can be imagined and are supported by some circumstantial evidence. For example, the ActII-ORF4 protein might be bound by various *act* biosynthetic gene promotors with significantly different affinities; a modest decrease in ActII-ORF4 (as in the *abs-542* mutant) might then result in titration of the protein by stronger-binding *act* promotors and a disproportionate scarcity at weaker binding sites. If the *act*VI-ORF4 promotor were a weak binding site, transcription from that gene could be severely reduced in this situation. Differential regulation of *act* biosynthetic genes is supported by a comparison of the often substantial *act*VI-ORF1 transcripts observed in the *abs* mutants in this work with the complete lack of detectable expression from an *actI*::xylE fusion in those mutants (the latter experiment described in Aceti and Champness, 1998). In addition, Arias *et al.* (12) found different ActII-ORF4 binding efficiencies *in vitro* for DNA fragments containing *act* biosynthetic gene promotors (one with the divergent promotors for *act*VI-ORF1 and *act*VI-ORFA, the other with *act*III and *act*I-ORF1 divergent promotors). In a second scenario, disproportionate expression of *act*VI-ORF1 could be caused by a cooperative regulatory mechanism. This idea is

based on the multiple ActII-ORF4 binding sites discovered in that gene (12); footprinting studies identified a presumed primary site located within the promotor region and a secondary site approximately 20 bp downstream of the first and within the transcribed region. A cooperative mechanism could result in a sigmoidal expression curve such that changes in ActII-ORF4 concentrations over a certain range would have disproportionately large effects. Such a mechanism is exemplified by the *E. coli deo* operon. Binding of a regulatory protein to either the *deo*P1 or *deo*P2 operator leads to a 2-fold effect on transcription while binding to both sites results in a 20 to 30-fold effect (58); in this way, a modest decrease in regulatory protein concentration could severely reduce transcription from the regulated gene.

The second discrepancy (lack of detectable Act in cultures with significant levels of *act*VI-ORF1 biosynthetic gene transcript) can also be explained by differential regulation of the *act* biosynthetic genes. A more severe reduction in expression of <u>some</u> *act* biosynthetic genes could block Act production. Again, such a reduction appears to be demonstrated in both *abs* mutants by the *actI*::*xylE* fusion. The difference between the models on this issue is the identity of the differentially regulating effector: ActII-ORF4 in the "ASR target" model, AbsA2~P and AbsB or their downstream effectors in the "downstream target" model.

In Chapter 3, the behavior of *act*II-ORF4::*xylE* fusions in *absA** and *absB* mutant strains is described. It should be noted that, in recent years, confidence in the accuracy of *xylE* as a reporter gene has declined

considerably among many researchers studying streptomycetes (44). The importance attached to these results should be adjusted accordingly.

The *act*II-ORF4::*xylE* fusions exhibited an approximately 2-fold decrease in activity in the *absB*⁻ strain, a result consistent with results of S1 nuclease protection assays. However, the fusions showed essentially no response to the *absA** mutation; i.e., Act synthesis was completely blocked while *act*II-ORF4 transcript levels were unaffected. If accurate, this result poses a problem for the "ASR target" model. In balancing the reliability of the xylE fusion assays versus S1 nuclease protection assays, however, it must be concluded that this result is most likely an artifact.

It is not clear why the fusion would respond inaccurately to an *absA** mutation while reporting with (apparent) accuracy in J1501 and *absB* mutant backgrounds. In one scenario, if *absA* targeted *act*II-ORF4 transcript stability, the replacement of the 3-prime end of that transcript with *xylE* mRNA could render it unresponsive to regulation by *absA*. Alternatively, a regulatory site targeted by absA could be missing from the fusion construct while sites for *absB* (and possibly other regulators) were present. Since greater than 200 bp of sequence downstream and about 1400 bp of upstream of the transcriptional start site are included in the construct, this would require an *absA* target distant from the *act*II-ORF4 gene. Regulatory mechanisms operating at such a distance are not unknown (58). In either case, lack of regulation by *absA* would be expected to result in *above normal* levels of the transcript reminiscent of a Pha-type mutant; however, it is

possible that the approximately 2-fold increase expected would not be distinguishable in this assay.

