

TWO TRANSCRIPTIONAL REGULATORS REQUIRED FOR PROPER MORPHOGENIC
DEVELOPMENT AND ANTIBIOTIC PRODUCTION IN *STREPTOMYCES COELICOLOR*

by

BRANDAN HILLERICH

(Under the Direction of Janet Westpheling)

ABSTRACT

The primary objective of this work was to further characterize two transcriptional regulators that were previously identified in our lab. One regulator, Agl3R, belongs to the GntR family of transcriptional regulators. During this study I showed that Agl3R was a repressor of its own transcription as well as that of the adjacent ATP-binding cassette transporter, encoded by *agl3ABC*. A transposon insertion mutant of the *agl3R* open reading frame exhibited a *whi* phenotype and failed to produce the antibiotic actinorhodin. Deletion of the *agl3ABC* cluster restored the wild-type phenotype to an *agl3R* mutant leading to the suggestion that overexpression of the ABC transporter was responsible for the phenotype, and that excessive expression of *agl3ABC* may allow for improper importation of a developmental signal. The second regulator, XdhR, is a member of the TetR family of transcriptional regulators and is located directly adjacent to a molybdopterin binding cluster, *xdhABC*, encoding an enzyme with xanthine dehydrogenase (XDH) activity. A deletion mutant for the *xdhR* open reading frame was *blu* and overproduced the blue pigment associated with actinorhodin production. I determined that XdhR was a transcriptional repressor of its own synthesis as well of that of the adjacent *xdhABC* cluster. I suggested that over expression of an enzyme with XDH activity might cause a signaling breakdown due to manipulation of the GTP pool and the amount of ppGpp(p) present in the cell.

Index Words: *Streptomyces*, ATP-binding cassette transporter, xanthine dehydrogenase, GntR regulator, TetR regulator

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Chapter 1

Introduction

***Streptomyces* life cycle**

Streptomyces are gram positive, soil dwelling microorganisms that produce many industrially significant antibiotics (37). Along with production of antibiotics, *Streptomyces* have an interesting developmental cycle that has been the object of much research (Fig 1.1). The *Streptomyces* life cycle begins with germination from uninucleoid spores under favorable growth conditions. The bacteria grow as thick masses of branching substrate mycelia as long as conditions are favorable. Upon sensing environmental stress and nutrient depletion, extracellular signals are produced that lead to a developmental shift (64, 65, 72, 93). The substrate mycelia begin to lyse and provide nutrients for the growth of aerial hyphae. Antibiotic production begins at the same time that the developmental shift begins. Small molecule signals are also responsible for the regulation of antibiotic and secondary metabolite production. The coincidence of aerial hyphal growth and secondary metabolite production is thought to act as a mechanism to protect the remaining nutrients from being used by other microorganisms. As the aerial hyphae continue to grow, they coil and septate to produce uninucleoid spores for dispersal (16).

***Streptomyces* morphogenesis**

The study of morphogenesis in *Streptomyces* has been predominately carried out using the model organism *Streptomyces coelicolor*. A genetic manipulation system for *S. coelicolor* was developed by D. A. Hopwood over forty years ago, and with the publication

of the 8.67MB genome has since been expanded upon to include an efficient system for targeted mutagenesis (30, 37). In recent years, there has been much advancement in the understanding of *S. coelicolor* development due to the discovery of previously unidentified developmental genes as well as further characterization of genes already known to be involved in morphogenesis (6, 29, 30). Our understanding of the developmental process has come mainly through identification of mutations that cause deficiencies in morphogenesis. Such mutations are typically divided into two categories: *bld*, mutants lacking the ability to produce aerial hyphae, and *whi*, mutants that produce aerial hyphae but fail to produce mature, uninucleoid spores (50).

The *bld* genes of *S. coelicolor* participate in a regulatory cascade that culminates in the production and excretion of the surfactant SapB, a lantibiotic-like lanthionine-containing peptide (52). Unlike other lantibiotic compounds, SapB lacks antibiotic activity and acts as a surfactant at the air/liquid interface to decrease the surface tension required for the erection of aerial hyphae (65, 93). Failure to produce SapB results in mutants that are unable to produce aerial hyphae and are thus developmentally deficient. It has been shown that addition of SapB to plate-grown cultures causes erection of substrate hyphae but is not sufficient for the formation of morphologically distinguishable aerial hyphae (89, 95). Extracellular complementation experiments using different *bld* mutants plated in close proximity showed the ability of certain *bld* mutants to complement one another. This led to the suggestion that the genes functioned in a regulatory cascade (Figure 1.2) (50, 93).

The *bld* mutants that have been characterized are diverse in function, showing the complex nature of developmental regulation in *S. coelicolor*. The *bldA* gene encodes the only tRNA for the translation of the rare leucine codon, UUA, that is found in several genes

required for morphogenesis and antibiotic production (56, 57). The TTA codon is found in the gene identified by the *bldH* mutant, which is a homolog of *adpA* from *Streptomyces griseus* (88). In *S. griseus* the *adpA* gene was shown to encode an AraC-like transcriptional regulator that functions as the master regulator of *S. griseus* development. The TTA codon is also present in genes involved in secondary metabolite production in *S. coelicolor*, such as *actII-ORF4*, a gene involved in actinorhodin production, and *redZ*, a regulator of undecylprodigiosin production (25, 91). The *bldB*, *bldC*, *bldD*, and *bldM* genes encode DNA-binding proteins (21, 23, 42, 60, 71). The *bldB* gene encodes a protein containing a helix-turn-helix motif for DNA binding, but shows no significant similarity to any characterized proteins (93). The *bldC* gene encodes a putative transcriptional activator that is a member of the MerR family. Mutations in the *bldC* gene also result in failure to produce actinorhodin and undecylprodigiosin due to the required activation by BldC of the *actII-ORF4* and *redZ* genes (42). The *bldD* gene encodes a small DNA-binding protein that acts to repress the transcription of the sigma factors *bldN*, *whiG*, and *sigH* (21, 23, 51). Both sigma-bldN and sigma-whiG are involved regulation of morphogenesis (21, 23), while sigmaH is active during heat and ethanol stress (51). Sigma-whiG has been shown to be involved in the first step that leads to aerial hyphae formation (77). Sigma-bldN is responsible for transcription of *bldM*, which encodes a response regulator required for aerial hyphae development (7, 62). The *bldG* gene encodes a putative anti-anti-sigma factor that is a homolog of a *Bacillus subtilis* protein that is involved in regulation of sigma factors involved in stress response and sporulation (8, 9). The *bldG* gene product requires activation by post-translational phosphorylation (9). The *bldJ* gene encodes an extracellular peptide that when processed acts to govern aerial mycelium production and is imported by the oligopeptide

permease encoded by *bldK* ((64, 65). The genes encoded in the *bldI* and *bldL* mutants have yet to be characterized (14, 58, 66).

Based on complementation experiments performed to date, the *bld* genes fit into the developmental cascades as follows: *bldJ* < (*bldK,bldL*) < (*bldA,bldH*) < *bldG* < *bldC* < (*bldD,bldM*) -> SapB (Figure 1.2) (63). It is of note that the only signaling molecule encoded by the identified *bld* genes is BldJ, and importation of the mature 655 Da extracellular signal triggers the beginning of the cascade (64). The fact that two of the identified *bld* mutants, *bldB* and *bldI*, do not fit neatly into the proposed cascade suggests that there are other *bld* loci yet to be discovered (50, 93).

The *bld* mutants fail to produce SapB and thus fail to erect aerial hyphae on rich media containing glucose as a carbon source. The addition of SapB extracellularly to *bld* mutants allows for aerial development (92). Still, there has yet to be a direct connection made between the *bld* genes and the gene cluster responsible for production and secretion of SapB. The gene cluster responsible for synthesis and exportation of SapB is the *ramCSAB* cluster which is under control of the gene product of *ramR*, a positive regulator (Figure 1.2) (48, 67). The lantibiotic-like peptide, SapB, is 42-amino acids in length and undergoes significant post-translational modification in order to become active and is encoded by the *ramS* gene. The C-terminus is modified by dehydration of four alanine residues and the introduction of two lanthionine bridges. The modified peptide is exported from the cells and the leader sequence is cleaved to leave the mature 21-amino acid SapB molecule (52, 94). The product of the *ramC* gene encodes an enzyme that is believed to catalyze the dehydration and cyclization of the SapB precursor (52). The *ramAB* genes encode an ATP-binding cassette transporter that is likely involved in exporting SapB (52, 94). RamR is a response regulator

that has been shown to bind to the promoter region of the *ramCSAB* gene cluster, however there is nothing currently known about activation of RamR during the onset of aerial hyphal growth (48, 63).

Another group of eight genes encoding secreted surfactant proteins, the chaplins, was recently shown to play a role in proper aerial mycelium development (22). Each of the eight chaplin proteins, ChpA-H, contain an N-terminal secretion signal and a conserved hydrophobic domain termed the “chaplin domain”. The chaplins have been placed into two groups, the long chaplins (ChpA-C) and the short chaplins (ChpD-H). The *chpABC* genes encode peptides containing a signal peptide domain, two chaplin domains, and a C-terminal domain that is used as a target for the sortase enzyme that attaches them covalently to the cell wall. The *chpDEFGH* genes encode the short chaplins which contain only the signal peptide and a single chaplin domain (18, 22). It has been shown that deletion of individual chaplin genes is not sufficient for effecting morphogenesis. Deletion of at least four chaplin genes is required before effects on morphogenesis become apparent (22).

The effects of many of the *bld* mutants including the *ram* mutants have been shown to be media dependent, with the chaplin mutants being a notable exception (18, 22, 50, 63). When grown on rich media containing glucose as the primary carbon source, the *bld* mutants fail to erect aerial mycelium. This phenotype is at least partially rescued in most *bld* mutants when they are grown on minimal media containing mannitol, galactose, glycerol, or maltose as the sole carbon source (15, 50). This evidence coupled with the lack of SapB production in wild type *S. coelicolor* on minimal media suggests that there is a second, SapB-independent pathway for aerial mycelium initiation (92). It has been recently suggested by

Elliot, et. al. that the chaplins may act as the surfactant that is required for aerial hyphae emergence on minimal media (22).

The *whi* mutants of *S. coelicolor* are characterized by their inability to produce mature spores from aerial hyphae as evidenced by the lack of gray spore pigment. Mutations in these genes thus result in the aerial hyphae remaining white. The *whi* mutants can be further categorized as either early *whi* mutants, encoding genes required for full septa formation, or late *whi* mutants, encoding genes required for final spore maturation (16).

The early *whi* genes, *whiA*, *whiB*, *whiG*, *whiH*, and *whiI*, are required for transcription of the late *whi* genes as well as for proper septation (Figure 1.3). The *whiG* mutant identifies a gene for a FliA-like sigma factor that is responsible for the initiation of the developmental program that leads to spore formation from aerial hyphae (49). Mutations in *whiG* result in aerial hyphae that are long and straight. Transcription of two regulatory genes, *whiH* and *whiI*, is directly dependent upon Sigma-WhiG (2, 77). The protein encoded by *whiH* belongs to the GntR-like transcriptional regulator family, and mutations in this gene result in loosely coiled aerial hyphae containing partially condensed DNA (26, 77). A mutation in the response regulator encoding *whiI* gene results in a phenotype similar to that of *whiH*, however lacking any DNA condensation that accompanies septation (2). The other two early *whi* genes, *whiA* and *whiB*, are transcribed from promoters that are not Sigma-WhiG dependent. The *whiA* gene encodes a protein of unknown function, while the *whiB* gene encodes a transcriptional regulator that is only found in Actinomycetes and is thought to respond to oxidative stress (3, 43, 44). Both *whiA* and *whiB* mutants have phenotypes that are nearly indistinguishable from *whiI* (26).

Transcriptional regulators

Signal transduction plays a crucial role in how organisms adapt to changes within the environment in which they live. As previously discussed, signaling and gene regulation leads to antibiotic production and morphogenic development in *S. coelicolor*. Two of the major classes of transcriptional regulators found in *S. coelicolor* are GntR-like regulators with 57 predicted family members and TetR-like regulators with 151 predicted family members (6). Members of both classes have been shown to play roles in regulation of morphogenesis and antibiotic production in *Streptomyces sp.*

The first family of proteins, the GntR family of transcriptional regulators, includes more than 1,300 (75) members that are distributed among a diverse group of bacteria and are involved in the regulation of a variety of different biological processes. The first characterized member was GntR, a negative regulator of the gluconate operon of *B. subtilis* (28). Members of this family contain similar N-terminal DNA-binding domains with HTH motifs but lack significant similarity in regions involved in effector binding or oligomerization (32). Typically, oligomerization between regulatory subunits and/or conformational changes resulting from the binding or removal of inducing/repressing molecules allows correct HTH disposition and confers DNA binding ability to the protein as a whole. While many GntR family proteins have been shown to act as repressor proteins that are responsive to carboxylate-containing intermediates in carbon metabolism, some have been shown to bind sites other than typical operator sequences and at least one, FadR, can act as a transcriptional activator (19).

