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DEVELOPMENTAL REGULATION OF STREPTOMYCES COELICOLOR ANTIBIOTIC SYNTHESIS BY THE AbsA TWO-COMPONENT SYSTEM

presented by

Todd B. Anderson

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Microbiology

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DEVELOPMENTAL REGULATON OF *Streptomyces coelicolor* ANTIBIOTIC SYNTHESIS BY THE AbsA TWO-COMPONENT SYSTEM

By

Todd B. Anderson

A DISSERTATION

Submitted to
Michigan State University
In partial fulfillment of the requirements
For the degree of

DOCTOR OF PHILOSOPHY

Department of Microbiology

2000

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ABSTRACT

DEVELOPMENTAL REGULATON OF *Streptomyces coelicolor* ANTIBIOTIC SYNTHESIS BY THE AbsA TWO-COMPONENT SYSTEM

By

Todd B. Anderson

Streptomycetes synthesize antibiotics in a growth-phase dependent manner temporally commensurate with, but spatially independent of sporulation. In *Streptomyces coelicolor*, the *absA* locus encodes a two-component signal transduction system which globally regulates antibiotic production independent of morphogenesis. Mutations in *absA* produce drastically opposing phenotypes. Mutations responsible for an Abs$^-$ (antibiotic synthesis deficient) phenotype were previously localized to the *absA1* histidine kinase gene. In this study Abs$^-$ mutants C542 and C577 were shown to contain point mutations in the region of *absA1* encoding the transmitter domain; these were proposed to lock AbsA1 into a kinase dominant, phosphatase deficient enzymatic state. Second-site suppressor mutations, *sab* (suppressor of *abs*), that restored antibiotic synthesis to the Abs$^-$ mutants were previously mapped very near to the *absA* locus. Sequence analysis identified several *sab* mutations in the *absA1* or *absA2* genes; the latter encodes a two-component response regulator. A genetic analysis of the *absA* locus was performed to examine the mechanism of AbsA regulation. A disruption in *absA2* caused the precocious hyperproduction of pigmented antibiotics (Pha phenotype), demonstrating that AbsA2 negatively regulates antibiotic synthesis. Gene replacements in *absA*, that disrupted phosphoryl-group transfer, also caused the Pha phenotype, demonstrating that the phosphorylated form of AbsA2 is the active negative regulator of antibiotic synthesis.

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These Pha strains demonstrated temporal acceleration of calcium-dependent antibiotic synthesis, in addition to previously characterized acceleration of pigmented antibiotics. *In-vitro* phosphorylation experiments utilizing a maltose-binding protein fusion to truncated AbsA1 ('AbsA1) and a His-tag fusion to AbsA2 provided preliminary biochemical evidence supporting AbsA1 autokinase activity and AbsA1-AbsA2 phosphoryl-group signal transduction. High-copy expression of *absA1* alleles in a wild type background resulted in a Pha phenotype, providing genetic evidence that AbsA1 also possesses AbsA2-P phosphatase activity. S1 nuclease mapping of the *absA* locus demonstrated leaderless cotranscription of *absA1* and *absA2*. Transcription of *absA* was growth-phase regulated, experiencing a dramatic increase prior to the appearance of pigmented antibiotics. Transcription of the *absA* locus was also autoregulated. In contrast to its activity as a negative regulator of antibiotics, phospho-AbsA2 appeared to activate transcription of *absA*. AbsA regulation of pathway-specific regulators *redD*, *redZ*, and *cdaR* was examined by S1 nuclease protection assays. Expression of *redD* was temporally retarded with respect to *absA* and was clearly regulated by AbsA2 in a phosphorylation-dependent manner. Conversely, *redZ* transcription was expressed at high levels coincident with *absA* expression and showed no dependence on AbsA. Transcription of the *cdaR* pathway-specific regulator was also temporally regulated in parallel with the *absA* time course. Transcription of *cdaR* does not appear to depend on AbsA. The possible role and mechanism of AbsA-mediated regulation of antibiotic synthesis in the *S. coelicolor* life cycle is discussed. Preliminary evidence supporting the conservation of *absA* in other species of *Streptomyces* is presented.

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I dedicate this work to my beautiful boys, Todd and David, who helped me keep a healthy perspective and balance in my life over the course of this project. This work is also dedicated to my wife, Marywbska, whose support and sacrifice allowed me to achieve this goal; and to my mother, Alice Anderson, and the memory of my father, Ellef Anderson, in appreciation of the values and work ethic they helped instill in me. Finally, I dedicate this work to my sister, Pamela, whose commitment to good science has always motivated me.

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I would like to thank my mentor, Dr. Wendy Champness, for her guidance, patience and support during this period of my professional development. Thanks to Renqiu Kong, Marywbska Calderón, and Mark Kazmierczak for valuable technical support. I thank Paul Brian for the lab training that helped me get up and running. I am also grateful to N. Jamie Ryding, Gary Brown, and David Aceti for their sharing of knowledge and ideas. My appreciation goes out to the members of my thesis committee, Dr. David Arnosti, Dr. Michael Bagdasarian, Dr. Michael Thomashow, and Dr. Suzanne Thiem for helpful guidance and discussion. Thank you to Mervyn Bibb for providing plasmids used in this study.

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LIST OF ABREVIATIONS

Abs$^-$	antibiotic synthesis deficient
Act	actinorhodin
CDA	calcium-dependent antibiotic
HK	histidine kinase
HTH	helix-turn-helix
Mmy	methylenomycin
ONA	Oxoid nutrient agar
Pha	precocious hyperproduction of antibiotics
PSR	pathway-specific regulator
Red	undecylprodigiosin
RR	response regulator
sab	suppressor of *abs*
SARP	*Streptomyces* antibiotic regulatory proteins
TM	transmembrane

CHAPTER 1

INTRODUCTION

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The genus *Streptomyces* is composed of high-G+C, Gram-positive bacteria of the order *Actinomycetales*. *Streptomyces* are most noted for their ability to produce a diverse array of biologically active secondary metabolites. This genus accounts for over one-half of the thousands of naturally produced antibiotics discovered and is the source of over half of the bio-active compounds currently in clinical use (165). *Streptomyces* metabolites important to health and agriculture include antibacterial, antifungal, antiparasitic, and antitumor drugs, immunosuppressive agents, insecticides, and even herbicides. The abundance of naturally occurring compounds produced by this genera, together with the relative ease of culturing streptomycetes (in comparison to fungi or other actinomycetes), makes them a prime target for bioexploration. Numerous efforts are also underway to produce novel metabolites through genetic engineering of well characterized *Streptomyces* biosynthetic pathways (82). Given the alarming increase in the incidence of antibiotic resistance in common human pathogens, coupled with the continual emergence of new human, animal and plant pathogens, it is critical that *Streptomyces* continue to be exploited as a source of new drugs. Unfortunately, at present we are able to culture only a handful of *Streptomyces*. Therefore, to fully harness the fabulous diversity of chemical compounds synthesized by this genus, and other little known actinomycetes, we must gain a better understanding of the biological processes driving their growth and development.

Streptomyces are notable for their morphological similarity to filamentous fungi. Spore germination and germ tube extension is followed by vegetative growth through hyphal extension and branching to form a dense weave of substrate mycelium. Vegetative growth frequently ends in response to some nutrient limitation (90a) at which

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time the cell enters the second phase of biphasic biomass accumulation coincident with the production of aerial mycelium. DNA replication continues throughout the period of biomass accumulation (90a; 112). Prior to the appearance of antibiotics and aerial mycelium, the colony undergoes the initial stages of a complex program of cellular differentiation which has been divided into two distinct processes - morphological and physiological differentiation (31). During morphological differentiation, aerial hyphae emerge from the substrate mycelium, terminally septate, and develop spores (reviewed by 34; 37). Meanwhile, in the substrate mycelium, the temporally parallel but spatially and metabolically distinct process of physiological differentiation results in the synthesis of secondary metabolites.

Streptomyces produce several chemically distinct categories of antibiotics (reviewed by 150; 173; 163). In spite of this tremendous diversity, there are certain consistencies in their genetic organization and regulation that suggest substantial evolutionary conservation in the mechanism controlling their synthesis. Organizationally, biosynthetic genes encoding a particular antibiotic are clustered together on the chromosome. Since first demonstrating the clustered organization of actinorhodin genes (109), a great many *Streptomyces* antibiotic gene clusters have been identified (cataloged by 119; 72). Clusters occupy approximately 15 to 100 kb and include biosynthetic genes, resistance determinants, and regulatory genes. Antibiotic synthesis is growth-phase dependent, occurring during the stationary phase. Temporal expression of most regulatory genes for antibiotic production are upregulated during transition phase growth. Cluster-specific regulatory features are common throughout the genus (30). Likewise, preliminary evidence using PCR and Southern hybridization

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techniques suggests that homologs of many antibiotic pleiotropic regulatory genes are present in numerous streptomycetes (*absB*, (136); *afsQ*, (84); *afsR*, (115) ; *cutRS*, (32); *bldA*, (99); *absA2*, see Chapter 6). Moreover, cloned antibiotic gene clusters that were introduced into heterologous host streptomycetes showed proper temporal expression, suggesting some level of regulatory conservation (109; reviewed in 119). Taken together, these results imply that regulatory mechanisms governing antibiotic production may be conserved at various stages of development. The remainder of this chapter will review advances in the field of *Streptomyces* antibiotic regulation with a focus on model organism *S. coelicolor*. Special attention will also be given to the topic of this study, the AbsA two-component signal transduction system, and its contribution to regulation of antibiotic production in *S. coelicolor*.

Regulation of Cellular Differentiation and Antibiotic Production

Regulation of antibiotic production in streptomycetes can be divided into three tiers. At the earliest stages of development, antibiotic synthesis and sporulation are jointly regulated. The precise timing of the uncoupling of joint regulation during the vegetative and/or transition phases has not been widely studied. Uncoupling of antibiotic synthesis from morphogenesis is characterized by two levels of regulation; pathway-specific regulators control the synthesis of a single antibiotic, where as pleiotropic regulators influence the production of two or more. Expression of these regulators increases greatly during the transition phase and continues well into stationary phase growth, which is characterized by the onset of antibiotic accumulation. In order to understand the role of any individual regulator on differentiation, it is valuable to have an

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Factors triggering differentiation

It has been well established that exhaustion of one or more nutrients such as carbon, nitrogen, phosphorous, or trace elements leads to the onset of secondary metabolism (reviewed in 152). Easing of catabolite repression was demonstrated as one mechanism of initiating antibiotic synthesis through derepression of specific enzymes required in secondary metabolism. Production of numerous *Streptomyces* antibiotics was repressed by growth on glucose, and certain other carbon sources (reviewed by 46). Cell-free assays with antibiotic synthases showed that a number of these were repressed in the presence of glucose. In contrast to the inducible enzymes of enteric bacteria, cyclic-AMP did not appear to play a role in relieving carbon source repression in *Streptomyces* (45 and references therein). Readily assimilable ammonium salts also negatively affected antibiotic synthesis in numerous strains (reviewed by 151; 46). In tylosin producing *S. fradie*, ammonium repressed several enzymes associated with catabolism of amino acids used to generate propionate and butyrate precursors of the macrolide ring (103). Conversely, addition of branched chain amino acids promoted tylosin production in this strain. In general, greater production of antibiotics was favored by growth on complex, slowly assimilable nitrogen sources. The biosynthesis of a large number of antibiotics was subject to regulation by phosphate (reviewed by 111). Those chemical classes of

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antibiotics especially sensitive to phosphate included aminoglycosides, tetracyclines, macrolides and polyenes, while antibiotics directly assembled from amino acids were much less sensitive. Phosphate was shown to repress phosphatases required in biosynthetic steps of aminoglycosides, and several other biosynthetic enzymes from other classes of antibiotics. However, in the synthesis of macrolides, phosphate seemed to function at the level of primary metabolism by inhibiting precursor formation. Thus, early in colony development, growth-phase dependent regulation of antibiotic synthesis may be due in part to catabolite repression of antibiotic biosynthetic and/or regulatory genes, or repression of pathways involved in precursor formation.

Given that antibiotic production is associated with the low growth rates of stationary phase metabolism, it was thought that nutrient deprivation may also induce secondary metabolism through a growth-rate sensitive response. Several studies examined whether antibiotics could be induced by low growth rate alone in the absence of nutrient deprivation. In continuous culture experiments with *S. cattleya* (106) and *S. venezuelae* (172), synthesis of cephamycin C and cloramphenicol, respectively, were both induced at low growth rates independent of nutrient limitations. Likewise, granaticin was produced by *S. thermocyolaceus* in response to low growth rate in the absence of nutrient deficiencies (85). In contrast, Demain and Fang (1995) cite various cases where some form of nutritional stress was required along with low growth rate to induce antibiotic synthesis.

Stringent response is a common mechanism involved in bacterial adaptation in response to low growth-rate or nutrient deprivation. In *Enterobacteriaceae*, the stringent response is associated with the accumulation of the intracellular effector molecules

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(p)ppGpp (reviewed by 24). In *Streptomyces*, the involvement of ppGpp-dependent regulation of antibiotic synthesis and sporulation appeared to be species specific and in some cases metabolite specific. Mutations in the *relC* gene of *S. coelicolor* (128) and *S. griseus* (129) produced "relaxed" mutants with altered L11 protein in the 50S ribosomal subunit that were impaired in their ability to bind RelA - an enzyme that catalyzes the synthesis of pppGpp from GTP (or GDP) and ATP during the stringent response . The *S. coelicolor* relaxed mutants were deficient in actinorhodin (Act) and undecylprodigiosin (Red) synthesis and unable to form aerial hyphae. Similarly, the *S. griseus relC* mutant was unable to produce streptomycin or form aerial hyphae. Unfortunately, these mutants were also impaired in growth so that it was uncertain whether their phenotypes were due to reduced levels of (p)ppGpp or general growth deficiencies. More recently, deletions were made in the *relA* gene of *S. coelicolor* (113; 114; 27; 26) that completely eliminated ppGpp synthesis. Martinez-Costa, et al. (1996) found that the *relA* deletion reduced sporulation and severely affected Act production, but had little or no effect on Red and CDA synthesis. Chakraburtty and Bibb (1997) reported that the *relA* null mutant failed to make Act or Red; however, only under nitrogen limiting conditions. A similar conditional dependence on ppGpp may also extend to streptomycin production in *S. griseus*, since contrary to the finding of Ochi (1990b) mentioned above, Neumann et al. (1996) witnessed substantial streptomycin production without any significant accumulation of ppGpp in *S. griseus* when grown on minimal medium. Likewise, there was no obligatory relationship between antibiotic production and ppGpp accumulation in *S. clavuligerus* when grown in defined or complex medium and subjected to nutritional shift-down (12). Therefore, (p)ppGpp may be required to trigger sporulation and/or

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synthesis of certain antibiotics under a particular set of culture conditions; however, its role appears to be species dependent, nutritionally conditional, and limited to certain products of secondary metabolism.

Quorum sensing, cell-density-dependent gene expression, is another common mechanism for the initiation of developmental responses. In quorum-sensing systems, effector molecules accumulate in the medium until such time that they reach threshold levels and elicit a response. In Gram-negative bacteria the effector molecules are normally acyl-homoserine lactone autoinducers that are able to diffuse across the cytoplasmic membrane (58). A well characterized example of Gram-negative quorum sensing is found in autoinducer regulated gene expression of bioluminescence in *Vibrio harveyi*. This organism produces two signaling molecules, AI-1 and AI-2, that accumulate in the medium during vegetative growth (13). In the absence of autoinducer, the phosphorylated response regulator protein, LuxO, represses bioilluminescence. Upon reaching threshold concentrations, AI-1 and AI-2 activate the phosphatase activity of their independent cognate sensor molecules LuxN and LuxQ (hybrid two-component sensor kinases) that are able to integrate their signal through LuxO to derepress expression of bioilluminescence genes (56).

Quorum sensing is also a common mechanism among Gram-positive bacteria for regulating developmental gene expression. Examples of the best characterized processes are reviewed by Kleerebezem, et al. (1997) and include genetic competence in *Bacillus subtilis* and *Staphylococcus pneumoniae*, virulence response in *Staphylococcus aureus*, and the production of peptide antibiotics by various species of Gram-positive bacteria. In contrast to their Gram-negative counterparts, these cell-density dependent systems

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incorporate a post-translationally processed peptide as a signaling molecule. Unlike the diffusible N-acyl homoserine lactones, these peptide pheromones are secreted by dedicated ATP-binding-cassette exporters. Another common characteristic of these systems which is not as ubiquitous in the Gram-negative mechanisms is the integration of two-component signal transduction systems as sensor and response mechanisms to regulate gene expression. The histidine kinase (HK) of these systems is thought to sense the processed peptide pheromone and activate response regulator-mediated expression of the peptide pheromone, the two-component system, and the genes responsible for the cell-density dependent phenotype (96). A peptide pheromone quorum-sensing mechanism has yet to be conclusively demonstrated in *Streptomyces*; however, a hierarchical regulatory cascade controlling aerial mycelium formation in S. coelicolor does appear to incorporate small peptide signaling molecules (185; 126; discussed below). Likewise, the *S. coelicolor* genome sequencing project (http://www.sanger.ac.uk/Projects/S_coelicolor/) has uncovered numerous homologs to ABC transporters with as yet unassigned functions.

Interestingly, a variant of the Gram-positive quorum sensing systems not involving peptide pheromones is found in the genus *Streptomyces*. N-acyl homoserine lactone analogs, γ-butyrolactones, have been implicated as cell-density-dependent signaling molecules in morphological and/or physiological differentiation in several species (e.g., *S. virginiae* (189; 188); *S. viridochromo* (61); *S. bikiniensis* and *S. cyaneofuscatus* (60)). The best known example of butyrolactone regulation of differentiation is found in *S. griseus* where A-factor autoregulator is essential for the induction of aerial mycelium formation and streptomycin production (reviewed by 75;

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130). A-factor is described as a microbial hormone that is able to diffuse freely across the cytoplasmic membrane. It was originally discovered by its ability to cause sporulation in a *bld* mutant (deficient in sporulation and antibiotic production) of *S. griseus* (92) when wild type and mutant were streaked side-by-side. A-factor exerts its regulatory function through an interaction with A-factor binding protein (ArpA), present in the cytoplasm of the cell. In the absence of A-factor, ArpA acts as a repressor of independent loci required for activation of aerial mycelia and streptomycin genes. Subsequent binding of A-factor to ArpA causes dissociation of the repressor from the promoter region of *adpA*. AdpA then activates expression of the streptomycin pathway-specific activator StrR and is also implicated in activating aerial mycelium formation (177; 130). A-factor is produced in low concentrations during the "decision phase" of vegetative growth, described by Neumann, et al. (1996), as an integral part of a genetically programmed pathway required for the onset of differentiation. Later, it accumulates to much higher concentrations during the transition and stationary phases where it acts on ArpA. This is in contrast to VB, the γ-butyrolactone signaling molecule of *S. virginiae*, which induces production of the antibiotic virginiamycin (but not aerial mycelium) and is synthesized just prior to the antibiotic itself (94).

It is uncertain how widespread γ-butyrolactone signaling is in the regulation of *Streptomyces* differentiation. *S. coelicolor* produces six such molecules (8) that are distinct from A-factor, but are able to complement an A-factor deficient mutant in cross-feeding experiments (66). One of these, expressed only during transition and stationary phase growth, was purified and shown to stimulate both Act and Red production in *S. coelicolor* (14). Horinouchi and Beppu (1992) predicted γ-butyrolactone hormonal

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regulators to be general constituents of morphological and/or secondary metabolite regulatory cascades. More conservatively, Yamada, et al. (1997) estimated that about 60% of streptomycetes produce butyrolactone signaling molecules. It is probably safe to say that as more systems are studied, this type of quorum-sensing signaling mechanism will be found to act both as global inducers of differentiation and as signals for a particular regulatory cascade of a single antibiotic.

Whereas stringent response and easing of catabolite repression represent induction of differentiation by way of a particular stress, synthesis of A-factor, as a quorum-sensing signal or microbial hormone, seems to induce cellular differentiation through a programmed developmental cycle; a regulatory cascade that progresses as the cell ages. Evidence for this type of programmed developmental cycle has been convincingly demonstrated in *S. griseus*. Inconclusive, but growing, evidence also exists for programmed development in *S. coelicolor*.

The idea that *S. griseus* differentiation was under the control of a timed developmental program was first proposed by Ensign (1988). He demonstrated that cells became committed to sporulation at 10-12 hours after spore germination in the absence of nutrient limitations and that this timing was not varied by 10X additions of any individual nutrient to defined medium. That study was further elaborated by Neumann, et al. (1996) who postulated that A-factor-induced aerial mycelium formation and streptomycin production was initiated during the first stage (the "decision phase") of diauxic exponential growth in *S. griseus*. The authors demonstrated, using an A-factor deficient mutant, that low concentrations of A-factor were required during the first stage of diauxic growth (up to 10 hours in the growth medium tested) for cellular differentiation

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to take place, even though appreciable quantities were not produced until the culture entered stationary phase at 24 hours. The earlier that A-factor was supplied during the decision phase, the higher the final yield of streptomycin, although yield was not affected by the concentration of A-factor during this phase. Addition of A-factor after the diauxic lag (10-24hr), during the second stage of exponential growth (the "execution phase"), did not induce differentiation. In the same manner, known inhibitors of streptomycin production, L-valine and staurosporin (a kinase inhibitor), were only effective if added prior to the diauxic lag (i.e., during the "decision phase"). Like Ensign (1988), Neumann, et al. (1996) noted that the onset of the diauxic lag occurred in the absence of any perceivable nutritional or physical perturbation, and its timing could not be altered by doubling the concentration of any individual nutrient or all nutrients. The authors proposed that A-factor is an inducer of cellular differentiation that forms part of a programmed differentiation cycle in which the decision by the cell to switch on morphological and physiological differentiation is made during the first phase of diauxic exponential growth.

A second example of programmed development may come from the hierarchical pattern of complementation witnessed in the *bld* (bald) mutants of *S. coelicolor* (185). *Streptomyces bld* mutants are so called because they are deficient in aerial mycelium formation; most are also blocked in antibiotics (reviewed by 31; 35). Losick and collaborators (184; 185; 127; 126) believe that many of the *bld* mutants are involved in a hierarchical cascade of extracellular signaling that controls SapB synthesis - a small protein required for the initiation of aerial mycelium formation. This hypothesis is based on the ability of certain *bld* mutants to complement the aerial mycelium deficiency of

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other *bld* strains through "cross feeding" when grown next to one another on solid medium (184; 185), or by preconditioning agar medium with one *bld* mutant prior to cultivation of another (185; 126). The hierarchy of steps in the regulatory cascade are ordered by mutations in *bld261*, *-K*, *-A*, *-H*, *-G*, *-C*, and *-D*. Briefly, *bld 261* is required (and possibly encodes) for the synthesis of an extracellular factor that is believed to be the first signal in the cascade (126). The *bldK* gene encodes an ABC membrane-spanning transporter (127) that is possibly involved in importing the extracellular signal associated with *bld261*. The most extensively studied *bld* gene, *bldA*, encodes the only tRNA that translates the rare UUA leucine codon of *Streptomyces* (101; 104). It is yet to be determined what function BldA has in this cascade, but UUA codons have been identified in antibiotic pathway-specific regulators *redZ* (182) and *actII-ORF4* (52). The *bldG* mutations are complemented by a locus that shows similarity to antisigma/anti-antisigma genes of *Bacillus subtilis* (28; 30 and references therein). The final gene of the hierarchy, *bldD*, is thought to code for or regulate synthesis of an extracellular molecule that is heat and protease resistant and capable of restoring aerial mycelium formation to all the other *bld* mutants of the cascade, presumably by its ability to restore SapB synthesis (185). Interestingly, these complementation studies demonstrated the ability to suppress morphological differentiation without pleiotropic suppression of antibiotic defects.

Several characteristics of the *bld* mutants suggest their involvement in a programmed developmental cycle. First, *bld* mutations tend to be pleiotropic for morphological and physiological differentiation, and therefore represent early components of the developmental process where these pathways are still coupled

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(Figure 1). There are many other regulators of either sporulation or antibiotic production that are uncoupled from each other (discussed below). *whi* mutants (impaired in sporulation) are able to extracellularly compliment *bld* mutations (e.g., *whiF* C99, 33; 184). Likewise, overexpression of antibiotic pleiotropic or pathway specific regulators can suppress *bld* mutations (29; 65), demonstrating that *bld* genes control cellular differentiation upstream of uncoupled sporulation or antibiotic regulatory pathways. Very few transcript studies have been performed on the *bld* genes. Of those mentioned above, *bldD* transcription was most prominent at the earliest time point tested, 15 hr, although this was predicted to have already been in transition phase since aerial mycelia began to appear by 18 hr and antibiotics by 24 hr (48). Antibiotic regulatory genes are predominantly up-regulated during the transition phase, which likely corresponds to the second leg of the diauxic curve ("execution phase") described by Neumann, et al. (1996). Therefore, given that the *bld* genes act upstream of both *whi* genes and antibiotic regulatory genes, and the latter are expressed during transition and stationary phase, the signaling cascade proposed by Willey, et al. (1993) may form part of a genetic program that is active during the decision phase to commit the culture to differentiation. Obviously, there is still much work to be done to define whether the proposed cascade is part of a developmentally programmed pathway or is induced by nutritional or physical stress. Likewise, it will be exciting to see how this pathway is integrated into the global regulation of antibiotics.

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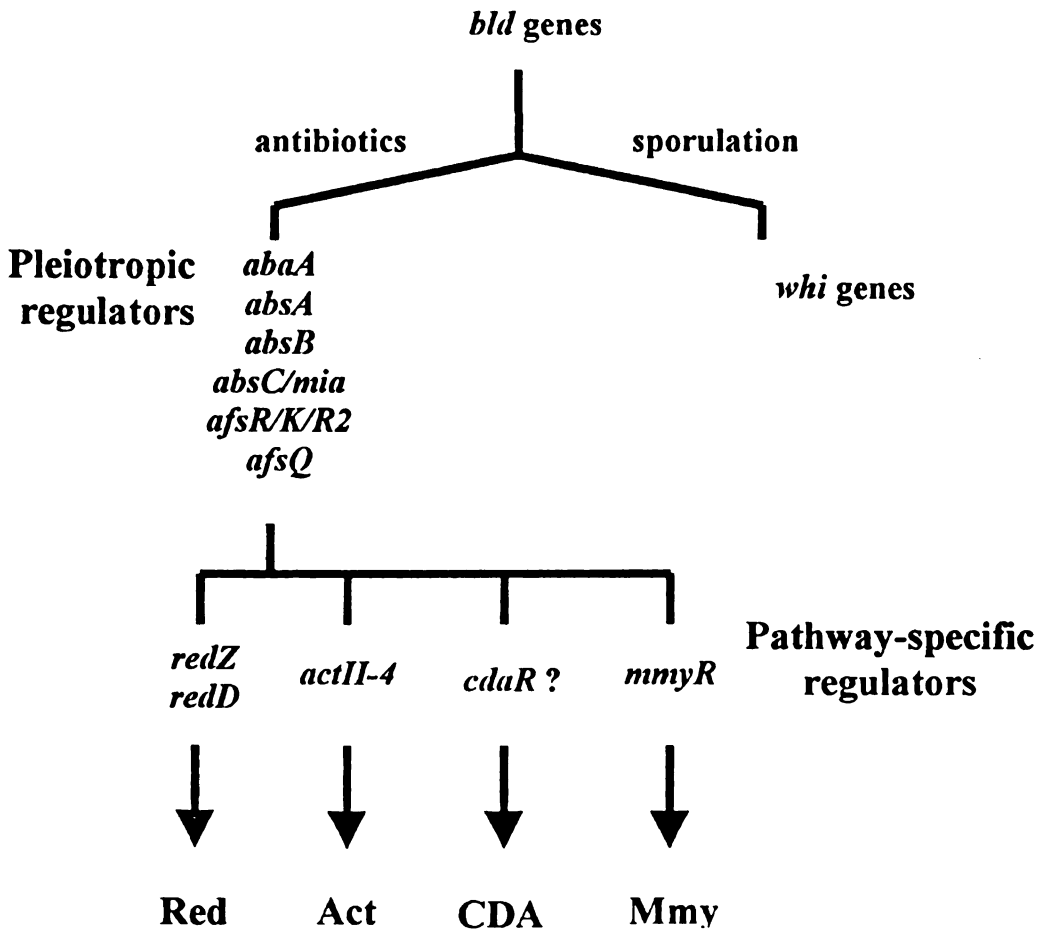

Figure 1. Hierarchy of antibiotic regulatory loci. Genetic loci involved in the regulation of antibiotic production in *S. coelicolor* can be divided into a three-tier hierarchy. Mutations in many *bld* genes block both antibiotic production and sporulation. Pleiotropic regulators influence the production of more than one antibiotic. Genes for the pathway-specific regulators are linked to the biosynthetic genes of their respective antibiotic gene clusters and regulate only the antibiotic that they are associated with.

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Uncoupling of differentiation: regulation of antibiotics

In addition to the joint regulation of morphological and physiological differentiation demonstrated by *bld* mutants, sporulation and antibiotic production are also subject to uncoupled regulatory mechanisms. These have been studied in greatest detail in model organism *S. coelicolor*. Numerous genes uniquely involved in sporulation, *whi* genes, have recently been reviewed by Chater (1998) and Chater and Losick (1997). This section will concentrate principally on the regulation of antibiotic production centered around mechanisms discovered in *S. coelicolor* and closely related *S. lividans*, but will also incorporate examples from other species to highlight similarities or differences that will aid in conferring the current state of understanding of this complex process to other species within the genus. Reviews on this topic can be found in Champness (1999); Bibb (1996); Chater and Bibb (1997); and Hopwood, et al. (1995).

Streptomyces coelicolor synthesizes four distinct antibiotics, actinorhodin (Act), undecylprodigiosin (Red), calcium-dependent antibiotic (CDA), and methylenomycin (Mmy). The gene clusters for Act and Red have been cloned (109; 110) and the biochemical pathway for Act synthesis has been extensively studied (reviewed by 72). An advantageous characteristic of Act and Red is that they are pigmented (blue and red, respectively, at alkaline pH) which facilitates phenotypic screens for factors affecting their synthesis. The sequence of *cda* genes has recently been revealed through the ongoing *Streptomyces* sequencing project (http://www.sanger.ac.uk/Projects/S_coelicolor/). Previous work had partially cloned and characterized this locus (39), but its regulatory and biochemical characteristics are still largely unknown. Methylenomycin carries the distinction of being the only

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Streptomyces antibiotic whose sequence is carried on a plasmid, SCP1, a 350 kb linear plasmid (95) present at a copy number of four. The *mmy* gene cluster was localized to a 20 kb region and found to contain a regulatory gene, *mmyR* (36).

A common characteristic of antibiotic gene clusters is that they encode for one or more pathway-specific regulators (PSR) that solely influence the expression of genes for that antibiotic. Champness (1999) lists PSRs for antibiotics produced in numerous species. Most antibiotic clusters encode a single PSR, although, the daunorubicin cluster of *S. peucetius* has three, *dnrI*, *dnrN*, and *dnrO*, and the Red cluster contains two, *redD* and *redZ*. Most PSRs contain a DNA-binding motif that transcriptionally activates one or more of the operons carrying antibiotic biosynthetic genes; although at least one, MmyR, of the methylenomycin pathway is a repressor (36). Where multiple PSRs exist, apparently only one interacts with biosynthetic gene promoters (e.g., RedD and DnrI) while the others regulate expression of the first. For example, Red Z and DnrN are required for expression of *red* and *dnr* biosynthetic genes because they activate *redD* and *dnrI* transcription, respectively (65; 59). They do not bypass the need for RedD and DnrI. RedZ and DnrN show extensive full-length sequence similarity to two-component response regulators (RR) of the FixJ subfamily, but have lost the ability to phosphorylate (65; 59). A number of other PSRs, RedD, ActII-ORF4, DnrI, SnoA, and CcaR, and one pleiotropic regulator, AfsR, have recently been classified as a special group of *Streptomyces* antibiotic regulatory proteins (SARPs) based on amino acid similarities that suggest they contain a conserved C-terminal DNA-binding motif like the one found in the two-component response regulator OmpR (183). Similar heptameric direct repeats in act and *dnr* biosynthetic promoters have been predicted as binding sites for ActII-ORF4 and

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DnrI, respectively, and although these two SARPs can apparently substitute for one another (166), SARPs generally only regulate genes of the antibiotic they are associated with. One final observation about PSRs is that their overexpression at relatively low copy number results in dramatic increases in antibiotic production (162; 62) which may suggest that their expression is tightly controlled by the cell.