These "ASR target" and "downstream target" models can also be compared with respect the degree of precedent for regulation at each level. Many examples of apparent regulation at the ASR level exist. Few indications of regulation at the downstream level exist, one being the disruption of *afsR*, which resulted in a severe decrease in *act*III transcripts but no effect on *act*II-ORF4 transcripts (69). However, the most relevant precedent was recently provided by Ryding and Champness (187), who have discovered that *cda* biosynthetic genes are apparently controlled directly by the *absA* regulatory pathway, not through the putative ASR for that cluster (*cdaR*). While *cdaR* has not been conclusively proven to be an ASR, other possible regulatory genes in the *cda* cluster were tested and no evidence was found for regulation of those genes by *absA*. Given the likelihood that *absA* evolved to regulate CDA production, it can be argued that its easiest evolutionary course to regulation of Act and Red would be through analogous routes in those systems. These recent discoveries regarding regulation of the *cda* cluster by *absA*, therefore, tend to favor the "downstream targets" model in the *act* and *red* systems.

In conclusion, neither the "ASR target" nor the "downstream target" model can be ruled out at present. Arguments discussed early in this chapter are relevant to both *absA* and *absB* and tend to favor ASR targets. The final two arguments (regarding the behavior of *act*II-ORF4::*xylE* in *absA* mutants

and the precedent of cda regulation) are specific to *absA*. The first must be given relatively little weight due to the reputation of the *xylE* reporter for unreliability, and the second is speculative. At this time, therefore, preference must be given to the theory that the *abs* loci target ASR transcripts.

These questions could be further explored in a number of ways. The ability of *act* and *red* biosynthetic and regulatory gene promoters to bind AbsA~P can be tested; although it is not known whether AbsA~P is the terminal repressor in the *absA* pathway, this is the simplest case and the only testable one at this time. If AbsA~P is not the terminal repressor, identification of the other component(s) of this regulatory pathway is a very high priority. Assay of transcripts from a greater number of *act* biosynthetic genes (and from *red* biosynthetic genes, which are now better characterized) would address the hypothesis of differential regulation. The possible existence of a feedback mechanism could be tested relatively easily by determining whether *act*II-ORF4 and *redD* transcripts are reduced in various biosynthetic gene mutants that already exist.

Do the *bld A, B, D, G, H, I,* or *A95* loci control ASR transcript abundance? Expression from *act*II-ORF4::xylE fusions in a *bldA* mutant strain was not significantly altered compared to expression in the parental type; this result was expected, as the *bldA* mutation is known to affect translation, not transcription, of *act*II-ORF4. The *bldD* and *bldH-181* mutants produced significant and minor quantities of Act, respectively, under the

conditions of the experiment; therefore, it was not surprising that the *act*II-ORF4::xylE fusion was expressed in those strains.

In contrast, no prior information was available on the remaining *bld* mutants tested in this study (*bld*s *B*, *G*, *I*, and *A95*) to enable prediction of the results. Expression from the *act*II-ORF4 fusion was not significantly different in those strains when compared to the parental type. Again, suspicions about the accuracy of the xylE fusions must be considered; however, the only available data at this time suggests that the effects of the bld loci on Act synthesis takes place downstream from *act*II-ORF4 transcription, perhaps at translation or activity of the ActII-ORF4 protein or biosynthetic gene expression. The *bldB* gene has been predicted to encode a regulatory protein; therefore, if it directly targets antibiotic synthesis, it is likely to be at the level of biosynthetic gene transcription. However, because the *bldB* mutation has pleiotropic effects including loss of sporulation, it is likely to target higher order regulators. Mutations in *bldB* and in *bldH* are known to affect ADP-ribosylation of a number of proteins (197) and their effects on antibiotic synthesis might be moderated through this mechanism. The *bldG* gene is predicted to encode an anti-sigma or anti-anti-sigma factor (23); the pleiotropic effects caused by the *bldG* mutation leads to the prediction that it too is likely to target higher order regulators instead of antibiotic biosynthetic genes directly.