GntR-like transcriptional regulators have been shown to act as environmental sensors for controlling genes involved in responding to external stimuli. DasR is a GntR family

protein that regulates cell-cell communication in *Streptomyces griseus* (82) independent of A-factor, a member of the γ -butyrolactone family of microbial hormone morphogens. The *whiH* gene of *S. coelicolor* encodes a GntR family member that plays a crucial role in the septation of aerial hyphae. WhiH mutants fail to produce the grey spore pigment associated with mature spores. WhiH is required for the transcription of a number of genes that constitute a regulatory cascade for differentiation of *S. coelicolor* (76, 77). Recently, another GntR-like transcriptional regulator, DevA, has been characterized in *S. coelicolor*. It has been shown that *devA* mutants fail to properly develop mature spores, producing short spore chains with misplaced septa (38). During a random transposon mutagenesis screen, our lab previously identified a mutant in the *SCO7168* gene which encodes a predicted GntR-like transcriptional regulator family member. The *SCO7168* disruption mutant exhibited a *whi* phenotype and failed to produce the blue pigment associated with actinorhodin production (Figure 1.4) (85). The *SCO7168* gene is located directly adjacent to but in the opposite orientation of a predicted ATP-binding cassette transporter.

The second family, the TetR-family of transcriptional regulators, is one of the most common types of bacterial regulators with greater than 2,000 members predicted among bacteria, *S. coelicolor* alone contains 151 predicted members (6, 74). TetR-like regulators are identified by a conserved HTH DNA-binding domain and form homodimers. Members are generally repressors and function to regulate a wide range of cellular activities including drug efflux, antibiotic production, amino acid metabolism, and development (74). The first member of the group, TetR, was identified in *E. coli* and controls expression of the genes encoding a tetracycline efflux pump responsible for drug resistance conferred by Tn10. In the absence of tetracycline, TetR binds to the *tet* promoter and represses transcription of the

efflux pump genes. When tetracycline enters the cell it binds TetR and causes conformational changes within the protein that abolish protein binding, thus relieving repression (78). The characterized members in the *S. coelicolor* genome include two repressors of actinorhodin biosynthesis, ActII and CprB, two repressors of drug resistance genes, Pip and PqrA, and a repressor of gamma-butyrolactone synthesis, ScbR (1, 11, 17, 27, 87). Also identified in the transposon insertion screen in our lab was the *SCO1135* gene which encodes a predicted TetR-like transcriptional regulator. The *SCO1135* insertion mutant displayed a *bld* phenotype and overproduced actinorhodin compared to wild type (Figure 1.5) (85). The interesting thing about this mutant is the apparent uncoupling of morphogenesis and antibiotic production. The *SCO1135* gene is located directly adjacent to and oriented in the opposite direction from a gene cluster that is predicted to encode a molybdopterin-binding complex.

ATP-binding cassette transporters

The regulation of carbon utilization is central to the most interesting and important aspects of the biology of *Streptomyces*. As previously discussed, one aspect of the *bld* mutants observed early in their characterization is that growth on poor carbon sources is sufficient to partially restore the morphological and antibiotic defects of most these mutants (14). One of the major types of carbohydrate transporters in *S. coelicolor* are ATP-binding cassette (ABC) transporters.

ABC transporters are composed of a membrane-bound complex comprised of the two hydrophobic membrane spanning subunits and two ATPase subunits. In addition, a substrate-binding protein is essential for import activity. Substrate-loaded protein interacts

with the integral membrane subunits that form a translocation pore. This interaction stimulates conformational changes in the membrane-spanning domains allowing for the opening of the transport channel. ATP hydrolysis by the ATPases provides the energy for transport and overcoming the binding protein's tight interaction with the substrate once through the membrane. (36). It has been shown that ATP hydrolysis alternates equally between the two ATPases thus showing that the subunits work co-operatively (40). The hydrolysis of high energy ATP to lower energy ADP results in relaxation that is coupled to the movement of the solute through the translocation pore (81).

ABC transporters can act as importers or exporters. The majority of prokaryotic transporters act as importers and transport small solutes such as maltose, histidine, ribose, or small peptides. Import systems can be distinguished from export systems in several ways. First, the ATPase protein is usually encoded by a gene that is separate from the genes encoding the integral membrane subunits. In *Streptomyces*, it has been shown that the *msiK* gene encodes the likely ATPase that is used for ABC-transporters (79). The second difference is the requirement of a dedicated binding protein for delivery of the solute to the membrane transport domain. In Gram-negative bacteria, such as *E. coli*, the substrate binding proteins are soluble and allowed to move freely throughout the periplasm, only attaching to the transporter after binding their substrate. In Gram-positive bacteria, the substrate binding proteins are lipoproteins that are exposed on the cell membrane and anchored to the cytoplasmic membrane (20). Lastly, the motif for binding of the membrane domains to the ATPases is highly conserved.

ABC transporters have a wide variety of functions in solute transport, including sugars, peptides, and amino acids (80), as well as drug efflux (90). The *bldK* gene cluster of

S. coelicolor, for example, encodes an ABC transporter that transports SapB, a small molecule morphogen that promotes sporulation in *S. coelicolor* on rich but not minimal medium (52, 65, 95). It also transports the drug bialaphos and *bldK* mutants that fail to respond to the morphogen and are also resistant to bialaphos (65). There are 81 predicted ABC transporters in the *S. coelicolor* genome and 45 are predicted to be involved in carbohydrate transport (6). Along with sugar transport, the *dasABC* gene cluster of *S. griseus* has also been implicated in development. DasABC encodes a probable sugar transporter but over expression of this cluster results in ectopic sporulation in substrate mycelia in response to glucose (82). The predicted ABC-transporter encoded by *SCO7167-65* and located adjacently to the GntR-like regulator identified by Sprusansky, et. al. in *S. coelicolor* showed little similarity to known ABC-transporters (85).

Molybdenum enzymes

Molybdenum containing enzymes perform a variety of functions all centered around oxidation or reduction reactions. These enzymes take advantage of the ability of molybdenum to use oxidation states IV, V, and IV under physiological conditions. This allows these enzymes to catalyze redox-reactions that require the movement of one or two electrons (35). Molybdenum enzymes, with the exception of nitrogenase, contain a molybdenum cofactor (Moco) in the active site. Moco is made of molybdenum covalently bound to a tricyclic pterin called molybdopterin (MPT) and is surrounded by oxygen and sulfur atoms (73). The role of the cofactor is to be able to control the redox properties of molybdenum through coordination with sulfur.

Moco biosynthesis is a process that has been studied extensively in *E. coli*, and has been shown to be highly conserved from bacteria to higher organisms such as plants and animals, including humans (73). Five gene clusters, referred to as *moa*, *mob*, *mod*, *moe*, and *mog* and containing more than 15 genes have been shown to be involved in Moco production in *E. coli* (83). The synthesis of Moco is a five step process that is marked by formation of the stable intermediates precursor Z, MPT, adenylated MPT, and Moco with the last step being insertion into the enzyme active site (73). In *E. coli*, synthesis of Moco begins with the conversion of GTP to precursor Z. This conversion is catalyzed by MoaA and MoaC. MoaA is a member of the S-adenosylmethionine (SAM)-dependent family of enzymes that catalyze the formation of substrate and protein radicals through cleavage of SAM using an iron-sulfur cluster (84). In the second step, precursor Z is converted into MPT by addition of two sulfur atoms in a reaction catalyzed by MPT synthase. In *E. coli*, MPT synthase made up of two large subunits encoded by *moaD* and two small subunits encoded by *moaE* (70). The next step involves adenylation of MPT to form MPT-AMP, a reaction that is catalyzed in *E. coli* by MogA. Adenylation is required before molybdenum can be inserted into the cofactor to form mature Moco. Also before insertion can occur, molybdenum in the form of molybdate must be actively transported into the cell. This is accomplished in *E. coli* by an ATP-binding cassette transporter encoded by the *modABCE* operon. ModA is a periplasmic substrate binding protein with a high affinity for molybdate. The transport system is further comprised of two integral membrane subunits encoded by *modB* and dimeric ATPase encoded by *modC*. The operon has been shown to be under the control of regulatory protein, ModE (4). Once molybdenum is present in the cell and MPT has been adenylated, mature Moco can be formed. The insertion of molybdenum into the cofactor is coupled to the hydrolysis of MPT-

AMP for which MoeA is required in *E. coli* (53). In the final step, Moco is inserted into the active site of the target enzyme. In *E. coli*, a number of proteins have been proposed to be involved in Moco incorporation including MogA, MoeA, MobA, and MobB (59). The exact method for insertion is still unknown at this time.

Molybdenum enzymes have been separated into three families, the xanthine dehydrogenase (XDH)/aldehyde oxidoreductase family (AOR), sulfite oxidase family, and DMSO reductase family. The first characterized member of the XDH/AOR family was xanthine oxidase from cow's milk (24). XDH is a molybdo-flavoenzyme that participates in purine catabolism and catalyzes the conversion of hypoxanthine to xanthine and xanthine to uric acid. XDH is a heterotrimeric protein comprised of two [2Fe-2S] cluster binding domains, a flavin-adenine (FAD) binding domain, and a domain for dimerization and Moco binding (10). Electrons from the substrate are carried from the Moco center to FAD by the two [2Fe-2S] clusters. Once electrons reach the FAD site, they are transferred either to molecular oxygen or to NAD⁺ to form NADH. In *E. coli* it has been shown that the conversion of hypoxanthine to xanthine by XDH plays a role in the purine salvage pathway in response to nitrogen limitation (96). Xanthine can be used to regenerate the guanosine triphosphate (GTP) by conversion to guanosine monophosphate (GMP) via inosine monophosphate (IMP). This provides a potential link between the activity of XDH and the intracellular level of GTP, a key starvation sensing mechanism in bacteria. In *S. coelicolor*, the predicted molybdopterin-binding cluster encoded by *SCO1132-34* located adjacently to the TetR-like transcriptional regulator identified by Sprusansky, et. al. shows similarity to members of the XDH/AOR family of molybdenum containing proteins (85). A second potential XDH cluster in *S. coelicolor* is encoded by *SCO4971* (Bentley).

Guanosine 3'-diphosphate 5'-diphosphate and guanosine 3'-diphosphate 5'-triphosphate

Bacteria commonly produce small molecule regulators in response to environmental stress. In response to starvation conditions, many bacteria produce ppGpp (guanosine 3'-diphosphate 5'-diphosphate) and pppGpp (guanosine 3'-diphosphate 5'-triphosphate). The RelA protein is the molecule responsible for the synthesis of (p)ppGpp from GTP. ATP is used by RelA as the donor of a phosphate group to the 3' end of either GTP to form (p)ppGpp. RelA is bound to the 50s ribosomal subunit during translation. Under starvation conditions and while mRNA is attached to the ribosome, codon specific uncharged tRNA molecules bind to the ribosome resulting in synthesis of (p)ppGpp by RelA (31). The binding of uncharged tRNA to the ribosome occurs presumably due to a lack of the charged species. Binding of uncharged tRNA causes peptide synthesis to stall, synthesis of (p)ppGpp is thought to cause the uncharged tRNA to be released in order to allow elongation to proceed. The increase of the intracellular level of (p)ppGpp is a signal for initiation of the stringent response. The stringent response involves arrest of new ribosome synthesis along with breakdown of ribosomal proteins causing an increase in available amino acids for protein synthesis (5). The advantage of using (p)ppGpp as a signaling molecule is that starvation conditions can be assayed after every single round of (p)ppGpp synthesis.

In *Streptomyces*, starvation is accompanied by initiation of morphogenesis and antibiotic production. The effect of (p)ppGpp synthesis varies among *Streptomyces* species and even among strains (12, 13, 39, 41, 45, 46, 54, 61). Ochi was the first to describe the effects of (p)ppGpp in *S. coelicolor* by using thiopeptin resistance as a screen to identify the *relC* mutant (68). The role of (p)ppGpp has been predominantly studied using mutants that

are deficient in (p)ppGpp production. Of these mutants, the most studied are *relA* disruption and deletion strains. It was shown that *relA* inactivation in *S. coelicolor* leads to abolishment of (p)ppGpp accumulation during transition phase (12, 13, 45, 46, 61).

In *S. coelicolor* A3(2), (p)ppGpp accumulation is required for initiation of antibiotic production during transition phase during nitrogen and glucose limitation (12, 47). *S. clavuligerus relA* mutants are defective in sporulation as well as antibiotic production (45, 46). The antibiotic production defects in *S. coelicolor* have been linked to lack of transcription of *redD* and *actII-orf4*, genes required for undecylprodigiosin and actinorhodin production respectively, in *relA* mutant strains (12, 13, 33, 34). While RelA appears to be required for antibiotic production during nitrogen and glucose limitation, there appears to be (p)ppGpp independent pathway for antibiotic production during phosphate limitation (47, 86). An attempt to determine whether this was due to *relA* independent (p)ppGpp production led to the identification of *rshA*, a gene that when overexpressed in a *relA* mutant restores antibiotic production (86). It was thus shown that *rshA* acts downstream of *relA* and while induced by (p)ppGpp, it has not effect on (p)ppGpp levels (86). The (p)ppGpp-dependent initiation of transcription of *redD* and *actII-orf4* has been suggested to be due to an interaction between (p)ppGpp and RpoB, the β -subunit of RNA polymerase. Rifampicin resistant mutants that restored actinorhodin production were identified in *S. coelicolor* and *S. lividans relA* deficient strains (41, 55, 97). In both instances, the assumption was made that the mutations in RpoB that allowed for rifampicin resistance mimicked the effects of the (p)ppGpp bound form of RNA polymerase.