Acting between the *bld* genes that regulated morphological and physiological differentiation, and the PSRs which influence the synthesis of individual antibiotics, are a number of genetic loci that pleiotropically regulate more than one antibiotic without affecting growth or sporulation. Remarkably, the only knowledge of antibiotic pleiotropic regulators in the genus *Streptomyces* comes from work in *S. coelicolor* and *S. lividans* (which like *S. coelicolor* produces Act and Red) due largely to the ability to perform visual screens for the pigmented antibiotics Act and Red in these strains. The loci which have so far been implicated in pleiotropic regulation are *abaA* (53), *absA* (2), *afsR/K/R2* (115; 116; 174), *mia* (28), and *micX* (143). With the exception of *absB* (a putative homolog of *E. coli* RNaseIII (136)) and *micX* (a possible antisense fragment (142)) each of the loci have at least a suspected involvement in phosphorylation signal-transduction pathways.

The *mia* and *abaA* loci were isolated because they produced a phenotype when cloned on a multicopy plasmid (28; 53). The 2.7 kb *abaA* clone isolated from *S. coelicolor* contained five open reading frames and caused overexpression of Act in both *S. lividans* and *S. coelicolor* when carried on a multicopy plasmid. The region responsible for Act overexpression was isolated to cotranscribed *orfA* and *orfB* (53). Fragments containing *orfA* and *orfB*, or *orfB* alone were able to overexpress Act.

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Furthermore, single cross-over disruption of *orfB* abolished production of Act and severely reduced that of Red and CDA while having no effect on Mmy. OrfA of this locus shows similarity to the transmitter region of two-component sensor kinases (146). OrfB did not show similarity to any proteins deduced from gene databases. Interestingly, there is significant structural organization between *abaA* and the *whiJ* locus of *S. coelicolor*, mutants of which are impaired in sporulation. Three open reading frames of *whiJ* show similarity to those in *abaA*, including Orf1 which is homologous to the putative OrfA kinase (146).

In contrast to *abaA*, the high copy clone of the *mia* locus abolished synthesis of all four antibiotics in *S. coelicolor* (28). The region responsible for the *mia* phenotype has been narrowed down to a 90 nt sequence of unknown function (30) which lies in an intergenic region, contains an open reading frame for a 20 amino acid peptide, and possesses sequence consistent with the formation of a large stem loop (29). The *mia* region has recently been included in the newly described *absC* locus (29) and is believed to contain the transcriptional start site for *orf1* of *absC* (146). The N-terminus of ORF1 shows sequence similarity to two-component histidine kinases just as in *abaA* ORFA; however, the C-terminal domain of ORF1 is similar to protein phosphatases (146). Gene replacement in *orf1* produced an increase in Act. Since the *mia* fragment lies in the promoter region of *absC orf1*, it is possible that the high-copy number *mia* phenotype is caused by the titration of a regulator of *orf1*. Therefore, both *abaA* and the *mia*-containing *absC* loci encode proteins that are not histidine kinases but possess two-component-like kinase domains and appear to be closely associated with the pleiotropic

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phenotypes obtained from strains overexpressing these regions. The role of these loci in phosphorylation signal-transduction pathways is currently under investigation.

The remaining pleiotropic regulatory loci all encode for proteins commonly associated with phosphorylation signal-transduction mechanisms. AfsR is a serine-threonine phosphoprotein that also was isolated by its ability to cause overexpression of Act and Red when expressed on multicopy plasmids in *S. lividans* and *S. coelicolor* (78; 54). In vitro, AfsR is phosphorylated by the downstream, convergently transcribed gene product of *afsK*, a serine-threonine protein kinase (115). The N-terminal domain of AfsR is 33% identical to the PSRs ActII-ORF4 and RedD (115), which have recently been predicted to contain an OmpR-like DNA-binding motif (183). The strain of *S. coelicolor* carrying multiple copies of *afsR* produced increased levels of *actII-4* and *redD* transcripts consistent with the effect of AfsR on these antibiotics. Similarly, AfsR was not able to restore synthesis of Act or Red in *ΔactII-4* or *ΔredD* mutants, demonstrating that it could not bypass the PSRs (54). A chromosomal in-frame deletion of *afsR* had a conditional effect on antibiotic synthesis (54). On minimal medium, the *afsR* mutant was essentially devoid of Act or Red, while on complex medium Act was only slightly delayed, and on rich medium Act and Red production were comparable to the wild-type strain. AfsR was also found to reduce CDA synthesis in a similar medium-dependent nature, but had no effect on Mmy. Thus, the AfsR Ser-Thr phosphoprotein appeared to regulate antibiotics through a direct or indirect interaction with the PSRs; however, it was only essential for Act and Red under certain nutritional conditions.

The final set of pleiotropic regulatory loci, *absA*, *afsQ*, and *cutRS*, all encode independent two-component signal transduction systems. CutRS is a negative regulator

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of Act synthesis in *S. lividans* (32). Gene disruptions in either *cutS* histidine kinase (HK) or *cutR* response regulator (RR) resulted in accelerated and increased production of Act, which could be suppressed by introducing the cloned *cutR* gene. Chang, et al. (1996) reported a similar phenotype upon creating a gene replacement in *cutS* of *S. coelicolor*; however, recent evidence suggests that this locus does not regulate antibiotic synthesis in *S. coelicolor* (15). No information has been given as to the effect of CutRS on Red production.

The *afsQ* locus of *S. coelicolor* was discovered by virtue of its ability to stimulate production of pigmented antibiotics and A-factor when introduced on a multicopy plasmid into *S. lividans* (84). This locus encoded the AfsQ1 RR and AfsQ2 HK. Surprisingly, no phenotype was obtained when either of these genes were disrupted on the *S. coelicolor* chromosome. In contrast, a low copy-number plasmid carrying *afsQ1* was able to suppress an *S. coelicolor absA* mutation (globally deficient in antibiotic synthesis). Given the ability of this gene to suppress the *absA* mutation in low copy number, the absence of a phenotype in the *afsQ* chromosomal disruptions may simply mean that, like AfsR, this system is only essential or active under certain nutritional conditions.

The final two-component system thus far identified as an antibiotic pleiotropic regulator in *S. coelicolor* is encoded by the *absA* locus. The *absA* locus was isolated from mutants that grew and sporulated normally but were deficient in pigmented antibiotics (2). Like the *absB* and *mia* strains, *absA* mutants were shown to be globally blocked in synthesis of all four *S. coelicolor* antibiotics. The *absA* locus was shown to encode the AbsA1 HK and the AbsA2 RR. It was later discovered that mutations in the

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AbsA1 HK were responsible for the Abs$^-$ phenotype exhibited by the original mutants (19). Gene replacement or disruption of *absA1*, believed to have a polar effect on the expression of downstream *absA2*, resulted in the opposite phenotype, early onset and overexpression of pigmented antibiotics (Pha phenotype; precocious hyperproduction of antibiotics) (19). Therefore, it was hypothesized that the AbsA two-component system was a negative regulator of antibiotic synthesis. Consistent with their antibiotic phenotypes, the Abs$^-$ and Pha *absA* mutants affected expression of the PSRs *redD* and *actII-ORFIV* such that their levels varied in agreement with the amount of antibiotic produced (1). Therefore, AbsA seemed to regulate antibiotic synthesis through the direct or indirect transcriptional control of the PSRs.

It is apparent from a review of antibiotic pleiotropic regulators that phosphorylation signal transduction plays an extremely important role in regulating the onset of antibiotic synthesis after this process has been uncoupled from that of sporulation. It is yet to be determined if these regulators comprise a linear regulatory cascade or are part of an integrated network of independent cascades either activating or repressing the production of multiple or individual antibiotics in response to numerous independent signals. Examples of two-component regulation of developmental processes in other Gram-positive organisms, in addition to data from *S. coelicolor* pleiotropic regulators, seems to support an integrated network of antibiotic regulation in the genus *Streptomyces*.

The best example of regulation of growth-phase dependent differentiation including sporulation and secondary metabolite formation comes for the Gram-positive soil bacterium *B. subtilis* (reviewed by 192). This organism demonstrates stationary-

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phase production of a small enzymatically synthesized peptide antibiotic surfactin. Surfactin synthesis is regulated by a complex integrated network of multiple positive and negative controls involving various two-component systems and other phosphorylation signal transduction molecules (e.g., aspartyl-phosphate phosphatases). In addition, various components of this regulatory web are under the control of independent effector molecules (e.g., CodY, ComX, Cfx), whose relative concentrations inside or outside the cell tip the whole mechanism toward overall positive or negative control. The surfactin regulatory network is not physiologically isolated; instead, the molecules active in this pathway are also involved in regulation of other stationary-phase processes such as competence, sporulation, and degradative enzyme synthesis. Thus, where numerous two-component and other phosphoprotein signal transduction regulators mediate a process (e.g., surfactin synthesis), there is precedent for integration of multiple pathways and signals rather than a single linear regulatory cascade.

Transcript studies suggest that pleiotropic regulators *absA*, *afsQ*, *afsR*, and *cutRS* are transcribed simultaneously during transition and stationary phase growth; however, a detailed examination of their simultaneous temporal expression is lacking, as is a detailed analysis of the epistatic relationship between them. AbsA and AfsR have been shown to affect the transcription of PSRs. In addition, multiple copies of *afsR* or *afsQ1* suppressed the *absA* mutation in an Abs^- strain to restore antibiotic synthesis. This would suggest that AfsR and AfsQ act either downstream of AbsA in a linear cascade or in a separate cascade that integrates into a global antibiotic regulatory pathway. In support of the latter, chromosomal disruptions of *afsQ1* and *afsR* did not affect antibiotics under conditions in which *absA* mutations do show a phenotype (84; 54; 2; 19). The fact that

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afsR and *afsQ* are not essential for antibiotic production under conditions in which AbsA demonstrated global control suggests that they may be part of independent integrated pathways responding to different environmental signals. Therefore, even though a great deal of data is missing on the interactions of pleiotropic regulators, that which exists suggests that they do not form a single linear regulatory cascade controlling antibiotic synthesis in *S. coelicolor*.

In order to begin constructing a unified model for antibiotic regulation in *S. coelicolor*, it is not sufficient to simply identify the regulatory molecules that are involved. Once identified, it is necessary to determine the biochemical mechanism of each independent system and establish the interactions that exist between systems to allow one to integrate these into the overall pathway. Of the multiple loci that have been identified based on their ability to produce a phenotype, very little is known about the biochemical mechanisms which account for their activity. With respect to the various two-component systems AbsA, AfsQ, and CutRS, questions of interest include: what is the signal that modifies HK activity; is phosphorelay the mechanism of signal transduction, and if so, what is its role in altering the activity of the RR; what is the target(s) of the RR and is its activity positive or negative; and, how is expression of this system regulated during the course of growth and in comparison to other genes of the pathway(s) they regulate?

Two-Component Signal Transduction Systems

Two-component signal transduction systems are ubiquitous throughout eubacteria as a means of regulating varied aspects of cell physiology in response to environmental

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conditions, including physical, behavioral, nutritional, and developmental responses. The paradigm two-component system (Figure 2) is composed of two proteins, a histidine kinase and response regulator. The mechanism of signal transduction in all two-component systems studied to date is phosphorelay. Extensive reviews of two-component protein structure and function are presented in Parkinson and Kafoid (1992), Stock, et al. (1995), and Volz (1995).

HK proteins are generally composed of two-domains, an N-terminal sensor domain and a C-terminal transmitter domain. Sensor domains frequently contain from two to eight transmembrane helices which anchor the HK to the cell membrane. This domain is highly divergent between systems, which may in part be due to the high signal specificity of each sensor. As the name implies the sensor domain is thought to contain a region responsible for signal molecule recognition, although this mechanism is poorly understood in most two-component systems. Among those HKs where signal sensing has been best characterized are NarX (25; 102), VirA (154), and PhoR (179). The HK transmitter domain is more highly conserved and is responsible for the enzymatic activity associated with this molecule. Several subdomains of the transmitter share sequence and functional homology and are conserved in their organization. The H-box, present in all two-component HKs, contains a conserved His residue that is the site of phosphorylation in these proteins. The transmitter domain is associated with two independent enzyme activities; autophosphorylation at the conserved His residue which is believed to be carried out in trans between the monomers of HK homodimers (161) and phospho-aspartate phosphatase activity directed at the phosphorylated form of the RR. The

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A. Histidine Kinase

Sensor Domain

Transmitter Domain

B. Response Regulator

Receiver Domain

Effector Domain

Figure 2. Two-component signal-transduction proteins. Primary structure diagrams of two-component histidine kinase and response regulator proteins demonstrate common features conserved among members of this family. (A.) The histidine kinase frequently consists of two domains. The N-terminal sensor domain contains between two and eight transmembrane helices that anchor the histidine kinase to the cell membrane. The sensor domain has also been implicated in signal sensing in some systems, which may account for the high sequence divergence of this domain among all histidine kinases. The C-terminal transmitter domain has several highly conserved subdomains indicated by boxes with the amino acid residue for which they are named. The H-box contains the histidine, which is the conserved site of phosphorylation in all histidine kinases. Other sub-domains have been implicated in nucleotide and Mg^{2+} binding required for enzymatic activity. The transmitter domain possesses the auto kinase and phosphatase activity of the histidine kinase. (B.) The response regulator normally consists of two domains. The receiver domain is highly conserved among all members of the two-component family. Three residues conserved in all response regulators are the Asp, Asp, and Lys, which form the acidic pocket in which the central Asp is phosphorylated. The C-terminal effector domain is responsible for the regulatory action of the protein. Most response regulators possess a DNA binding region in the effector domain, indicated here by a helix-turn-helix motif, which is consistent with the function of these proteins as transcriptional regulators.

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phosphorylated HK also acts as a donor for phosphoryl group transfer from the His of the HK to a conserved Asp of its cognate RR. While the majority of HKs fit the above description, there are also numerous hybrid HKs, most of which have one or more additional domains C-terminal to the transmitter domain. For example, hybrid HKs LuxN and LuxQ of *Vibrio harveyi* have response regulator receiver domains fused to the C-terminus of their transmitter domains (13). Likewise, BarA and ArcB have an additional receiver domain, but also contain another C-terminal domain with a phospho-accepting histidine independent of the transmitter His (83). These extra phospho-accepting domains undoubtedly complicate signal transduction between the HK and RR.

The two-component RR is usually a two-domain cytoplasmic protein. The N-terminal receiver domain is highly conserved among virtually all members of this family, with three invariable residues, Asp, Asp, Lys, which form an acidic pocket in which the central Asp is the site of phosphorylation (Figure 2). The C-terminal effector domain most often contains a DNA-binding motif, such that most response regulators act in transcriptional regulation. Response regulators are grouped into subfamilies based on sequence similarity within their effector domains (reviewed by 161). The most common variants of RRs are proteins which possess only the receiver or effector domain, such as Spo0F of *B. subtilis* (191) and GerE of *E. coli* (40). Those proteins which contain only the receiver domain make up part of signal-transduction cascades in which they act as intermediate signal-transduction molecules between an orthodox HK and RR. Where the effector domain exists on its own, it is able to mediate transcriptional regulation in the absence of signal. An offshoot of the signal-independent GerE protein are pseudo

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response regulators such as RedZ and DnrN mentioned earlier (65; 59), which have dual-domain, full-length homology to RRs, but have lost the requirement for phosphorylation.

Phosphorylation is the mechanism of signal transduction in two-component systems (reviewed by 161). Under the two-component paradigm (Figure 3), the sensor region of the HK senses a signal which causes a conformational change across the cell membrane to stimulate autophosphorylation at the conserved His of the transmitter domain. Subsequently, the phosphoryl group is transferred to the Asp of its cognate RR. This causes a conformational change between the receiver and effector domains that permits recognition and binding of the effector domain to its target promoter and transcriptional activation of the target gene. A review of two-component systems suggests that most HKs are phosphatase dominant in the absence of signal. Exceptions that propose kinase default HKs in the absence of signal include the EnvZ/OmpR (135), Cpx (139), and LuxN/Q/O (56) systems; however, the status of the first two are debated by other researchers (55; 132). The difficulty in unequivocally determining the default activity for the great majority of two-component systems is the inability to define and isolate the signal. Nevertheless, with the exception of Lux , in all other systems where good signal identification data exists, the signal stimulates HK kinase activity.

While two-component phosphorylation-mediated signal transduction may appear at first glance to confer all or nothing regulation, a closer look at the mechanism reveals its ability to provide subtle, finely tuned responses. In part, this is due to the dual

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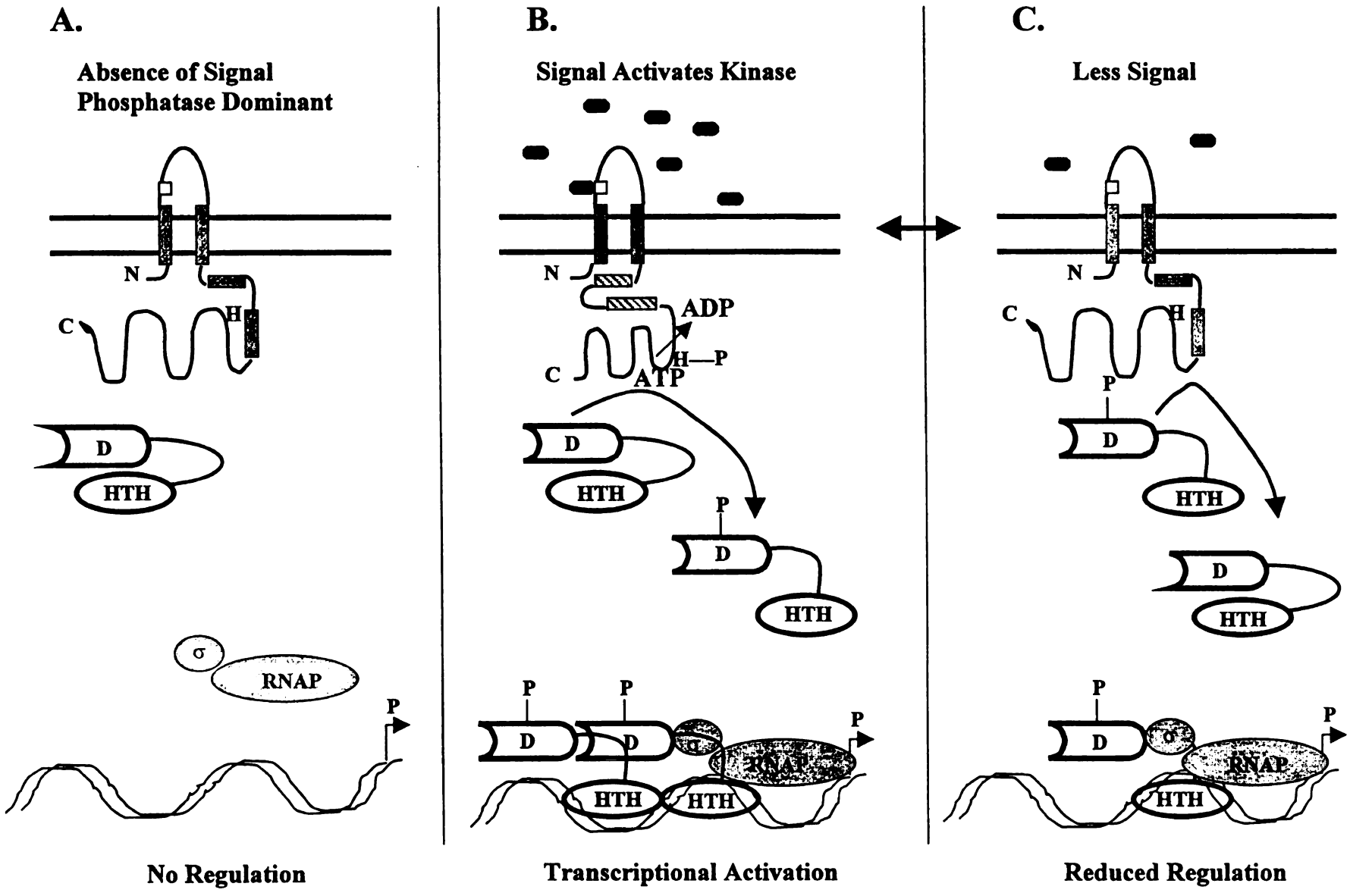
Figure 3. Paradigm two-component signal-transduction system activity. (A.) In the absence of signal most histidine kinases are thought to be in a phosphatase dominant state. Under these condition, the unphosphorylated receiver domain of the response regulator interacts with the effector domain to inhibit binding to the target promoter. If the response regulator is an activator, then transcription is blocked; indicated here by the inability of RNA polymerase holoenzyme to recognize the promoter. (B.) In the presence of signal, ligand binding to a recognition site in the sensor domain of the histidine kinase causes a conformational change across the membrane. The transmitter domain stimulates autokinase activity and phosphorylation occurs at the conserved histidine. The phosphoryl group is transferred to the Asp of its cognate response regulator. Again phosphorylation causes a conformational change, now allowing the HTH region to recognize its target promoter, which effects transcriptional regulation (depicted here as activation). (C.) Module C of this diagram depicts the hypothetical situation in which the concentration of signal decreases or is lower than that in module B. A signal-concentration dependent kinase/phosphatase equilibrium is established in which part of the histidine kinase population is in the kinase state, while the remainder possess phosphatase activity. Some phosphorylated response regulator molecules may be dephosphorylated such that the regulatory output at the target promoter reflects the proportion of phosphorylated to unphosphorylated response regulator. The corresponding output would be intermediate to signal saturation or signal deficient conditions.


A.
Absence of Signal
Phosphatase Dominant
B.
Signal Activates Kinase
C.
Less Signal


A.
Absence of Signal
Phosphatase Dominant
N
C
H
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HTH
σ
RNAP
P
No Regulation
B.
Signal Activates Kinase
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C
ATP
H—P
D
HTH
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P
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D
D
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HTH
HTH
Transcriptional Activation
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Less Signal
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kinase/phosphatase activities of the HK (161). In the two-component paradigm, the signal stimulates HK kinase activity. However, unless the signal is present at sensor-saturating levels, only part of the HK population is in the kinase mode, the rest still exercise phosphatase activity toward the phosphorylated response regulator. Therefore, a signal-concentration dependent equilibrium is established between the kinase and phosphatase activities of the HK that combine to produce a corresponding regulatory output, intermediate to that of the signal saturated or signal deficient extremes (145; 159).

In addition to the signal-concentration-dependent response, several other characteristics of two-component systems add to their complexity. Several systems have more than one signal that may vary in their ability to trigger autophosphorylation of the HK and, therefore, produce different degrees of response as was recently exemplified by NarX autophosphorylation in response to nitrate versus nitrite (102). In addition to the signal associated properties of the HK, the response of two-component regulators at their target promoters can also vary greatly. Promoter recognition sites can be extremely variable not only between different response regulators, but even for the same regulator. For example, the promoter region for at least eight operons regulated by NarL have been extensively characterized (reviewed by 159). While a weak heptameric consensus sequence has been proposed for NarL binding, heptameric sequences exhibit great diversity with respect to number, location, orientation, and spacing. Similarly, while OmpR recognizes multiple decameric sites in the *ompF* and *ompC* promoters, there is no similarity between C-box and F-box sequences (135). In addition, regulatory responses can require anywhere from a single to multiple copies of the regulator in only the

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phosphorylated state (e.g., Spo0A, reviewed by 157), or a combination of the phosphorylated and unphosphorylated states (e.g., OmpR (159) and UhpA (41)). Finally, while some two-component systems seem to be the only regulator of a particular response in simple pathways (e.g., Uhp transport system (89)), other pathways integrate multiple two-component systems together with other transcription factors and feedback mechanisms to fine tune a response through a combination of interacting regulators (e.g., competence gene expression in *B. subtilis* (121)). Therefore, while phosphorelay does appear to play a central role in the signal transduction of all two-component systems, outside of this mechanism enormous variability exists in the process of converting a signal to a response.

The AbsA two-component system of *S. colelicolor*

The *absA* locus encodes a two-component signal transduction system (Figure 4) that negatively regulates production of all four *S. coelicolor* antibiotics (19). The AbsA1 HK is predicted to be membrane bound and undergo phosphorylation at His202 of the highly conserved transmitter domain. AbsA1 also has a relatively large C-terminal domain (approximately 160 amino acids) that does not show sequence similarity to deduced proteins from gene databases and is of yet unknown function. A vector-borne allele of *absA1* used to complement the Abs$^-$ C542 and C577 mutants was truncated at the *Bam*HI site of *absA1*, which removed the last 69 amino acids of the C-terminal domain (19). Nevertheless, this truncated form of AbsA1 was able to restore wild type pigmentation to the Abs$^-$ mutants, calling into question the requirement

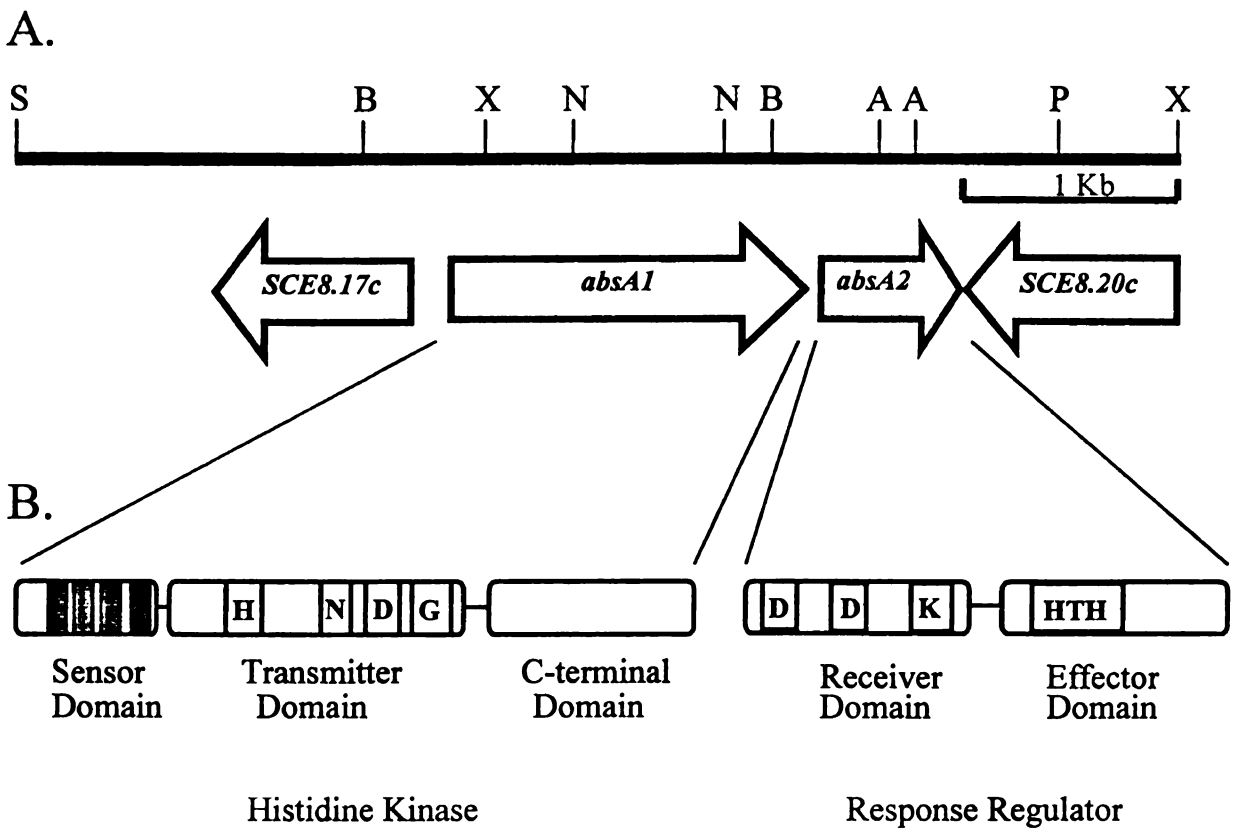


Figure 4. The AbsA two-component system. (A.) A physical map of the *absA* locus and surrounding genome; (B.) Primary sequence diagrams of the AbsA1 histidine kinase and AbsA2 response regulator. AbsA1 possesses three domains. The N-terminal sensor domain is predicted to contain four transmembratne helices. The transmitter domain contains sub-domains conserved in other histidine kinases including the H-box with the putative site of phosphorylation at His202. The unique C-terminal domain of AbsA1 is of unknown function and shows no similarity to other proteins deduced from gene databases. The AbsA2 response regulator consists of two domains. The receiver domain contains the highly conserved residues of other response regulators including a putative site of phosphorylation at Asp54. The C-terminal effector domain contains a helix-turn-helix DNA-binding motif, which allows AbsA2 to effect transcriptional regulation at a target promoter(s). Lettered boxes represent highly conserved sub-domains of functional importance named after a highly conserved residues or motifs found therein. Dark boxes represent transmembrane helices. Restriction sites are A, *Apa*I; B, *Bam*HI; N, *Nae*I; P, *Pst*I; S, *Sac*I; and X, *Xho*I.

of the C-terminal domain in AbsA1 signal sensing and transduction. The AbsA2 RR is a member of the FixJ subfamily of two-component regulators. It has two domains, an N-terminal receiver domain with the putative site of phosphorylation at Asp54, and a C-terminal effector domain containing a helix-turn-helix DNA-binding motif, which is predicted to regulate target promoter transcription.

The *absA* locus was originally identified in Abs$^-$ mutants that were globally deficient in antibiotic synthesis (2). The mutations responsible for the Abs$^-$ phenotype were localized to a 1.45 kb region of *absA1* (19) in two independent isolates, C542 and C577. Interestingly, *sab* (suppressors of Abs$^-$) mutants arose spontaneously at a relatively high frequency (0.1%) in C542 or C577 protoplasts (19). Also, a gene replacement and gene disruption of the *absA1* gene resulted in precocious hyperproduction of antibiotics (Pha), characterized by early onset and overproduction of antibiotics. The *absA1* gene knockouts were believed to have a polar effect on downstream *absA2* due to the close proximity of the two genes. Given these results, it was hypothesized that *absA2* encoded a negative regulator of antibiotics since its elimination resulted in the Pha phenotype (19). The Abs$^-$ and Pha mutants were found to affect the level of expression of *redD* and *actII-ORF4* in a manner which corresponded to their phenotypes (1). In relation to parental stain J1501, PSR expression was lower in the Abs$^-$ mutant and higher in the Pha strain. Therefore, AbsA appeared to mediate production of antibiotics through direct or indirect transcriptional regulation of the antibiotic pathway-specific regulator genes.

In this study I continued to examine basic characteristics of alleles and gene products of the *absA* locus in order to define in greater detail the mechanism of AbsA-mediated antibiotic regulation. Given previous demarcation of the mutations causing the Abs$^-$ phenotype to a 1.45 kb region of *absA1*, the C542 and C577 *absA1* mutant alleles were sequenced in order to characterize, at a molecular level, changes responsible for these phenotypes. Characterization of numerous *sab* mutants was also discussed, including the identification of various second-site suppressor mutations localized within the *absA* locus. The AbsA1 HK and the AbsA2 RR possess conserved residues consistent with the formation of active sites involved in phosphorelay signal transduction. Thus, a genetic and biochemical approach was taken to determine whether phosphorelay was active in this system, and to define the role of phosphorylation in mediating AbsA2 activity. Because antibiotic production is growth-phase dependent, a transcript analysis of *absA* was performed. Identification of the *absA* transcription start site permitted analysis of its promoter region. In addition, temporal expression of *absA* was determined, which allowed for qualitative characterization of its timing with respect to that of growth and antibiotic production. It was known from previous work that AbsA influenced the expression of *redD* and *actII-ORF4* PSRs. In this study I examined the temporal expression of the PSRs *redD*, *redZ*, and *cdaR* in comparison to that of *absA* and further examined the influence of AbsA on expression of these regulators as putative targets of AbsA2. Finally, preliminary results were presented which suggest that AbsA2 is conserved in other species of *Streptomyces*.