Additional insights into role of *absA*. What is the role in *S. coelicolor* of the signal transduction system encoded by *absA*? Does *absA* regulate the

timing (onset and/or shutdown) of antibiotic synthesis, or does it modulate quantities of antibiotics produced, or both? As discussed in Chapter 2, S1 nuclease protection experiments suggest that the timing of transcriptional onset of neither *act*II-ORF4 and *redD* is greatly affected by *abs* mutations. Additional data bearing on this question was presented in Chapter 3, in which *act*II-ORF4 transcription was monitored via *xylE* reporter gene fusions. The most relevant of these are comparisons of early timepoints in Figures 10B and 10D. Although one cannot be certain that the onset of transcription has been captured in these experiments (particularly that shown in Figure 10B), three of the four time courses appear to show the beginning of major transcription in abs strains within the same 12 hour period as in the parental strain. The exception is the *absB*⁻ C120 strain in Figure 10B, in which major transcription may be delayed.

Brian *et al.* (26) presented evidence from reporter gene fusion studies for a 6-12 hour shift in expression of the *actI* biosynthetic gene in an *absA* mutant background (in this case, a disruption of *absA*1 resulting in precocious hyperproduction of Act and an apparent shift to an earlier onset of *actI* transcription). Recently, Ryding and Champness (187) studied transcription of *cda* (53) genes as well as of *absA* itself using both S1 nuclease protection and RT-PCR. It was found that major transcription of *absA* does not occur until *after* transcription from *cda* biosynthetic gene promotors; thus, *absA* is apparently uninvolved in the timing of onset of *cda* biosynthesis unless this involvement occurs in late-expressing *cda* genes

that were not assayed. Mutations in *absA* do appear to affect both the quantities of transcript from *cda* biosynthetic gene transcripts as well as the shutdown of transcription at later timepoints. Transcription from *redD* and *absA* were also compared in these studies and found to reach significant levels within the same 6-hour period. Although a more precise study is needed in the case of *redD*, these results tend to support the idea that *absA* functions to modulate quantity, and possibly shutdown of synthesis, of both CDA and Red. Although there is firm evidence that both Act and CDA production are detectable earlier in *absA* Pha mutants (10, 26), this may be due to stronger (not earlier) expression, resulting in earlier detection of the antibiotics. The preponderance of evidence at this time, then, suggests that the *abs* genes do not significantly affect the timing of transcriptional onset from *act*, *red*, and *cda* genes.

If *absA* does function to modulate quantities of antibiotics produced, its ultimate purpose may be to balance the flow of energy or building blocks between antibiotic production and sporulation. The formation of spores is presumably more essential than antibiotic production to the survival of the organism; therefore, *absA* may repress antibiotic synthesis when energy or nutrients are in short supply. The behavior of Pha mutants, which overexpress antibiotics and are somewhat deficient in sporulation, supports this hypothesis.

APPENDIX

Reproductions of Figures 1A, 2, 3A, 4A, and 5.

Figure 1A.

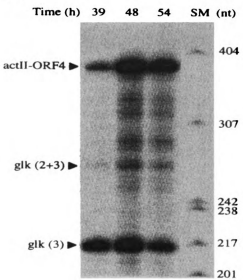


Figure 2

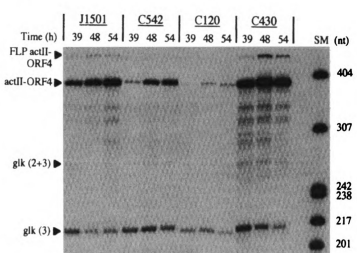
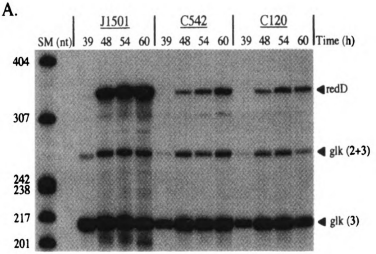


Figure 3 A.

Figure 4 A.



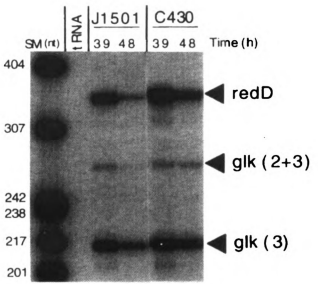

SM (nt)
tRNA
J1501
C430
39 48
39 48
Time (h)
404
307
242
238
217
201
redD
glk (2+3)
glk (3)

Figure 5

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