Accumulation of (p)ppGpp has an indirect effect on morphological development in *S. coelicolor*. The ATP-dependent conversion of GTP to (p)ppGpp by RelA causes a decrease

in the amount of GTP in the cell. A decrease cellular GTP levels has been shown to be sufficient to trigger sporulation in *S. coelicolor* (69, 86). This effect was shown to be mediated by the membrane-associated GTP-binding protein, Obg (69). The GTP-bound form is predominant during exponential growth, however, once the level of GTP decreases, the GDP-bound form becomes more prevalent and triggers sporulation (69). The effect of GTP depletion on development was mimicked by addition of the GMP-synthetase inhibitor, decoyinine (86). The addition of decoyinine did not affect antibiotic production.

Recently microarray analysis of a *S. coelicolor relA* mutant as compared to wild type gave support to the previous findings. It showed that active *relA* stimulated genes for antibiotic production, surfactants required for sporulation, alternative sigma factors, and some ribosomal proteins (33).

Project Goals

This project was undertaken in order to further elucidate the pathways that lead to morphogenesis and antibiotic production in *S. coelicolor*. The first stage was accomplished by identifying mutants displaying morphogenic and antibiotic production deficiency using a Tn5 transposon screen. My goals were to further characterize the genes identified by two of the mutants, SE69 and SE293, and to place them within the known regulatory cascades for development and antibiotic production. I chose this project because it allowed me to gain valuable research skills while working on a project that could potentially impact antibiotic production in an industrially important family of microorganisms.

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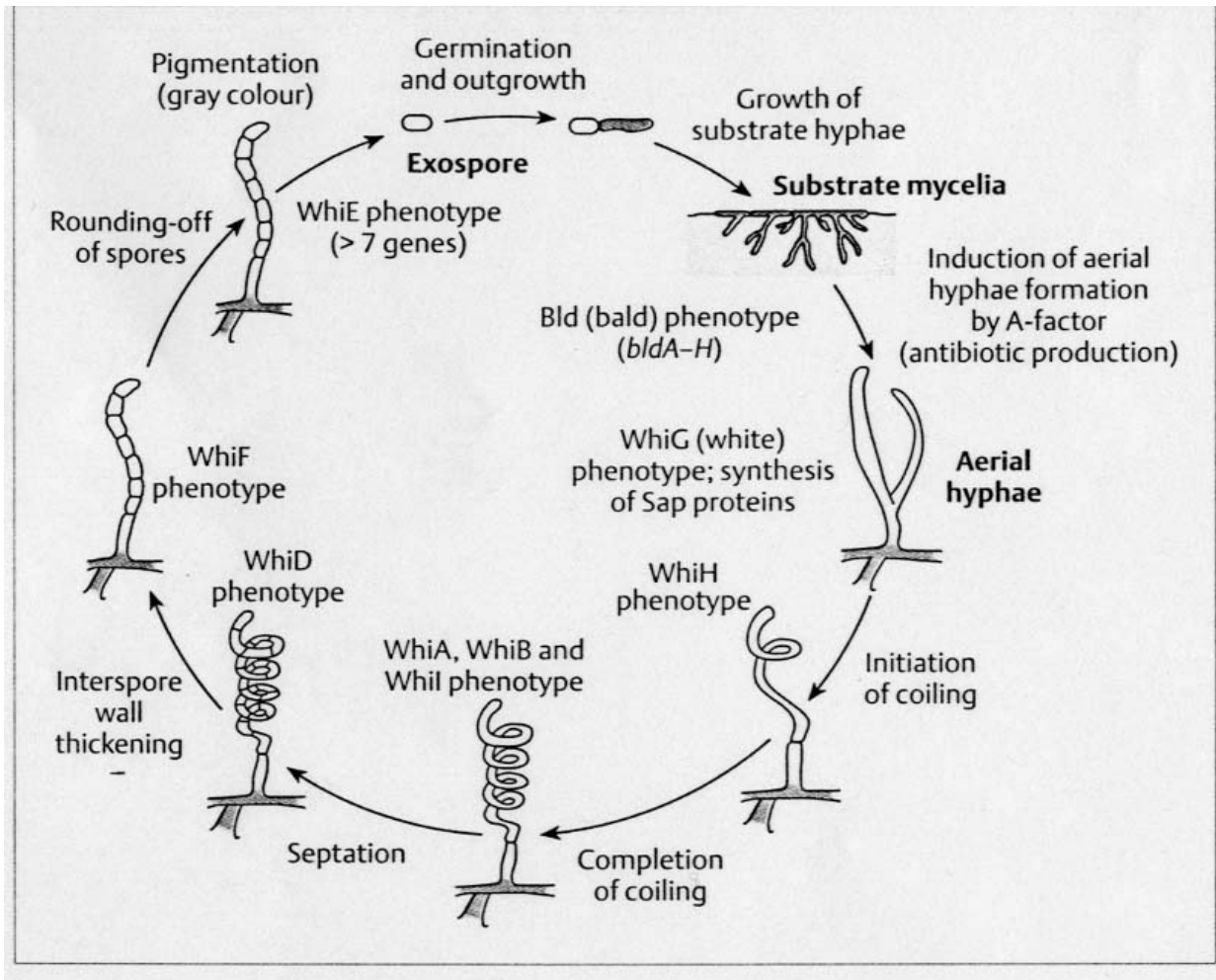


Figure 1.1 *Streptomyces* lifecycle beginning with germination of exospores and ending with mature spore formation. Various checkpoints for development are listed beside illustrations of their phenotypes.

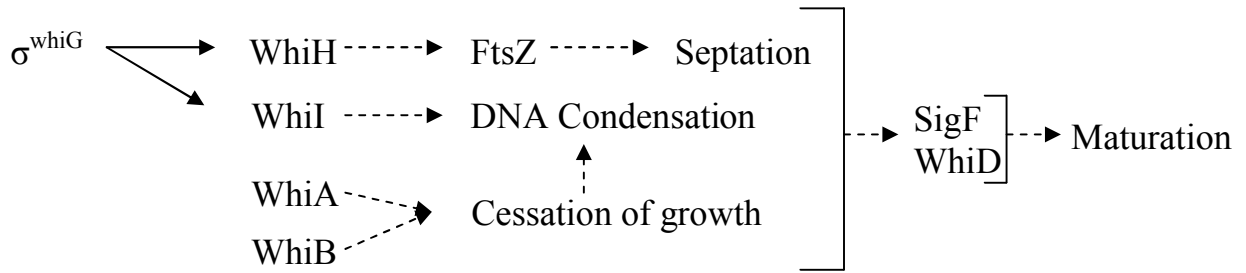


Figure 1.3 The known *whi* cluster for *S. coelicolor* beginning with initiation of aerial hyphae and proceeding to spore maturation. The only direct interaction known is between σ^{whiG} and *whiH* and *whiI*. *WhiH* is required for *FtsZ* expression and proper septation. *WhiI* is required for DNA condensation. *WhiA* and *WhiB* are required for cessation of elongation. All five early *whi* genes are required for *SigF* and *WhiD* production and proper spore maturation.

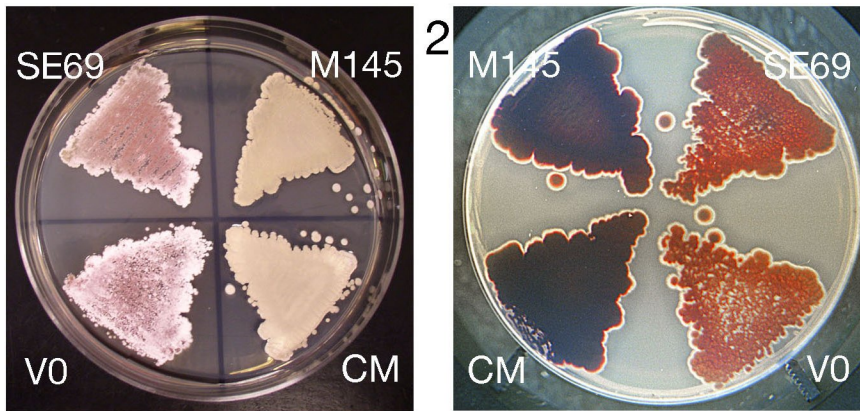


Figure 1.4 SCO7168 (SE69) mutant showing *whi* phenotype and lack of actinorhodin production. On the left is the morphogenic phenotype for SE69, wild type M145, the complimented mutant (CM), and the vector insert control (VO). On the right, the antibiotic production phenotypes of SE69, M145, CM, and VO. Adapted from Sprusansky, et. al. 2003.

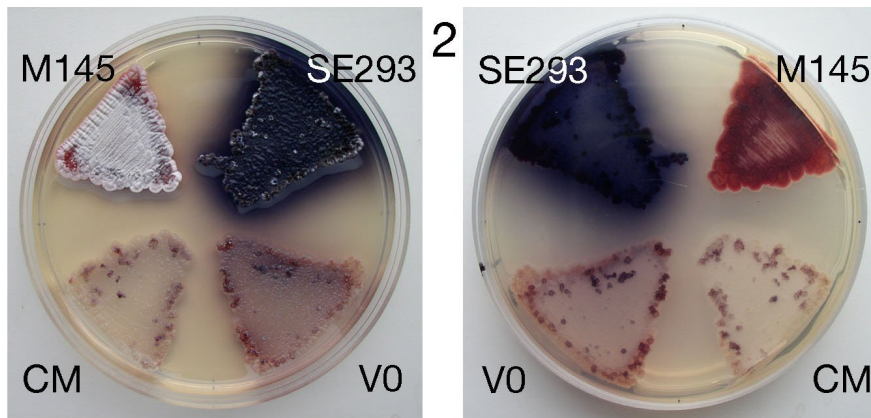


Figure 1.5 SCO1135 (SE293) mutant showing *bld* phenotype and overexpression of actinorhodin. On the left is the morphogenic phenotype for SE293, wild type M145, the complemented mutant (CM), and the vector insert control (VO). On the right, the antibiotic production phenotypes of SE293, M145, CM, and VO. It is of note that the mutant failed to complement. Adapted from Sprusansky, et. al. 2003.

Chapter 2

A New GntR Family Transcriptional Regulator in *Streptomyces coelicolor* is Required for Morphogenesis and Antibiotic Production and Controls Transcription of an ABC Transporter in Response to Carbon Source

Abstract

We recently reported the isolation and initial characterization of a transposon-generated mutation that resulted in defects in both morphogenesis and antibiotic production in *Streptomyces coelicolor*. The insertion identified the SCO7168 open reading frame whose predicted product is a GntR family transcriptional regulator. Here we show that this gene acts to repress transcription of itself as well as a series of genes immediately adjacent to it on the *S. coelicolor* chromosome that likely encode an ATP-binding cassette type (ABC) transporter for carbohydrate uptake. Transcription of this transporter is strongly induced by growth on relatively poor carbon sources such as trehalose and melibiose and weakly by lactose and glycerol but not glucose and induction is not repressed by the presence of glucose. Constructed deletions of the ABC transporter itself resulted in suppression of the original transposon mutation, suggesting that inappropriate expression of the ABC transporter is responsible, at least in part, for the mutant phenotype. Because this transporter responds to the presence of α -glucosides and has similarity to two other carbohydrate transporters of this class we have named the genes of the transporter *agl3E*, *agl3F* and *agl3G* and the GntR-like protein that regulates transcription of the transporter *agl3R* in accordance with established nomenclature. We suggest that *agl3R* is one of a number of homologous proteins in

Streptomyces (there are 57 putative GntR family regulators in the *S. coelicolor* genome) that respond to nutritional and/or environmental signals to control genes that affect morphogenesis and antibiotic production.

Introduction

The mechanisms by which cells sense environmental signals and transmit that information to changes in gene expression often involve the participation of transcription factors that respond either directly or indirectly to small molecules or substrates. One such family of proteins, the GntR family of transcriptional regulators, includes more than 1,300 (31) members that are distributed among a diverse group of bacteria and are involved in the regulation of a variety of different biological processes. These regulators have been shown to act as environmental sensors for controlling genes involved in responding to external stimuli. DasR, for example, is a GntR family protein that regulates cell-cell communication in *Streptomyces griseus* (40) independent of A-factor, a member of the γ -butyrolactone family of microbial hormone morphogens. Of special interest in this context is the GntR-like *whiH* gene of *S. coelicolor*, which plays a crucial role in the septation of aerial hyphae, and mutants fail to produce the grey spore pigment associated with mature spores. WhiH is required for the transcription of a number of genes that constitute a regulatory cascade for differentiation of *S. coelicolor* (32, 33).

Streptomyces sp. undergo an elaborate program of cellular development in response to environmental changes. When nutrients are plentiful, the organism grows as a branching “substrate” mycelium that penetrates and solubilize organic material in the soil. As food sources are exhausted, development begins with the erection of aerial hyphae that ultimately

septate to form uninucleoid spores. Many of the same signals that lead to the initiation of development trigger the production of a large number of medically important natural product antibiotics. Most of what is known about the regulation of pathways that contribute to morphogenesis and antibiotic production comes from the study of mutants defective in one or both of these processes (8, 13). Mutants blocked in the production of aerial hyphae are called Bald (*bld*) mutants, many of which are also defective in antibiotic production. Mutants able to initiate morphogenesis but defective in the ability to form mature spores are called White (*whi*) mutants. The genes identified by these *bld* and *whi* mutants are diverse in function and while a clear picture of how they participate is still emerging, it is clear that they hold the key to understanding both morphogenesis and antibiotic production in these complex bacteria.