CHAPTER 2

SEQUENCE ANALYSIS OF *absA* ALLELES OF Abs$^-$ AND *sab* MUTANTS

The *absA* locus of *S. coelicolor* was isolated from mutants that demonstrated an uncoupling of the temporally parallel processes of sporulation and antibiotic synthesis (2). Two independent isolates, C542 and C577, that mapped to the same region of the chromosome, were globally deficient in the synthesis of all four *S. coelicolor* antibiotics (Abs$^-$, antibiotic synthesis deficient) while unaffected in sporulation. The *absA* locus was later shown to encode a two-component signal transduction system comprised of the AbsA1 histidine kinase and AbsA2 response regulator (19). Marker exchange and marker rescue experiments in the C542 and C577 Abs$^-$ mutants localized the mutations responsible for this phenotype to a 1.45kb region of *absA1* (19). This phenotype was dramatically opposed to the early onset and overproduction of antibiotics (Pha phenotype) obtained from an *absA1* gene disruption and gene replacement (19). The Pha phenotype resulting from *absA1* disruptions was hypothesized to result from polar effects on downstream *absA2*. Therefore, it was proposed that AbsA2 encoded a negative regulator of antibiotics and that the C542 and C577 Abs$^-$ strains mutationally locked the AbsA system into a negative regulatory state.

Another characteristic of the C542 and C577 Abs$^-$ mutants was that they underwent apparent spontaneous reversion. Pseudorevertants of the Abs$^-$ phenotype, *sab* (suppressor of abs) mutants, which restored synthesis of all four antibiotics, spontaneously arose in the C542 and C577 *absA* mutant protoplasts at a frequency of 0.1% (19). The *sab* mutants were of considerable interest because identification of second site suppressors is a useful tool for finding additional members of a regulatory pathway. Alternatively, if localized to the *absA* locus, these pseudorevertants might provide insights into the mechanism of AbsA1/AbsA2 interactions.

In this study further characterization of the C542 and C577 Abs$^-$ mutants was undertaken. It was of considerable interest to sequence the *absA1* alleles of the Abs$^-$ mutants C542 and C577 to confirm that mutations responsible for the Abs$^-$ phenotype were indeed present. Additionally, it was hoped that these mutations would provide evidence, when analyzed together with *sab* mutations (below) and site-directed mutations (Chapter 3) into the biochemical mechanism that locks AbsA1 into a negatively acting state. This chapter was also concerned with the characterization of *sab* mutants, beginning with colony purification and phenotypic analysis on through to genetic mapping and sequence identification of various *sab* mutations within the *absA* locus. This work is presented in " Genetic suppression analysis of non-antibiotic-producing mutants of *Streptomyces coelicolor absA* locus" (6) . My contribution to this publication includes designing the sequencing strategy, amplification and preparation of DNA for sequencing, and analysis of raw sequencing data for the *absA1* and *sab* mutant alleles. Conclusions drawn from this work as a whole are reprinted from the text (6):

(i) Non-antibiotic-producing (Abs-) mutants of the *absA* locus, which seem to lock the AbsA regulatory system into a negatively regulating mode, contain point mutations in conserved domains of the AbsA1 histidine kinase sensor-transmitter protein.

(ii) The *absA1* mutants spontaneously acquire suppressive mutations that restore antibiotic synthesis.

(iii) Plasmid-mediated and protoplast fusion mapping techniques were useful for genetic analysis of suppressive (*sab*) mutations, locating some close to *absA*.

(iv) Actinophage ϕC31-derived vectors were useful for marker rescue and marker exchange experiments that verified the existence and location of *sab* mutations and allowed transduction of *sab* mutations from strain to strain.

(v) Sequence analysis defined *sab* mutant residues in the *absA* two-component system. Some *sab* alleles (Type I) restore a wild-type phenotype to Abs$^-$ mutants, whereas some (Type II) cause antibiotic overproduction.

(vi) Antibiotic overproduction in sab strains can result from deletion of *absA*, consistent with *absA*'s proposed role as a negative regulator, but the most strongly pigmented *sab* strain contains a point mutation in the AbsA2 response regulator, suggesting a complex role for the *absA* locus in production of antibiotics.

CHAPTER 3

GENETIC AND TRANSCRIPTIONAL ANALYSIS OF *absA*, AN ANTIBIOTIC GENE CLUSTER-LINKED TWO-COMPONENT SYSTEM THAT REGULATES MULTIPLE ANTIBIOTICS IN *S. coelicolor*

INTRODUCTION

Streptomycetes are notable among prokaryotes for their fungal-like developmental cycles. Early in the growth of a colony, multinucleoidal vegetative hyphae extend through the growth medium, branching extensively to form a mycelial mat. Later, in response to poorly understood signals, the vegetative hyphae initiate a program of multicellular differentiation. Morphological differentiation produces sporulating aerial hyphae on the colony surface (reviewed by 37; 34) while the temporally parallel but spatially distinct process of secondary metabolite ("antibiotic") production occurs in the substrate mycelium (reviewed by 31; 35).

Streptomycete antibiotic biosynthetic pathways involve multiple enzymes that are encoded in large clusters of genes. Each species typically contains several antibiotic gene clusters and these are subject to a complex network of regulation. Much of what is known about the regulation of antibiotic genes has come from genetic studies in *Streptomyces coelicolor*. One level of regulation that was discovered in *S. coelicolor*, and is now known to be common to most if not all streptomycetes, involves so-called "pathway-specific regulation," a mechanism in which a cluster-linked transcriptional regulator – usually an activator – regulates expression of numerous polycistronic transcripts in an antibiotic gene cluster. In the cases of the *S. coelicolor* antibiotics actinorhodin and undecylprodigiosin, which are especially well characterized, the pathway-specific activators are ActII-ORF4 and RedD, respectively (122; 167; 62; 57). Both are OmpR-like DNA binding proteins and are founding members of the SARP (for streptomycete antibiotic regulatory protein) family of regulators, which also includes many of the known cluster-linked regulators for other streptomycete antibiotics (183).

Studies of *S. coelicolor* antibiotics have been facilitated by the ease of assaying the antibiotics. Two are pigments: actinorhodin (Act) and undecylprodigiosin (Red) are blue and yellow, respectively, at alkaline pH; both are red at acidic pH. The other two *S. coelicolor* antibiotics, calcium-dependent antibiotic (CDA) and methylenomycin (Mmy), can be assayed in simple plate culture-inhibition assays. Production of the *S. coelicolor* antibiotic pigments can easily be seen to be growth-phase regulated in both plate and liquid cultures. It has been demonstrated that this temporal regulation results from growth-phase regulated expression of the pathway-specific activators (167; 62; 182). It is less well understood what regulates the pathway-specific activators. However, one such control involves the *absA* two-component system, which was discovered in a genetic analysis of global, or coordinate, antibiotic regulation. Mutants of *absA* were first identified because of their actinorhodin/undecylprodigiosin-minus, sporulation-plus phenotype; subsequently, they were shown to be calcium-dependent antibiotic-minus and methylenomycin-minus, as well (2). This phenotype was named Abs$^-$ and classical genetic mapping showed that the Abs$^-$ phenotype was attributable to mutations in the *absA* locus (2; 19) Further work showed a deficiency of *actII-ORF4* and *redD* transcription in *absA* mutants (1), explaining the Abs$^-$ phenotype, at least with respect to actinorhodin and undecylprodigiosin.

The genetic map location of *absA* was far from the *act* and *red* gene clusters, but was close to the only existing *cda* mutant. Recent genomic sequencing of *S. coelicolor* has revealed that *absA* is associated with the *cda* gene cluster (http://www.sanger.ac.uk/Project/S_coelicolor/). Previous to the genomic sequencing, only a peptide synthetase-encoding segment of the *cda* cluster had been defined (39).

Now, it is apparent that *absA* lies in a 12 kb region between the peptide synthetase genes and a putative SARP-like regulator for the *cda* cluster, *cdaR* (Figure 5). The function of *absA* as a regulator of multiple antibiotic clusters, while being genetically associated with one cluster, makes *absA* highly unusual among antibiotic regulators.

In typical two-component systems, a dimeric histidine kinase uses ATP to autophosphorylate, with one subunit transphosphorylating the other on a specific conserved histidine residue (reviewed by 161). The phosphoryl group is then transferred to an aspartate residue on a cognate response regulator, modulating its activity as a transcriptional regulator. The *absA*-encoded two-component system is highly "orthodox," including the features common to many of the better-studied two-component systems. The *absA1* gene is predicted to encode a histidine kinase and the adjacent, downstream gene, *absA2*, is predicted to encode a response regulator with a C-terminal helix-turn-helix DNA binding domain. Following the two component paradigm, sequence conservation predicts that the AbsA1 protein would autophosphorylate at His 202 and the phosphoryl group would transfer to Asp54 of AbsA2. AbsA2 is highly homologous to NarL of *E. coli* and the transmitter domain of AbsA1 is similar to the cognate kinases, NarX (63). Closely related two-component systems from *Bacillus subtilis* include DegS/DegU and ComP/ComA (reviewed by 121).

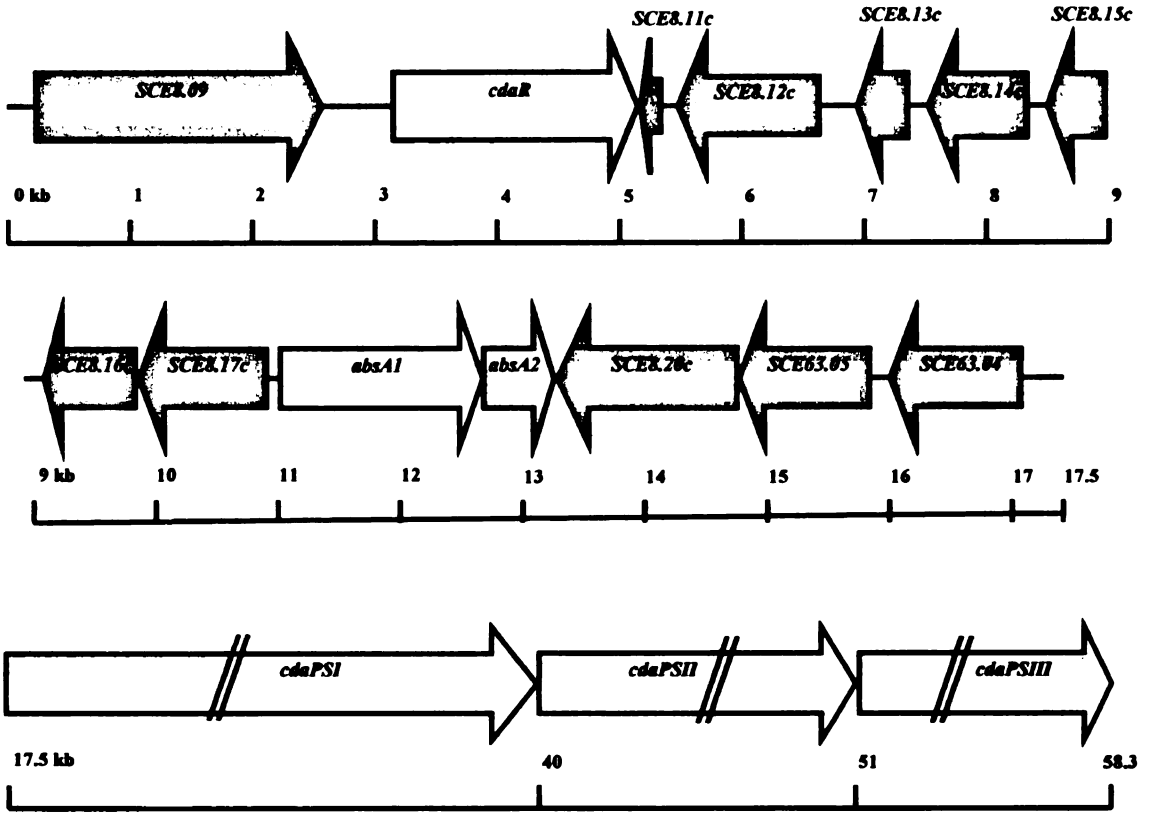


Figure 5. Position of *absA* with respect to the *cda* gene cluster. This 58.3 kb region of the *cda* cluster was reconstructed from sequence data made available by the *Streptomyces coelicolor* Sequencing Project (The Sanger Centre). Genes shown in white have been named and given putative functions based on genetic or functional analysis. *cdaR* is homologous to pathway-specific activators. Biosynthetic genes *cdaPSI*, *cdaPSII* and *cdaPSIII* encode peptide synthases which catalyze steps in the enzymatic synthesis of the lipopeptide antibiotic CDA. Shaded genes have been assigned putative functions based on sequence similarity to other proteins (annotated in http://www.sanger.ac.uk/Projects/S_coelicolor/).

Marker rescue experiments (19) and subsequent sequence analysis (6) of *absA* mutants located the mutations that were responsible for the Abs$^-$ phenotype to the transmitter domain of AbsA1. Below, we refer to these alleles as *absA1**. Additional genetic experiments revealed that *absA* could also mutate to a phenotype essentially opposite to Abs$^-$; this phenotype was characterized by an early onset and increased level of antibiotics (19). Antibiotic gene transcription was correspondingly increased in the

overproducing mutants (1). Two *absA* disruption mutations caused the overproduction phenotype, suggesting that the role of *absA* in antibiotic regulation was primarily negative (19).

We have undertaken a genetic dissection of the *absA* locus, which we describe here. This work evaluates the role of phosphorylation in *absA*-mediated regulation of actinorhodin, undecylprodigiosin, and CDA and establishes the genetic basis for the two opposing phenotypes observed in *absA* mutants. We also describe a transcriptional analysis of the *absA* genes which reveals autoregulatory behavior of AbsA2. Together, the results of these experiments have implications for the mechanism by which AbsA signal transduction regulates antibiotics during the *Streptomyces coelicolor* life cycle.

MATERIALS AND METHODS

Growth Conditions

Streptomyces strains were cultured in YEME broth (73) for use in plasmid and protoplast preparations. Cultures used for chromosomal DNA extraction were grown for two days either in YEME broth or on SpMR (91) plates overlaid with cellophane disks. Cultures used for RNA extraction were grown in 50 ml of SpMR broth in 300 ml baffled flasks, inoculated with 10^8 spores, and incubated at 30°C, 250 rpm for 18, 30 or 54 hours. Thiostrepton was added to obtain a final concentration of 10 μg/ml in liquid culture or 200 μg/ml in agar. Hygromycin (Hyg) was added to agar plates to a final concentration of 200 μg/ml. *Escherichia coli* was grown in L broth or L agar (147). Ampicillin was added to obtain a final concentration of 50 μg/ml in both agar and broth.

Antibiotics Assays

Assay conditions for the calcium-dependent antibiotic were as previously described (2). Strains were grown on Oxoid nutrient agar (ONA), or R5 (73), and were placed onto plates with or without added calcium (as $Ca(NO_3)_2$ to 12 mM). Soft ONA or ONA plus Ca was seeded with CDA-sensitive *Staphylococcus aureus* and was overlaid around the plugs. Plates were incubated overnight at 37^0C. Actinorhodin and undecylprodigiosin determinations were as previously described (2).

Plasmid and DNA Manipulations

All oligonucleotide primers used in this study (Table 1) were prepared by the Macromolecular Structure Facility at Michigan State University (East Lansing, MI, USA). *Streptomyces* plasmid preparations and transformations were performed as described by Hopwood, et al. (1985). *Streptomyces* chromosomal DNA was isolated using the method of Pospiech and Neumann (1995). *Escherichia coli* plasmid preparations were done by alkaline lysis (147) or using QIAprep spin columns (QIAGEN). All replicative plasmids shown in Figure 6 were constructed by first cloning the *S. coelicolor absA* region of interest into pBluscriptII SK+ (Stratagene) by standard cloning techniques (147). Inserts flanked by *Bam*HI sites were then subcloned directly into the *Bgl*II site of pIJ702 (73) as in the case of pCB220 and pTBA155. Inserts used to construct pCB520, pCB530, pCB540, pTBA156, and pTBA175 were first subcloned into pIJ2925 (86) and then excised as *Bgl*II fragments from the pIJ2925 polylinker for ligation into the *Bgl*II site of pIJ702. Replicative ligations were transformed into *S. lividans*

TABLE 1. Oligonucleotide primers used in this study.

Name	Nucleotide Sequence[1]	Plasmid or Probe Generated[2]	Priming Location[3,4]
***absA1* primers**			
WC12	CGCTACATCGCCGACCAG	n.a.	541 to 558 c.s.
WC13	GTTGACGACCGACACCACC	n.a.	999 to 1016 t.s.
WC14	GCAGGACATCCTCGACTCCCTGGGC	pTBA150	588 to 612 c.s.
WC16	CATCTGGGCGATCGGCAACGACCG	pTBA150	–82 to –59 c.s.
WC20	CATCGACGGCCACAGGTTC	*absA1* S1 probe	267 to 285 t.s.
WC24	CTGATACCGGCGGTGCTC	pTBA570	1399 to 1416 c.s.
WC30	GCCCAGGGAGTCGAGGATGTCCTGC	pTBA150	588 to 612 t.s.
WC64	TTTTAGATCTCCGCTTGGTCAGTCGGTCC	*absA1* S1 probe	–172 to –154 c.s.
***absA2* primers**			
P11	SSWSAGGCASSWSCCSCCSSWSGCSAC	n.a.	352 to 380 t.s.
WC8	TTTTAGATCTGACGACGAGACSATCATCCGSCGSGGG	pTBA 500	22 to 48 c.s.
WC9	TTTTAGATCTGTGSAGSCGCTGSGCGATCTCSGCG	pTBA 500	510 to 534 t.s.
WC15	CCCTGCTCGAGATCCGGATGCCG	pTBA570	152 to 174 c.s.
WC26	CGCGAATCATCCGATCGTTCCCTGGTG	pTBA150	–17 to 10 t.s.
WC28	TTTTCTGCAGAACGGCGGGGACTGCGGG	pTBA570	344 to 367 t.s.[5]
WC29	CGGCATCCGGATCTCGAGCAGGG	pTBA570	152 to 174 t.s.
WC35	CAGGGAAGGATCCGATGATTCGCG	n.a.	-14 to 10 t.s.
WC65	GGCCCTGCTCGCCATCCGGATGCCGG	pTBA516	150 to 175 c.s.
WC66	CCGGCATCCGGATGGCGAGCAGGGCC	pTBA516	150 to 175 t.s.
WC67	GGCCCTGCTCAACATCCGGATGCCGG	pTBA532	150 to 175 c.s.
WC68	CCGGCATCCGGATGTTGAGCAGGGCC	pTBA532	150 to 175 t.s.

[1] S = G or C; W = A, C, G, or T

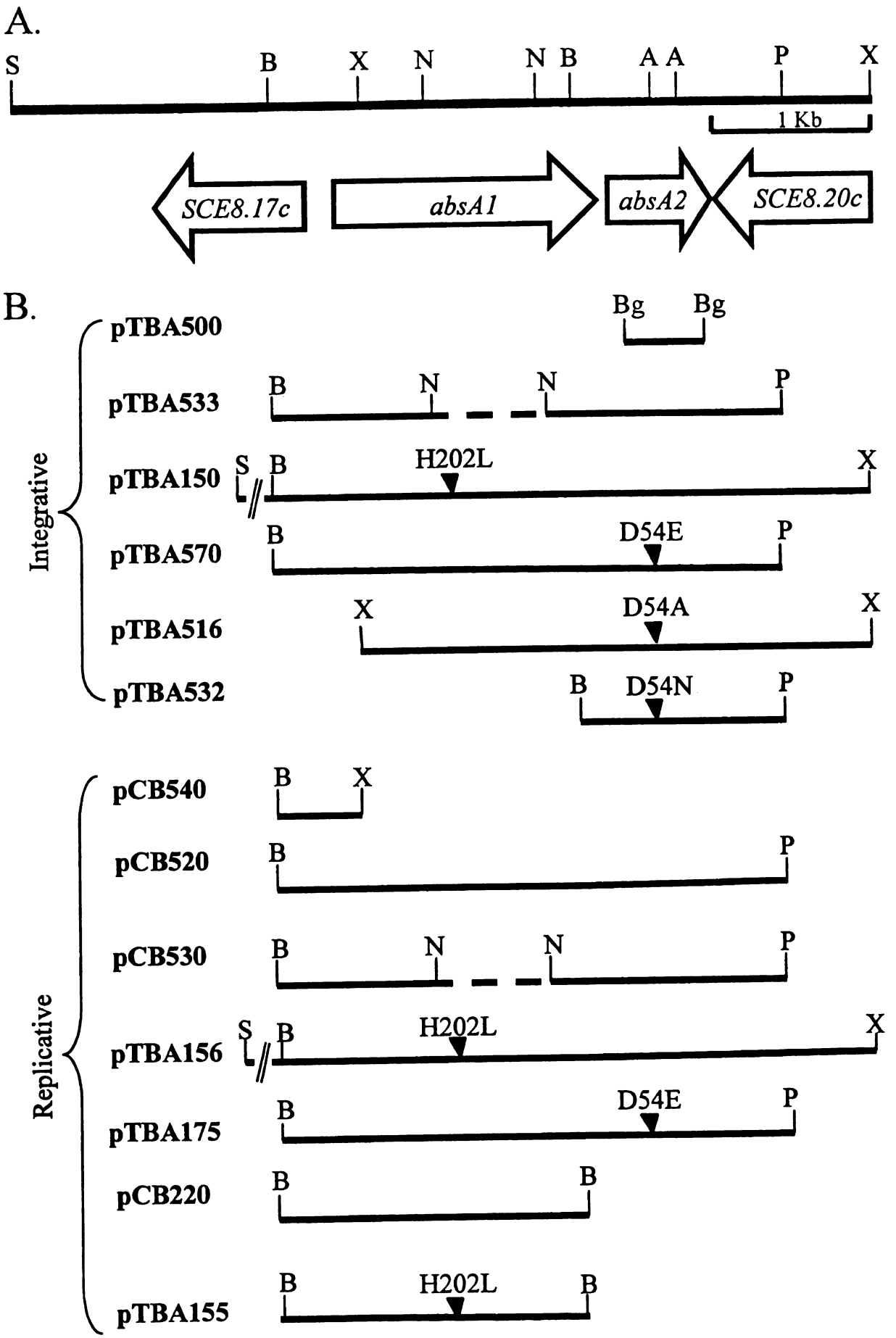
[2] n.a. = not applicable

[3] c.s. = coding strand; t.s. = template strand

[4] numbering begins at the first nucleotide of the translation start codon

[5] numbering begins at the first nucleotide of the translation stop codon

Figure 6. The *absA* locus and plasmid inserts based on *absA*. (A.) A restriction map of the *absA* locus and surrounding genome. (B.) Characteristics of the *absA*-based inserts used to create the integrative and replicative plasmids discussed in the text; details of vector construction are given in the Materials and Methods. The dashed line (---) indicates an in-frame deletion; ▼ represent the locations of amino acid substitutions. Restriction sites are: A, *Apa*I; B, *Bam*HI; Bg, *Bgl*II; N, *Nae*I; P, *Pst*I; S, *Sac*I; X, *Xho*I.


A.
S
B
X
N
N B
A A
P
X
1 Kb
SCE8.17c
absA1
absA2
SCE8.20c
B.
Integrative
pTBA500
Bg
Bg
pTBA533
B
N
N
P
pTBA150
S
B
H202L
X
pTBA570
B
D54E
P
pTBA516
X
D54A
X
pTBA532
B
D54N
P
Replicative
pCB540
B
X
pCB520
B
P
pCB530
B
N
N
P
pTBA156
S
B
H202L
X
pTBA175
B
D54E
P
pCB220
B
B
pTBA155
B
H202L
B

1326; plasmids recovered from these transformants were then transformed into *S. coelicolor* J1501.

Disruption of *absA2* in C500

The integrative plasmid pTBA500 (Figure 6) was constructed using an *absA2* fragment amplified by PCR from primers WC8 and WC9, both of which contained *Bgl*II restriction sites at their ends. The truncated region of AbsA2 encoded by the WC8/WC9 PCR product is illustrated in Figure 7. PCR amplification was carried out in a 100 µl reaction volume with 100 ng J1501 chromosomal DNA template, under buffer and thermal-cycler conditions for *absA2* amplification described in Anderson, et al. (1999). The *absA2* amplification product was purified on Wizard PCR preparatory columns (Promega) prior to and following digestion with *Bgl*II. The resulting 5'- and 3'-truncated *absA2* fragment was cloned as a *Bgl*II fragment into the *Bam*HI site of pIJ963 (86) to produce the integrative plasmid pTBA500.

Strain C500 possessing a chromosomal disruption in *absA2* was created by single cross-over integration of pTBA500. pTBA500 was passed through *dam*$^-$, *dcm*$^-$ *E. coli* ET12567 prior to transformation into *S. coelicolor* J1501. Hyg^r resistance was used to select for single-crossover recombinants; these displayed the Pha phenotype. Plasmid integration was analyzed using Southern hybridization with *absA*- and *hyg*-specific probes.

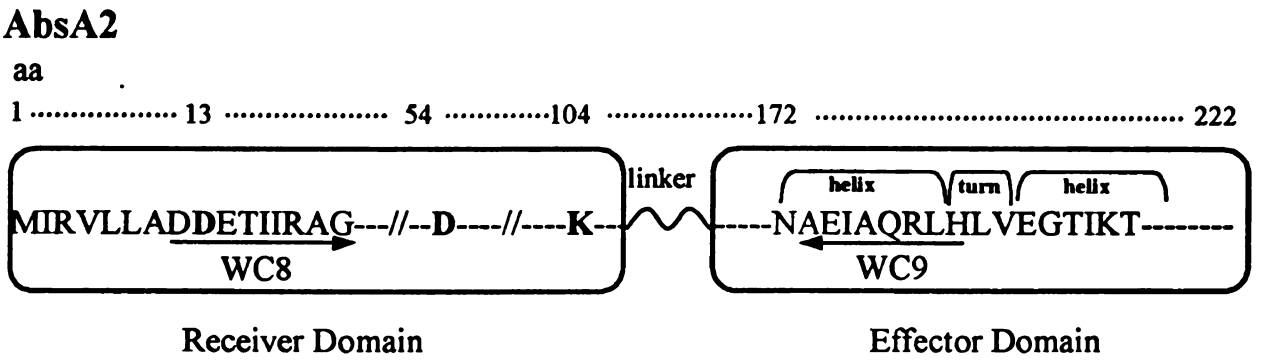


Figure 7. Creation of the *absA2* disruption in strain C500. An internal region of *absA2* was generated by PCR from primers WC8 and WC9. Primer WC8 recognized the region of *absA2* around the highly conserved Asp13 codon. Primer WC9 annealed to the region of *absA2* encoding the first helix of the helix-turn-helix DNA-binding motif.

Construction of an In-Frame Deletion in *absA1* in C530

The integrative plasmid pTBA533 (Figure 6) was constructed by first digesting pCB400 (pIJ2925 with a 2 kb *absA1* *Bam*HI fragment) with *Nae*I to remove the 0.8 kb fragment internal to *abaA1* (Figure 8) which created pCB420. The resulting 1.2 kb *Bam*HI *absA1* fragment was ligated into the *Bam*HI site of pCB300 (pSK+ with a 1.8 kb *Bam*HI/*Xho*I *absA2*-containing region) to produce pCB500. The 2.4 kb *Xho*I/*Pst*I region of pCB500 carrying the whole *absA* locus with the *absA1* in-frame deletion was subcloned from the pCB500 polylinker as a *Sac*I/*Pst*I fragment into pIJ2925 to create pCB501. The entire pCB501 insert was removed from the polylinker as a *Bgl*II fragment and ligated into *Bam*HI-digested pIJ963 to produce the integrative plasmid pTBA533.

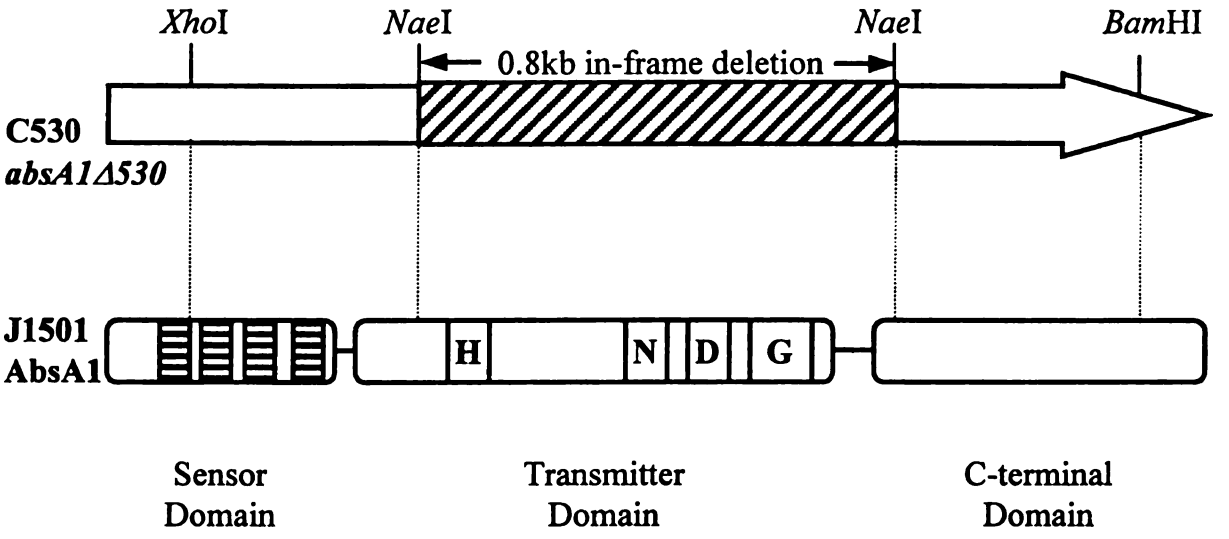


Figure 8. The *absA1Δ530* in-frame deletion (diagonal hatch) was created by the removal of a 0.8kb *Nae*I region internal to *absA1*. Horizontally hatched boxes in the sensor domain represent four transmembrane helices predicted for AbsA1. Lettered boxes of the transmitter domain symbolize highly conserved sub-domains of two-component histidine kinase transmitters. The H-box contains His202 which is the putative site of phosphorylation in AbsA1.

Initial attempts at gene replacement used a strain with a deletion/*ermE* replacement in *absA* (C430), but this strain transformed extremely poorly (19). Therefore, gene replacements were created in strain J1501, as follows. Strain C530 possessing a chromosomal *absA1* in-frame deletion was created through double cross-over gene replacement with integrative plasmid pTBA533. pTBA533 was passed through *dam*$^-$, *dcm*$^-$ *E. coli* DM-1 (GIBCO BRL) prior to transformation into *S. coelicolor* J1501. Hyg^r resistance was used to select for single-crossover recombinants. Plasmid integration was analyzed using Southern hybridization with the same *absA*- and *hyg*-specific probes described above (see Disruption of *absA2*). Single-crossover

recombinants were subjected to multiple rounds of propagation and spore isolation on solid and liquid media without Hyg to allow double-crossover curing of the plasmid. Single colonies were chosen for making spore preparations and chromosomal extractions. Initial screening for double crossovers was performed by PCR amplification from primers WC12/WC13, which were both internal to the in-frame deletion, and WC16/WC26, which produced a 1 kb product for *absA1* with the in-frame deletion, versus a 1.8 kb product for wild type *absA1*. If no PCR products corresponding to wild type *absA1* were amplified, then Southern hybridization was performed on an *Xho*I digest of chromosomal DNAs. Colonies which had successfully undergone double cross-over integration of the *absA1* allele carrying the in-frame deletion showed no signal for the *hyg* probe and a single signal of 2.4 kb for the *absA* probe. Final confirmation of the integrity and fidelity of the C530 *absA* locus was obtained by sequencing the entirety of *absA1* and *absA2*. Procedures for the amplification and sequencing of *absA1* and *absA2* are described elsewhere (6).