At times and under conditions when the wild type has produced abundant amounts of spores, the *agl3R* mutant produces no spores and relatively few aerial mycelia. It also fails to produce the blue pigment associated with actinohorodin production (41). On relatively poor media such as, MM-glucose, MM-mannitol and MM-maltose, the mutant grows more slowly than wild type and fails to produce pigment. Both the morphological and antibiotic production defects of the *agl3R* mutant are restored by complementation with the wild type allele (41). The *agl3R* open reading frame is located directly adjacent to a cluster of genes that show amino acid sequence similarity to ATP binding cassette (ABC) type membrane transporters (35, 41). ABC transporters have a wide variety of functions in solute transport, including sugars, peptides, and amino acids (39), as well as drug efflux (43). Perhaps the most relevant and interesting aspect of these transporters in this context is the fact that they are capable of transporting more than one type of molecule. The *bldK* gene cluster of *S. coelicolor*, for example, encodes an ABC transporter that transports SapB, a small molecule

morphogen that promotes sporulation in *S. coelicolor* on rich but not minimal medium (24, 28, 46). It also transports the drug bialaphos and mutants defective in the transporter fail to respond to the morphogen and are bialaphos resistant (28). While 45 of the 81 predicted ABC transporters in the *S. coelicolor* genome are predicted to be involved in carbohydrate transport (2), the *agl3EFG* gene cluster described here and the *dasABC* gene cluster of *S. griseus*, have also been implicated in development. DasABC encodes a probable sugar transporter but over expression of this cluster results in ectopic sporulation in substrate mycelia in response to glucose (40).

The *S. coelicolor* genome contains 57 putative GntR-like proteins (Streptomyces.org.uk ScoDB "GntR"), but only *whiH* (32) and the *agl3R* open reading frame have been identified by mutation. The product of the *whiH* gene is likely to be a transcriptional regulator but the direct target of its activity is not known, nor is anything known about the environmental conditions that *whiH* responds to. Here we show that the *agl3R*, also a GntR-like regulator, acts to repress transcription of itself as well as a cluster of genes directly adjacent to it that likely encode an ABC-type carbohydrate transporter. Reporter gene fusions to the promoter of the transporter showed that expression is strongly induced by growth on the α -glucosides, melibiose and trehalose, as sole carbon source and that induction is not repressed by glucose. Gel mobility shift experiments using a his-tagged version of *agl3R* showed that it binds the intergenic region between *agl3R* and *agl3E* *in vitro*. Transcript analysis of the affect of the *agl3R* mutation on other *whi* genes suggests that it does not fit into the previously proposed, *whiG* dependent, regulatory cascade for morphogenesis in *S. coelicolor*. We speculate that this GntR-like regulator plays a role in the regulation of morphogenesis and antibiotic production in response to the presence of complex carbohydrates as carbon source.

Materials and Methods

Bacterial strains and growth conditions. General techniques for bacterial growth were as in Sambrook and Russell (34) and Kieser *et al.* (23) for *E. coli* and *S. coelicolor* respectively. *S. coelicolor* strains were grown in YEME medium for genomic DNA isolation and Soy agar medium with the addition of 10 mmol MgCl₂ for mating experiments. Antibiotic selections were applied by overlay with soft nutrient agar. *Streptomyces* RNA was isolated from cells grown on cellophane discs placed on top of MYM agar. *E. coli* strains and growth conditions for preparation of cosmids for marker replacement in *Streptomyces* were as described (14). *Streptomyces* strains used in this work are listed in Table 2.1 with indicated source and (47). Primers used to amplify fragments for S1 mapping, construction of transcriptional fusions to the *xylE* gene (19), and construction of mutants are listed in Table 2.2.

Microscopic analysis of aerial mycelium development. The procedure used in this analysis was as previously described (32). Samples from colonies grown for 5 days on MYM agar medium were mounted on an aluminum stub with O.C.T. compound, submerged in liquid nitrogen slush at approximately -210° C, and transferred to a Gatan Alto 2500 cryostage and cryoprep chamber (Gatan UK, Ferrymills 3, Osney Mead, Oxford, OX2 0ES, UK) attached to a LEO 982 Field emission scanning electron microscope (FE-SEM, LEO Electron Microscopy, Inc., One Zeiss Drive, Thornwood, NY 10594). The sample was sublimated to remove surface frost at -95° C for 3 min, coated with platinum, placed on the cryostage in the main chamber of the microscope, at approximately -140° C, and viewed at 5.0 kV.

RNA isolation and S1 Nuclease Mapping. S1 nuclease mapping was carried out as described (25). RNA was isolated from cells after 16, 24, 36, 48, or 60 hours of growth on MYM agar plates overlaid with cellophane discs, and 40 µg was hybridized to approximately 0.05 pmol of [³²P] 5'-end-labeled DNA probe and incubated with 100 U of S1 nuclease (Roche). Probes were generated by PCR with Taq DNA polymerase (Qiagen) using *S. coelicolor* genomic DNA as template. The same oligonucleotides, S1-forward and S1-reverse, were used to generate a 305 bp probe for mapping the *agl3R* transcript (S1 Reverse labeled) and the *agl3E* transcript (S1 forward labeled). Primers were labelled using [γ -³²P]-ATP and OptiKinase according to the manufacturers instructions (USB) prior to the PCR reaction. The *hrdB* promoter probe was as described (22). The same labeled oligonucleotides were used in the fmol cycle DNA sequencing system (Promega) to generate the G, A, T, and C sequencing ladders. DNA fragments were separated on a 6% denaturing polyacrylamide gel and bands were visualized by autoradiography.

Construction of transcriptional fusions to the xyle reporter gene. Primers complimentary to the DNA sequence 542 bp upstream of the *agl3E* translational start site (*agl3E* Forward) and 97bp downstream of the *agl3E* ATG (S1 Forward) were used to generate a 639 bp fragment containing the *agl3E* promoter region. The *agl3E* Forward primer contained base changes that created a HindIII restriction site for subsequent cloning (Table 2.2). The PCR product was (13, 33) purified, digested with HindIII and ligated to pBluescript II+ that had been cut with HindIII and SphI. An aliquot of the ligation mixture was transformed into competent XL10 Gold *E. coli* cells and transformants were selected with ampicillin. Plasmid DNA was isolated, using a Qiagen Plasmid Mini Column, from transformants that had been grown

overnight in liquid LB broth containing 100 µg/mL ampicillin. Plasmid DNA was then digested with HindIII and BamHI and the promoter-containing fragments were ligated to HindIII/BamHI cut pXE4, placing the fragments upstream of the *xylE* gene and in the correct orientation for transcription to generate pXE7167 and pXE7168. Plasmid constructions were verified by restriction digestion. Each plasmid was digested with HindIII and EcoRI to generate a fragment that contained the promoter-*xylE* fusion which were then blunted using the Klenow fragment of PstI and ligated to EcoRV digested pHyg*oriT* (41) containing a hygromycin resistance gene. The ligated vector was transformed into chemically competent XL10 Gold *E. coli* cells and transformants were selected for using hygromycin on LB agar plates. The constructions were confirmed by using XbaI and EcoRI digestion. Plasmids were transformed into electro-competent *E. coli* Et12567 containing the non-transmissible helper plasmid pUZ8002 as previously described (14). Transformants were selected using hygromycin on LB agar plates and single colonies were used for conjugation with *S. coelicolor* M145 and *agl3R* (14). Exconjugants were selected on MS-agar plates overlaid with 50 µg/mL hygromycin after 16 hours.

XylE assays. Assays were performed on whole cells as previously described (4) with the following modifications. *S. coelicolor* strains BH10 and BH11 were grown for 36 hours on SMMS-maltose agar medium (23) overlaid with cellophane disks. After 36 hours cells were scraped from the cellophane, vortexed briefly with glass beads to disperse the cell mass and diluted with liquid minimal medium (23) containing the indicated sugar. Cultures were grown for 36 hours at 30° C. Cells were harvested by centrifugation for 10 minutes at 3000 rpm, washed once with sterile 50 mM K₂HPO₄, pH 7.4, and re-suspended in sterile 50 mM

K₂HPO₄ to an OD₆₀₀ of 0.5. 200 µl of the cell suspension was added to 400 µl of sterile 50 mM K₂HPO₄ containing 10 mM catechol. Absorbance was measured at OD₃₇₅ at 10 second intervals for 90 seconds.

Construction and confirmation of deletion mutants. The deletion mutants shown in Figure 2.1 were constructed using the PCR targeting method of Gust et. al. (14). The *Δagl3EFG*, *Δagl3EFGR*, mutants were made by replacing the chromosomal region of the deletion with the apramycin-resistance cassette (*acc(3) IV*). The extent and location of the deletions in each strain were confirmed by PCR. The presence of the apramycin resistance cassette was detected using specific primers, P1 and P2. The location of the cassette was determined using primers located outside the deleted sequence, SCO7168Out, SCO7165Out, and SCO7162Out. Primers within the deleted sequence, S1 Forward and S1 Reverse, were used as negative controls.

Gel retardation assays. To construct the his-tagged version of the Agl3R protein, the coding region was amplified by PCR from *S. coelicolor* genomic DNA using primers His₆-Agl3R Forward and His₆-Agl3R Reverse (Table 2). The fragment was digested with XmnI (Promega) and DdeI (Promega), treated with the Klenow fragment of DNA-polymerase (Promega), and cloned into the NdeI site of pET28a that had also been blunted. DNA sequencing confirmed the correct orientation and fusion to the his-tag. The resulting construction was transformed into *E. coli* BL21 (DE3) and cells were grown in 2 x YT (17) at 37°C to an OD₆₀₀ of 0.6, induced by the addition of 1 mM IPTG, grown an additional 18 hrs at 18° C, harvested by centrifugation and resuspended in phosphate buffer (20 mM

phosphate, 500 mM NaCl, 5% glycerol, 2 mM DTT, pH 8.0). Cells were lysed by sonication and centrifuged at 50,000 g for 20 min. The supernatant was passed over a Ni column, and protein eluted using a 10 mM to 300 mM imidazole gradient. Purified His₆-Agl3R Protein (5, 10 or 20 µg) or crude cell extract (20 µg total protein) was mixed with the same 305 bp PCR generated DNA fragment containing the intergenic region between *agl3E* and *agl3R* used for S1 nuclease mapping experiments. The fragment was 5'-end-labeled with [γ -³²P]ATP (MP Biomedicals) using T4-polynucleotide kinase (Promega) and purified on a 1% agarose gel. Labeled DNA fragment (1 ng, 6000 c.p.m.) was incubated with cells extracts or purified his-tagged Agl3R protein for 20 min at 30°C in 20 µl total volume of binding buffer (20 mM Tris, 10 mM MgCl₂, 1 mM EDTA, 1 mM DTT, pH 8.0) containing 1 µg sonicated salmon sperm DNA and 3 µg BSA. For competitive inhibition of the binding reaction, 100 ng of unlabeled fragment or 100 µg of sonicated salmon sperm were added to 5 µg purified His₆-Agl3R protein. Reactions were displayed on a non-denaturing 6% acrylamide/TBE gel and visualized by autoradiography.

RT-PCR. RNA was isolated from *S. coelicolor* M145 and the *aglR* mutant after 24, 36, 48 and 60 hours of growth on MYM agar medium overlaid with cellophane discs as for S1 nuclease mapping. The One-Step PCR kit (Qiagen) was used with primers specific for each gene. Forward and reverse primers for *whiG*, *whiB*, *whiH*, *whiE*, *sigF*, *alg3A*, *agl3R* and *hrdB* are shown in Table 2.2. Reaction mixtures contained 10 pmol of each primer and 150 ng of RNA in a total volume of 20 µL. Each primer was first tested using chromosomal DNA as template and without a reverse transcription cycle to test for DNA contamination in

the RNA. HrdB was used as a control for RNA concentration. Products were displayed on a 1% agarose gel and visualized by staining with EtBr.

Results

An insertion into the agl3R open reading frame results in a white mutant phenotype. A diagram showing the location of SCO7168 (SC9A4.30), the site of the transposon insertion, and the annotated region of the chromosome at this address are shown in Figure 2.1. As previously reported (41), the SCO7168/SE69 mutant (referred to hereafter as *agl3R*) was defective in sporulation and production of blue pigment associated with antibiotic production in *S. coelicolor*. The predicted 224 amino acid protein encoded by this open reading frame shows sequence similarity to the GntR family of transcriptional regulators. A DNA fragment containing the single SCO7168 open reading frame complemented both the morphological and antibiotic production phenotypes of the mutant suggesting that both aspects of the mutant phenotype were the result of a single mutation. To more fully characterize the morphological phenotype of this mutant, cells were grown on the sporulation medium, MYM, and visualized with scanning electron microscopy. After 5 days of growth on agar plates, visual inspection of colonies showed that the morphologically wild type M145 strain produced a dense covering of aerial mycelium and grey-pigmented spores, while the mutant remained white and failed to produce the grey pigment associated with mature spores. Such mutants in *Streptomyces* species are called *whi* mutants. As shown in Figure 2.2, scanning electron microscopy revealed that the aerial hyphae made by this mutant did not coil or septate and no spores were visible. At the same stage of growth in the wild type, coiled, fully septated aerial hyphae as well as mature spores are clearly present.

The original collection of *whi* mutants isolated by Chater and Hopwood (8), were not significantly reduced in vegetative growth and made aerial mycelia at the same time as wild type while failing to produce spores on prolonged incubation. When compared to other, well characterized, *whi* mutants, the morphological defects of *agl3R* are similar to those of *whiH* but perhaps most closely resemble those of *whiG* (13). *whiG* mutants make relatively straight aerial hyphae that completely lack sporulation septa. *whiH* mutants make loosely coiled aerial hyphae with infrequent but obvious sporulation septa. Interestingly, like *agl3R*, the *whiH* gene encodes a GntR-like transcriptional regulator but the *agl3R* mutant has a more severe morphological phenotype than *whiH* and *whiH* mutants are not defective in antibiotic production. Neither the *agl3R* mutant nor *whiH* mutants produce the grey pigment associated with mature spores. *whiA* or *whiB* mutants have abnormally long and coiled aerial hyphae almost devoid of septation. The products of the *whiG*, *whiA* and *whiB* genes apparently act early in aerial hyphae formation and may participate in the decision to enter sporulation (9). Mutations in *whiG* are, in fact, epistatic to those in *whiH* (7, 33). Analysis of the phenotypes of the *agl3R* and *whiH* mutants suggests that the *agl3R* gene product participates in the maturation of aerial hyphae rather than in the earliest stages of the decision to enter sporulation.

The agl3R gene product acts to repress transcription of an adjacent gene cluster that encodes a putative ABC-type transporter. A gene cluster directly adjacent to *agl3R* and transcribed in the opposite direction contains a number of open reading frames (Figure 2.1), three of which show similarity to ABC-type transporters (41). In accordance with established nomenclature these open reading frames are designated *agl3E*, *agl3F*, and *agl3G*.

To test whether a mutation in *agl3R* affected transcription of this cluster, high resolution S1 nuclease mapping experiments were performed on RNA isolated from cells at different stages of development using a PCR generated probe overlapping the beginning of the *agl3E* open reading frame (Figure 2.3, Panel A). RNA was isolated from morphologically wild type, M145, and *agl3R* mutant cells grown on MYM sporulation agar and harvested at different stages of development. This solid medium supports morphological development of the wild type and the stages of development are clearly detectable. Cells harvested after 16 hours were growing vegetatively (no aerial mycelia present). Aerial mycelia began to appear between 24 and 36 hours and were abundant between 36 and 48 hours. The grey pigment associated with mature spores was evident at 60 hours. As shown in Figure 2.3, Panel B, no transcript was detected from *agl3E* in wild type cells suggesting that it was not expressed under the conditions and on the media from which the cells were harvested. In the *agl3R* mutant strain transcription of *agl3E* was readily detected and two peaks of apparent transcriptional activity were observed, one at 36 hours and another at 60 hours. This pattern of expression was detected in three independent experiments and the absence of transcript at 48 hours was evident in every case. In fact, two independent experiments are shown in Figure 2.3, Panel B. S1 nuclease mapping was also performed on RNA isolated from a strain in which the *agl3R* mutation had been complemented in trans with a wild type copy of the *agl3R* open reading frame. The presence of a wild type copy of *agl3R* restored repression of the abundant *agl3E* transcripts appearing at 36 and 60 hours of growth. The same RNA preparation used for analysis of *agl3E* was used for detection of the *hrdB* transcript, which served as a control for the level of RNA (Figure 2.3, Panel C). These data suggest that the *agl3R* gene product acts to repress, either directly or indirectly, transcription of *agl3E* and

perhaps some genes in the downstream gene cluster, that are likely to be translationally coupled to *agl3E*. It is also of note that transcription of *agl3E* is apparently temporally regulated and is detected at 24 to 36 hours and again at 60 hours with a lower level at 48 hours. This observation suggests that simple repression by the *agl3R* gene product may not fully explain transcriptional regulation of *agl3E*. If, in fact, transcription of *agl3E* is constitutive in the mutant, differences in message stability might account for differences in the levels of RNA observed. If the appearance of RNA at the onset of aerial hyphae production represents new initiation, either another repressor or an activator of transcription, in addition to the *agl3R* gene product may be involved.

Inappropriate expression of the gene cluster beginning with agl3E in the agl3R mutant is responsible, at least in part, for the mutant phenotype. To establish a direct connection between the gene cluster beginning with *agl3E* and the defects in morphogenesis and antibiotic production observed in the *agl3R* mutant, various deletions of the gene cluster beginning with *agl3E* were constructed in both the wild type and an *agl3R* mutant (Figure 2.1). Because construction of each mutant was generated by marker replacement with a constructed deletion, it was not possible to construct a deletion of the ABC transporter in the original *agl3R* transposon mutant. Four deletions were constructed, two different deletions of the downstream gene cluster and two double mutants that deleted both the ABC transporter and the *agl3R* open reading frame. The genes downstream of *agl3EFG* do not show homology with ABC transporters or sugar transport proteins but are included in this analysis because of their proximity to the transporter and the fact that there is the possibility that they are translationally coupled to the transporter encoded by *agl3EFG*. All of the

mutants were compared to wild type after growth on maltose as carbon source (the same conditions used to isolate RNA for the S1 nuclease mapping experiments).

As there is no detectable expression of the transporter in the wild type strain, a deletion of the gene cluster in a wild type strain should have no phenotype. In fact, mutants deleted for *agl3EFG* and/or the downstream gene cluster were indistinguishable from wild type. If inappropriate expression of the gene cluster beginning with *agl3E* in the *agl3R* mutant were responsible for the mutant phenotype, deletion of this cluster in an *agl3R* mutant should suppress the *agl3R* mutant phenotype resulting in a wild type phenotype. Deletions of the downstream gene cluster were, in fact, indistinguishable from each other, the single mutants or the wild type strain. We conclude from this analysis that inappropriate expression of the transporter in the *agl3R* mutant is responsible, at least in part, for the mutant phenotype.

The agl3R gene product represses transcription of its own synthesis. Some GntR-like regulators (notably *whiH*) serve to repress their own synthesis (33). To test whether a mutation in *agl3R* affected transcription of itself, high resolution S1 nuclease mapping experiments were performed using a PCR generated probe overlapping the beginning of the *agl3R* open reading frame (Figure 2.3, Panel A). RNA was isolated from morphologically wild type, M145, and *agl3R* mutant cells grown on MYM sporulation agar and harvested at the same stages of development as those used to analyze *agl3R* transcription. As shown in Figure 2.3, Panel D, in wild type cells a transcript originating upstream of *agl3R* is detected at approximately the same low level throughout growth and development. In the *agl3R* mutant, transcription of *agl3E* is approximately the same as wild type until 60 hours of

growth when abundant RNA was detected. While 60 hours of growth is well after the onset of aerial mycelia production, the *agl3R* mutant does not produce spores so it is difficult to correlate this stage of development with the wild type. At 60 hours, however, under the same conditions M145 was clearly beginning to produce the grey pigment associated with mature spores. S1 nuclease mapping was also performed on RNA isolated from a strain in which the *agl3R* mutation had been complemented in trans with a wild type copy of the *agl3R* open reading frame. As shown in Figure 2.3, the presence of a wild type copy of *agl3R* restored repression of the *agl3R* transcript. The same RNA preparation used for analysis of *agl3R* was used for detection of the *hrdB* transcript that served as a control for the level of RNA (Figure 2.3, Panel E). These data suggest that the *agl3R* gene product acts to repress its own transcription, either directly or indirectly. Interestingly, expression of *agl3R* in the *agl3R* mutant is apparently temporally regulated with a peak of transcriptional activity appearing as the cells enter late stages of sporulation.

The Agl3R protein binds the agl3R/agl3E promoter region in vitro. To address whether the regulatory effect of the *agl3R* gene product involved direct interaction with the *agl3R/agl3E* promoter region, a His₆-Agl3R protein was constructed and expressed in *E. coli* and used in gel mobility shift assays with a DNA fragment containing the intergenic region between *agl3R* and *agl3E*. As shown in Figure 2.2.4, crude extracts from *E. coli* cells expressing the His₆-Agl3R protein retarded the promoter-containing fragment, whereas extracts from uninduced cells did not. Furthermore, the amount of probe shifted in the retardation assay was reduced in the presence of unlabelled probe but not nonspecific DNA suggesting that binding of Agl3R to the promoter-containing fragment was sequence or motif specific.

DNA sequences located upstream of the apparent transcription start sites for *agl3E* and *agl3R* are shown in Figure 2.3, Panel F. The transcription start site of *agl3R* was mapped to a guanine residue 23 nucleotides upstream of the annotated translational start. Sequences centered around -10 and -35 base pairs upstream of the start site show little homology to any known prokaryotic RNA polymerase binding site suggesting that either this promoter is recognized by a novel sigma factor or transcriptional regulators facilitate RNA polymerase binding. The transcription start site of *agl3E* was mapped to a purine residue only 13 nucleotides upstream of the annotated translational start. The apparent leader regions of these mRNAs is somewhat shorter than for typical messages, especially for the transcript beginning with *agl3E*. There are, however, purine rich sequences likely to serve as ribosome binding sites in both transcripts located between -10 and -6 with respect to the translation start site (Figure 2.3). The distance between the putative ATG translation start sites of *agl3E* and *agl3R* is 135 bp with 18 bp between the apparent -35 regions.

Transcription of agl3E is induced by growth on relatively poor carbon sources. To identify conditions that might lead to induction of the transporter, a transcriptional fusion between the promoter of *agl3E* and the *xylE* reporter gene was constructed. The *xylE* gene encodes a catechol dioxygenase that converts colorless catechol to a bright yellow oxidation product. The *agl3E* promoter/*xylE* fusion was constructed on a non-replicating vector containing a ϕ C31 attachment site and introduced into the wild type and *agl3R* mutant strains allowing insertion of the fusion into the *S. coelicolor* chromosome. Cells were then grown in liquid medium containing various carbohydrate carbon sources and monitored for catechol dioxygenase activity (4, 19). As shown in Figure 2.5, transcription from the *agl3E-xylE*

fusion was not detected from cells grown on glucose or lactose but was readily detected from cells grown on melibiose or trehalose. Transcription was constitutive in an *agl3R* mutant supporting the notion that the *agl3R* gene product serves to repress transcription of the transporter. Expression of the *agl3E-xylE* fusion during growth on melibiose or trehalose was not affected by the presence of glucose. While no transcription from the *agl3E* promoter was detected by S1 nuclease mapping from cells grown on maltose as carbon source on plates, some transcription was detected from cells grown on maltose in liquid medium. We conclude from these data that transcription of the transporter is induced by growth on relatively poor carbon sources and that induction is not repressed by the presence of glucose. These data also suggest that these carbon sources relieve repression by the *agl3R* gene product either by direct interaction with the protein itself or by allowing the expression of a factor that leads to its ability to function.

The agl3R mutant has no apparent affect on the expression of whiE, whiG, whiB, whiH, or sigF, nor is its expression dependent on whiG, whiH or whiE. A model for how known *whi* genes interact has been proposed (33). To investigate whether *agl3R* fits into this cascade, RT-PCR was performed to determine whether a mutation in *agl3R* affected the expression of other genes known to be involved in morphogenesis and antibiotic production and whether its expression was dependent on the activity of other *whi* genes. RNA was isolated from *S. coelicolor* M145 (Figure 2.6A, left) and *agl3R* (Figure 2.6A, right) at 24, 36, 48 and 60 hours and used as template for detection of *whiG*, *whiB*, *whiH*, *sigF*, and *whiE* orf 1 transcription. The constitutively expressed *hrdB* transcript was used as a control for the presence of RNA. We emphasize that this method is not quantitative but does allow detection of the presence or

absence of transcripts. This analysis confirms the observations from S1 nuclease mapping showing that *agl3R* transcription is repressed in the wild type and constitutive in the *agl3R* mutant. There is no detectable difference in the expression of any of the *whi* genes tested in the *agl3R* mutant (Figure 2.6A). While *sigF* and *whiE* transcripts were not detected in this analysis (these genes are transcribed late in development and their transcripts would not be present at the times examined here), the fact that no transcript from these genes was detected in the *agl3R* mutant suggests that it is unlikely to be a repressor of their transcription. While *whiH* transcription has been shown to be dependent on the activity of *whiG* (33), transcription of *agl3R* is not, suggesting that it is not part of the proposed *whiG* dependent regulatory cascade.

Discussion

agl3R controls transcription of itself and a group of genes likely involved in carbohydrate transport. S1 nuclease mapping of transcripts originating upstream of the GntR-like protein, *agl3R*, and the first open reading frame of the ABC-type transporter, *agl3E*, suggested that transcription of these genes is repressed by the *agl3R* protein and gel mobility shift experiments using His₆-Agl3R protein and the *agl3R-agl3E* intergenic region showed that the *agl3R* protein binds this region directly *in vitro*. Analysis of a reporter gene fusion to the *agl3E* promoter indicated that expression of the transporter is strongly induced by growth on relatively poor carbon sources such as trehalose and melibiose and weakly by lactose and glycerol. Induction was not repressed by the presence of glucose.