In each of the gene replacements described in this study, double-crossover integration of the mutant allele required propagation of single-crossover transformants for numerous generations under nonselective conditions. It should be noted that although single-crossover transformants demonstrated Hyg sensitivity after only a few generations of growth in the absence of antibiotic, many of these still possessed the *hyg* marker as determined by Southern analysis. Similarly, PCR screening that indicated complete resolution of the double crossover was frequently contradicted by Southern analysis. Final confirmation of successful gene replacement required careful analysis by Southern hybridization and sequencing.

Site-Directed Mutagenesis

The *absA2* D54E allele was generated using PCR amplification with mutagenic primers. Separate upstream and downstream *absA2* fragments, with an overlapping region centered at the site of the D54E-encoded mutation, were amplified from pCB460 (pSK+ carrying a 3.9 kb *Bam*HI/*Xho*I fragment with the entire *absA* locus) using primer pairs WC24/WC29 and WC15/WC28 (Table 1). The resulting GAC to GAG change also introduced an *Xho*I site into the PCR products. Thus, the D54E-containing fragments were digested with *Xho*I and an additional restriction enzyme, the site for which was present in the upstream or downstream region surrounding *absA2*: *Bam*HI for the (WC24/WC29) product upstream of *absA2*, and *Pst*I for the downstream (WC15/WC28) product. A three-way ligation between these fragments and pSK+ *Bam*HI/*Pst*I produced pTBA160 containing a 1.2 kb insert with the entire *absA2* D54E allele. Confirmation of the site-directed change was obtained by sequence analysis of the entire *absA2* D54E allele from pTBA160. Subsequently, a 2 kb *Bam*HI *absA1* region was ligated into pTBA160 *Bam*HI to create pTBA162. The 3.2 kb *Bam*HI/*Pst*I insert of pTBA162 containing the entire *absA* locus was removed as a *Xba*I/*Kpn*I fragment from the pTBA162 polylinker for ligation into pIJ2925 to produce pTBA166. The same 3.2 kb insert was excised from the pTBA166 polylinker as a *Bgl*II fragment and ligated into pIJ963 *Bam*HI to generate the integrative plasmid pTBA570 (Figure 6).

Strain C570 possessing a chromosomal *absA2* D54E mutation was created through double-crossover gene replacement with integrative plasmid pTBA570. pTBA570 was demethylated as described above prior to transformation into *S. coelicolor*

J1501. Hyg^r resistance was used to select for single-crossover recombinants. Plasmid integration was analyzed using Southern hybridization with the same *absA*- and *hyg*-specific probes described above (see Disruption of *absA2*). Single-crossover recombinants were subjected to multiple rounds of propagation on solid and liquid media without Hyg to allow double-crossover curing of the plasmid. Single colonies were chosen for making spore preparations and chromosomal extractions. Initial screening for plasmid curing was performed by PCR amplification from primers WC30/WC28, which amplified a 1.2 kb product containing the entire *absA2* allele. Only the *absA2* D54E allele was susceptible to digestion with *Xho*I. Therefore, if no PCR products corresponding to wild type *absA2* were amplified, then Southern hybridization was performed on *Xho*I digests of chromosomal DNAs. Colonies which had successfully undergone double cross-over integration of the *absA2* D54E allele showed no signal for the *hyg* probe and signals of 1.7 and 1.5 kb for the *absA* probe. Final confirmation of the integrity and fidelity of the C570 *absA* locus was obtained by sequencing the entirety of *absA1* and *absA2* as described (6).

The D54A and D54N alleles of *absA2* were created by PCR-mediated introduction of these mutations using the QuickChange Site-Directed Mutagenesis Kit (Stratagene). The D54A mutation was introduced into *absA2* through a single nucleotide change (GAC to GCC) with complimentary primers WC65 and WC66. Likewise, complimentary primers WC67 and WC68 produced a single mismatch (GAC to AAC) in *absA2* to generate the D54N mutation. The mutagenesis reactions were carried out on 50 ng of pTBA400 (pSK+ carrying a 1.8 kb *Bam*HI/*Xho*I *absA2*-containing fragment) under the manufacturer-prescribed buffer conditions with the addition of 5% glycerol and 2.5%

dimethyl sulfoxide (DMSO). The thermal cycler conditions were 95°C for 5 min, followed by 12 cycles of 95°C for 1 min, 65°C for 45 sec, and 72°C for 12 minutes. The PCR products were digested with *Dpn*I and transformed into *E. coli* DH5α (GIBCO BRL) to create pTBA410 (containing *absA2* [D54A]) and pTBA430 (containing *absA2* [D54N]). Both the D54A and D54N mutations removed a *Taq*I restriction site at the mutagenized codon. Therefore, to screen for successful incorporation of site-directed mutations, plasmid preparations from transformant colonies were used as template in PCR reactions with primers WC35 and P11, which amplified a 360 nt region internal to *absA2*. Amplification products were digested with *Taq*I and analyzed on 1.2% agarose gel. In each case, over 90% of the transformants tested screened positive for the mutation. Confirmation of the desired mutations was obtained by sequencing the *absA1* and *absA2* portions of the mutagenized plasmids, pTBA410 and pTBA430. Integrative plasmid pTBA532 (Figure 6) encoding the *absA2* D54N mutation was made by subcloning the 1.8 kb *Bam*HI/*Kpn*I fragment from the polylinker of pTBA430 into pIJ963. In order to construct integrative plasmid pTBA516 (Figure 6), the pTBA410 insert was increased in size to 3.8 kb by cloning in a 2 kb *Bam*HI fragment containing the upstream portion of *absA1* to produce pTBA516. The 3.2 kb *Xho*I fragment of pTBA516 (containing *absA2* [D54A]) was then cloned into the *Sal*I site of pIJ2927 (86) to create pTBA414. This same 3.2 kb insert was then removed as a 3.2 kb *Bgl*II fragment from the polylinker of pTBA414 and cloned into pIJ963 *Bam*HI to create the integrative plasmid pTBA516.

Strains C516 and C532, possessing chromosomal mutations *absA2* [D54A] and *absA2* [D54N], respectively, were created through double-crossover gene replacements

with integrative plasmids pTBA516 and pTBA532, respectively. Single- and double-crossover integration procedures were the same as described for C570 gene replacement. Initial screening for plasmid curing was performed by PCR amplification of *Taq*I restriction digest analysis of the WC35/P11 *absA2* products. Chromosomal DNA from strains that did not amplify wild type *absA2* alleles were digested with *Xho*I and analyzed by Southern hybridization with *hyg* and *absA* probes as described above. Colonies which had successfully undergone gene replacement with the *absA2* (D54A) and (D54N) alleles showed no signal for the *hyg* probe and signals of 3.2 kb for the *absA* probe. Final confirmation of the integrity and fidelity of the C516 and C532 *absA* loci was obtained by sequencing the entirety of *absA1* and *absA2* using methods described previously (6).

The *absA1* H202L allele was generated using PCR overlap extension (171). Separate upstream and downstream *absA1* fragments, with an overlapping region centered at the site of the H202L mutation, were amplified from pCB460 (pSK+ carrying a 3.9 kb *Bam*HI/*Xho*I fragment with the entire *absA* locus) using primer pairs WC16/WC30 and WC14/WC26 (Table 1). Then, the full-length *absA1* H202L allele was amplified by combining 5 to 10 ng of the upstream and downstream PCR products as template together with primers WC16 and WC26. PCR amplifications were carried out in 50 µl reactions using high-fidelity *Pfu* DNA polymerase (Stratagene). Buffer conditions and thermal cycler settings were as previously described (6) except that the extension time was increased from 1 to 4 minutes. The expected size product was agarose-gel purified and digested with *Xho*I and *Bam*HI to produce a 1.45 kb fragment that was ligated into pSK+ to create pTBA140. Confirmation of the site-directed change was obtained by sequence analysis of the *absA1 Xho*I/*Bam*HI fragment from pTBA140.

Subsequently, a 5 kb *Bam*HI fragment (containing the 3' region of *absA1* and all of *absA2*) was ligated into pTBA140 *Bam*HI to produce pTBA142. The 3.2 kb *Xho*I region (containing most of the *absA1* H202L allele and all of *absA2*) was excised from pTBA142 and ligated into *Xho*I-digested pCB360 (pIJ2925 containing a 1.6 kb *Sac*I/*Xho*I insert with the 5' region of *absA1* and upstream *SCE8.17c*) to create pTBA144. The entire 5.4 kb insert was removed from pTBA144 as a *Bgl*II fragment for ligation into pIJ963 *Bam*HI to create the integrative plasmid pTBA150 (Figure 6).

Strain C550 possessing a chromosomal *absA1* H202L mutation was created by gene replacement with pTBA150. Single- and double-crossover integration procedures were the same as described for the C570 gene replacement. Initial screening for plasmid curing took advantage of a *Taq*I restriction site introduced by the H202L mutation. An internal region of *absA1* was amplified from chromosomal DNA of putative C550 strains using primers WC12 and WC13, followed by *Taq*I digest analysis. Chromosomal DNA from strains that did not amplify wild type *absA1* were digested with *Xho*I and analyzed by Southern hybridization with *hyg* and *absA* probes as described above. Colonies which had successfully undergone gene replacement with the *absA1* H202L showed no signal for the *hyg* probe and a signal of 3.2 kb for the *absA* probe. Final confirmation of the integrity and fidelity of the C550 *absA* locus was obtained by sequencing all of *absA1* and *absA2* using methods described previously (6).

RNA Isolation

Streptomyces RNA isolation was carried out as described by Hopwood, et al. (1985) using the preparation method for dot-blotting and northern blotting . Two

independent isolations at 18, 30 and 54 hours of growth were performed for *S. coelicolor* strains J1501 (*hisA1 uraA1 strA1* SCP1$^-$ SCP2$^-$ Pgl$^-$), C542 (*absA1-542* (6)), and C570 (*absA2* [D54E] *hisA1 uraA1 strA1* SCP1$^-$ SCP2$^-$Pgl$^-$). Four 50 ml cultures were pooled for each 18 hour RNA preparation, whereas two 50 ml cultures were pooled for 30 and 54 hr samples. The concentration, purity and integrity of the RNA samples was evaluated by spectrophotometry and agarose gel electrophoresis. Isolation of *E. coli* RNA, for use as a negative control, was performed with an RNAeasy RNA-purification column (QIAGEN).

S1 Nuclease Protection Assays

All experiments were performed using 50 μg of RNA and 60,000-100,000 cpm of ^{32}P-end-labeled double-stranded DNA probe. The *absA* transcript time-course analysis incorporated an *absA1* probe together with a *glk* (glucose kinase (7)) probe - which served as an internal standard for normalizing the quantity of RNA in each assay; (1) The 455 bp *absA1* probe was generated by PCR using the WC64 forward primer and the 5'-^{32}P-end-labeled WC20 reverse primer. The template for *absA1* probe synthesis was pCB400, containing the 2 kb *Bam*HI region of the *absA* locus cloned into pIJ2925. A 309 bp ^{32}P-end-labeled *glk* probe was also generated by PCR from the primers and template described by Aceti and Champness (1998). Primers (50 pmoles) were end-labeled by the T4 polynucleotide kinase (Promega) forward reaction as described by the manufacturer with minor modifications. Prior to initiating the end-labeling reaction, each primer was incubated with spermidine (10 mM final concentration) at 70°C for 10 minutes. Likewise, ethanol precipitation of the labeled oligos was facilitated by the

addition of 2 μg of glycogen. The labeled oligo was divided between duplicate 50 μl PCR reactions. The reaction mix contained 20 pmoles of each primer, 100 ng template, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 5% glycerol, 2.5% DMSO, 1% formamide and 1.25 U *Taq* polymerase (Perkin Elmer). Thermal-cycler conditions were 95°C for 5 min, followed by 30 cycles of 95°C for 1 min, 65°C for 45 sec, and 72°C for 1 min, and a final extension at 72°C for 10 minutes. The S1 nuclease protection assay was performed as previously described (1). Replicate RNA isolates were tested in independent S1 experiments. Time-course experiments included *E. coli* RNA as a negative control. In addition, the presence of excess probe was verified by treating an RNA sample with 2-fold concentrations of each probe and comparing their signals to the same sample treated with normal levels of probe. Results were analyzed by electrophoresis on 6% polyacrylamide sequencing gels (147) and autoradiography. Transcript sizes were estimated by running ^{32}P-end-labeled ϕX174/*Hinf*I molecular weight markers (Promega) on the same gel. To map the *absA1* transcription start site, a sequencing ladder was generated from primer WC20 using the *fmol*® DNA Sequencing System (Promega) and compared to S1-treated 18 hr C542 RNA hybridized to the *absA1* probe.

The region upstream of the *absA2* translation start site was examined for promoter activity using a 504 nt probe. The *absA2* probe protected the region from 330 nt upstream of the *absA2* translational start to 174 nt downstream into the coding region. The *absA2* probe was generated by PCR using forward primer WC24 and 5'-^{32}P-labeled WC29 reverse primer. The PCR template was pCB460 (pSK+ carrying a 3.9 kb *Bam*HI/*Xho*I insert with the entire *absA* locus). Primer end-labeling, PCR reaction

conditions, and probe purification were performed as described for the *absA1* probe. The *absA2* probe was used in S1 nuclease protection assays with 18 and 30 hr C542 RNA.

RESULTS

Negative Regulation of Antibiotics by the *absA2*-Encoded Response Regulator and *absA1* Histidine Kinase

In previous genetic studies of the *absA* locus, certain mutations that disrupted the locus suggested that the *absA*-encoded two-component system functioned as a negative regulator of antibiotics (19). These mutations caused a visible phenotype of early, enhanced production of the actinorhodin and undecylprodigiosin antibiotics; we refer to this phenotype as Pha, for precocious hyperproduction of antibiotics. The Pha mutant alleles were created by insertions into the *absA1* gene, which is upstream in a putative *absA1-absA2* operon. The phenotype in these mutants may have resulted from disruption of *absA1* or from polar effects on expression of *absA2*. To distinguish between these possibilities, we directly tested the function of *absA2* by specifically disrupting the *absA2* gene. A fragment internal to *absA2* (Figure 6) was cloned into the nonreplicating plasmid pIJ963 to create pTBA500, which was then integrated into the *absA* locus of strain J1501. The resulting strain, C500, was *absA1*$^+$ *absA2*::pTBA500, with *absA2* truncated upstream of the predicted helix-turn-helix domain (Figure 7). Disruption of *absA2* in C500 caused a Pha phenotype (Figure 9), thereby demonstrating the involvement of *absA2* in negative regulation of antibiotic production. Both repressor and activator functions are well-documented for two-component response regulators;

functional regions of the transmitter domain of histidine kinases, including the conserved H, N, D, and G boxes. The phenotype of the *absA1Δ530*, strain C530, was Pha (Figure 9) and, moreover, was identical to that of C500. This result implicated the protein kinase activity of AbsA1 in the negative regulation effected by AbsA2.

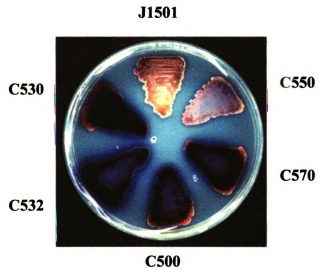


Figure 9. The effect of an *absA* gene disruption and gene replacements on antibiotic production. Strains were grown for 4 days on SpMR agar. Strains are *S. coelicolor* J1501 (wild type), C550 (*absA1* [H202L]), C570 (*absA2* [D54E]), C500 (*absA2*::pTBA500), C532 (*absA2* [D54N]), and C530 (*absA1Δ530*). Actinorhodin and undecylprodigiosin pigments were assessed as described in Materials and Methods. Images in this dissertation are presented in color.

Genetic Evaluation of the Role of Phosphorylation in AbsA2-Mediated Regulation

For most response regulators, phosphorylation of a conserved aspartate residue is essential for the regulatory functions of the proteins *in vivo*. Following this precedent, the AbsA2 regulatory activity would likely require that AbsA2 be phosphorylated; AbsA1 would likely be responsible for AbsA2 phosphorylation. The Pha phenotypes of C500 and C530 would be consistent with this scenario, but it was important to consider the additional factor that many of the characterized two-component system histidine kinases are bifunctional enzymes that possess both kinase and phosphatase activities; the phosphatase activity dephosphorylates the phosphorylated response regulator. In the case of AbsA1, the in-frame deletion in C530 would remove AbsA2-specific phosphatase activity, as well as the kinase activity associated with the transmitter domain. Thus, in strain C530, phospho-AbsA2 may be present if AbsA2 can be phosphorylated by an alternative kinase or low molecular weight phosphate donors, and the C530 phenotype might be caused by a lack of the AbsA1 phosphatase and a resulting overabundance of phospho-AbsA2. In this case the negatively regulating form of AbsA2 would be the unphosphorylated form.

In order to distinguish whether phospho-AbsA2 or unphosphorylated AbsA2 functions as the negative regulator, we constructed several mutants with site-directed changes to the chromosomal *absA2* gene (Figure 6), altering the AbsA2 aspartate residue (D54) that is analogous to the conserved phosphorylated aspartate of response regulators. Separate gene replacements created strains C570, C516, and C532 with AbsA2 amino acid replacements, D54E, D54A, and D54N respectively. These aspartate substitutions have been shown to prevent phosphorylation of numerous response regulators (88; 42;

22). All three mutant strains exhibited the Pha phenotype (Figure 9; C516 not shown). Thus, these results supported the hypothesis that phospho-AbsA2 functions as the negative regulator.

The histidine residue in AbsA1 that corresponds to the site of phosphorylation in well-characterized members of the histidine kinase family is His 202 (Figure 6). A site-directed mutation, H202L, was made in the chromosomal *absA1* gene of J1501, creating strain C550. Strain C550 exhibited a Pha phenotype, a result consistent with a requirement for histidine kinase activity in negative regulation. However, the phenotype differed from that of C530 (*ΔabsA1*) in several respects. First, C550 visibly produced undecylprodigiosin earlier than actinorhodin, whereas C530 produced both antibiotics precociously. Second, hyperproduction of antibiotics, relative to strain J1501, never reached the levels seen for C530 (Figure 9). The reason for the weaker Pha phenotype of C550 is not clear at this time. We considered the possibility that AbsA1 (H202L) contained a second site of phosphorylation; however a fusion protein containing the AbsA1 (H202L) transmitter domain fused to maltose-binding protein did not demonstrate autokinase activity *in vitro*, whereas an MBP:: AbsA1^{+} fusion did (Chapter 5).

Precocious Hyperproduction of Calcium-Dependent Antibiotic, Undecylprodigiosin and Actinorhodin in *absA* Mutants

We sought to determine whether Pha mutations affected synthesis of calcium dependent antibiotic, in addition to actinorhodin and undecylprodigiosin. To assess CDA activity, plugs from plate grown cultures were tested for anti-*Staphylococcus aureus* activity. In the presence of added calcium, the lipopeptide CDA is active, damaging cell

membranes (100). For CDA assays, culture plugs were tested on plates with and without added calcium. In a 2-day time course, shown in Figure 10, CDA activity was detected in Pha mutants at least 7 hrs earlier than in J1501. Pha mutants C550 and C530 are shown. Similar results were obtained with C550, C530 and C570 on R5 media (data not shown). These results showed that AbsA negatively regulates CDA as well as actinorhodin and undecylprodigiosin.

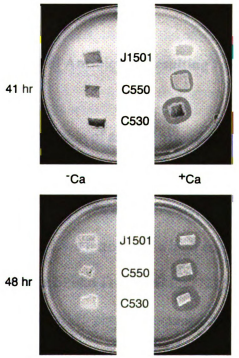


Figure 10. Calcium-dependent antibiotic assays in Pha mutants. Growth conditions are described in Methods. Plugs were taken from ONA plates at the times indicated. CDA activity is detected in the presence of calcium, right.

Over the course of cultivating Pha mutants we have observed variability in how much earlier a given Pha mutant produces antibiotics compared to J1501. The amount of acceleration has ranged from at least 7 hours to several days on different media, *e.g.* R5, SpMR and ONA. In addition, in quantitative assays of actinorhodin and undecylprodigiosin, the Pha-related overproduction has varied from 5-fold to more than 60-fold (data not shown). An exploration of this phenomenon will be reported in more detail elsewhere. Besides the effect on antibiotics, the Pha phenotype includes a defect in morphology. Pha mutants produce aerial hyphae relatively sparsely and their colony surfaces are notably crenulated.

Precocious Hyperproduction of Antibiotics Resulting from AbsA Domain Overexpression

In some cases, overexpression of an unphosphorylated response regulator can mimic the regulation of target promoters that normally is effected by a phosphorylated response regulator (e.g., 180). To evaluate whether overexpression of unphosphorylated AbsA2 could regulate antibiotics, we introduced a high copy clone of the *absA2* (D54E) mutant allele (pTBA175; Figure 6) into J1501 and C577S25, a strain deleted for *absA2* and most of *absA1* (6). The pTBA175 plasmid included *absA1*$^+$ and the *absA* promoter region. If unphosphorylated AbsA2 could negatively regulate antibiotics we might have observed a delay of antibiotics in the Pha C577S25 strain. However, we observed no change in the Pha phenotype (data not shown) suggesting that phosphorylation of AbsA2 is required even at high protein abundance, for negative regulation.

When pTBA175 was introduced into J1501, the resulting phenotype was Pha, indicating an interference with normal AbsA-mediated regulation (Figure 11B). To further examine the phenomenon, we evaluated the effects of overexpressing selected domains of the AbsA1 and AbsA2 proteins (Figure 6). First, we excluded an effect of the *absA* promoter region by introducing plasmid pCB540; this plasmid did not alter the J1501 phenotype (Figure 11A). Second, we observed that multiple copies of the entire *absA* locus, in pCB520, produced no change in the Abs^+ phenotype (Figure 11A). Next, we evaluated a set of high-copy plasmids that expressed the wild-type AbsA2 but carried phosphorylation-minus *absA1* alleles; these included pCB530, carrying the in-frame deletion of *absA1Δ530*, and pTBA156, carrying the *absA1* [H202L] allele. These produced no change in the Abs^+ phenotype (Figure 11). In contrast, a Pha phenotype resulted from plasmids that lacked *absA2+*, but contained *absA1* sequences. Two such plasmids were pCB220 and pTBA155. A pattern that emerged from these results was that an increase in gene dosage of $absA2^+$, with or without an increase in $absA1^+$, did not alter antibiotic regulation. However, an increase in *absA1* sequences without an increase in $absA2^+$ deregulated antibiotics. One interpretation of these results is that a high *absA1* gene dosage causes a shift in the ratio of AbsA1 kinase to phosphatase activity to favor the phosphatase activity, and relatively low expression of $AbsA2^+$ may not allow sufficient AbsA2-P accumulation to down-regulate antibiotics.

What would cause the ratio of AbsA1 phosphatase to kinase activity to be higher than normal in these strains? In the cases of the pCB220 and pTBA155 plasmids, the C-terminal 69 aa of *absA1* are truncated. In previous complementation analyses, the

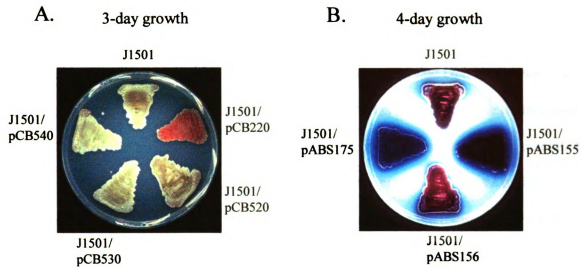


Figure 11. The effects of high-copy expression of *absA* alleles on antibiotic production. All plasmids were derivatives of pIJ702 expressed in a *S. coelicolor* J1501 background. Plasmid inserts are shown in Figure 6. Strains were grown for 3 or 4 days on SpMR agar. Early onset of both Red and Act are characteristics of the Pha phenotype. Frame A demonstrates the early production of Red in the Pha phenotype, whereas frame B shows the early onset of Act synthesis.

pCB220 *absA1* allele was capable of restoring a wild-type phenotype to *absA1**, but it is possible that this allele and the H202L version in pTBA155 have higher than normal phosphatase activities. However, *absA1* is wild-type on pTBA175. One speculation about the observed effects might be that the AbsA1-kinase activity is normally activated by the binding of a low-abundance signal molecule and the AbsA1 polypeptides produced by the high-copy constructs are present in quantities sufficient to titrate the ligand; thus the population of AbsA1 molecules may be predominantly in the phosphatase mode.

High Resolution S1 Nuclease Mapping of the *absA* Transcription Start Site

The *absA1* and *absA2* ORFs are separated by only 17 nt and are likely cotranscribed. To define the transcription start site for *absA*, high resolution S1 nuclease mapping was performed. First, a PCR-generated double-stranded DNA probe specific to the predicted promoter region for *absA1* (Figure 12B) was used to map the transcription start site of *absA1* by analyzing the S1 product alongside a sequencing ladder generated from the same ^{32}P-labeled primer used to synthesize the probe. The *absA1* probe protected a single product of 291 nt (Figure 12A), identifying the transcription start site for *absA1* as the adenosine nucleotide that is also predicted to be the putative translation start site. To evaluate cotranscription of *absA1* and *absA2*, the region upstream of *absA2* was probed with a 504 bp double-stranded DNA probe (Materials and Methods). The S1 product showed no indication of independent promoter activity for *absA2* (data not shown). These results indicated that *absA1* and *absA2* are transcribed as a single, leaderless transcript. Leaderless transcripts are not uncommon in actinomycetes, as documented by Strohl (1992), who reported that 11 of 139 promoters analyzed produced leaderless transcripts.

Inspection of the sequence upstream of *absA1* revealed a –10 region with the sequence TAGCGT (Figure 12); this is similar to the consensus sequence proposed by Strohl (1992) for transcription from *Streptomyces* RNA polymerase that contains an *E. coli*-like $E\sigma^{70}$ sigma factor, *e.g.* HrdB or HrdD (23). There was, however, no recognizable consensus sequence in the –35 region.

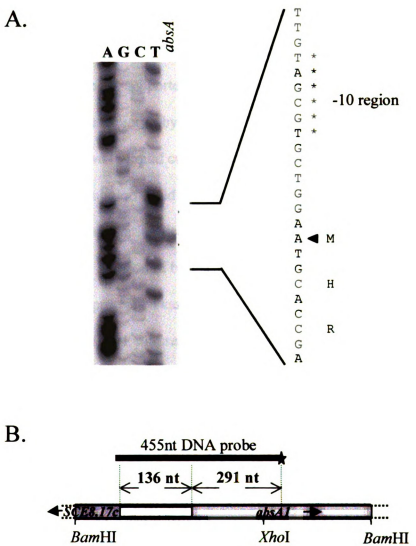


Figure 12. S1 nuclease protection mapping of the *absA* locus. (A.) High resolution S1 nuclease protection mapping on total RNA isolated from an 18 hour culture of *S. coelicolor* C542 grown in SpMR liquid medium. The AGCT sequencing ladder was generated from 5'-labeled oligonucleotide WC20 (see Materials and Methods section). The transcription start site (○) and the hexameric -10 promoter region (*) are shown. (B.) The *absA* probe was a 455 bp PCR product amplified from primer WC64 (Table 1) and primer WC20 - uniquely labeled with ^{32}P at the 5' end. The shaded areas represent coding regions of *absA1* and *SCE8.17c* contained on pCB400. The *absA* probe extends 291 nt downstream of the putative translation start site and 136 nt upstream.

Growth-Phase Dependent Expression and Autoregulation of *absA*

To evaluate the temporal profile of *absA* expression, RNA was isolated over a 54 hr time course from cultures grown in liquid media. The media used, SpMR (see Materials and Methods), supported production of the actinorhodin and undecylprodigiosin antibiotics by strain J1501. *Streptomyces coelicolor* does not sporulate when grown in liquid cultures, so temporal comparisons of antibiotic production and sporulation could not be made in this experiment. However, as is generally observed, the antibiotics showed growth-phase dependent production kinetics, appearing only after a period of biomass accumulation.

S1 nuclease protection assays were performed on the *absA* promoter region from RNA isolated from J1501 cultures grown for 18 hours, 30 hours, and 54 hours. Antibiotics were not produced in the 18 hour culture but were visible in the 30 hr J1501 culture. Figure 13 shows that the *absA* transcript was present in the 18 hr culture, and it increased significantly in abundance from 18 hours to 30 hours. The transcript then remained at a constant level through 54 hours. Comparisons of transcript abundance in different cultures were aided by the addition of a probe for the *glk* (glucose kinase) gene to each S1 assay. The *absA* signal increased about five-fold relative to the *glk* signal over the course of culture growth.

Figure 13 also includes S1 nuclease protection assays of RNAs isolated from two strains that are mutant for the *absA* locus. One strain was C542, an Abs$^-$ strain mutant in *absA1* (i.e., an *absA1** strain), as described above. The second was C570, the Pha strain carrying the D54E mutation in *absA2* that was described in Figure 6. The profile of *absA* expression was altered in both *absA* mutants. In C570, the *absA* transcript abundance

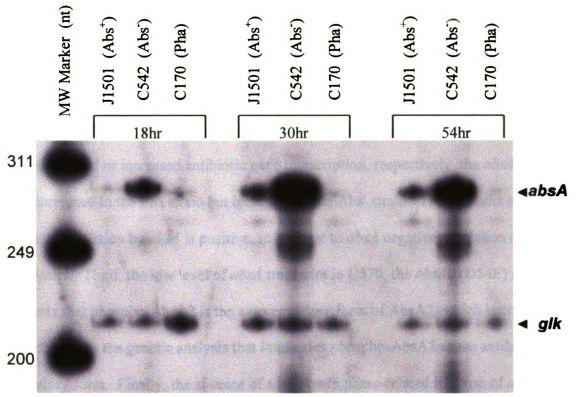


Figure 13. High resolution S1 nuclease protection analysis of the *absA* transcript, using RNA isolated from 18, 30 and 54 hour *S. coelicolor* cultures in SpMR liquid medium. *S. coelicolor* strains are J1501 (*absA*$^+$), C542 (*absA1-542*, (6)), and C570 (*absA2* [D54E]). The *absA* probe was the 455 bp probe in Figure 12. Glucose kinase (*glk*) was measured to normalize the amount of RNA assayed at each time point (1).

was very low at all time points. In contrast, in C542, the *absA* transcript was several-fold more abundant than in J1501, at all time points.

The S1 protection assays revealed several aspects of *absA* regulation. First, the effects of the *absA* mutations indicated that *absA* expression is autoregulated. Second, the mutant effects on the *absA* transcript were opposite to the previously-observed effects on antibiotic transcripts: whereas the Abs$^-$ and Pha phenotypes were found to correlate with decreased or increased antibiotic gene transcription, respectively, the *absA* transcript was decreased in the Pha strain but increased in the Abs$^-$ strain. These results suggest that autoregulation by *absA* is positive, in contrast to *absA* negative regulation of antibiotics. Third, the low level of *absA* transcript in C570, the *absA2* (D54E) mutant, suggests that phospho-AbsA2 is the autoregulatory form of AbsA2, which is consistent with data from the genetic analysis that implicates phospho-AbsA2 as the antibiotic-regulatory form. Finally, the absence of any growth phase-related increase of *absA* transcript in C570 suggests that phospho-AbsA2 was responsible for the growth-phase regulation observed in J1501 and C542. Thus, the growth phase regulation of *absA* appears to result from phosphorylation-dependent AbsA2-mediated autoregulation. We have not determined at this time whether the *absA* autoregulation is direct or indirect.

DISCUSSION

In this paper we have described a genetic and transcriptional analysis of the *absA* locus which further characterizes aspects of the mechanism of AbsA-mediated regulation of antibiotic production. Disruptions in the *absA1* and *absA2* genes demonstrated that the AbsA two-component system is a negative regulator of the multiple antibiotics produced

by *S. coelicolor*, including calcium-dependent antibiotic, actinorhodin and undecylprodigiosin. In addition, gene replacements in the *absA* locus altered the putative sites of phosphorylation of AbsA1 or AbsA2. As predicted from sequence conservation with other two-component systems, both the His at position 202 of AbsA1 and the Asp residue at position 54 of AbsA2 were required for normal regulation of antibiotic synthesis: each of the gene replacement strains tested attained an antibiotic overproducing phenotype (Pha) consistent with a mechanism in which the phosphorylated form of AbsA2 is the active negative regulator of antibiotic synthesis. Our results did not, however, distinguish whether or not AbsA2~P is a direct repressor of the antibiotic genes or whether it is the activator of a repressor.