Most sugar transport in *Streptomyces* relies on either ABC-type transporters/permeases or phospho-transferases (PTS). A recent *in silico* study of the *S.*

coelicolor genome revealed 53 possible carbohydrate transport systems (3), most of which were ABC permeases. Carbohydrate uptake by ABC transporters has been described for cellobiose, cellotriose (37), and trehalose in *S. reticuli* (36), cellobiose and xylobiose in *S. lividans* (18), and maltose and maltodextrans in *S. coelicolor* (45). In addition, ABC transporters for maltodextran, cellobiose, cellotriose, β -xylosides, α -glucosides, xylose, chitobiose, lactose, sugar alcohols, ribose, maltose, maltodextrans, arabinose, cyclodextrans, trehalose, sorbitol, palatinose, and mannitol were identified based on similarity to previously characterized ABC clusters known to transport these sugars (3). The *agl3EFG* gene cluster was noted in that study as a likely sugar transporter, but was not assigned to a specific carbohydrate group and no transporters showing similarity to specific melibiose transporters were identified in that analysis. *agl3E* also shows similarity to the *msmE*, the corresponding sugar binding protein in the *Streptococcus mutans*, *msmEFG* transporter, a multiple sugar importer, one of which is melibiose (42). Here we show that the *agl3EFG* transporter, which has some weak similarity to the melibiose class of transporter responds to the presence of α -glucosides.

Most of the predicted regulatory genes for ABC transporters show similarity to the LacI/GalR family of transcriptional regulators. Transcription of the *malEFG* operon in *S. coelicolor*, for example, which encodes an ABC transporter specific for maltose and maltodextrans is controlled by MalR, a repressor located adjacent to the *malEFG* operon. The *malEFG* operon has been shown to be induced by the presence of amylase and maltotriose and is repressed by glucose (38). There is, however, an example of an ABC transporter, *dasABC* in *S. griseus*, that is controlled by the GntR-like repressor DasR (40). Members of this family contain similar N-terminal DNA-binding domains with HTH motifs

but lack significant similarity in regions involved in effector binding or oligomerization (16). Typically, oligomerization between regulatory subunits and/or conformational changes resulting from the binding or removal of inducing/repressing molecules allows correct HTH disposition and confers DNA binding ability to the protein as a whole. While many GntR family proteins have been shown to act as repressor proteins that are responsive to carboxylate-containing intermediates in carbon metabolism, some have been shown to bind sites other than typical operator sequences and at least one, FadR, can act as a transcriptional activator (12).

Inappropriate expression of an ABC transporter leads to defects in morphogenesis and antibiotic production. Constructed deletions of the ABC transporter encoded by *agl3EFG* resulted in suppression of the original transposon mutation, suggesting that inappropriate expression of the ABC transporter is responsible, at least in part, for the mutant phenotype. Mutations in *agl3EFG* alone have no growth phenotype probably because of redundancy in function with other α -glucoside transporters such as *agl1EFG* and *agl2EFG* and no morphological phenotype.

Interestingly, both *agl3R* and *whiH*, the two GntR-like regulators identified by mutation in *S. coelicolor*, encode transcriptional repressors and while the target of *whiH* is unknown, presumably the inappropriate expression of the genes it controls is responsible, at least in part, for the mutant phenotype. The organization of the *agl3EFG* gene cluster is similar to that of *dasABC* in *S. griseus* that like *agl3EFG*, is controlled by a GntR-like transcriptional regulator, *dasR*. Constructed deletions of *dasR* resulted in over expression of *dasABC* and ectopic sporulation when cells were grown on glucose. Over expression of *dasA*

itself showed the same phenotype suggesting that inappropriate expression this ABC transporter affects morphogenesis in this strain, as well.

The morphological phenotype of *agl3R* is similar to *whi* mutants of *S. coelicolor* in that it initiates morphogenesis, as evidenced by the emergence of aerial hyphae, but fails to develop the grey spore pigment associated with mature spores. Transcription of *whiH*, the other characterized GntR-like regulator in *S. coelicolor*, is dependent on *whiG* and *whiH* mutants are defective in the expression of some late sporulation genes, including *whiE* and *sigF*. The *whiH* gene product has also been implicated in the regulation of its own synthesis (33). The *whiG*, *whiA* and *whiB* genes apparently act early in aerial hyphae formation and participate in the decision to enter sporulation (13). They are absolutely required for sporulation septation, and for all visible signs of nucleoid condensation and partitioning as well as other changes associated with later stages of sporulation (13). In addition, *whiG*, *whiA* and *whiB* are epistatic to *whiH* in that they prevent these structures from forming in double mutants (7, 13). These spore-like features and the synthesis of clearly detectable levels of the *whiE*-directed grey spore pigment were not due to any residual activity of previously studied *whiH* alleles since they were retained by a constructed *whiH* null mutant. Interestingly, there is a potential ABC transporter located directly upstream of the *whiH* gene and initial BLAST searches show weak similarity to transporters of the amino acids glycine and proline (with E-values of 40% (6e-16) and 46% (2e-15), respectively) and multi-drug transporters (with E-values of 45% (3e-16) and 53% (2e-15), respectively) but not carbohydrate transporters. In fact, of the 57 probable GntR-like proteins in the *S. coelicolor* genome, five are adjacent to predicted ABC-type transporters, *Agl3R*, *WhiH*, *SCO0823*, *SCO6246* and *SCO6294*.

agl3R is apparently independent of the *whiG* regulatory pathway for the morphogenesis.

Several models have been proposed for the participation of the *whi* genes in aerial hyphae development (13, 33). The sigma factor encoded by the *whiG* gene is pivotal in the initiation of a series of steps that involve *whiH*, whose expression is *whiG* dependent. Expression of a late stage sigma factor encoded by *sigF* is dependent on *whiH*. Deletion of *agl3R* has no effect on transcription of *whiG*, *whiB*, *whiH*, *whiE* or *sigF* when compared to the wild type M145 (Figure 2.6) and transcription of *agl3R* was not dependent on *whiG*, *whiH* or *whiE* suggesting it does not participate in the *whiG* dependent regulatory cascade previously proposed.

What is the connection between the expression of the agl3EFG transporter, carbon utilization and the regulation of development? The regulation of carbon utilization is central to the most interesting and important aspects of the biology of *Streptomyces*. One aspect of the *bld* mutants, observed early in their characterization, is that growth on poor carbon sources is sufficient to partially restore the morphological and antibiotic defects of these mutants (5). With the exception of *bldB* (5, 6, 27, 30), when grown on minimal medium agar plates containing glucose as carbon source, *bld* mutants fail to erect aerial hyphae and are also defective in antibiotic production. When grown on minimal medium containing mannitol, aerial hyphae and spore production is partially restored. While growth on mannitol partially rescues the morphogenic defect of *bldA* mutants, the cells remain deficient in antibiotic production (5). In contrast, growth on mannitol rescues both sporulation and antibiotic production in *bldH* mutants (5). The most severe of the *bld* mutants, *bldB*, remains

both morphologically and physiologically defective, failing to sporulate or produce antibiotics, regardless of carbon source.

While these observations clearly suggest a connection between the initiation of morphogenesis and antibiotic production and the regulation of carbon utilization, the fact that the phenotype of some morphological mutants is carbon source dependence has been difficult to interpret. Are there several independent pathways for the initiation of morphogenesis, some of which are repressed by glucose? Does relief of carbon catabolite repression in *bld* mutants result in the expression of genes that affect morphogen transport? Does carbon source play a more direct role in initiation by, for example, inducing morphogen synthesis or morphogen transport?

The mechanism of carbon catabolite repression in *Streptomyces* is fundamentally different from that in Gram-negative bacteria and even different from low G+C Gram positive bacteria. While *Streptomyces* do produce cyclic-AMP and cyclic-AMP receptor protein (CRP) there is no clear link between these molecules and glucose mediated carbon catabolite repression (11). In *S. coelicolor*, glucose is not transported by a phosphotransferase and uptake depends on the activity of glucose kinase (1, 44). A number of genes have been implicated in the global regulation of carbon utilization in *Streptomyces* but no clear picture of how they interact or interconnect is evident from present studies. As in yeast, glucose kinase clearly plays a role in catabolite repression in *Streptomyces* independent of its kinase activity (26) perhaps involving other regulators (15). While the PTS does not transport glucose, Enzyme IIA-glucose plays a role in general sugar transport and inducer exclusion (21). A mutation in *ccrA1* leads to relief of glucose repression of a number of genes in *S. coelicolor* (20) suggesting that repression rather than activation controls the expression of

many genes involved in carbon utilization. Genes with similarity to *ccpA* have been identified but no clear homologue exists and efforts to detect serine-47 phosphorylated Hpr in *Streptomyces* have not been successful (29).

Interestingly, many studies focused on the regulation of catabolite repression have identified genes that also play a role in morphogenesis and antibiotic production. For example, levels of cAMP change during growth and development and reach a peak during the transition from vegetative growth to aerial mycelium production in *S. coelicolor* and mutations in either adenylate cyclase or CRP result in defects in spore germination and delays in sporulation and antibiotic production (10, 11).

Many ABC transporters have multiple functions, most notably *bldK* in *S. coelicolor* which transports the drug bialaphos and a small molecule morphogen, SapB. Drug transport is apparently irrelevant to the morphogenic defects of *bldK* mutants and the fact that the same transporter serves to import both molecules may be coincidental. Like *bldK*, the *agl3EFG* transporter may serve two functions, carbohydrate transport and morphogen signaling. The two bursts of transcription of *agl3E* seen in the mutant, correspond to the timing of two major decision points during development, one at the initiation of aerial hyphae formation and the other late in development as spore maturation begins. The fact that expression of this transporter is induced by complex carbohydrates may provide a direct connection between carbon utilization, morphogenesis and antibiotic production.

An alternative model for the effects we observe is that morphogen transport by this permease might be an accident of its binding chemistry and what we are detecting is a perturbation of the signaling cascade rather than a direct effect on development. Inappropriate and/or high level expression of the transporter, as in an *agl3R* mutant, might

lead to the transport of one or perhaps several signaling molecules, not normal substrates for this permease, literally giving the organism mixed signals as it enters stationary phase. This model would also provide an explanation for why the relief of carbon catabolite repression in *bld* mutants, and the resulting inappropriate expression of carbohydrate utilization genes, might lead to defects in morphogenesis. The pathways leading to morphogenesis and antibiotic production in these complex bacteria are clearly complicated, highly regulated and interconnected. Any serious perturbation in the progression of check points that result in normal development would result in defects in one or both of these processes.

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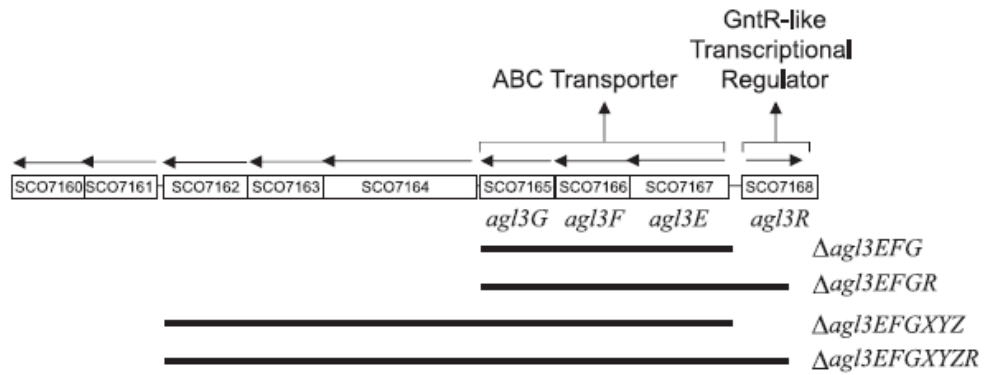


Figure 2.1 Organization of the *S. coelicolor* genome showing the *agl3R* open reading frame identified by transposon insertion and adjacent genes. Gene notations are based on the Sanger Centre Genome Sequencing Projects (www.sanger.ac.uk/Projects/S_coelicolor). Below the gene map is a diagram showing the limits of the DNA deleted (black lines) in constructed mutants.

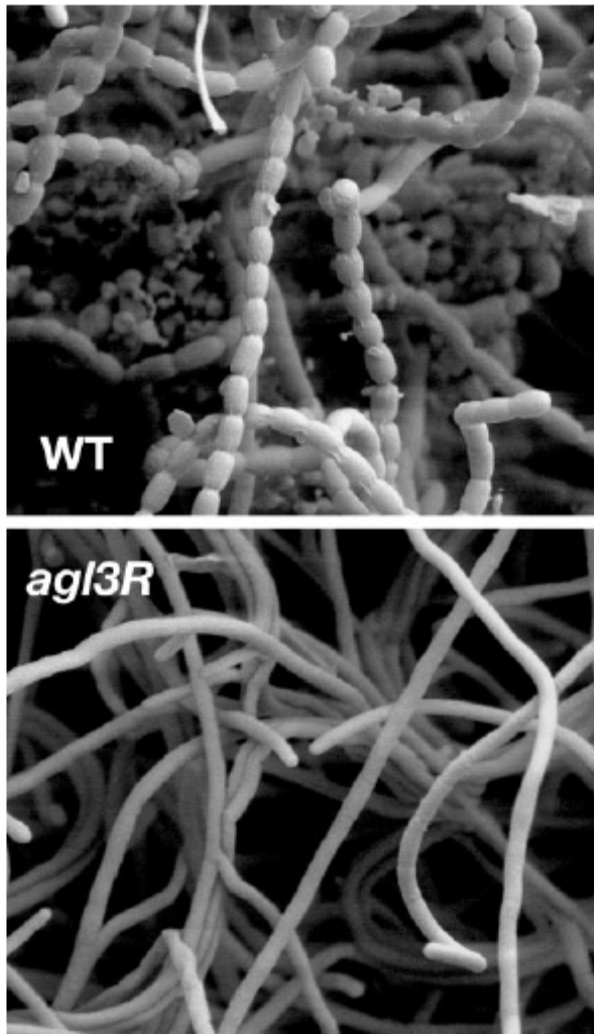


Figure 2.2 Scanning electron micrographs of aerial hyphae from wild-type *S. coelicolor* (M145) and the *agl3R* mutant at 5000 fold magnification. Both strains were grown for five days at 30° on MYM media.