Without *absA* regulation, the timing of antibiotic production is advanced but, even in Pha cultures, a period of approximately 2 days passes before antibiotics appear. One interpretation of this observation is that the appearance of antibiotics in Pha cultures indicates the time at which the culture enters an antibiotic production-competent state, but the AbsA system normally imposes a delay on production. The heterogeneities in a growing mycelial biomass complicate distinctions of growth phases, but for the purposes of further discussion, we refer to the postulated "AbsA-repressed" period as the "transition stage." We can envision several models for how AbsA, as a signal transduction system, could modulate production of antibiotics during culture growth.

One model, which accommodates both the genetic and transcriptional data, supposes the following. Early in growth, a culture is not competent for antibiotic synthesis; also, the *absA* genes are expressed at a low level. Following a period of growth, the culture enters the "transition stage." During this time, the signal that

regulates AbsA may be present at significant levels. If AbsA1 is like many sensor kinase/phosphatases, it will require signal binding to activate the kinase function, and exist in a phosphatase-dominant mode if signal is absent (131; 69; 186). Once the signal is present, and AbsA1 is shifted to a kinase-dominant form, AbsA2-P will accumulate and negatively regulate antibiotics and also positively autoregulate, accounting for the AbsA2-P-dependent, growth-phase-related increase of *absA* transcript seen in J1501. Easing of AbsA-repression may require that the signal be depleted or degraded, allowing AbsA1 to switch to the phosphatase form and dephosphorylate AbsA2, allowing antibiotic gene expression. At present, we have no information regarding the nature of the signal hypothesized to regulate AbsA1.

If the normal function of the AbsA system is in negative regulation of antibiotics, what explains the Abs$^-$ phenotype in the mutants that first defined the *absA* locus? We hypothesize that these *absA1** alleles lock the AbsA system into the negatively-regulating mode, *i.e.*, in which AbsA2 is phosphorylated. In support of this notion, the Abs$^-$ phenotype requires *absA2*$^+$ (6). The mutant AbsA1* proteins might be constitutively kinase-dominant forms, either lacking phosphatase capability or functioning as signal-independent kinases. The latter possibility would be most consistent with the increased level of *absA* transcript observed in C542, *e.g.* AbsA2-P would be present even in young cultures lacking signal and would autoregulate. Another observation that could be explained by signal-independent AbsA1 kinase activity is the persistence of the Abs$^-$ phenotype over the life of mutant cultures: even colonies that grow for several weeks remain unpigmented.

An alternative model for signal regulation in the AbsA system is that AbsA1 is a kinase in the absence of signal and a phosphatase in the presence of signal, as a few sensor kinase-phosphatases are proposed to function (135; 139; 138; 56). In this case, the transition stage culture lacks the signal regulating AbsA1 and AbsA1-kinase activity would generate AbsA2-P. Later a signal would switch AbsA1 to the AbsA1-phosphatase mode so it could dephosphorylate AbsA2-P, allowing antibiotic synthesis. We consider this model to be less compelling than the first because the AbsA2-P-dependent transcription profiles are more simply explained if the AbsA1-kinase activity is activated by a transition stage signal.

What purpose does AbsA regulation of antibiotics serve in the *S. coelicolor* life cycle? One relevant observation is the substantial perturbation of morphogenesis observed in most Pha mutants: these mutants usually produce only sparse aerial hyphae. Conversely, antibiotic production is not altered in Abs$^-$ strains. One possibility is that precocious antibiotic synthesis *per se* is deleterious to normal sporulation. Calcium-dependent antibiotic may be especially inhibitory as suggested by strain C577S25 (6; Champness, unpublished). This strain demonstrates a strong Pha phenotype for pigmented antibiotic production, but is blocked in synthesis of CDA due to a deletion in this gene cluster. In contrast to other Pha mutants, C577S25 is wildtype for sporulation. Thus, it may be that *S. coelicolor* acquires competence for antibiotic production before the sporulation process has proceeded adequately and the function of the AbsA system is to delay antibiotic production to allow optimal sporulation.

What factors establish the state that we have referred to as "antibiotic-production competent"? Likely candidates include the genes that have been identified on the basis of

mutant defects in antibiotic synthesis. Among these is a second gene found in Abs$^-$ mutant hunts, *absB*, which encodes the *S. coelicolor* homolog of RNase III (136). Another large group of genes is known to regulate both antibiotic synthesis and the onset of sporulation. Some genes in this group are the *bld* genes, several of which encode regulators of gene expression (reviewed by 30). Another is *relA*, which encodes pppGpp synthetase (27; 26; 113; 114). Also important are the components of γ-butyrolactone signaling pathways (188; 130).

Additional antibiotic regulatory genes have been isolated on the basis of multi-copy stimulation of antibiotic production. The best characterized of these are the AfsQ1/Q2 two-component system (84) and the AfsR/K serine-threonine phosphoprotein/kinase pair (76; 77; 71; 115; 116; 54). Mutations in the *afsQ1/Q2* genes cause no phenotype, but disruptions to the *afsR/K* locus conditionally reduce antibiotic synthesis, especially on high phosphate media (115; 54). The multicopy effect of *afsR/K* has been shown to correlate with increased antibiotic pathway-specific activator transcription (54). Multi-copy clones of the *afsR/K* locus can restore antibiotic synthesis to Abs$^-$ *absA1** mutants (28), and overexpression of the AfsQ response regulator does the same (84). These observations imply that these genes can compete against the postulated persistent negative regulation imposed by *absA1** alleles.

It is widely observed that phosphorylation of response regulators modifies their activities, likely causing conformational changes that affect promoter recognition or cooperative binding at the target promoter (161). However, the extent to which phosphorylation is required for DNA binding and transcriptional regulation *in vivo* varies for different response regulators. In the Nar system of *E. coli* (reviewed by 159), which

regulates nitrate/nitrite-responsive anaerobic respiratory pathways, phosphorylation of NarL is absolutely required for DNA binding and regulatory activity (11). Conversely, in *E. coli* UhpA-mediated regulation of sugar-phosphate uptake, high-copy expression of the unphosphorylated UhpA D54N protein allowed phosphorylation-independent activation of the *uhpT* promoter (180; 181). If unphosphorylated AbsA2 was functional *in vivo*, high-copy expression of the *absA2* D54E allele, on plasmid pTBA175 (Figure 6), might have repressed antibiotic synthesis. Since it did not (Figure 11B), it appears that AbsA2 regulatory activity is strongly dependent on phosphorylation.

It is noteworthy to contrast AbsA2 with several other recently discovered antibiotic cluster-linked regulators that are closely related in sequence. One such protein is RedZ, a *red*-cluster-linked activator of *redD* transcription (182). The amino acid sequence of RedZ has end-to-end similarity to AbsA2, including the putative helix-turn-helix region, with 27% identical residues overall. However RedZ lacks the conserved aspartate residue that is normally the site of phosphorylation in response regulators (65). A homolog of RedZ, DnrN, is found in the daunorubicin gene cluster of *S. peucetius*, where it regulates *dnrI*, which encodes a SARP pathway-specific regulator of the *dnr* cluster. Although the DnrN protein sequence has retained the conserved aspartate, other residues of the phosphorylation pocket are not conserved and phosphorylation appears to not be involved in DnrN function *in vivo* (59). Thus DnrN and RedZ appear to serve as regulators in the unphosphorylated state. It is not known if a modification other than phosphorylation regulates the activity of RedZ or DnrN.

Our results have shown that the AbsA two-component system is a negative regulator of the calcium-dependent antibiotic, actinorhodin, and undecylprodigiosin. For

the later two antibiotics, *absA* negatively regulates the SARP pathway-specific activator genes. It will be important to determine whether AbsA2 regulates the SARP genes directly. For the *cda* cluster, interesting questions are whether AbsA2 regulates the SARP homolog, *cdaR*, or whether it regulates another, as yet unidentified *cda* regulator, or directly represses *cda* biosynthetic genes.

CHAPTER 4

TEMPORAL EXPRESSION OF *red* AND *cda* PATHWAY-SPECIFIC REGULATORS AND THEIR DEPENDENCE ON AbsA

INTRODUCTION

Global negative regulation of antibiotics by AbsA has been demonstrated to act through transcriptional control of *redD* and *actII-4* pathway-specific regulators (PSRs) (1). While these genes constitute possible targets of AbsA2, the red gene cluster contains a second PSR, *redZ*, which is required for expression of *redD*, but can not activate *red* biosynthetic gene expression in the absence of *redD* (182). The effect of AbsA on *redZ* expression is unknown. Another plausible target of AbsA2 is the putative PSR of the *cda* gene cluster, *cdaR*, the sequence of which was recently made available by the *S. coelicolor* genome sequencing project (http://www.sanger.ac.uk/Projects/S_coelicolor/).

Very little is known about the temporal expression of the *cda* gene cluster of *S. coelicolor*. The deduced *cdaR* gene product shows sequence similarity to the N-terminal region of *Streptomyces* antibiotic regulatory proteins (146), including *redD* and *actII-4*, that contain an OmpR-like DNA-binding motif (183). A chromosomal disruption of *cdaR* was found to eliminate CDA production, suggesting it had a positive regulatory function (156). Sequencing of the *cda* cluster also revealed that the *absA* locus lies within it (Figure 14). The significance of *absA* association with the *cda* genes is not yet known. The only other *Streptomyces* antibiotic gene cluster known to encode a two-component signal transduction system is the rapamycin gene cluster of *S. hygroscopicus*, which contains genes encoding a putative two-component system of as yet unknown function (149). However, various Gram-positive bacteria which produce class I and class II antimicrobial peptide (AMP) antibiotics do contain two-component genes as part of the antibiotic gene cluster

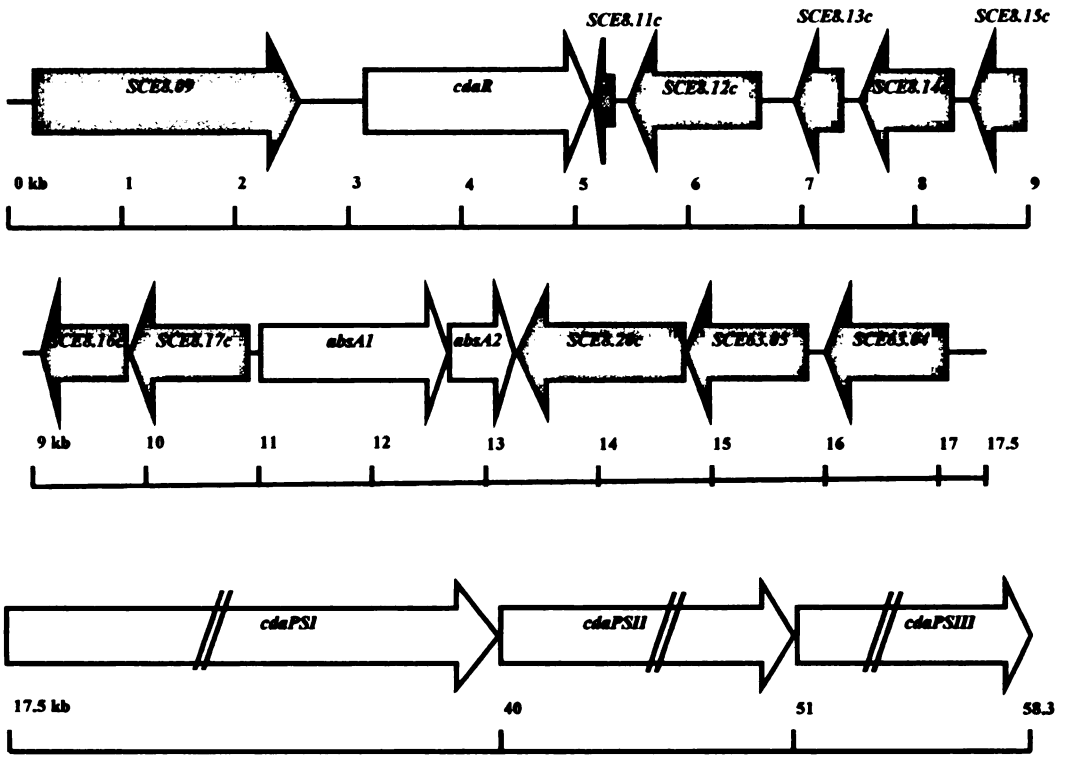


Figure 14. Position of *absA* with respect to the *cda* gene cluster. This 58.3 kb region of the *cda* cluster was reconstructed from sequence data made available by the *Streptomyces coelicolor* Sequencing Project (The Sanger Centre). Genes shown in white have been named and given putative functions based on genetic or functional analysis. *cdaR* is homologous to pathway-specific activators. Biosynthetic genes *cdaPSI*, *cdaPSII* and *cdaPSIII* encode peptide synthases which catalyze steps in the enzymatic synthesis of the lipopeptide antibiotic CDA. Shaded genes have been assigned putative functions based on sequence similarity to other proteins (annotated in http://www.sanger.ac.uk/Projects/S_coelicolor/).

(reviewed by 123; 96). AMPs have a conserved regulatory gene organization in which an open-reading frame encoding an autoinducing peptide pheromone precedes the genes of a two-component system. While demonstration of response regulator binding to operons within AMP clusters is still pending, disruption of either the autoinducer or two-component genes abolishes AMP production (47; 10; 98). It is hypothesized that two-component systems regulate most or all of the regulatory, biosynthetic and immunity/transport operons of AMP clusters (123; 96). Although the precise target(s) of AMP two-component systems remains to be elucidated, unlike AbsA, these systems have not been implicated in regulation of genes outside of the cluster in which they are found.

Given the recent revelation of the association of *absA* with the *cda* cluster, and dependence of other PSRs on AbsA, it was of interest to determine whether inhibition of CDA synthesis in the Abs$^-$ mutant was correlated with transcriptional regulation of *cdaR*. Similarly, I examined the effect of AbsA mutants on the expression of *redZ* and the possibility that AbsA-mediated regulation of *redD* was a consequence of its effect on *redZ*. Finally, growth-phase dependent expression of PSRs was compared to that of *absA* to establish temporal relationships between AbsA activity and the appearance of PSRs of the *red* and *cda* clusters.

MATERIALS AND METHODS

Time-course analyses of pathway specific regulators *redD*, *redZ*, and *cdaR* transcripts were performed using high-resolution S1 nuclease protection assays. The same RNA samples used for S1 analyses of the *absA* locus were used here, therefore,

growth conditions and RNA isolation procedures were as described in the Materials and Methods of Chapter 3. Double-stranded DNA probes were also labeled and synthesized under the same conditions described above (Chapter 3, Materials and Methods). A *redD* probe of 497 bp, with a predicted 330 nt S1 product (167; 1), was generated from primers and template described in Aceti and Champness (1998). The expression of *redZ* was evaluated with a 405 bp probe synthesized from template pIJ4132 (White and Bibb, unpublished) with forward primer WC43 (5' AGATCTTGGAGCGGGAACTCTC CCTGC) and ^{32}P-labeled reverse primer WC96 (5' GTCGCAGCACACACCAGGA CACG). Previous studies of this gene predict this probe would produce an S1 product of 141 nt (65). A *cdaR* probe of 584 bp was amplified from *S. coelicolor* J1501 chromosomal DNA with forward primer WC106 (5' GGCGCACTGACGAAA GCAAGGGC) and ^{32}P-labeled reverse primer WC94 (5' CCGCCCACCGTAAGACC TCGGCC). The transcription start site for this locus had not previously been determined. S1 nuclease assay conditions were identical to those used for *absA*. Likewise, the same *glk* probe was used to normalize RNA loading, and S1 product sizes were estimated alongside ^{32}P-labeled ϕX174/*Hinf*I molecular weight markers (Promega).

RESULTS

Dependence of Pathway-Specific Regulators *redZ* and *redD* on AbsA.

Red antibiotic synthesis is under the control of two pathway-specific regulators, RedD and RedZ (reviewed by 35; 31). The RedD activator is required for Red synthesis (144; 50) and overexpression of *redD* causes an increase in *red* biosynthetic gene expression and Red production (122; 167). Expression of *redZ* is required for both Red

synthesis and *redD* expression, suggesting that RedZ is an activator of *redD* transcription (182). Negative regulation of Red synthesis by AbsA was previously demonstrated to act through transcriptional regulation of *redD* (1). Therefore, it was of interest to see if the dependence of *redD* on AbsA2 was mediated through *redZ*, a possible target of AbsA2. Similarly, while *redD* (167), *redZ* (182) and *absA* (Chapter 3) have all been shown to be temporally regulated, I sought to gain a better understanding of the temporal relationship between the global regulator AbsA2 and expression of pathway-specific regulators which are possibly under its control. As such, S1 nuclease protection assays were performed to evaluate *redD* and *redZ* transcription with the same RNA samples used to analyze *absA*. These were extracted from 18, 30, and 54 hr cultures representing time points prior to and during antibiotic synthesis. AbsA-mediated regulation of *red* PSRs was tested in wild type (J1501) versus *absA* mutant strains C542 (Abs$^-$) and C570 (Pha).

The *redD* transcript gave a single product of 330nt (Figure 15) with a temporal pattern and AbsA2 dependence in agreement with that observed previously (1). The 18 hr cultures although turbid, still had rather sparse growth and no pigmentation. As expected, no *redD* transcript was discernable. *redD* was not present until 30 hours when it was strongest in J1501, coinciding with the Red pigment observed in this culture at that time. The level of *redD* in 30 hour C570 was surprisingly low since this strain reproducibly demonstrated early onset of Red on SpMR agar plates.

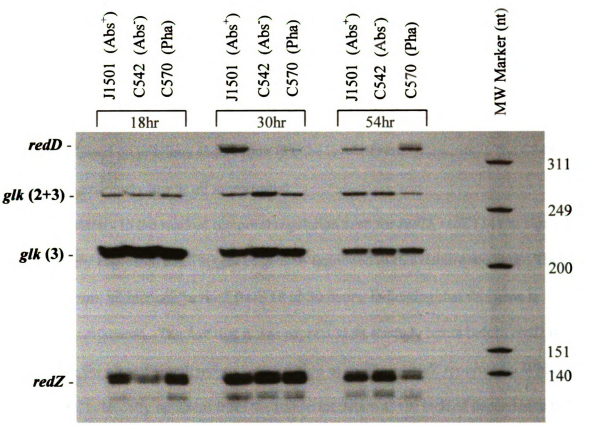


Figure 15. High-resolution S1 nuclease protection analysis of *redD* and *redZ* transcripts. Temporal regulation of *redD* and *redZ* was followed by analyzing RNA samples isolated from *S. coelicolor* strains grown for 18, 30 and 54 hours in liquid SpMR. The dependence of *redD* and *redZ* expression on the AbsA two-component system was tested by examining their transcription in strains J1501 (wild type), C542 (Abs$^-$), and C570 (Pha) at each time point. Glucose kinase (*glk*) transcript was measured to normalize the quantities of RNA loaded at each time point.

Nevertheless, the low level of signal did coincided with the lack of pigmentation observed in this culture. By 54 hours, J1501 cultures had attained a strong red hue, while the C570 cultures were maroon, having overtaken J1501 in Red as well as visibly producing Act. At no point was there any pigmentation in the Abs$^-$ C542 cultures. As expected, based on previous observation (1), the level of *redD* transcript in the C542 Abs$^-$ strain was extremely low at all times tested.

Contrary to the marked temporal regulation seen for *redD*, *redZ* (141 nt signal) was strongly expressed at all stages of growth examined in this culture medium. There was, however, an increase in *redZ* from 18 to 30 hours, indicating that this gene is temporally regulated. The fact that it was expressed so strongly hours before *redD* is in contrast to the almost simultaneous upregulation of *redD* and *redZ* reported by White and Bibb (1997). Equally apparent from the transcript data was the lack of dependence that *redZ* had on AbsA2, as witnessed by the similarity in signal intensities for each of the strains tested.

Dependence of *cdaR* Expression on AbsA

No previous transcript data was available for *cdaR*, but its expression was hypothesized to be growth-phase dependent like other antibiotic PSRs. The intergenic region between the translation start codon of the deduced open reading frame for *cdaR* and the stop codon of upstream gene SCE8.09 is 744 bp. S1 nuclease protection assays of *cdaR* transcript employed a 584 bp double-stranded DNA probe that protected a region 439 nt upstream of the predicted translation start codon. Expression of *cdaR* was evaluated at 18, 30, and 54 hours in strains J1501 (wild type), C542 (Abs-) and C570

(Pha). S1 nuclease protection results presented in Figure 16 revealed a major product of approximately 500 nt, corresponding to a transcription start site about 380 nt upstream of the translation start codon. Although undigested probe was present in some samples, its inconsistency lead me to believe that it did not represent an additional transcription start site beyond that of 380 nt. There were some minor signals of smaller size that could represent additional promoters of this gene; however, these were not further analyzed in this study. Inspection of sequence upstream from the region encompassing the transcription start point identified hexamers around -10 and -35 with strong similarities to consensus sequences proposed by Strohl (1992) to be recognized by *Streptomyces* RNA polymerase containing and *E. coli* σ^{70}-like sigma factor (data not shown). Comparison of *cdaR* expression in J1501 between 18 and 30 hours suggested that this gene was under growth-phase dependent regulation. It also appeared as though *cdaR* was expressed considerably earlier than *redD* under these growth conditions, but not earlier than *redZ*.

It was not clear from the results presented in Figure 16 whether AbsA regulated the expression of *cdaR*. A comparison of 18 hour J1501 and C542 transcripts indicated that there was a strong effect. Given that C542 is an Abs$^-$ strain that does not produce antibiotics and *cdaR* expression was dramatically increased in the Abs$^-$ strain, the transcript results suggested that CdaR was a negative regulator. In contrast, a chromosomal disruption of *cdaR* reportedly blocked synthesis of CDA (156) implying that CdaR was an activator of CDA. An additional point of confusion was that the 18 hour C570 signal was also stronger than that of J1501. C570 is an overproducing Pha strain, but like the Abs$^-$ strain showed increased expression of *cdaR* in comparison to

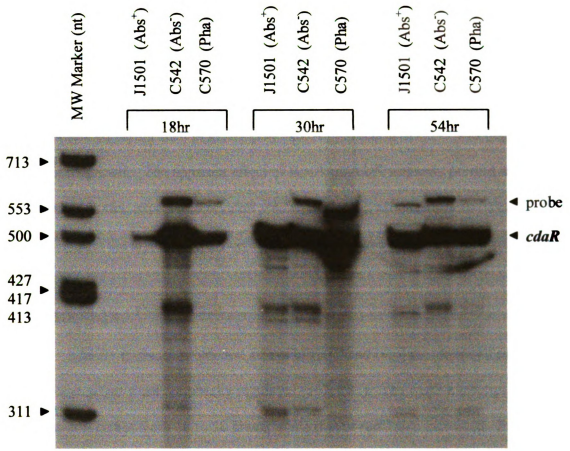


Figure 16. High-resolution S1 nuclease protection analysis of the *cdaR* transcript. Temporal regulation of *cdaR* was followed by analyzing RNA samples isolated from *S. coelicolor* strains grown for 18, 30 and 54 hours in liquid SpMR. The dependence of *cdaR* expression on the AbsA two-component system was tested by examining its transcription in strains J1501 (wild type), C542 (Abs$^-$), and C570 (Pha) at each time point.

J1501. Thus, the conundrum lies in that both C542 (Abs$^-$ with constitutively phosphorylated AbsA2) and C570 (Pha with constitutively unphosphorylated AbsA2) appeared to enhance expression of *cdaR* at 18 hours. CDA production, as measured in zone of inhibition assays, was positive in C570 and, as demonstrated for Act and Red, was produced earlier than in J1501 (29). CDA production in C542 was negative over the course of growth. The apparent effect of AbsA on *cdaR* was only present at 18 hours; at 30 and 54 hours, expression of *cdaR* was equivalent in all three strains. Therefore, it is possible that the effect of AbsA on *cdaR* expression was artifactual, as discussed below.

DISCUSSION

The S1 nuclease protection assays of *redD* and *redZ* corroborated earlier reports that these genes were temporally regulated (167; 182). The *redD* transcript underwent a dramatic increase from 18 to 30 hours, while that of *redZ* also increased. Previous assays of *redD* and *redZ* expression in cultures grown on minimal medium demonstrated very modest expression of *redZ* prior to that of *redD* (182). In the SpMR liquid cultures used in this study, *redZ* is strongly expressed at the earliest time point, several hours before the appearance of *redD*. SpMR is a good sporulation medium due partially to its low phosphate concentration (91). Phosphate limitation is well documented as a trigger for differentiation (111) and was hypothesized to account for Act and Red dependence on pleiotropic regulator AfsR (54). Thus, the robust levels of *redZ* in the 18 hour time point of Figure 15 may represent a medium-dependent effect that caused earlier and increased expression of *redZ* in relation to cultures grown in minimal medium.

Expression of *redD* was previously shown to depend upon AbsA (1). That finding was also corroborated here as seen by a comparison of *redD* transcripts from J1501 and C542. Lack of Red production in the C542 mutant was presumably a result of the extremely low level of *redD* expression in this strain. Not previously known was whether AbsA was exercising its effect on *redD* through regulation of *redZ*, which activates transcription of *redD*. The results presented here showed that this was not the case, AbsA has no apparent regulatory effect on *redZ* transcription. Therefore, the *redZ* promoter is not a likely target of AbsA2. A possible scenario is one of competitive regulation between AbsA2 and RedZ at the *redD* promoter. Likewise, AbsA2 could act indirectly by regulating transcription of a positive or negative regulator of *redD*.

RedZ is a pseudo response regulator showing full length homology to AbsA2, but possessing changes in residues of its active site that result in its inability to become phosphorylated (65). *redZ* also contains a rare UUA codon which is recognized by *bldA*-encoded leucinyl-tRNA. *S. coelicolor bldA* mutants are defective in aerial mycelium synthesis and production of at least three antibiotics, including Red. It has been suggested that BldA is a translational regulator of morphogenesis and secondary metabolism in *S. coelicolor* (reviewed by 74). Transcript studies performed here revealed that *redZ* was present at robust levels well before the appearance of *redD*. In C542 there was also significant expression of *absA* at 18 hours, such that high levels of AbsA2-P may have blocked expression of *redD*. However, robust levels of *redZ* transcript in the absence of AbsA2 in the C570 strain, both in total concentration and in the active phosphorylated form, was not sufficient to trigger early expression of *redD*. This suggests that the activation of *redD* transcription is not simply dependent upon the

concentration of *redZ* transcript, but that some other growth-phase dependent, post-transcriptional event may regulate RedZ activation of *redD* expression.

Joint consideration of *absA* and *red* transcript data together with the role of phosphorylation on AbsA activity brings to light some interesting observations and conjecture. We know that C542 is blocked in antibiotic synthesis and likely produces constitutively phosphorylated AbsA2. C542 expressed the greatest levels of *absA* and virtually no *redD*, coincident with the absence of Red antibiotic. We may conclude from these observations that in the presence of elevated levels of AbsA2-P (C542) there is efficient and essentially complete inhibition of antibiotic synthesis exercised through repression at the transcriptional level of certain pathway specific regulators. In the J1501 wild type strain there was considerable expression of *redD* and antibiotic production by 30 hours. According to the genetic and transcript data presented in Chapter 3, the effectiveness with which AbsA2 represses antibiotic synthesis would also depend upon the level of AbsA2 phosphorylation, which in agreement with the two-component signal transduction mechanism would be dictated by the equilibrium between the signal-dependent kinase and phosphatase modes of AbsA1. So in J1501, AbsA2 may retard Red production but it does not completely inhibit it due to the absence of signal saturation, which would mimic the C542 phenotype. Interestingly, while J1501 *absA* expression was greatest at 30 hours, so was that of *redD*, and Red was present in the culture at this time. Thus, although AbsA2 is capable of globally inhibiting antibiotic synthesis, as witnessed in strain C542, under most physiological conditions its purpose may instead be to moderate synthesis of antibiotics through competitive binding at PSR promoters.

Transcript data for *cdaR* demonstrated that like *redD*, *redZ* and *absA*, its expression was growth-phase dependent. It was not clear whether AbsA was regulating expression of *cdaR*. Both C542 (Abs$^-$) and C570 (Pha) had higher levels of *cdaR* transcript than wild type J1501. The difference in *cdaR* expression between the three strains was only seen at 18 hours. By 30 hours expression was essentially equal, although there was still no CDA produced in C542. Since each of *cdaR*, *redZ*, and *absA* were expressed at 18 hours, it is likely that this time point represented transition phase growth. It is possible that the 18 hour samples represented a window during the transition growth period when *cdaR* expression is strongly upregulated. If one sample represented an early time point in this window (e.g. J1501) while another represented a later time point (e.g. C542), a large variation in expression could be seen. The similarity in *cdaR* transcription in each of the strains at 30 and 54 hours does not coincide with the effects of AbsA-mediated regulation witnessed for *redD* and *actII-4* (here; 1). Nevertheless, the lack of CDA synthesis in Abs$^-$ strain C542 and the presence of *absA* within the *cda* cluster suggests that AbsA does regulate this antibiotic. Thus, it is possible that the target of AbsA2 in the *cda* cluster is not *cdaR*. In an ongoing parallel study, several *cda* biosynthetic genes were shown to be tightly repressed in the C542 Abs$^-$ strain over the course of growth and pigmented antibiotic production (146). The same transcripts were clearly present at each time point tested in J1501. These results coincide with the lack of CDA synthesis in the Abs$^-$ mutants and further bring into question whether *cdaR* is a target of AbsA regulation.

Given growth by hyphal extension and branching and mycelial clumping and wall growth, synchronous growth with well defined metabolic boundaries is difficult to

achieve in Streptomyces. In this study cultures were begun by inoculating with identical spore titers and grown simultaneously under identical conditions. Biomass or culture turbidity were not measured. Thus, the 18 hour *cdaR* transcript results could represent somewhat different points during transition phase growth resulting in signal differences that are artifacts of asynchronous growth. Similarly, the surprisingly low level of *redD* expression in the 30 hour culture could be due to slower growth of this culture in comparison to J1501. Therefore, while the results presented in this chapter do add to the understanding of antibiotic regulation and the role of AbsA therein, uncertainties arose which warrant a more precise analysis of *absA* and PSR expression from late exponential to early stationary phase growth. Such an analysis would seek to correlate samples to a growth curve and more accurately establish expression profiles of various PSRs and pleiotropic regulators and their interdependence.

CHAPTER 5

OVEREXPRESSION, PURIFICATION, AND PHOSPHORYLATION OF AbsA1 AND AbsA2 PROTEINS

INTRODUCTION

Overexpression and purification of two-component proteins has been an important tool for analyzing biochemical properties of histidine kinases (HK) and response regulators (RR), and for obtaining mechanistic details on these systems. Typically the protein expression systems employed allow for high levels of expression from specialized promoters (e.g., T7 or *tac*) and rapid affinity column purification by means of a peptide or protein fusion tag. Since the RR is cytoplasmic, the entire protein is usually included in the fusion product. Conversely, recovery of HK fusion protein from the soluble phase of a heterologous expression system often requires that the N-terminal transmembrane-containing domain of the HK be deleted from the fusion protein. Single column affinity purification schemes frequently results in greater than 90% purity of recovered fusion proteins.

A common use of purified two-component proteins has been to examine the role of phosphoryl-group transfer in signal transduction between the HK and RR. The mechanism of communication in two-component systems has routinely been shown to involve phosphorylation events linked to highly conserved residues and subdomains of RRs and HKs (reviewed in 160; 131). Numerous studies demonstrated *in vitro* phosphorylation of RRs by small molecular weight phosphate donors such as acetyl phosphate or phosphoramidate (107; 51; 117; 148; 41).

The importance of phosphorylation in mediating RR activity was demonstrated by mutations at the RR aspartate residue that is the site of phosphorylation, which correlated the inability for *in-vitro* phosphorylation with phenotypic defects *in vivo* (CheY (18), NtrC (120), OmpR (20); (44)). Similarly, site-directed changes to the site of

phosphorylation of HKs, an invariable His that lies within the highly conserved H-box subdomain, eliminated *in-vitro* phosphorylation by MgATP and the *in-vivo* phenotype associated with their phosphorylated state (190; 125; 187).