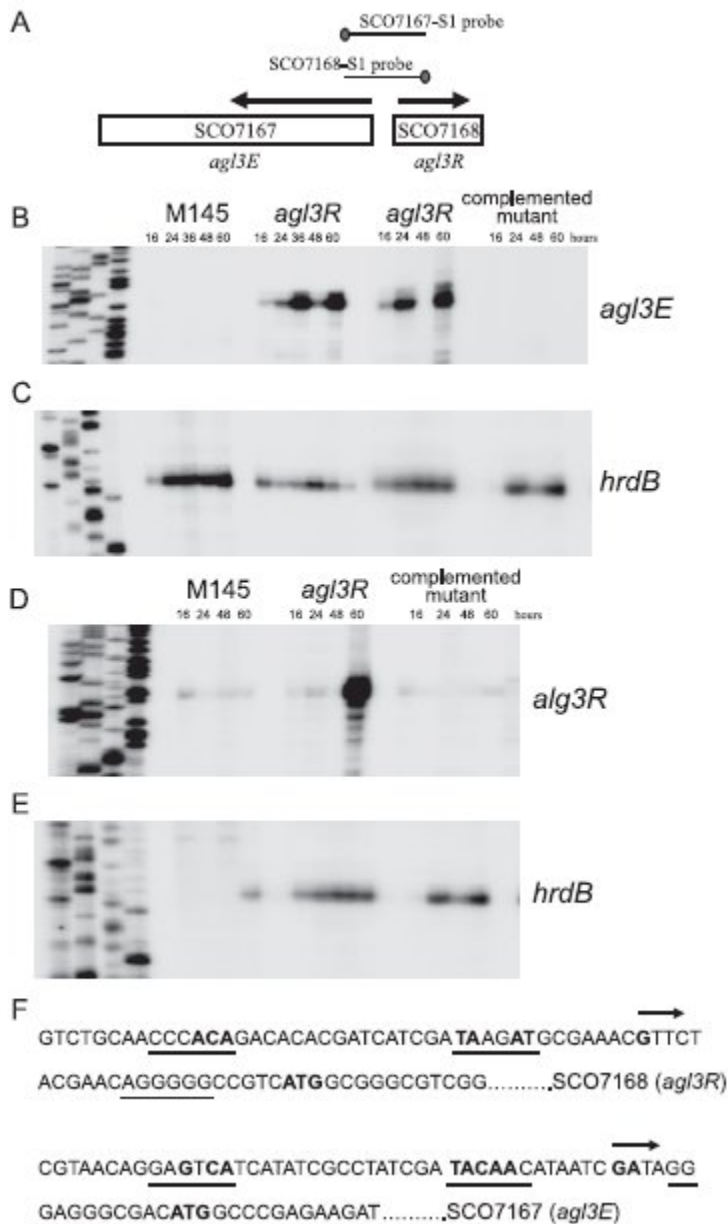


Figure 2.3 S1 nuclease mapping of transcripts originating upstream of *agl3R* and *agl3E*. RNA was isolated from the wild type (M145), the *agl3R* mutant and the *agl3R* mutant containing a wild type copy of the *agl3R* gene. Panel A shows the position of the probe used for detection of *agl3R* and *agl3E* transcripts. Panel B shows the protected RNA of a transcript originating upstream of *agl3R* isolated from the wild type, the *agl3R* mutant (two different experiments) and the *agl3R* mutant containing the *agl3R* wild type allele. Panel D shows the protected RNA of a transcript originating upstream of *agl3E* isolated from the wild type, the *agl3R* mutant and the *agl3R* mutant containing the *agl3R* wild type allele. Panels C and E show transcripts originating upstream of the *hrdB* gene as a control for RNA. In all cases the sequencing reactions were loaded A, G, C, T. Panel F. DNA sequence upstream of the apparent transcription start sites (indicated by bold letters and an arrow) for *agl3R* and

agl3E. The potential -35 and -10 region RNA polymerase recognition sequences and bases with consensus to TTGACA and TACAAT prototypical of vegetative promoters of *Streptomyces* are in bold and underlined. Putative ribosome binding sites are underlined and the annotated translation start sites (ATG for each) are also in bold letters.

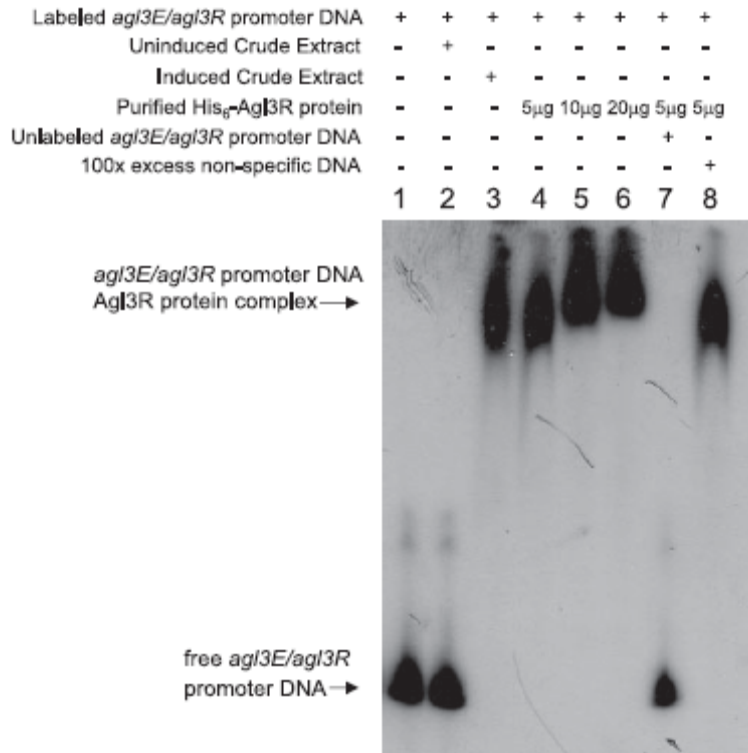


Figure 2.2.4 Gel mobility shift assays using a DNA fragment containing the intergenic region between *agl3E* and *agl3R* and His₆-Agl3R protein. Lane 1, labeled fragment; lane 2, labeled fragment with crude extract from uninduced cells containing the His₆-Agl3R construction; lanes 3, labeled fragment with crude extract from induced cells containing the His₆-Agl3R construction; lanes 4-6, labeled fragment with 5 µg, 10 µg, 20 µg of purified His₆-Agl3R protein; lane 7, labeled fragment with 100 ng unlabeled promoter containing fragment and 5 µg purified His₆-Agl3R protein, lane 9, labeled fragment with 100 µg sonicated salmon sperm DNA and 5 µg purified His₆-Agl3R protein

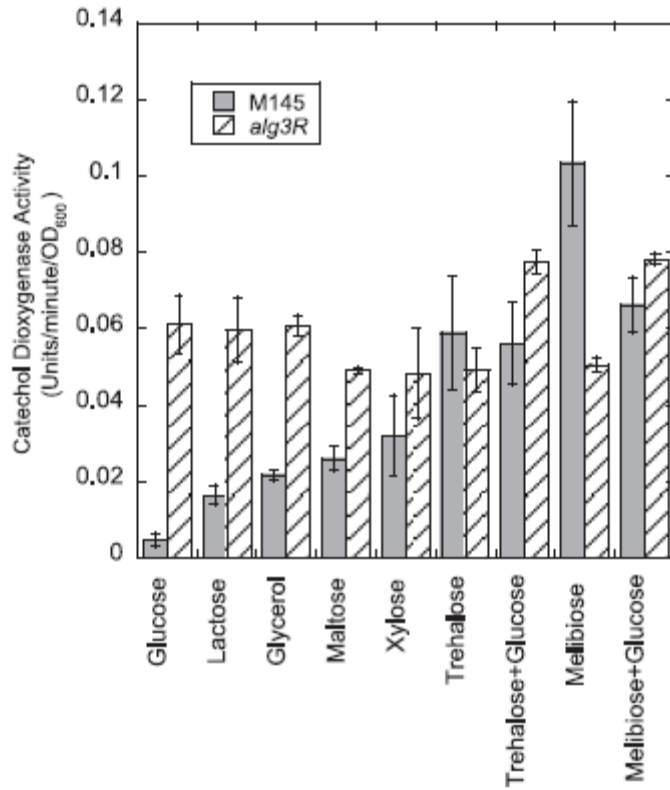


Figure 2.2.5 Histogram showing the results of quantitative catechol dioxygenase assays from cells grown on various carbon sources. The darkly shaded bars are from wild type (M145) cells and hatched bars are assays from the *agl3R* mutant, each containing a transcriptional fusion between the *xyIE* reporter gene and the promoter region of *agl3E*.

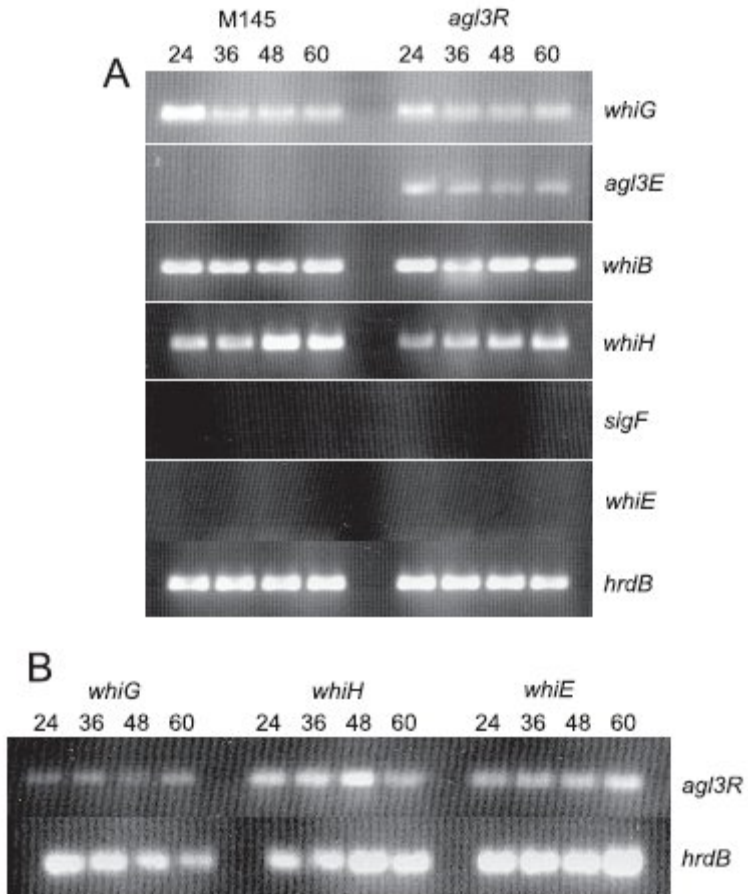


Figure 2.2.6 RT-PCR analysis of transcripts from wild type *S. coelicolor* and various *whi* mutants. Panel A, transcripts from *whiG*, *agl3E*, *whiB*, *whiH*, *sigF*, *whiE* in the wild type, M145, and the *agl3R* mutant. Panel B, transcripts from the *agl3R* in *whiG*, *whiH* and *whiE* mutants. *hrdB* transcript was used as a control for RNA.

Strain	Description	Reference or source
M145	SCP1 ⁻ SCP2 ⁻	23
<i>agl3R</i>	M145 SCO7168::Tn5:: <i>apr</i>	41
BH5	<i>agl3R</i> containing the <i>agl3R</i> wild-type allele	41
BH6	Deletion of SCO7167-SCO7165	This work
BH7	Deletion of SCO7167-SCO7162	This work
BH8	Deletion of SCO7167-SCO7165 and Δ SCO7168	This work
BH9	Deletion of SCO7167-SCO7162 and Δ SCO7168	This work
BH10	M145 containing the <i>aglE-xylE</i> fusion	This work
BH11	<i>agl3R</i> containing the <i>aglE-xylE</i> fusion	This work
J2408	<i>whiH::ermE</i>	13
J2400	<i>whiG::hyg</i>	13
YU105	<i>pro arg red act::ermE whiE::hyg</i>	47

Table 2.1 Strains used in this study.