Purified HKs and RRs have also been useful in examining two-component protein interactions and enzyme activities. Numerous examples of *in-vitro* phosphorelay between purified cognate HKs and RRs have been reported (e.g., NarQ/X/L (148), EnvZ/OmpR (5); (80), DegS/U (42), PhoR/P (153)). The HK is frequently said to catalyze "trans-phosphorylation" of the RR. However, evidence that small molecular weight phosphate donors readily phosphorylated RRs suggested that phosphotransfer from HK phosphohistidine to Asp of the RR was largely catalyzed by the RR (161). Nevertheless, the rate of RR phosphorylation was greatly increased *in vitro* in the presence of its cognate phosphorylated HK, suggesting that the latter was a preferred substrate. HKs have also been shown to demonstrate HK phosphatase activity toward the phosphoaspartate of their cognate RRs *in vitro* (NarL (178); (148), EnvZ (5); (80)).

Purification of RRs has aided in examining the role of phosphorylation in RR target recognition and binding. RR phosphorylation alters its output activity by causing a conformational change between the receiver and effector domains (161). Consistent with this role for phosphorylation, deletion of the N-terminal receiver domain of several RRs resulted in constitutive effector domain activity associated with the phosphorylated form of the intact protein (155; 90; 64). Similarly, gel-shift DNA-binding assays with purified RR have consistently shown either an absolute requirement for phosphorylation, or that phosphorylation increases affinity for the RR target (OmpR (81), NarL (11), UhpA (41), FixJ (3); (141)). In addition, purified RRs have been useful in DNaseI and hydroxyl

radical footprint assays for identifying the location, orientation, arrangement, and consensus sequence of RR binding sites on their target promoters (Cpx (132), UhpA (41), NarL/P reviewed by 159, OmpR reviewed by 135).

Based on the usefulness of purified two-component proteins in defining mechanisms and targets, I sought to purify AbsA1 and AbsA2 proteins. Heterologous expression systems were used to overexpress His-tag fusions of AbsA2 and AbsA2 D54E, and maltose-binding-protein fusions of AbsA1 and AbsA1 H202L. A *Streptomyces* expression system was also used to produce His-tagged AbsA2. In-vitro phosphorylation of AbsA2 was attempted with acetyl phosphate and AbsA1 phosphate donors in order to support *in-vivo* genetic analyses that suggested phosphorelay signal transduction between His202 of AbsA1 and Asp54 of AbsA2.

MATERIALS AND METHODS

Bacterial Strains

All *E. coli* strains used were derivatives of strain K-12. Strain JM109 (Stratagene) served as the host for plasmid preparation and maintenance of 'AbsA1 and AbsA2 heterologous expression plasmids. Heterologous expression of MBP-'AbsA1 proteins was performed in strain JM109, while heterologous expression of AbsA2-His_{10} proteins was carried out in strain BL21(DE3) (Novagen). *Streptomyces lividans* 1326 (73) was used for preparation, maintenance, and expression of AbsA2-His_6 from a *Streptomyces* expression vector. *In-vivo* functional analysis of the AbsA2-His_6 fusion protein was performed in strain C570 (see Chapter 3, Materials and Methods).

Construction of AbsA2-His_{10} and AbsA2-His_6 Expression Plasmids

AbsA2 encoding expression plasmids (Figure 17) were constructed using *absA2* alleles generated by PCR from upstream primers WC36 (5' CCAGGGAAGGATCATAT GATTCGCGTAC), which introduced an *Nde*I restriction site at the translation start site of *absA2* or WC35 (5' CAGGGAAGGATCCGATGATTCGCG), which introduced a *Bam*HI restriction site 2 bp upstream from the *absA2* (D54E) translation start site, and downstream primer WC37 (5' GGGCGACCGGCGGATCCGCCTC), which introduced a *Bam*HI restriction site 90 bp downstream from the *absA2* translation stop codon. The template for amplification of wild type *absA2* was *S. coelicolor* J1501 chromosomal DNA, whereas *absA2* encoding a D54E mutation was amplified from pTBA160 (pBluescript II SK+ [Stratagene] harboring a 1.2 kb *Bam*HI/*Pst*I region of the *absA* locus encoding the D54E mutation). PCR conditions were as described for amplification of *absA2* (6). PCR products were digested as indicated by the manufacturer (*Nde*I and *Bam*HI, Gibco) and digest products were purified on preparatory columns (Wizard PCR Preps, Promega) prior to ligation. The *absA2 Nde*I/*Bam*HI fragment was cloned into pET16b *Nde*I/*Bam*HI (Novagen) and pIJ4123 *Nde*I/*Bam*HI (168) to produce pTBA235 and pTBA270, respectively. The *absA2* D54E *Bam*HI fragment was cloned into pET16b *Bam*HI to produce pTBA240. Plasmids pTBA235 and pTBA240 encoded His_{10} fusions at the N-terminus of AbsA2, while pTBA270 encoded a His_6 fusion at the N-terminus of AbsA2. Sequence fidelity and the frame of expression of the *absA2* alleles in these constructs were confirmed by sequencing pTBA235 and pTBA240 from primers

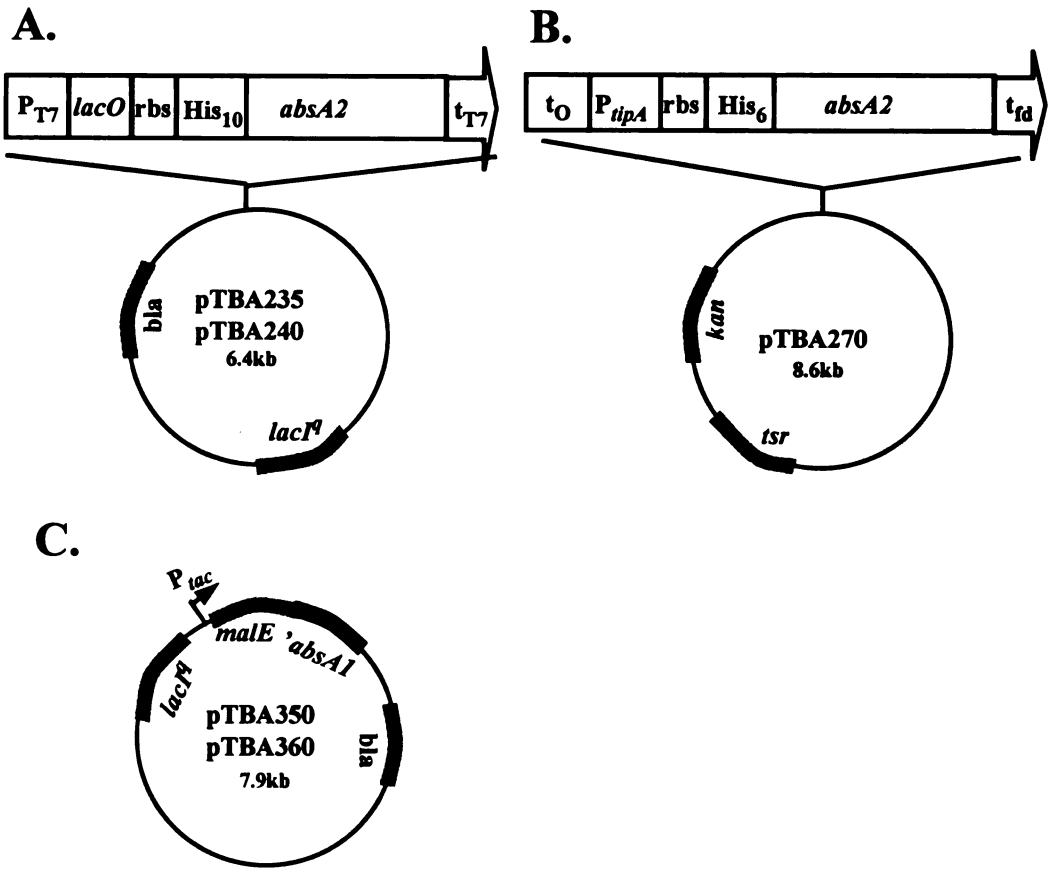


Figure 17. Plasmids for '*absA1* and *absA2* overexpression. (A.) Plasmids pTBA235 containing *absA2* and pTBA240 containing *absA2* (D54E) are derivatives of pET16b (Novagen). Overexpression of the *absA2* alleles is under the control of a T7/*lac* promoter. An in-frame His_{10}-encoding tag is fused upstream of *absA2*. These plasmids are expressed in *E. coli* BL21(DE3), which has a chromosomal copy of the gene for T7 RNA polymerase also under the control of the Lac repressor, such that *absA2* expression is IPTG-inducible. A T7 transcriptional terminator is situated downstream of *absA2*. Genes for the Lac repressor (*laqI*^{q}) and ampicillin resistance (*bla*) are also present. (B.) Plasmid pTBA270 is a derivative of the *Streptomyces* overexpression plasmid pIJ6021. Expression of *absA2* is under the control of the *tipA* promoter, which is thiostrepton inducible. An in-frame His_6-encoded tag is fused upstream of *absA2*. Transcriptional terminators t_O and t_{fd} lie on either side of the overexpressed allele. Plasmid pTBA270 carries resistance genes for thiostrepton (*tsr*) and kanamycin (*kan*). (C.) Plasmids pTBA350 carrying '*absA1* and pTBA360 carrying '*absA1* (H202L) are derivatives of pMAL-c2 (New England Biolabs). Overexpression of 'AbsA1 is under the control of the IPTG-inducible *tac* promoter. The *malE* gene encoding maltose-binding protein is fused to the 5' end of '*absA1*. These plasmids carry genes for the Lac repressor (*lacI*^{q}) and ampicillin resistance (*bla*).

PETFOR and PETREV (supplied by Iowa State Sequencing Facility, Aimes, Iowa), which were specific to regions of the vector upstream and downstream from the *absA2* insert. Sequence fidelity and the frame of expression of the *absA2* allele in pTBA270 was confirmed by sequencing from primers WC51 (5' GGACGGCGTCAGAGAAGGG AGCGG), which recognized a sequence on the vector upstream of the fusion protein start site, and WC37. Sequencing was performed by the Iowa State Sequencing Facility (Ames, Iowa). All PCR primers were synthesized by the Michigan State University Macromolecular Structure Facility (East Lansing, Michigan). Plasmids pTBA235 and pTBA240 were transformed into BL21(DE3). The AbsA2 overexpression plasmid pTBA270 was transformed into *S. lividans* 1326.

Construction of MBP-'AbsA1 Overexpression Plasmids

The 'AbsA1 overexpression plasmids (Figure 17) encode a truncated form of AbsA1 lacking the N-terminal transmembrane domain. The *'absA1* alleles were generated by PCR from primers WC69 (5' GGCCACCGTCGACCAGGGCAAGG), which introduced a *Sal*I restriction site at the region encoding the predicted linker between the transmembrane and transmitter domains, and WC70 (5' GGTCTCG TCGTCGACGAGCAGTACGC), which introduced a *Sal*I restriction site 37 bp downstream of the *absA1* stop codon. The template for generation of the 1.3 kb wild type *'absA1* insert was pTBA115 (pIJ2925 (86)) carrying a 3.2 kb *Bam*HI/*Pst*I insert encompassing the entire *absA* locus). The *'absA1* insert encoding a H202L mutation was amplified from pTBA142 (pSK+ with a 6.5 kb *Xho*I/*Bam*HI fragment including the entire *absA* locus encoding AbsA1 H202L). PCR conditions were as described for

amplification of *absA1* (6). PCR products were digested with *Sal*I (Promega), separated by low melting point agarose gel electrophoresis, and purified with Wizard preparatory columns (Promega) prior to ligation. The *'absA1 Sal*I insets were cloned into pMAL-c2 *Sal*I (New England BioLabs) to generate plasmids expressing N-terminal maltose-binding protein (MBP) fusions to the transmitter domain of 'AbsA1. Plasmid pTBA350 encoded wild type *'absA1*, where as pTBA360 encoded 'AbsA1 H202L. Confirmation of sequence fidelity and reading frame of the *'absA1* inserts was obtained by sequencing pTBA350 and pTBA360 from primers WC21 (5' GCCGCGTCCTTCATCCTG), WC23 (5' GCCGAGGAAGGCCACGATG), WC12 (Table 1, Chapter 3), and WC24 (Table 1, Chapter 3). The sequence of *'absA1* (H202L) was without error. However, the *'absA1* insert of pTBA350 encoded a L478P mutation 14 residues upstream of the *absA1* stop codon in the C-terminal domain of unknown function. In previous complementation analyses (19), a plasmid containing an *absA1* allele encoding a 69 aa C-terminal truncation was effective in restoring wild-type levels of regulation to Abs$^-$ strains carrying mutations in the transmitter domain of *absA1*. Therefore the MBP-'AbsA1 fusion possessing the L478P mutation was hypothesized to be unaffected in kinase activity and was used for preliminary *in-vitro* phosphorylation reactions while a new wild type MBP-'AbsA1 construct was being prepared. Plamids pTBA350 and pTBA360 were transformed into JM109.

Culture Media and Growth Conditions.

Heterologous expression of AbsA2-His_{10} from BL21(DE3)/pTBA235 and BL21(DE3)/pTBA240, and MBP-'AbsA1 from JM109/pTBA350 and JM109/pTBA360

was carried out in duplicate 1 L baffled flasks with 200 ml each of LB broth supplemented with 100 μg/ml carbenicillin. Production flasks were inoculated at 1% v/v from 8 hr seed cultures and incubated at 37°C and 250 rpm to an OD_{600} of 0.6 to 1. BL21(DE3)/pTBA235 and BL21(DE3)/pTBA240 cultures were induced with 100 mM IPTG to a final concentration of 0.1 to 1 mM. JM109/pTBA350 and JM109/pTBA360 cultures were induced with IPTG to a final concentration of 0.4 mM. Cultures were grown for 3 hr post induction at 30°C and 250 rpm prior to harvesting.

AbsA2 overexpression in TBA270 was carried out in quadruplicate 300 ml baffled flasks containing 50 ml 1:1 SpMR:YEME broth (91; 73) plus 12 μg/ml kanamycin. Each flask was inoculated with 10^9 spores and incubated 16 hr at 30°C and 250 rpm. Flasks were induced with 50 mg/ml thiostrepton to a final concentration of 10 μg/ml and were spiked with an additional 10 μg/ml of kanamycin. 1326/pTBA270 cultures were incubated at 30°C and 250 rpm for 12 hr post induction prior to harvesting.

Purification of MBP-'AbsA1 Proteins

JM109/pTBA350 and JM109/pTBA360 cultures were pooled and harvested by centrifugation for 10 min at 5000 x g and 4°C. Each pellet was washed with 20 ml T buffer (40 mM Tris-HCl, pH 7.5, 200 mM KCl, 20 mM $MgCl_2$) and centrifuged. The washed pellets were resuspended in 10 ml T buffer at 4°C and sonicated on ice for 6 x 20 sec bursts at an output of 10 Watts. The sonicated suspension was placed at -20°C overnight, thawed on ice, and sonicated again for 4 x 20 sec bursts at 10 Watts. The final sonicated suspension was centrifuged for 20 min at 20,000 x g and 4°C. The supernatant was loaded on a column containing 5 ml amylose resin (New England BioLabs). The

column was washed with 6 volumes T buffer and MBP-'AbsA1 was eluted with 10 mM maltose in T buffer + 20% glycerol. The eluted protein was evaluated by SDS-PAGE on a 10% polyacrylamide gel, and stored at -20°C.

Purification of AbsA2-His Proteins

BL21(DE3)/pTBA235 and BL21(DE3)/pTBA240 cultures were harvested and sonicated as above. 1326/pTBA270 cultures were harvested in a similar manner except that harvest and wash centrifugations were performed at 8000 x g. Post-sonication supernatants from BL21(DE3)/pTBA235 and BL21(DE3)/pTBA240, and 1326/pTBA270 were applied to a 5ml column containing 2.5ml Ni^{2+} resin (His-Bind, Novagen). Column washes and elution were performed as indicated in the pET Expression System Manual (Novagen) with the exception that 400 mM imidazole instead of 1 M was used for elution. Since AbsA2-His_{10} from BL21(DE3)/pTBA235 and BL21(DE3)/pTBA240 was localized almost entirely in insoluble inclusion bodies, the post-sonication pellet was solubilized by resuspension in 2.5 ml loading buffer plus 6 M urea, and incubated on ice for 2 hr with periodic vortexing. This suspension was centrifuged once again at 20,000 x g prior to loading on the nickel column. Wash and elution buffers also contained 6 M urea.

Protein refolding for 6 M urea solubilized AbsA2-His_{10} was performed by two methods. Eluate containing AbsA2-His_{10} was diluted to 1 mg/ml in Buffer A (50 mM Tris-HCl, pH 7.9, 50 mM NaCl, 5% glycerol) plus 6 M urea and 1ml was placed in 1/4" dialysis tubing with a molecular weight exclusion limit of 10,000-12,000 Da (Gibco BRL). The sample was sequentially dialyzed against 2 x 100ml volumes each of Buffer

A + 6 M urea, Buffer A + 4 M urea, Buffer A + 2 M urea, and Buffer A for periods ranging from 4 to 11 hours. Dialysis was performed at 4°C with stirring.

Refolding of AbsA2-His_{10} was also attempted while still bound to the nickel column following the method of Kleman-Leyer, et al. (1997). AbsA2-His_{10} solubilized with 6 M urea was loaded on the Ni^{2+} column and washed as above. The column was then washed with 4 volumes of Buffer D (40 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$, 0.5M KCl, 20% Glycerol) + 6 M urea. Protein refolding was performed by applying a 20 ml linear gradient of Buffer D from 6 M to 0 M urea. The column was washed with an additional 2 volumes of Buffer D and AbsA2-His_{10} was eluted with Buffer D + 400 mM imidazole. Sample concentration and buffer exchange in order to reduce salt and imidazole concentrations was performed on 4 ml Ultrafree 10K NMWL centrifugal filters (Millipore).

'AbsA1 and AbsA2 Phosphorylation Assays.

Phosphorylation of AbsA2-$His_{6/10}$ was attempted using enzymatically synthesized ^{32}P-labeled acetyl phosphate as described by Quon, et al. (1996). To 7.5 µl of AKP buffer (137) containing ^{32}P-acetyl phosphate was added 2.5 µl purified AbsA2-$His_{6/10}$ (0-5 µg); incubation was for 15 minutes at 37°C. The reaction was stopped by the addition of 3.3 µl 4X stop buffer (240 mM Tris-HCl, pH 6.8, 8% SDS, 40% glycerol, 0.004% Bromophenol blue, 100 mM EDTA). Samples were analyzed by SDS-PAGE on a 10-12% polyacrylamide gel followed by autoradiography.

MBP-'AbsA1 and MBP-'AbsA1 H202L were phosphorylated as fusion proteins and after proteolytic cleavage with Factor Xa based on the method of Jin et al. (1990b)

with modifications. Proteolysis of MBP-'AbsA1 proceeded by reacting 28 µl MBP-'AbsA1 (~50 µg protein in 40 mM Tris-HCl, pH 7.5, 0.2M KCl, 50 mM NaCl, 20 mM $MgCl_2$, 2 mM $CaCl_2$) with 2 µl Factor Xa (2 µg, New England BioLabs) overnight at 8°C. Phosphorylation reactions combined 1 to 5 µl of fusion or cleaved MBP-'AbsA1 (5-20 µg), 1 µl of 250 mM $MgCl_2$, T Buffer (40mM Tris-HCl, pH 7.5, 200 mM KCl, 20 mM $MgCl_2$) to a volume of 19.5 µl, and 0.5 µl of [γ-^{32}P] ATP (3000 Ci/mmole, DuPont NEN) at room temperature for 10 minutes.

Phosphorylation of AbsA2-$His_{6/10}$ from ^{32}P-'AbsA1 generated above was carried out by phosphorylating the 'AbsA1 Factor Xa cleavage product, as described above, in the absence of additional T Buffer. After the 10 min room temperature incubation purified AbsA2-$His_{6/10}$ was added to bring the reaction volume to 20 µl. The reaction was incubated 2 min at room temperature and stopped by addition of 20 µl 2X SDS-loading buffer (200 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 2% 2-mercaptoethanol, 0.05% Bromophenol blue). Samples were boiled 2 min and stored on ice prior to analysis on 10-12% SDS-PAGE gel and autoradiography.

RESULTS

Overexpression and Purification of AbsA2-His_{10} from *E. coli*

AbsA2 is a cytoplasmic protein. Sequence similarity to other RRs suggested that the site of phosphorylation of AbsA2 was Asp54. *In-vivo* genetic analysis of chromosomal site-directed mutations demonstrated a phenotype for the AbsA2 D54E mutant consistent with Asp54 as the site of phosphorylation (see Chapter 3). Therefore,

AbsA2 and AbsA2 D54E were overexpressed as His-tag fusion proteins in a pET-based *E. coli* heterologous expression system to examine the effect of the D54E mutation on phosphorylation of AbsA2 *in vitro*. Full length *absA2* alleles were fused at the 5' end to sequence encoding a His_{10} tag under the control of the strong T7*lac* promoter. Plasmids pTBA235 (*absA2*) and pTBA240 (*absA2* [D54E]) (Figure 17) were expressed in *E. coli* BL21(DE3) host, which has a chromosomal copy of the gene encoding T7 RNA polymerase under the control of the *lac* operator. The resulting strains producing AbsA2-His_{10} proteins were grown for 3 hours after induction with IPTG. After harvesting and lysis, analysis of total cellular protein by SDS-PAGE and Coomassie staining revealed a new major protein band with an M_r of ~29 kDa (Figure 18A, lanes 2 and 3), which closely agreed with the predicted size of 26.5 kDa for AbsA2-His_{10}. Separation of soluble and particulate cell lysate showed that little or no AbsA2-His_{10} was present in the soluble fraction (Figure 18A, lanes 4 and 5). In addition to the 29 kDa protein, a second major band with and M_r of ~58 kDa was also present in the insoluble fraction. As this product was not produced in BL21(DE3)/pET16, it appeared to be a product of pTBA240 expression. It was later noted that this product disappeared when β-mercaptoethanol was added prior to sample loading; therefore, this product was likely due to Cys-Cys covalent bonds or Ni^{2+} aggregation of AbsA2 monomers. Other response regulator His-tag fusions have also resulted in expression of protein in the insoluble fraction (137; 132; 108). Insoluble fractions of AbsA2-His_{10} proteins were denatured with 6 M urea and purified by affinity binding to Ni2+ columns. Eluted AbsA2-His_{10} proteins were greater than 95% pure (Figure 18A, lane 9; Figure 18B, lanes 2 and 3).

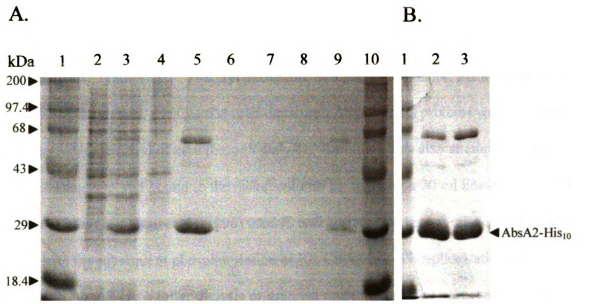


Figure 18. Purification of AbsA2 proteins overexpressed in *E. coli*.
(A.) SDS-PAGE (12%) analysis of His_{10}-tagged AbsA2 D54E protein purified from *E. coli* BL21(DE3) containing pTBA240. Lanes: (1) Molecular weight markers; (2) Whole-cell lysate of BL21(DE3)/pET16b, 3 hours post-induction; (3) Whole-cell lysate of BL21(DE3)/pTBA240, 3 hours post-induction; (4) Souble phase of BL21(DE3)/pTBA240 whole-cell lysate; (5) 6M urea-solubilized cell lysate pellet from BL21(DE3)/pTBA240; (6) Ni^{2+}-column flow through of 6M urea-solubilized pellet; (7) Ni^{2+}-column wash with binding buffer; (8) Ni^{2+}-column wash with wash buffer (60mM imidazole); (9) Dilute eluate of 6M urea-solubilized pellet from BL21(DE3)/pTBA240; (10) Molecular weight markers. (B.) SDS-PAGE (12%) analysis of Ni^{2+}-column purified His_{10}-tagged AbsA2 and AbsA2 D54E. Lanes: (1) Molecular weight markers; (2) Eluate of 6M urea-solubilized AbsA2-His_{10} from BL21(DE3)/pTBA235; (3) Eluate of 6M urea-solubilized AbsA2(D54E)-His_{10} from BL21(DE3)/pTBA240.

In-Vitro Phosphorylation of AbsA2-His_{10} with Acetyl Phosphate

Response regulators can be readily phosphorylated by small molecular weight phosphate donors such as acetyl phosphate (107; 117). Moreover, many cases exist where His-tagged RRs retained both *in-vitro* phosphorylation and *in-vivo* physiological activity without the necessity of cleaving the fusion tag (137; 132; 70; 105; 108). Thus, I

sought to examine the requirement of Asp54 for the phosphorylation of AbsA2 by *in-vitro* phosphorylation assays with AbsA2-His_{10} and AbsA2 (D54E)-His_{10} using enzymatically synthesized ^{32}P-acetyl phosphate as a phosphate donor. Prior to use in *in-vitro* phosphorylation, purified 6M urea-denatured AbsA2-His_{10} proteins were refolded by the gradual removal of urea through dialysis. Refolding was also attempted while AbsA2-His_{10} was still bound to the nickel column by applying a 20 ml linear gradient of 6 M to 0 M urea. Quon, et al. (1996) used *E. coli* acetate kinase to synthesize ^{32}P-acetyl phosphate for subsequent phosphorylation of RR CtrA-His_6. Phosphorylation of renatured AbsA2-His_{10} (after dialysis or gradient renaturation) was unsuccessful by this method. However, from the controls that were included in the assay it did appear as though the enzymatic synthesis of ^{32}P-acetyl phosphate was proceeding to completion. Thus, it was suspected that my attempts to refold the denatured AbsA2-His_{10} was not producing an active conformer of AbsA2.

Overexpression and Purification of AbsA2-His_6 from *S. lividans*

Unsuccessful phosphorylation of heterologously produced renatured AbsA2-His_{10} led me to overproduce AbsA2 in a *Streptomyces* expression system. It was hypothesized that weaker expression from the *tipA* promoter, together with production in its natural environment, might favor accumulation of AbsA2-His_6 in a soluble and active form. The same allele of *absA2* used to construct pTBA235 was ligated into pIJ4123 (168) to create pTBA270, which encoded an N-terminal His_6 fusion to AbsA2. Fusion protein expression was under the control of the thiostrepton-inducible *tipA* promoter. Given the slow growth of *Streptomyces* relative to *E. coli*, *S. lividans* 1326/pTBA270 was

grown for 16 hours prior to induction and an additional 12 hours post induction. Comparison of the whole-cell lysates from strains 1326 and 1326/pTBA270 did not clearly indicate a band due to overproduced AbsA2-His_6 fusion protein (Figure 19, lanes 1 and 2). Nevertheless, there was a significant product with an M_r of ~29kDa in the whole cell lysate and the soluble and particulate fractions of the lysate which could be the fusion protein (Figure 19, lanes 2, 3 and 4). When the soluble phase of the lysate was purified on a nickel column, a 29 kDa protein was eluted at better that 90% purity (Figure 19, lanes 8 and 9), which was in close agreement with the predicted size of 26.2 kDa for AbsA2-His_6.

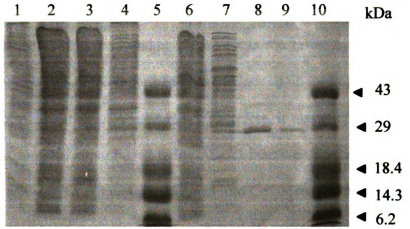


Figure 19. Purification of AbsA2-His_6 overexpressed in *S. lividans*. SDS-PAGE (12%) analysis of His_6-tagged AbsA2 purified from *S. lividans* 1326 containing pTBA270. Lanes: (1) Uninduced 16-hour *S. lividans* 1326; (2) Whole-cell lysate of 12-hour post induction *S. lividans* 1326/pTBA270; (3) Soluble phase of 1326/pTBA270; (4) 6 M urea-solubilized pellet from 1326/pTBA270; (5) Molecular weight markers; (6) Ni^{2+}-column flow through from soluble phase; (7) Ni^{2+}-column wash with 60 mM imidazole; (8) Ni^{2+}-column soluble-phase post-wash eluate (Fraction 1); (9) Soluble-phase eluate (Fraction 2); (10) Molecular weight markers.

It was noticed after repeated purification of 1326/pTBA270 cultures that very low yields of AbsA2-His_6 were obtained in the final eluted fraction. Examination of a low imidazole wash from the nickel column revealed that most of the 29 kDa AbsA2-His_6 was eluted in this fraction, suggesting that at least part of the reduction in yield was due to deterioration of the nickel-column affinity matrix from repeated use. Therefore, the wash sample was dialyzed and analyzed by SDS-PAGE, revealing that AbsA2-His_6 represented the major single band and approximately 30% of the total protein in this sample.

In-Vitro and *In-vivo* Analysis of AbsA2-His_6

Purified AbsA2-His_6 produced in *Streptomyces* was tested by *in-vitro* phosphorylation experiments as described above. Once again I was unable to demonstrate phosphorylation of AbsA2-His_6. Therefore, it was of concern that the His_6 and His_{10} fusion peptides might be rendering AbsA2 inactive. To test the activity of AbsA2-His_6 *in vivo*, pTBA270 was transformed into C570 (see Chapter 3), which carried a chromosomal allele that encoded AbsA2 with a D54E mutation at the proposed site of phosphorylation. Strain C570 caused an antibiotic overproducing phenotype (Pha) which has been associated with an inactive form of AbsA2. When pTBA270 was introduced into C570, antibiotic production was regulated at the wild type level (Figure 20). Conversely, when pIJ6021 (*tipA*-containing overexpression vector without insert (168) was introduced into C570, there was no effect on overproduction of antibiotics (Figure 20). This suggests that the AbsA2-His_6 fusion protein encoded by pTBA270 was

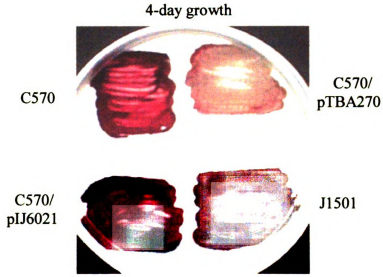


Figure 20. *In-vivo* analysis of pTBA270. The biological function of AbsA2-His_6 encoded by pTBA270 was tested by its ability to complement the Pha phenotype of strain C570, which carries a chromosomal *absA2* (D54E) mutation. Plasmid pTBA270 was transformed into strain C570. Antibiotic production of C570/pTBA270 was compared to that of C570 and wild type J1501. As a control, pIJ6021 (His_6 overexpression plasmid identical to pTBA270 without the *absA2* insert) was also transformed into C570 to test its ability to complement the Pha phenotype. Strains were analyzed after 4 days of growth on SpMR agar.

functional when expressed *in vivo*. Therefore, it was hypothesized that the inability to phosphorylate AbsA2-His_6 *in vitro* may have been due to an inherent instability of this protein under the purification or assay conditions, or a faulty phosphorylation reaction. An alternative to the enzymatic generation of ^{32}P-acetyl phosphate used in this study is the chemical synthesis of ^{32}P-labeled small molecular weight phosphate donors. The latter method has been more widely used for *in-vitro* phosphorylation of RRs (107; 117) and will be implemented in future trials with AbsA2.

Overexpression and Purification of 'AbsA1 from *E. coli*

Although small molecular weight phosphate donors have proven useful for *in-vitro* phosphorylation of various response regulators, there is at least one report in which it did not serve as a suitable donor (107). A preferred approach with several two-component systems has been the purification of cognate HK for use as an *in-vitro* phosphate donor (148; 178; 153; 42). This approach allows for more specific and efficient phosphorylation of RRs (161) as well as the opportunity to study properties of the HK and HK-RR interactions. Given difficulties phosphorylating AbsA2-His proteins with acetyl phosphate, I decided to overproduce and purify AbsA1 to use as a phosphate donor *in vitro*.

Since AbsA1 is membrane bound, a truncated form, 'AbsA1, which lacks the N-terminal transmembrane domain but possesses the entire transmitter and C-terminal domains, was expressed to favor accumulation in the cytoplasm. The truncated alleles of wild type *'absA1* and *'absA1* (H202L), which encodes an H202L change at the putative site of phosphorylation, were ligated into pMAL-C2 to produce N-terminal maltose-binding protein fusions (MBP-'AbsA1) (Figure 17). Protein expression from pTBA350 (MBP-'AbsA1) and pTBA360 (MBP-'AbsA1 [H202L]) was IPTG inducible from a *tac* promoter. The MBP-'AbsA1 fusions have a predicted M_r of 90 kDa. The whole cell lysates from these cultures demonstrated a band of minor intensity at about 90 kDa in comparison to the same strain carrying the pMal-c2 vector with no insert (data not shown). The soluble phase of the JM109/TBA350 cell lysate (Figure 21, lane 1) was purified on an amylose resin column as described in Materials and Methods. Purified MBP-'AbsA1 fusion protein with a predicted M_r of 90 kDa produced a diffuse band of

products ranging from about 75 to 90 kDa (Figure 21, lane 2) suggesting possible proteolysis in the host strain *E. coli* JM109. pMAL-c2-generated fusion proteins contain a Factor Xa recognition sequence situated between MBP and 'AbsA1. Cleavage of purified MBP-'AbsA1 with Factor Xa (Figure 21, lane 4) produced a major band in the range of ~42 to 47 kDa, which was probably composed principally of the liberated MBP domain (~42 kDa) and some 'AbsA1 (predicted M_r of 47 kDa). Another major band represented a product of ~38 kDa and there was also one minor band at ~30 kDa, again suggesting either proteolysis of the fusion protein in the host strain or secondary cleavage sites within 'AbsA1 that were recognized by Factor Xa.

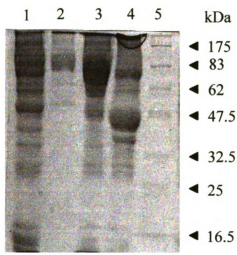


Figure 21. Purification of 'AbsA1 expressed in *E. coli*. SDS-PAGE (10%) analysis of maltose binding protein fusions to truncated *absA1* (*'absA1*) purified from *E. coli* JM109 containing pTBA350. Lanes: (1) Soluble-phase whole-cell lysate from 3-hour post-induction JM109/pTBA350; (2) Post-wash eluate of soluble-phase JM109/pTBA350 purified on an amylose resin column; (3) Concentrated JM109/pTBA350 eluate; (4) Concentrated JM109/pTBA350 eluate treated with Factor Xa; (5) Molecular weight markers.

Autophosphorylation of 'AbsA1 and Phosphorylation of AbsA2-His$_6$

Autokinase activity of MBP-'AbsA1 and the 'AbsA1 Factor Xa cleavage products was tested by addition of [γ-^{32}P] ATP. The major phosphorylation product in the untreated MBP-'AbsA1 sample (Figure 22, lane 1) corresponded to the same diffuse band from ~75 to 90 kDa observed by Coomassie blue staining (Figure 21, lane 2). After proteolysis with Factor Xa, the major phosphorylation products corresponded to the 38 and 30 kDa proteins (Figure 22, lane 4) previously observed by Coomassie blue staining (Figure 21, lane 4). An equivalent concentration of MBP-'AbsA1 H202L was treated

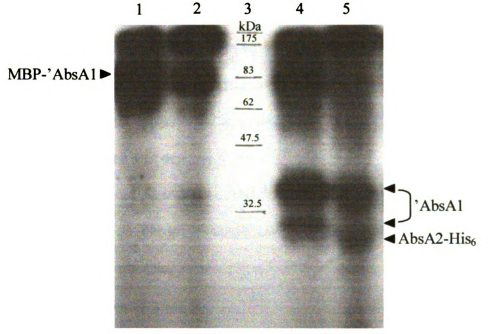


Figure 22. In-vitro phosphorylation of AbsA2-His$_6$ by 'AbsA1-phosphate. Radioactively labeled MBP-'AbsA1-phosphate and 'AbsA1-phosphate were prepared by incubating purified MBP-'AbsA1 and 'AbsA1 (cleaved from MBP by Factor Xa) with [γ-^{32}P] ATP in the presence of Mg^{2+}. After incubation, semi-purified soluble-phase AbsA2-His$_6$ from *S. lividans* was added to each sample. Samples were analyzed by SDS-PAGE (10%) and autoradiography. Lanes: (1) MBP-'AbsA1 + [γ-^{32}P] ATP; (2) MBP-'AbsA1-^{32}P + AbsA2-His$_6$; (3) Molecular weight marker; (4) Factor Xa-treated MBP-'AbsA1 + [γ-^{32}P] ATP; (5) 'AbsA1-^{32}P + AbsA2-His$_6$.

with Factor Xa and subjected to the same phosphorylation conditions, demonstrating no phosphorylation of the 'AbsA1 H202L mutant (data not shown). These results indicate that phosphorylation in MBP-'AbsA1 is occurring in the 'AbsA1 portion of the fusion protein, and that His202 of AbsA1 is required for phosphorylation.

MBP-'AbsA1 and the 'AbsA1 cleavage product were phosphorylated with [γ-^{32}P] ATP and used as donors for the *in-vitro* phosphorylation of AbsA2-His_6 produced in 1326/pTBA270. Semi-purified AbsA2-His_6 was added to each of MBP-'AbsA1-phosphate (Figure 22, lane 2) and 'AbsA1-phosphate (Figure 22, lane 5). Addition of AbsA2-His_6 to 'AbsA1-phosphate produced a strong signal at ~29 kDa (Figure 22, lane 5), which coincided with the size of AbsA2-His_6 in the semi-purified sample. The fact that this product did not appear when incubated with MBP-'AbsA1-phosphate suggested that its phosphorylation was dependent upon the liberated 'AbsA1 domain. In contrast, heterologously produced AbsA2-His_{10}, renatured by dialysis after 6 M urea solubilization and purification, was not phosphorylated under similar conditions. Addition of AbsA2-His_6 to MBP-'AbsA1-phosphate produced a faint signal at ~36 kDa (Figure 22, lane 2). This same signal appeared when [γ-^{32}P] ATP was added to soluble phase 1326/pTBA270 whole-cell lysate in the absence of MBP-'AbsA1 or 'AbsA1 (data not shown). Therefore, this signal appeared to represent a contaminating protein whose phosphorylation did not depend upon the presence of 'AbsA1. Preliminary evidence from these experiments suggests that AbsA2-His_6 could be phosphorylated from 'AbsA1-phosphate after cleavage of the MBP fusion domain.

DISCUSSION

Purification of AbsA2 and AbsA2 D54E was pursued for use in *in-vitro* phosphorylation experiments to support genetic evidence for the role of Asp54 in AbsA2 phosphorylation and activity. Highly purified AbsA2-His_{10} proteins renatured from the insoluble fraction of *E. coli* cultures, and soluble phase AbsA2-His_6 from *S. lividans* were not phosphorylated *in vitro* by acetyl phosphate. Subsequent overproduction of MBP-'AbsA1 fusions was performed to provide an alternative phosphate donor for AbsA2-His fusions. When 'AbsA1-phosphate was reacted with semi-purified AbsA2-His_6 from C270, preliminary results suggested cognate HK-RR phosphoryl-group transfer to form AbsA2-His_6-phosphate. Formation of an active phosphorylated conformer by the AbsA2-His_6 fusion was further supported by its ability to complement mutant AbsA2 D54E *in vivo*. In addition, comparison of autokinase activity by 'AbsA1 and 'AbsA1 H202L provided evidence that His202 is the site of phosphorylation in AbsA1 due to the inability of 'AbsA1 H202L to become phosphorylated under identical conditions in which wild type 'AbsA1 was readily phosphorylated.

Numerous two-component regulators overexpressed in *E. coli* and renatured after 6 M urea solubilization have been successfully phosphorylated *in vitro* by low molecular weight phosphate donors (137; 132; 108). Initial attempts to phosphorylate AbsA2-His_{10}, produced in *E. coli* and renatured after 6 M urea solubilization, were unsuccessful utilizing an enzymatic preparation of ^{32}P- acetyl phosphate. Similarly, AbsA2-His_6 purified from the soluble phase of *S. lividans* was not phosphorylated in this reaction. It is uncertain at this time if these experiments were unsuccessful because the AbsA2-His fusion conformers were inactive as a result of purification or reaction conditions, if there

was a problem with the enzymatic synthesis of acetyl phosphate, or if AbsA2 can not utilize acetyl phosphate as a phosphate donor. The affinities of response regulators to small molecular weight phosphate donors are low (161) and differences in the reactivities of RRs to acetyl phosphate have been reported (118). At least one RR, CheB, has been reported that can not use acetyl phosphate as a phosphate donor (107). An alternative method to that presented in the Materials and Methods for *in-vitro* phosphorylation of RRs utilizes chemically synthesized ^{32}P-labeled small molecular weight phosphate donors (107; 117). Given that certain studies of two-component protein behavior are facilitated by phosphorylation with species such as acetyl phosphate (e.g., AbsA2-P DNA-binding, AbsA1 phosphatase activity), we will continue to investigate expression, purification, and reaction conditions necessary to achieve small molecular weight donor *in-vitro* phosphorylation of AbsA2.

An alternative method that offers specific and efficient *in-vitro* phosphorylation of RRs has been routinely demonstrated using purified truncations of cognate HKs (148; 178; 153). Preliminary data presented here suggests that AbsA2-His_6 was successfully phosphorylated *in vitro* by 'AbsA1-phosphate. Nevertheless, further work needs to be done to optimize overexpression and purification of 'AbsA1 and AbsA2-His_6. The conditions for consistent recovery of highly-purified AbsA2-His_6 from S. lividans need to be reestablished. Similarly, multiple phosphorylation products due to proteolysis of MBP-'AbsA1 tend to confound the results of the *in-vitro* phosphorylation reactions. Proteolysis of MBP-'AbsA1 might be avoided by expression of pTBA350 in an alternative host, such as BL21 which has protease gene knockouts, or possibly by

expressing only the transmitter domain of AbsA1 instead of the transmitter and C-terminal domains.

Finally, while the scope of the experiments presented in this chapter were originally oriented at analyzing the role of Asp54 in phosphorylation and activity of AbsA2, the utility of obtaining purified AbsA1 and AbsA2, and establishing the conditions for *in-vitro* phosphorylation, project into various future pursuits of this project. I have already mentioned determining the phosphatase activity of AbsA1. In addition, AbsA2 in the phosphorylated and unphosphorylated states will be used in gel-shift and footprint assays to determine targets and a possible consensus binding sequence(s) of AbsA2, as well as the requirement for phosphorylation on DNA binding. Another projected use for purified AbsA2 is in the preparation of antibodies for immunoprecipitation and *in-vivo* phosphorylation studies aimed at differentiating between the kinase and phosphatase default models of the AbsA mechanism discussed in Chapter 3.

CHAPTER 6

AbsA2 HOMOLOGUES IN OTHER STRAINS OF *STREPTOMYCES*

INTRODUCTION

Streptomyces are presently the single greatest natural source of chemotherapeutic agents for health and agriculture (165). Nevertheless, a paucity of knowledge on the genetic regulation of antibiotic synthesis in the genus as a whole has traditionally led industry to depended on expensive and laborious random mutagenesis and screening programs to achieve improvements in product yield. Currently, numerous groups are working in the model organism *S. coelicolor* to piece together the complex array of pathways involved in antibiotic regulation (reviewed in Chapter 1; 30; 74; 35). It is hoped that many of the regulatory processes uncovered in *S. coelicolor* will be conserved in other streptomycetes such that a general model for the regulation of antibiotic synthesis can be constructed. New data presented here, along with existing evidence from *Streptomyces* and other genera, lend support to generalized conservation of developmentally regulated processes.

Precedent for the evolutionary conservation of genetic regulation of developmental processes such as sporulation and antibiotic synthesis exists in other Gram-positive bacteria. Producers of class II antimicrobial peptides (AMP) from the genera *Carnobacterium* and *Lactobacillus* show remarkable similarities in the organization and function of genes within clusters encoding their function (reviewed by 96). Regulatory features conserved in these clusters include genes encoding a peptide pheromone precursor, a two-component system, and an ATP-binding cassette (ABC) exporter. Similarly, gene clusters for the regulation and synthesis of lanbiotics by species of *Lactobacillus*, *Bacillus*, and *Staphylococcus* show pronounced conservation of gene organization and function (reviewed by 96). Once again, conserved regulatory elements

include signal precursors, two-component regulators, and transport systems. While these examples demonstrate conserved regulation of the products of a single gene cluster within which both regulatory and biosynthetic genes are found, more complex regulatory schemes may also be conserved.

Conservation of a two-component regulator that functions pleiotropically in differentiation is exemplified by Spo0A. The phosphorylated state of Spo0A triggers the initiation of sporulation in *B. subtilis* by activating and/or repressing transcription of several other regulators encoded on independent operons (reviewed by 68). Youngman and collaborators (21) presented compelling evidence for evolutionary conservation of Spo0A in phylogenetically diverse species of *Bacillus* and *Clostridium*. Based on the similarity of partial or complete DNA sequences, *spoOA* was proposed to be conserved in each of 8 *Bacillus* and 6 *Clostridium* species analyzed. Furthermore, *spo0A* homolog gene disruptions performed in *B. anthracis* and *C. acetobutylicum* demonstrated sporulation deficient phenotypes similar to *spo0A* mutants of *B. subtilis*. Thus, both structural and functional homology were shown to be conserved.

In addition to the examples sited in other Gram-positive bacteria, several observations suggest that numerous aspects of antibiotic regulation may be conserved among streptomycetes. First, throughout the genus *Streptomyces*, antibiotic synthesis is growth-phase dependent, which logically suggests an evolutionarily conserved genetic basis for their temporal regulation. A possible link to temporal regulation is the cell-density dependent accumulation of structurally similar γ-butyrolactones, which has been implicated in signaling the onset of morphological and physiological differentiation in several species of *Streptomyces* (reviewed in Chapter 1). As seen in the production of

peptide antibiotics in other Gram-positive bacteria, a routinely conserved feature of *Streptomyces* antibiotic gene clusters is the presence of one or more pathway specific regulators (reviewed by 30). On a broader scale, preliminary evidence for the conservation of many *S. coelicolor* pleiotropic antibiotic regulatory genes in other streptomycetes has been implied from PCR and Southern hybridization data (*absB* (136); *afsQ* (84); *afsR* (115); *cutRS* (32); *bldA* (101)).

In this chapter, I examine the conservation of the global antibiotic regulator AbsA2 in the genus *Streptomyces*. An attractive feature of AbsA2 is that it acts as a global negative regulator of antibiotics, so that by knocking it out, antibiotic production begins earlier and reaches greater concentrations. If this mechanism were conserved, it could have important economic implications in industrial relevant strains. Directed mutagenesis of AbsA2 homologs would offer an alternative to traditional random mutagenesis and screening protocols for increasing antibiotic yields. Here, I present preliminary data that suggests the existence of AbsA2 homologs in each of ten industrial strains of *Streptomyces* examined.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Streptomyces strains utilized in this study were *S. coelicolor* M600, *S. albus*, *S. ambofaciens* 2035, *S. clavuligerus*, *S. halstedii* JM8, *S. halstedii* 2581, *S. lincolnensis*, and *S. peucetius*. All studies were done with laboratory stocks of chromosomal DNA or samples received from other labs.

Escherichia coli K12 strain DH5α was used for proliferation of plasmids that carried inserts to be sequenced. *E. coli* was grown in culture tubes containing 10 ml of L broth supplemented with 100 μg/ml ampicillin. Cultures were incubated for 12 to 16 hours at 37°C and 250 rpm prior to plasmid extraction and purification on QIAprep spin columns (Qiagen).

PCR Amplification of Putative *absA2* Homologs

Amplification of *absA2* homologs was based on the strategy for *spo0A* homolog amplification (21). Members of the response regulator superfamily share extensive structural (primary, secondary and tertiary) similarity in the N-terminal receiver domain (176; 11). Sequence similarities are even stronger within subfamilies, such that a primer designed to a conserved region of the receiver domain would be expected to prime PCR synthesis from most response regulators of this subfamily. The C-terminal effector domain of RRs is more highly divergent such that primers specific to important functional regions of this domain (e.g., the helix-turn-helix [HTH] DNA-binding motif) would be expected to prime more specifically for homologs of *absA2*. In addition, streptomycetes have a codon preference favoring a high GC content, especially at position three which has a G or C over 90% of the time (16). Consequently, where a codon allowed either G or C at position three, degeneracy was designed into the *absA2* homolog primers to provide this option. Implementing these parameters, 5' forward primers were designed to conserved blocks around residues Asp9 (P10, 5' GCS GAC GAC GAG ACS ATC ATC CGS GCS; where S = G or C) and Asp54 (P9, 5' GCS CTS CTS GAC ATC CGS ATG CCS G) of the receiver domain (Figure 25). To impart

specificity toward *absA2* homologs, a 3' reverse primer (P8, G TGS AGS CGC TGS GCG ATC TCS GCG) was designed around the HTH motif of the effector domain. Primer P8 is specific to the first helix of the HTH motif. While this is not the so-called 'recognition helix', it is projected to possess residues that are important in DNA recognition and binding (67; 11). Five of the eight codons recognized by P8 encode amino acids that are moderately to highly divergent with respect to a HTH motif alignment of RRs of the same subfamily (11). Of these, the most highly divergent codons lie at the 3' and 5' ends of P8.

PCR amplification was carried out in a 100 µl reaction volume containing 100 ng chromosomal DNA template, 1X PCR buffer (with 1.5mM $MgCl_2$), 5% glycerol, 0.2 mM of each dNTP, 40 pmole of each primer, and 2.5U Taq polymerase. Thermal cycler conditions were: denaturation at 95°C for 5 min, followed by 30 cycles of 1 min at 94°C, 1.5 min at 60°C, and 1 min at 72°C, and a final extension at 72°C for 10 min. PCR amplification products were sized by separation on a 1.5% agarose gel with comparison to pBR322 *Hae*III DNA molecular size marker (Boehringer Mannheim).

absA2 Homolog Identification and Sequencing

Southern hybridization of PCR amplification products was utilized to identify putative *absA2* homologs. PCR amplification products were separated on a 1.5% agarose gel. DNA was transferred from the gel to a positively charged nylon membrane (Hybond-N+, Amersham) by capillary transfer (147). DNA was fixed to the membrane by U.V. crosslinking. Hybridization and colorometric detection of an *absA2*-dioxigenin labeled probe was performed as recommended (The Genius System User's Guide for

Filter Hybridization, Boehringer Mannheim) with the exception that all washes were performed at room temperature. Probes were prepared by purifying *S. coelicolor absA2* PCR products with Wizard PCR preparatory columns (Promega) and using random primed DNA labeling of 100 to 300 ng of *absA2* DNA overnight as recommended (The Genius System). In addition to probes prepared from the *S. coelicolor* P8/P9 and P8/P10 PCR products, another primer, P11 (5' SSWSAGGC ASSWSCCSCCSSWSGCSAC; S = G or C; W = A, C, G, or T), was used in combination with P9 to amplify a region of the AbsA2 receiver domain internal to the P8/P10 product to use as a probe against P8/P10 products.

Sequencing of putative *absA2* homologs was performed by cutting the P8/P10 PCR product of interest from 1% low-melting-point agarose gel and purifying with a QIAquick Gel Extraction Kit (Qiagen). The purified product (10 ng) was used as template for PCR amplification with primers WC8 (5' TTT TAG ACT TGA CGA CGA GAC SAT CAT CCG SGC SGG G) and WC9 (5' TTT TAG ATC TGT GSA GSC GCT GSG CGA TCT CSG CG), which are identical to primers P10 and P8, respectively, except that they contain *Blg*II restriction sites on their ends. The WC8/WC9 PCR products were digested with *Bgl*II and purified on Wizard PCR preparatory columns (Promega). The *absA2 Bgl*II homologs were ligated into *Bam*HI-digested pBluescript II SK+ (Stratagene) sequencing vectors and sequenced as described by Anderson, et al. (1999). The resulting sequence was compiled, analyzed and compared to that of *S. coelicolor absA2* using the Wisconsin GCG software package.

RESULTS

PCR Amplification of Putative *absA2* Homologs from Heterologous DNA

Primers specific to highly conserved regions of the receiver domain and the HTH motif of the effector domain were used to amplify putative *absA2* homologs from chromosomal DNA of industrially important strains of *Streptomyces*. Receiver domains of the same subfamily of RRs show extensive sequence similarity. Therefore, primers P10 and P9 were expected to prime numerous RRs of the AbsA2-containing subfamily since they were specific to highly conserved regions around codons for Asp9 and Asp54 of AbsA2. Conversely, primer P8, which was specific to the more highly divergent HTH encoding region of the effector domain, was predicted to permit more specific priming of *absA2* homologs. Furthermore, G-C base degeneracy was designed into the third position of codons where either base encoded the same residue to allow for silent mutations in evolutionary divergence, while maintaining the high G-C codon preference of streptomycetes.

The PCR products obtained using primer combinations P8/P9 and P8/P10 on chromosomal templates from *S. coelicolor* and eight industrial stains are shown in Figure 23. *S. coelicolor* DNA generated a single product of expected molecular size from each primer combination (P8/P9, 360 nt, Figure 23A lane 2; P8/P10, 522 nt, Figure 23B lane 2). Conversely, an assortment of products of various sizes and intensities were amplified from the other strains. Every strain except *S. halstedii* JM8 and *S. albus* generated a product of the same size as *S. coelicolor* M600 from primers P8/P9, some of which were very concentrated (Figure 23A). Primer combination P8/P10 only generated a product equivalent in size to M600 from *S. ambofaciens*, although both *S. griseus* and *S. albus*

generated products only slightly smaller or larger (Figure 23B). In addition, each of these P8/P10 products was of very modest abundance in comparison to many of those amplified with P8/P9.

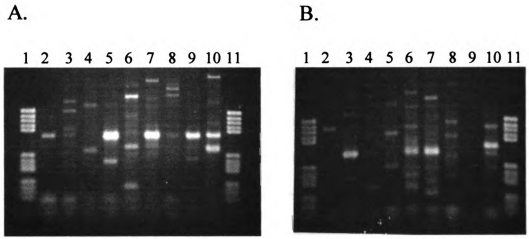


Figure 23. PCR amplification of putative *absA2* homologs. Putative homologs of *absA2* were amplified with primer pairs P8/P9 (A.) and P8/P10 (B.). Reactions were carried out in 100 µl volumes containing 100 ng chromosomal DNA template, 1X PCR buffer (with 1.5 mM $MgCl_2$), 5% glycerol, 0.2 mM of each dNTP, 40 pmole of each primer, and 2.5U Taq polymerase. Thermal cycler conditions were: denaturation at 95°C for 5 min, followed by 30 cycles of 1 min at 94°C, 1.5 min at 60°C, and 1 min at 72°C, and a final extension at 72°C for 10 min. PCR amplification products were sized by separation on a 1.5% agarose gel. Lanes; (1) DNA molecular weight marker pBR322 *Hae*III; (2) *S. coelicolor* M600; (3) *S. lincolnensis*; (4) *S. halstedii* JM8; (5) *S. griseus*; (6) *S. albus*; (7) *S. avermitilis*; (8) *S. cinnemonium*; (9) *S. halstedii* 2581; (10) *S. ambofaciens*; (11) DNA molecular weight marker pBR322 *Hae*III.

Identification of Putative *absA2* Homologs

Although major amplification products from P8/P9 corresponded in size to *absA2*, there were numerous other products of significant abundance generated in almost every

strain. Moreover, the major products amplified from P8/P10 were not of expected size. Therefore, in order to determine if any of these products represented possible *absA2* homologs, they were transferred to nylon membranes and hybridized against *S. coelicolor* P8/P9 or P8/P10 *absA2* probes.

The Southern blot of P8/P9-generated products provided strong evidence that genes of similar size and sequence to that of *absA2* were amplified from each of the strains tested (Figure 24A). Not only was hybridization specific to fragments of the expected size on these blots, but the *absA2* probe revealed products of similar size in *S. halstedii* JM8 and *S. albus* that were not visible on the gel alone. The lack of hybridization to fragments of other sizes together with the appearance of signals in *S. halstedii* JM8 and *S. albus*, suggested high annealing specificity for full-length products of similar sequence to that of *absA2*.

In marked contrast, the Southern blot of P8/P10-generated products (Figure 24B) demonstrated a hybridization pattern virtually identical to the pattern of amplification products seen on the gel. There was no apparent specificity for products of equivalent size to P8/P10 *absA2*. It was suspected that hybridization conditions for this blot were not stringent enough to exclude annealing based solely on primer recognition. Therefore, a fragment internal to P8/P10 was generated from M600 and hybridized to the P8/P10 products. The new P9/P11 probe recognized a 210 bp region of the receiver domain of *absA2*. Remarkably, when this probe was hybridized to P8/P10 PCR products, essentially all signals corresponded in size to *absA2* (Figure 24C). As observed in the P8/P9 blot, hybridization signals were revealed for *S. lincolnensis*, *S. halstedii* JM8, and *S. cinnemonium* that were not visible on the gel. In their initial rounds of PCR

amplification of *spo0A* homologs using a similar approach, Brown et al. (1996) obtained products of similar size to those of *spo0A* from four out of eight *Bacillus* species. Here, Southern hybridization of PCR products suggested that homologs of similar size to *absA2* existed in each of the strains tested. Indeed, sequence analysis of the amplified region of four putative homolog genes confirmed that these were of similar size to the same region of *absA2* (see below).

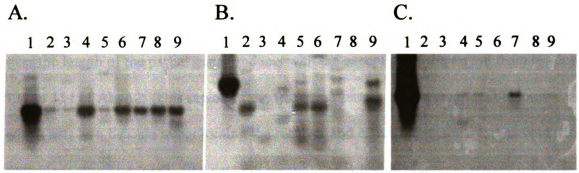


Figure 24. Southern blots of putative *absA2* homologs. Putative *absA2* homologs generated by PCR from primer pairs P8/P9 and P8/P10 were hybridized to *absA2* probes generated from *S. coelicolor* M600. (A.) P8/P9 PCR products hybridized to a P8/P9 *absA2* probe; (B.) P8/P10 PCR products hybridized to a P8/P10 *absA2* probe; (C.) P8/P10 PCR products hybridized to a P9/P11 *absA2* probe. Lanes: (1) *S. coelicolor* M600; (2) *S. lincolnensis*; (3) *S. halstedii* JM8; (4) *S. griseus*; (5) *S. albus*; (6) *S. avermitilis*; (7) *S. cinnemonium*; (8) *S. halstedii* 2581; (9) *S. ambofaciens*.

Sequence Analysis of Putative Homologs

Given the promising results obtained from Southern hybridizations, several PCR products were sequenced to examine their similarity to *absA2*. Although products from P8/P9 were more abundant, I chose to sequence P8/P10 products because they were 180 nt larger, encoding a region from the extreme N-terminus through to the HTH motif of AbsA2. PCR products from *S. griseus* and *S. ambofaciens* were gel purified and used as

templates for amplification from primers WC8 and WC9, which were identical to P8 and P10 but had *BglII* restriction sites designed into their ends to facilitate cloning of the amplified genes. In addition, it was found that P8/P10 PCR products of similar size to that of *absA2* could be generated from *S. peucetius* and *S. clavuligerus* (data not shown). The sequence of these products was also determined.

An alignment of the translated sequences from *S. ambofaciens*, *S. griseus*, *S. peucetius*, and *S. clavuligerus* with that of AbsA2 are shown in Figure 25. Inspection of the consensus sequence from this alignment revealed substantial amino acid similarity over the length of the partial gene products with the exception of the region from residue 138 to 154 of AbsA2. This region of the RR encompasses a solvent accessible loop which acts as a flexible tether joining the receiver and effector domains. It is devoid of secondary structure (prediction data not shown) and is highly divergent among response regulators (131; 11). A second apparently divergent region was observed between residues 67 and 81 of AbsA2; however, closer inspection revealed that only a four amino acid stretch from position 70 to 73 was highly variable. Comparison with crystolgraphic data from NarL RR predicted this small region to encompass a loop between helix α-3 and strand β-4 of the receiver domain (11; AbsA2 modeling data not shown).

Although the alignment shows significant sequence similarity over most of the amplified region, receiver domains from different RRs of the same sub-family tend to be

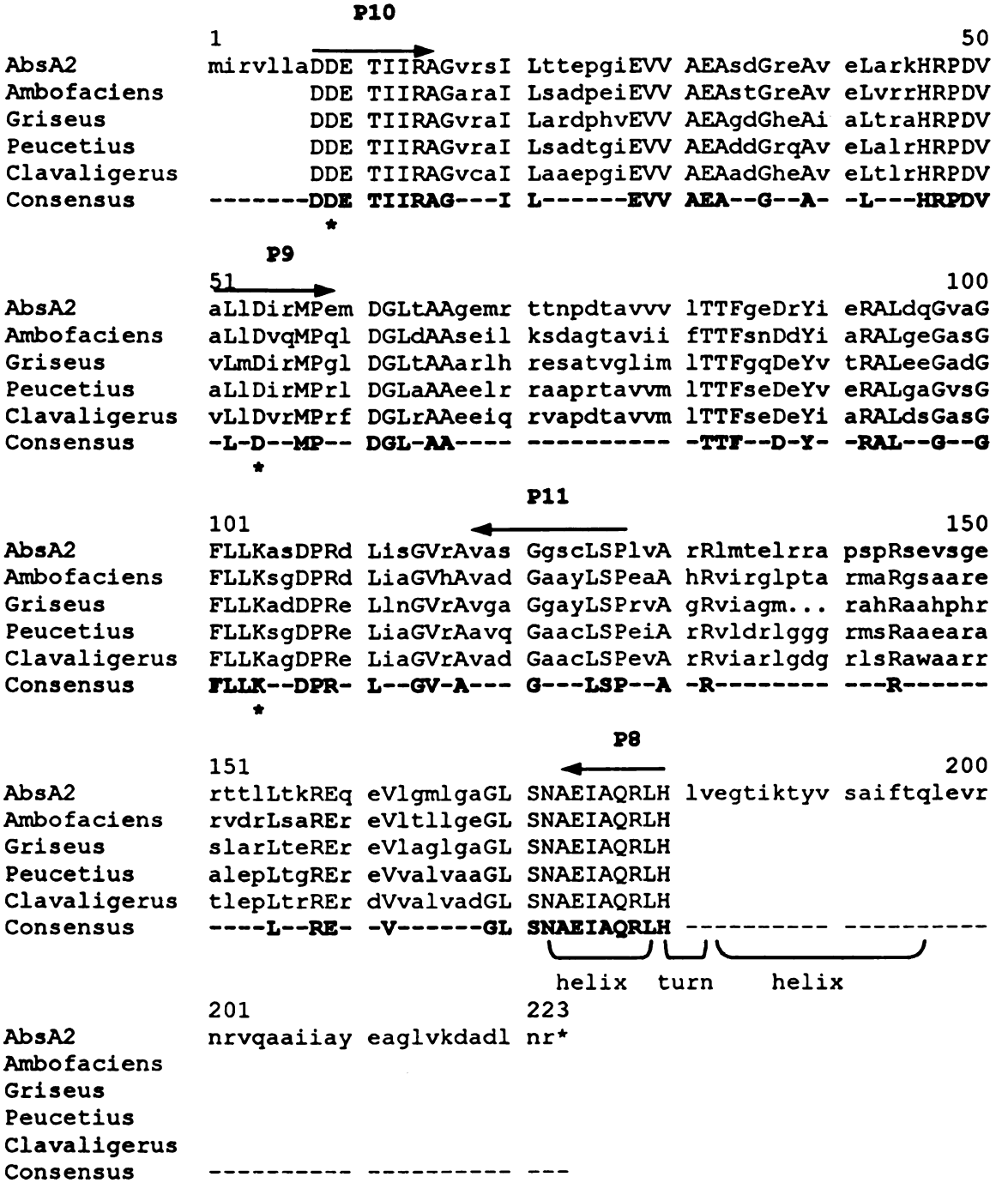


Figure 25. Amino acid sequence alignment of *S. coelicolor* AbsA2 with putative homologs from *S. amobofaciens*, *S. griseus*, *S. peucetius*, and *S. clavuligerus*. A consensus sequence of residues conserved in all five strains are in bold type. Primers P8 and P10 were used to amplify these genes. The orientation and location of amino acid condons recognized by primers P8, P9, P10, and P11 are indicated by arrows. Highly conserved residues within the acidic pocket are indicated (*). The predicted location of the helix-turn-helix DNA-binding region of AbsA2 is also identified.

the most highly conserved region of the RR. Therefore, regions of similarity were sought that might set the AbsA2 homolog apart from other RRs. Various residues were conserved among the putative AbsA2 homologs that were highly variable in RRs of the same sub-family as AbsA2. For example, amino acids H46 and R47 of AbsA2 were highly divergent not only among RRs from different genera, but also from an alignment of eleven randomly chosen *S. coelicolor* RRs of the same subfamily as AbsA2 (data not shown). These residues were predicted by homology modeling of AbsA2 to the NarL crystalography structure to represent the last amino acid of helix α-2 and the first amino acid of the α-2 to β-3 loop (11), which is one of the most highly solvent exposed regions of the receiver domain (176). It is tempting to speculate that these and possibly other uniquely conserved residues are important in cognate HK-RR recognition, or some other system-specific function.

A comparison of each homolog sequence with that of AbsA2 produced the following amino acid similarities and identities: *S. griseus*, 78% similarity, 60% identity; *S. amobofaciens*, 78% similarity, 61% identity; *S. clavuligerus*, 67% similarity, 63% identity; and *S. peucetius*, 69% similarity, 63% identity. Excluding the highly divergent linker region, which constituted about 10% of the sequence, amino acid identities would have been around 70%. Brown, et al. (1996) did not report amino acid identities between *B. subtilis* Spo0A and its homologs retrieved by PCR. They did, however, mention that DNA sequence identity for *spo0A* homologs from different strains of *C. acetobutylicum* was as low as 66%. DNA sequence identity between putative *absA2* homologs from different species tested here ranged from 70 to 72%. Sequence identity between homologous proteins of *Streptomyces* has been shown to vary greatly. The sigma factor,

σ^F, required for sporulation in streptomycetes was found to be 87% identical between *S. coelicolor* and *S. aureofaciens* (134). In another study, five polyketide synthase (PKS) genes encoding the type II polyketide antibiotic frenolicin in *S. roseofulvus*, demonstrated approximately 40 to 70% amino acid identity with similar PKS proteins from four other species of *Streptomyces* (17). Finally, a BLAST search of the nearly complete *Streptomyces* genome sequence (http://www.sanger.ac.uk/Projects/S_coelicolor/) recognized 52 RRs (in addition to *absA2*) with full-length similarity to AbsA2. Amino acid identities for all but one RR (excluding AbsA2) ranged from 26 to 49%, well below the 60 to 63% identity obtained here from putative *absA2* homologs from other species of *Streptomyces*. However, one RR found on cosmid St8D11 was 55% identical to AbsA2 and contained residues highly conserved in the putative homolog proteins. This locus will be discussed below.

DISCUSSION

Evidence exists for the evolutionary conservation of developmental regulation in Gram-positive bacteria. The well characterized Spo0A response regulator, which triggers sporulation in *B. subtilis*, was shown to be conserved in all species of highly diverse *Bacillus* and *Clostridium* tested (21). Using a strategy similar to that employed for Spo0A, degenerate PCR primers with varying specificities for *absA2* successfully amplified nucleotide sequences of the same size as *absA2* from ten industrial strains of *Streptomyces*. Southern hybridization analysis demonstrated that only those products that were the same size as *absA2* annealed with *absA2* probes. Yet, it was quite possible that RR genes with full-length sequence similarity to *absA2*, but different biological function,

were being primed and amplified. Therefore, the next step in confirming whether or not these were actual homologs of *absA2* was to inspect their sequence.

Sequence analysis of putative homologs from *S. griseus*, *S. ambofaciens*, *S. clavuligerus*, and *S. peucetius* revealed amino acid identities with *absA2* of approximately 60%. By comparison, *S. coelicolor* and *S. aureofaciens* homologs of the highly conserved σ^F sigma factor, required for transcription of late sporulation genes, shared 87% sequence identity (134). In contrast, homologous PKS genes, that synthesize different polyketide antibiotics through similar enzymatic reactions, have amino acid similarities that commonly range from just 40 to 60% (17). Moreover, only one *S. coelicolor* gene encoding a RR with full-length similarity to AbsA2 has yet demonstrated greater than 49% amino acid identity. Therefore, the fact that partial gene sequences from heterologous strains demonstrate at least 60% identity is suggestive evidence that these genes encode AbsA2 homologs.

A blast search conducted on the nearly complete *S. coelicolor* genome identified a RR from cosmid St8D11 that is 55% identical to AbsA2. The St8D11 RR possesses the highly conserved residues of putative AbsA2 homologs that are missing from other RRs of the same sub-family. Also, the HTH region of St8D11 RR shows 54% identity and 83% similarity to that of AbsA2. Not only is the St8D11 RR highly similar to AbsA2, but the St8D11 HK is a remarkable 44% identical to full-length AbsA1. These HKs share significant similarity in the C-terminus of the sensor domain, which is normally highly divergent among HKs. Moreover, the St8D11 HK has a C-terminal domain that is approximately 40% identical to that of AbsA1, making it the only protein from any database to demonstrate similarity to this region of AbsA1.

Streptomyces have linear chromosomes, the ends of which are genetically unstable, undergoing deletion and duplication events at a rather high frequency (reviewed by 175). As a likely consequence of this instability, no *S. coelicolor* housekeeping genes are found within at least several hundred kilobases of the chromosomal ends. Cosmid St8D11 lies very close to the end of the chromosome on restriction fragment AseI-A (140). According to Kieser et al. (1992), this region of the J1501 chromosome seems to have undergone several deletions and possibly duplication events in comparison to strain M145, which is the progenitor of J1501 and the strain currently being sequenced. Thus, it is uncertain whether the St8D11 two-component system is present in *S. coelicolor* J1501, the strain from which *absA* was isolated and the strain used in this study. Given its remarkable similarity to AbsA, it is possible that the St8D11 two-component system functions in antibiotic regulation, but is missing from J1501. Studies are currently underway to determine whether the St8D11 two-component system is present in J1501 and whether it is a regulator of antibiotic synthesis or sporulation.

Future amplification of *absA* homologs might take advantage of the unique C-terminal domain of AbsA1. This domain is about 160 amino acids long, is of unknown function, and shows homology to only one other *S. coelicolor* HK. Assuming that this domain is conserved, it could serve as a criterion for amplifying *absA* homologs. One strategy would be to prime for regions of the RR receiver domain and the HK transmitter domain that are highly conserved and of specialized function in two-component systems. This should reduce the amplification of spurious products of varying size. At the same time, putative homologs with a C-terminal *absA1* domain would generate products

approximately 500 bp larger than those of two-component systems with 'orthodox' primary structure.

The recently revealed association of *absA* within the *cda* gene cluster raises obvious questions about the possible conservation of *absA* throughout the genus *Streptomyces*. Can it be simply coincidental that a global regulator of antibiotics is located in an antibiotic gene cluster? If AbsA was originally associated only with *cda* regulation, how did it expand its range to regulate all *S. coelicolor* antibiotics? If AbsA was originally specific to *cda* regulation, in a manner similar to pathway-specific regulators, why don't more antibiotic gene clusters possess two-component systems? It is still not clear whether the AbsA two-component system is an idiosyncrasy of *S. coelicolor* or a highly conserved mechanism of antibiotic regulation throughout streptomycetes. Further analysis into the conservation of *absA* may help establish whether specific mechanisms of pleiotropic antibiotic regulation tied to multicellular development are highly conserved in *Streptomyces* or predominantly unique due to random evolutionary pressures of the organisms environment.

CHAPTER 7

CONCLUSIONS AND FUTURE RESEARCH

Prior to this study the AbsA two-component signal transduction system had been shown to globally regulate antibiotics of *S. coelicolor*. Interestingly, *absA* mutants were associated with dramatically opposing phenotypes. Mutations mapped to the *absA1* histidine kinase gene caused global inhibition of all four *S. coelicolor* antibiotics (Abs$^-$ phenotype), while gene disruptions of *absA* caused the early onset and overproduction of Act and Red (Pha phenotype) (19). Given the probable cotranscription of *absA1* and *absA2*, it was hypothesized that AbsA2 was a negative regulator of antibiotic production since gene disruptions in *absA* caused the Pha phenotype. This study sought to understand the basis for the dramatically opposed phenotypes obtained from *absA* mutants through further molecular genetic characterization of the *absA* locus and an examination of basic aspects of the AbsA two-component mechanism. Definition of AbsA2 as a positive or negative regulator was essential to more fully understanding any possible interactions with other antibiotic pathway-specific or pleiotropic regulators. Likewise, knowledge of the biochemical mechanism of signal transduction was considered prerequisite to defining more complex characteristics of the AbsA mechanism such as target binding and signal sensing.

Molecular Genetic Characterization of *absA1* Mutations Responsible for the Abs$^-$ Phenotype and Certain *sab* Suppressors of Abs$^-$

Mutations responsible for the Abs$^-$ phenotype had previously been localized to the *absA1* histidine kinase gene (2; 19). Sequence analysis of *absA1* from two independently isolate Abs$^-$ mutants, C542 and C577, identified point mutations that caused amino acid substitutions to the transmitter domain of AbsA1. The mutations to C542 (I360L and

R365Q) were contained within the G-box, which is proposed to play a critical role in phosphotransfer (158). The C577 mutation (L253R) was found in a region of moderate conservation among histidine kinases termed the X-box (79). X-box mutations in EnvZ (79), NtrB (9) and DegS (169) lock each of these HKs into a kinase dominant, phosphatase deficient state. Sequence characteristics of the Abs$^-$ mutations, taken together with results obtained from the genetic and transcript analyses of the *absA* locus (Chapter 3), lead to the hypothesis that mutations identified in C542 and C577 lock AbsA1 into a kinase dominant, phosphatase deficient state which causes constitutive negative regulation of antibiotics. It is uncertain at this point whether the Abs$^-$ mutants are signal-independent; however, the early accumulation of *absA* transcript together with the stability of the Abs$^-$ phenotype over days and weeks suggests signal-independent behavior.

Abs$^-$ stains C577 and C542 undergo apparent pseudoreversion to attain wild type (Type I) or Pha (Type II) levels of antibiotic production. Genetic mapping of five *sab* (suppressor of *abs*) mutants placed the mutations responsible for this phenotype very close to the *absA* locus (Appendix A). Marker exchange experiments demonstrated that restoration of antibiotic production was due to second-site suppressors of the Abs$^-$ mutations. The mapping data suggested that the *sab* mutations were located downstream of the *absA1* mutations. Therefore *absA2* was sequenced in the five mapped *sab* strains plus six unmapped *sab* mutants. Two *sab* mutations were identified in *absA2* while a third contained a deletion that included most of *absA1* and all of downstream *absA2*. The *absA1* gene was then sequenced in 2 of the 4 remaining mapped *sab* strains. Both of these were found to contain a *sab* mutation. Six remaining *sab* mutants that are wild type

for *absA2*, have not been sequenced for *absA1*. Second-site suppressors are a useful tool for finding additional members of a regulatory pathway. Identifying whether the remaining *sab* mutations lie in *absA1* would provide valuable insight into whether another locus is involved in the AbsA regulatory pathway. If all *sab* mutations were localized to *absA*, the likelyhood that AbsA functions through an intermediate regulator (e.g., that AbsA2 is an activator of a repressor) would be diminished. If a mutation was found to lie outside *absA*, then evidence would exist for the involvement of a second gene product. Four *sab* mutants that do not possess mutations in *absA2* are Type II (Pha); three of these are unmapped. An initial experiment to test whether these mutants possess a *sab* mutation in *absA1* would be to transform them with pCB200 (19) containing a truncated, functional form of *absA1*, or with pCB520 (Chapter 3) carrying the entire *absA* locus. If a *sab* mutation causing the Pha phenotype lies outside of *absA1*, the *absA* clones would not be expected to complement the mutation. Sab mutants not complemented by the *absA* clones could first be sequenced for *absA1* to assure that there is not a dominant Pha-producing mutant, and then studied through mapping and complementation to isolate the suppressor locus.

The Role of Phosphorylation in the AbsA Regulatory Mechanism

Disruptions in the *absA1* and *absA2* genes demonstrated that the AbsA two-component system is a negative regulator of the multiple antibiotics produced by *S. coelicolor*, including calcium-dependent antibiotic, actinorhodin and undecylprodigiosin. While synthesis of Act and Red had previously been shown to be accelerated in *absA* disruptions (19), this is the first demonstration that *absA* mutants also cause early onset

and overproduction of CDA. The *absA* locus was recently found to lie within the *cda* gene cluster. Thus, AbsA is also the first antibiotic-gene-cluster-associated regulator that demonstrates regulatory activity outside of its cluster.

Much of the attention of this study was focused on defining the role of phosphorylation in AbsA2-mediated negative regulation. Gene replacements in the *absA* locus altered the putative sites of phosphorylation of AbsA1 or AbsA2. As predicted from sequence conservation with other two-component systems, both the His at position 202 of AbsA1 and the Asp residue at position 54 of AbsA2 were required for normal regulation of antibiotic synthesis. Furthermore, each of the gene replacement strains tested attained an antibiotic overproducing phenotype (Pha) consistent with a mechanism in which AbsA2-P is the active negative regulator of antibiotic synthesis. In addition, high-copy expression of AbsA2 D54E was not able to complement the Pha phenotype of an *absAΔ* strain; moreover, it caused overexpression of antibiotics in a *absA* wild type background. Taken together, these results suggest that AbsA2 activity is strongly dependent upon phosphorylation.

Transcription of the *absA* locus also demonstrated phosphorylation-dependent autoregulation of *absA* expression. Transcript studies of *absA* suggest either positive autoregulation by AbsA2-P or negative autoregulation by unphosphorylated AbsA2. Data from this study coupled with precedent from other two-component systems favors a model for phospho-AbsA2-mediated positive autoregulation. First, the genetic data presented in Chapter 3 showed AbsA2-P to be the active regulator of antibiotics with a strong dependence on phosphorylation for activity. Likewise, the absence of growth-phase-related change in the *absA* transcript in C570 Pha strain (Chapter 3) suggests that

AbsA2-P was responsible for the growth-phase increase observed in J1501 and C542. Finally, the majority of response regulators from other two-component systems are activated by phosphorylation (161). Therefore, I predict that AbsA2-P is not only the active negative regulator of antibiotic synthesis, but that it also positively autoregulates its own expression. An experiment that could resolve whether *absA* autoregulation is positive or negative would examine *absA* expression in *absAΔ* strain C577S25 (Appendix A). C577S25 possesses a deletion in all of *absA2* and most of *absA1*, excluding the promoter region and part of the 5' region of *absA1* encoding the sensor domain. If *absA* positively autoregulates its own expression, this strain would be expected to contain low levels of transcript over the course of growth, similar to C570. Conversely, if *absA* expression is negatively autoregulated, C577S25 *absAΔ* should produce elevated levels of transcript similar to that of C542.

Model Summary

A working model which accommodates both the genetic and transcriptional data summarized above is illustrated in Figure 26 and supposes the following. Early in growth, a culture is not competent for antibiotic synthesis and the *absA* genes are expressed at a basal level. In the absence of signal, AbsA1 is in a phosphatase dominant form. Following a period of growth, the culture enters the "transition stage." During this time, the signal that regulates AbsA may be present at significant levels. Once the signal is present, AbsA1 is shifted to a kinase-dominant form and AbsA2-P will accumulate. AbsA2-P negatively regulates antibiotics and also positively autoregulates *absA* expression, accounting for the AbsA2-P-dependent, growth-phase-related increase of

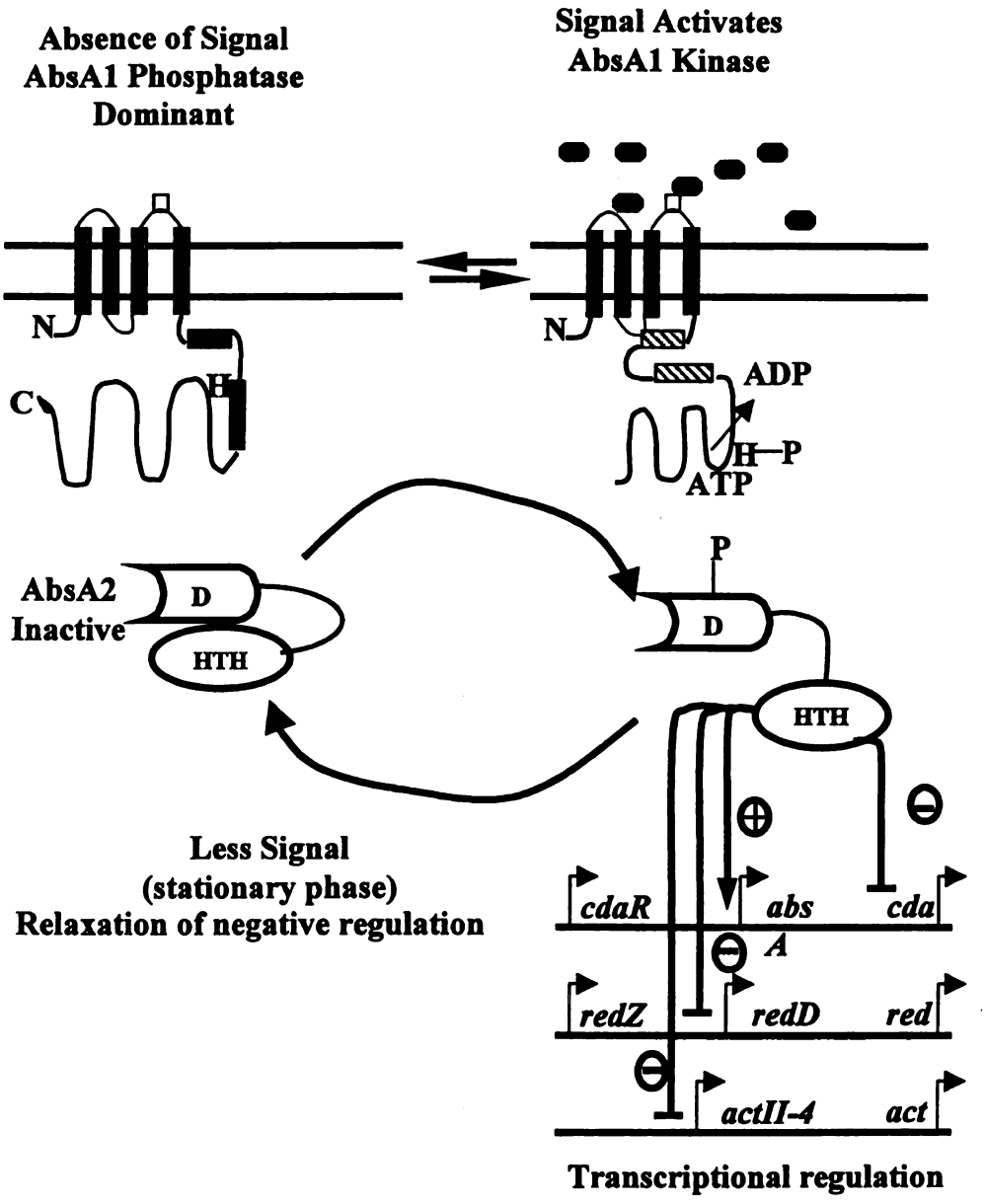


Figure 26. Model of AbsA-mediated regulation of antibiotic production in S. coelicolor.

absA transcript seen in J1501. A signal-concentration-dependent equilibrium is established between the kinase and phosphatase forms of AbsA1, such that the level of signal determines the extent to which antibiotics are repressed. Easing of AbsA-repression may require that the signal be depleted or degraded. As signal decreases, the AbsA1 kinase/phosphatase equilibrium would shift toward the phosphatase form, causing dephosphorylation of AbsA2 and a decrease in the negative regulation of antibiotic gene expression.

This model proposes the establishment of an AbsA1 signal-dependent kinase/phosphatase equilibrium. Genetic evidence for AbsA1 autokinase activity was demonstrated by loss of function phenotypes in gene replacements that targeted the transmitter domain or His202 of AbsA1. Furthermore, purified MBP-'AbsA1 (H202L) was not phosphorylated in vitro under the same conditions in which wild type MBP-'AbsA1 was readily phosphorylated, suggesting that AbsA1 is phosphorylated at this residue. Similar direct evidence for AbsA1 phosphatase activity toward AbsA2-P has yet to be demonstrated. However, genetic evidence from two experiments can be interpreted to support phosphatase activity by AbsA1. First, overexpression of wild type or an H202L mutant of AbsA1 in a wild type *absA* background caused a Pha phenotype (Chapter 3). These results are consistent with a decrease in the proportion of AbsA2-P, and thus an increase in the number of AbsA1 molecules exercising phosphatase activity. Second, in a recent experiment, pTBA155 carrying *absA1** (H202L) (Chapter 3) was transformed into C542. If AbsA1* H202L possesses phosphatase activity, I would expect some level of restoration of antibiotic production to strain C542, which is hypothesized to be locked in a kinase dominant, phosphatase deficient state. The

C542/pTBA155 strain attained wild type levels of antibiotic regulation, which suggests AbsA1 H202L phosphatase activity. The assumption that AbsA1 H202L can possesses phosphatase activity while being kinase deficient is consistent with phosphatase activity demonstrated by other HKs carrying mutations at the conserved histidine residue (80). A more direct analysis of AbsA1 phosphatase activity would test purified 'AbsA1 (Chapter 5) in-vitro phosphatase activity against purified AbsA2-His_6-P.

The principle focus for the immediate future of this project will be to refine and extend the model of AbsA-mediated regulation to include the identification of targets of AbsA2 regulation and the signal-sensing mechanism of AbsA1. A discussion of additional data generated in this study will be presented in the context of future work in these two areas.

AbsA2 Targets

A previous study demonstrated that mutations responsible for Abs$^-$ and Pha phenotypes correspondingly affected transcription of *redD* and *actII-ORF4* (1), thus establishing these pathway-specific activators as part of the AbsA regulatory pathway and possible targets of AbsA2. RedZ is a second pathway-specific regulator of the *red* biosynthetic pathway which appears to activate expression of *redD* (182). I found that mutations in *absA* had no affect on the expression of *redZ*, suggesting that it is not a target of AbsA2 regulation. Interestingly, RedZ is a "pseudo response regulator" which possesses full-length sequence similarity to AbsA2, but has lost the requirement for phosphorylation (65). RedZ possesses a HTH motif in its effector domain, making it possible that AbsA2 and RedZ compete for binding at the *redD* promoter, although

probably recognizing different sequences. Exclusion of *redZ* from the AbsA regulatory pathway makes the *redD* promoter an attractive potential target of AbsA2-mediated regulation of Red synthesis.

The effect of AbsA mutants was also tested on the expression of *cdaR*, a putative pathway-specific activator of the *cda* gene cluster (156). While *redD* and *actII-4* were consistently repressed in Abs$^-$ mutants over the course of growth (1; Chapter 4), the expression of *cdaR* was equivalent in Abs$^-$, Abs$^+$ and Pha cultures by the time these reached the stationary phase (Chapter 4). Only at the early 18 hr time point was there a difference in the levels of expression of *cdaR*; however, both the Abs$^-$ and Pha cultures had more abundant levels of *cdaR* transcript than J1501. The Abs$^-$ strain does not produce CDA, so if *cdaR* is an activator of CDA biosynthesis and a target of AbsA2, I would expect it to be repressed in the Abs$^-$ mutant. New evidence has demonstrated that Abs$^-$ mutants dramatically reduce expression of various *cda* biosynthetic operons over the course of growth (Ryding, unpublished), explaining the CDA$^-$ phenotype of Abs$^-$ mutants. Inspection of the *S. coelicolor* genome sequence reveals potential additional regulatory genes adjacent to the *cda* cluster (146). Thus, AbsA may be acting at a target other than *cdaR* in the *cda* gene cluster. Nonetheless, CDA production is evident several hours before the appearance of Red, and *cdaR* expression was evident at the earliest time point in which *absA* was witnessed. Therefore, I can not yet exclude that AbsA may be regulating the early expression of *cdaR*. As such, *cdaR* expression will be examined at closely grouped time points between mid-exponential and stationary phase growth of Abs$^-$, Abs$^+$, and Pha strains. Growth curves will be generated to assure that similar time points from each culture represent the same stage of development. Likewise, assays will

be conducted to determine the timing and abundance of CDA in the Abs^+ and Pha cultures. At the same time, S1 analyses will be performed on other putative regulators of the *cda* gene cluster to determine their dependence on AbsA and possible involvement in the AbsA regulatory pathway.

It is uncertain at this time whether the autoregulation of *absA* expression is direct or indirect. Nevertheless, an obvious target to begin testing for AbsA2 binding is the *absA* promoter region. The *absA* promoter has an imperfect heptameric inverted repeat, a motif frequently recognized by response regulators. The sequence and arrangement of the heptameric repeat sequence is loosely conserved in other possible targets of AbsA2 (e.g., *redD* and *actII-4*). Initial targets to be used in gel-shift AbsA2 DNA-binding assays will include the promoter regions of *absA*, *redD*, and *actII-4*. Successful target identification with gel-shift assays could be followed by DNaseI or hydroxy-radical footprint analyses to identify a possible AbsA2 consensus binding sequence, the identification of which could be valuable in the search for other targets of AbsA2, for example in the *cda* cluster.

Gel-shift assays will utilize purified AbsA2. Purification of AbsA2 and AbsA2 D54E was pursued for use in *in-vitro* phosphorylation experiments to support genetic evidence for the role of Asp54 in AbsA2 phosphorylation and activity. AbsA2-His-tag proteins purified from *E. coli* and *S. lividans* were not phosphorylated *in vitro* with enzymatically synthesized ^{32}P-acetyl phosphate under the conditions tested. Genetic data strongly suggest that AbsA2-P is required for target recognition. Therefore, it may prove necessary to resolve the problems with overexpression and *in-vitro* phosphorylation of AbsA2 in order to perform *in-vitro* DNA-binding studies. The following strategy is

being pursued. AbsA2-His_6 overexpression will concentrate on the *Streptomyces* overexpression system since, i.) AbsA2-His_6 was shown to be functional *in vivo* when expressed in *S. coelicolor* C570; ii.) a good yield of soluble phase AbsA2-His_6 was produced in the soluble phase, thus avoiding denaturation and renaturation (i.e., excess handling); and iii.) preliminary evidence exists for the *in-vitro* phosphorylation of AbsA2-His_6 by purified phospho-'AbsA1. A steady decrease in product yield was seen over time in the *Streptomyces*/Ni^{2+} expression/purification system. Suspected causes include plasmid instability in the *S. lividans* host and purification column degradation. I am currently examining the stability of the pTBA270 AbsA2 expression plasmid in strain C570 and establishing overexpression and purification conditions that will lead to consistent recovery of soluble phase AbsA2-His_6.

Both AbsA2 DNA-binding studies and determination of AbsA1 phosphatase activity would be facilitated by AbsA2-His_6 *in-vitro* phosphorylation by small molecular weight phosphate donors. Once reproducible recovery of AbsA2-His_6 has been achieved, *in-vitro* phosphorylation trials will be conducted using chemical synthesis of ^{32}P-acetyl phosphate. This method (118) has been employed more frequently and for a greater number of response regulators than the enzymatic preparation of ^{32}P-acetyl phosphate discussed in Chapter 5. Phosphorylation of AbsA2-His_6 utilizing purified 'AbsA1 as a phosphate donor will be pursued simultaneously. Preliminary experiments with MBP-'AbsA1 provided evidence that the Factor Xa-liberated phospho-'AbsA1 could serve as a phosphate donor for AbsA2-His_6 from *S. lividans*. Nonetheless, the MBP-'AbsA1 fusion protein appears to undergo proteolysis in the *E. coli* JM109 host, leading to purification products of multiple sizes. Two alternatives are currently being pursued to eliminate

proteolysis of MBP-'AbsA1. First, the fusion protein will be expressed in *E. coli* BL21, a strain containing *ompT* and *lon* protease knockouts. A second alternative is to overexpress only the transmitter domain of 'AbsA1 versus the current truncation which includes both the transmitter domain and the C-terminal domain of unknown function. The size of the MBP-'AbsA1 fusion products suggests that proteolysis is occurring in the C-terminal domain of AbsA1. Removal of the C-terminal domain should not affect the function of the transmitter domain since a 69 aa truncation of the AbsA1 C-terminus has previously been shown to complement Abs$^-$ mutants (19).

The AbsA Signal and Signal-Sensing Mechanism

Identification of the signal and signal-sensing mechanism has proven elusive for most two-component systems. Understanding the signal-sensing mechanism of AbsA1 would help define whether AbsA1 is kinase or phosphatase default in the absence of signal (i.e., whether the signal activates or relieves negative regulation of antibiotics). Likewise, identifying the signal recognized by AbsA1 may provide insight into what external environmental or cell-generated factors are involved in the onset of antibiotic synthesis and how this is coordinated with other developmentally regulated processes such as sporulation.

Transcriptional analysis of *absA* expression revealed that it was growth-phase regulated, experiencing dramatic upregulation prior to the appearance of antibiotics in a wild type culture. The temporal profile of *absA* expression suggests that the signal recognized by AbsA1 increases during transition phase growth. At this time, the signal that AbsA1 senses is not known. Thus, a more immediate focus on elucidating the

AbsA1 signal-sensing mechanism could serve to further refine the AbsA mechanism and possibly provide clues into the type of signal recognized by AbsA1.

Precedent from other two-component systems establishes two general trends for HK signal sensing mechanisms. The first class of HK proteins, exemplified by EnvZ (reviewed by 55) and NarX (25), have a large periplasmic loop with a proposed ligand-binding box lying between two transmembrane (TM) helices in the N-terminal sensor domain. Ligand (signal) binding is proposed to cause movement of one transmembrane helix with respect to the other, resulting in modulation of enzymatic activity in the transmitter domain (38). Alternatively, HKs such as FixL sense the signal on a cytoplasmic domain of the HK situated between the transmembrane and transmitter domains (reviewed by (4)). Deletion of the membrane-bound sensor domain of NarX locks the HK into a signal-independent kinase dominant state (25). Conversely, deletion of the transmembrane domain of FixL does not alter its signal sensitivity (43). An initial approach to identifying the region of AbsA1 involved in signal sensing could implement a series of in-frame deletions and amino acid substitutions. Unlike EnvZ and NarX, AbsA1 is predicted to contain four TM helices with relatively small external loops. Deletion mutations might include the entire TM domain or the two central TM helices. If the signal sensing region is contained within the deletions, I would expect an Abs$^-$ phenotype consistent with constitutive kinase activity demonstrated *in vivo* by truncations of HKs with the signal-sensing region contained within the transmembrane domain of the HK (170; 25). A recently identified HK from the *S. coelicolor* genome sequence of cosmid St8D11 is 40% identical to *absA1* and shows unusual similarity in the C-terminus of the normally highly divergent sensor domain. Striking within the alignment of this

region are 5 consecutive residues conserved in a predicted external solvent exposed loop between TM helix 3 and TM helix 4, which is also the largest of the helical loops. It is tempting to speculate that this highly conserved region represents a ligand-binding box within the sensor domain. Site-directed amino acid substitutions could provide useful in examining this region's functional significance.

This study has broadened our knowledge of the AbsA two-component system and allowed us to establish a working model which sets clear goals for future research. Whether the AbsA system is part of a genetically-programmed developmental cycle or an isolated component of an integrated network subject to external environmental stimuli will become more clear as the targets and signal-sensing mechanism of AbsA are elucidated. Finally, as approximately eighty novel two-component systems emerge from the *Streptomyces* Sequencing Project, knowledge of the structural features and mechanism of AbsA will surely provide insight into the function of other *Streptomyces* two-component systems.

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