Primer and purpose	Sequence ^a
To construct probes for S1 nuclease mapping	
<i>agl3E/agl3R</i> Forward	CGCTGCCCGCCGACGCGGTGGTCA
<i>agl3E/agl3R</i> Reverse	TGAGGTCCCGTTGACGATGAGACC
HrdB Forward	CGGCCGCAAGGTACGAGTTGATGA
HrdB Reverse	CCATGACAGAGACGGACTCGGCG
To amplify the promoter fragment for XylE fusions	
<i>agl3E/agl3R</i> Forward	TCCGCGTCCCGGA AGCTT CCGATGTCGCCG (wild-type sequence, AA GCGT)
<i>agl3E/agl3R</i> Reverse	GAGATAGGGGAGA AGCTT GTCGTTGTGCCG (wt sequence, GACCTT)
To construct deletion mutations	
SCO7167 Upstream	ATCGCCTATCGATAGAACATAATCGATAGGGAGGGCGACATTCCG GGGATCCGTCGACC
SCO7165 Downstream	TTCCGCCCGGTGGCGGGGATGTCGTGTCGTGCCCTCATGTGTAGG CTGGAGCTGCTTC
SCO7162 Downstream	GCCCTGGTCTGTGTCCACCTGTGGCCCGGCGCCCGCTGTGTAGGC TGGAGCTGCTTC
SCO7168 Upstream	CCGCGTACACGCTCCGCTCAGCCGTGGCCGTGATCGTGAATTCCG GGGATCCGTCGACC
To confirm deletion mutations	
<i>agl3R</i> Forward	CGACCGGCTCACGACCGGCCCGCGA
SCO7165 Reverse	CGCCGACGCGGGAGTCCCAGCGGA
SCO7162 Reverse	AGGGCCAGGGCCAGGGCCAGGACCG
P1	ATTCCGGGGATCCGTCGACCTGCA
P2	TGTAGGCTGGAGCTGCTTCGAAGT
To construct His ₆ -tagged <i>agl3R</i> protein	
His ₆ -Agl3R Forward	TTGTTGTAGAAGACGAGGGA
His ₆ -Agl3R Reverse	GGGGCCGGCTCAGATCTCTT
For RT-PCR	
WhiB Forward	GTCGACGACGCGGACGAGGAA
WhiB Reverse	AGATGCCGAAGCGCTCGTCGT
WhiG Forward	TGTGGCGGTGTCACAAGCGA
WhiG Reverse	ATCGCGTACGTCTCGAACTTG
WhiH Forward	AGCTGGGCCAGATGATCGTCT
WhiH Reverse	AAGGCACGCCATTGATGATG
WhiE Forward	TCTTCATGTCCGGCAACCGGA
WhiE Reverse	TAGAGCAGCCGACGCGTTCC
SigF Forward	GGAGGTGCTGTCTGCATCGA
SigF Reverse	GGAAACTACGTGCCAGTAGCC
Agl3R Forward	ACTGCTGTCCGACCGAGTGTA
Agl3R Reverse	CCGCGAACTCCTTCGATGATG
Agl3E Forward	AACATCACACCGAACCTGACC
Agl3E Reverse	AGGGGAGGACCTTGTGTTGT
HrdB Forward	GCCTTCGAAGCTGACCAGATT
HrdB Reverse	CGTCCCTTCTGCTGGTCA

^a Underlining indicates the HindIII restriction site; boldface indicates altered bases.

Table 2.2 Primers used in this study.

Chapter 3

A New TetR Family Transcriptional Regulator Required for Morphogenesis in *Streptomyces* *coelicolor*

Abstract

The interpretation of environmental signals plays a key role in the ability of an organism to survive starvation, adapt to environmental changes and initiate new programs of development. Both morphogenesis and antibiotic production in the *Streptomyces* are initiated in response to starvation and these events are coupled. We previously described a transposon generated mutant, SE293, which resulted in a *bld* strain that overproduced the antibiotic actinorhodin. The SE293 mutant also required arginine for growth on minimal medium. The SCO1135 open reading frame identified by the insertion encodes a member of the TetR family of transcriptional regulators. Here we show that a constructed deletion of the SCO1135 open reading frame resulted in the same phenotype as the insertion mutant but was prototrophic suggesting that the auxotrophy of the original mutant resulted from a second, unrelated mutation. The constructed deletion also resulted in constitutive expression of SCO1135 transcript as well as that of the gene cluster immediately adjacent to it, SCO1134-1132, which encodes a molybdopterin binding complex. A His₆-tagged version of the SCO1135 protein product was shown to bind the intergenic region between SCO1135 and SCO1134, which contains the apparent transcription start sites for each gene mapped by primer extension analysis. Increased expression of the SCO1134-1132 transcript in the SCO1135 deletion mutant also resulted in increased gene expression of xanthine dehydrogenase activity confirming the predictions about these open reading framed based on protein similarity. We have designated the SCO1134-1142 gene cluster *xdhABC* and the

regulator encoded by SCO1135 *xdhR*. We speculate that the inappropriate expression of xanthine dehydrogenase affects purine salvaging pathways at the onset of development creating artificially high concentrations of both GTP and ppGpp and perturbing the pathways these molecules participate in for the initiation of morphogenesis and antibiotic production.

Introduction

Bacteria must respond quickly and effectively to the constantly changing environments in which they live and regulatory proteins that respond to small molecule signals or morphogens serve to activate or repress the transcription of genes that allow the organism to adapt. The regulator proteins control interconnected and often complex networks that involve the interaction of multiple signaling pathways. Signal transduction typically involves the binding of a small molecule to a transcriptional regulator to modulate its activity on gene expression. The TetR-family is a common class of transcriptional regulator with more than 2000 members found in a wide variety of bacteria but only about 100 have been fully characterized (21). The first member of the group, TetR, was identified in *E. coli* and controls expression of the genes encoding a tetracycline efflux pump responsible for drug resistance conferred by Tn10. In the absence of tetracycline, TetR binds to the *tet* promoter and represses transcription of the efflux pump genes. When tetracycline enters the cell it binds TetR and causes conformational changes within the protein that abolish protein binding, thus relieving repression (23). RsrA, another member of this class has been shown to activate transcription of *rpoS*, a sigma factor responsible for transcription of stationary phase genes in *Pseudomonas putida*, in response to cell density (3).

TetR-like regulators contain a conserved HTH DNA-binding domain, form homodimers and generally act as repressors of transcription. They function to regulate a wide range of cellular activities including drug efflux, antibiotic production, amino acid metabolism, and development (21). There are 151 predicted TetR-like transcriptional regulators in the *Streptomyces coelicolor* genome (2, 21). Two are repressors of actinorhodin biosynthesis, ActII and CprB, two are repressors of drug resistance genes, Pip and PqrA, and one is a repressor of γ -butyrolactone synthesis, ScbR (1, 6-8, 27).

Both morphogenesis and antibiotic production in the streptomycetes are initiated in response to starvation. Upon sensing starvation, the substrate mycelia release small molecules that act as signals for the initiation of aerial hyphal growth as well as for the production of antibiotics (29, 30). As the aerial hyphae grow, they coil and septate into uninucleoid cells that give rise to spores. Most of what is known about this process comes from study of mutants that fail to produce aerial hyphae, called *bld* mutants, or those that initiate aerial hyphal growth but fail to produce mature spores, called *whi* mutants (13). We previously described a transposon generated mutant, SE293, that resulted in a *bld* strain that overproduced the antibiotic actinorhodin (25). The SE293 mutant also required arginine for growth on minimal medium (25). The SCO1135 open reading frame identified by the insertion encodes a member of the TetR family of transcriptional regulators. Here we show that a constructed deletion of the SCO1135 open reading frame resulted in the same phenotype as the insertion mutant but was prototrophic suggesting that the auxotrophy of the original mutant resulted from a second, unrelated mutation. The constructed deletion resulted in constitutive expression of SCO1135 and the gene cluster immediately adjacent to it, SCO1134-1132. The gene product of SCO1135 was shown to bind specifically to the

intergenic region between SCO1134 and SCO1134 which contains the transcription start sites for both genes as determined by primer extension analysis. The increased levels of transcription of SCO1134-32 also resulted in increased xanthine dehydrogenase activity as compared to the wild type. We chose to designate the SCO1134-1142 gene cluster *xdhABC* and the TetR-like regulator encoded by SCO1135 *xdhR*.

Methods and Materials

Strains and growth conditions. General techniques for bacterial growth were performed as described previously for *S. coelicolor* (16) and *Escherichia coli* (24) respectively. *S. coelicolor* strains were grown on mannitol-soya flour (MS) agar medium with the addition of 10 mmol MgCl₂ for mating experiments. Antibiotic selections were applied by overlay with soft nutrient agar (NSA). *Streptomyces* RNA was isolated from cells grown on cellophane discs placed on top of maltose-yeast extract-malt extract (MYM) agar. *E. coli* strains and growth conditions for the preparation of cosmids for marker replacement in *Streptomyces* were as described (9). The *S. coelicolor* strains used in this study were M145 (SCP1⁻, SCP2⁻), SE293/*xdhR* (M145 SCO1135::Tn5::*apr*), and Δ *xdhR*::*aac(3)IV* (M145 SCO1135::*acc(3)IV*). *E. coli* strains ET 12576 (*dam*⁻, *dcm*⁻) containing the non-transmissible helper plasmid pUZ8002 and BL21(DE3) (F⁻*dcm ompT hsdS* (rB- mB-) *gal* λ (DE3)) were used for mutant construction and protein expression respectively. Primers used to amplify fragments for construction of the His-tag fusion, construction of mutants, primer extension reactions, and reverse transcriptase (RT) PCR reactions are listed in Table 1.

Construction and confirmation of deletion mutants. A deletion of the SCO1135 open reading frame was constructed using the PCR targeting method described by Gust et al. The *ΔxdhR::aac(3)IV* mutant was made by replacing the SCO1135 open reading frame with the apramycin resistance cassette [acc(3)IV]. The presence and location of the apramycin resistance cassette was detected using specific primers, P1 and P2. The extent and location of the deletion was also confirmed by PCR. Marker replacement of the deletion with a wild type copy of *xdhR* was accomplished by the same method using cosmid 2STG38.

Scanning Electron Microscopy. The procedure used in this analysis was as previously described (22). Samples from colonies grown for 5 days on MYM agar plates were mounted on an aluminum stub with O.C.T. compound, submerged in liquid nitrogen slush at approximately -210° C, and transferred to a Gatan Alto 2500 cryostage and cryoprep chamber (Gatan UK, Ferrymills 3, Osney Mead, Oxford, OX2 0ES, UK) attached to a LEO 982 Field emission scanning electron microscope (FE-SEM, LEO Electron Microscopy, Inc. One Zeiss Drive, Thornwood, NY 10594). Samples were sublimated to remove surface frost at -95° C for 3 min, coated with platinum, placed on the cryostage in the main chamber of the microscope, at approximately -140° C, and viewed at 5.0 kV.

RT-PCR. RNA was isolated from *S. coelicolor* M145 and the *ΔxdhR::aac(3)IV* mutant after 24, 48, and 72 h of growth on MYM agar medium overlaid with cellophane discs as for primer extension analysis. The One-Step PCR kit (QIAGEN) was used with primers specific for each gene. Forward and reverse primers for *xdhA*, *xdhR*, *whiG*, *whiB*, *whiH*, and *hrdB* are listed in Table 1. Reaction mixtures contained 10 pmol of each primer and 100 ng of RNA in

a total volume of 20 μ l. Each primer was first tested using chromosomal DNA as template and without a reverse transcription (RT) cycle to test for DNA contamination in the RNA. HrdB was used as a control for RNA concentration. Products were displayed on a 1% agarose gel and visualized by staining with ethidium bromide.

Gel retardation assays. To construct a His-tagged version of the XdhR protein, the coding region was amplified by PCR from *S. coelicolor* genomic DNA using primers XdhR-His Forward and XdhR-His Reverse (Table 1). The fragment was digested with NdeI (NEB) and EcoRI (NEB) and cloned into NdeI/EcoRI digested pET28a. XbaI and HindIII digestion confirmed the correct orientation and fusion to the His-tag. Expression and purification of the His₆-XdhR protein from *E. coli* was as previously described (12). Purified His₆-XdhR protein (5, 10, or 20 μ g) or crude cell extract (20 μ g total protein) was mixed with the 305-bp PCR-generated DNA fragment containing the intergenic region between SCO1134 and SCO1135 used for primer extension experiments. The fragment was 5'-end labeled with [γ -³²P]ATP (MP Biomedicals) using T4 polynucleotide kinase (Promega) and purified on a 1% agarose gel. The labeled DNA fragment (1 ng; 6,000 cpm) was incubated with cell extracts or purified His-tagged XdhR protein for 20 min at 30° C in 20 μ l (total volume) of binding buffer (20 mM Tris, 10 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, pH 8.0) containing 1 μ g sonicated salmon sperm DNA and 3 μ g bovine serum albumin. For competitive inhibition of the binding reaction, 100 ng of unlabeled fragment or 100 μ g of sonicated salmon sperm was added to 5 μ g purified His₆-XdhR protein. Reactions were displayed on a non-denaturing 6% acrylamide Tris-borate-EDTA gel and visualized by autoradiography.

RNA isolation and primer extension analysis. Primer extension reactions were carried out as described previously (24). RNA was isolated from cells grown for 48 h on MYM agar plates overlaid with cellophane discs. Primers were labeled using [γ - ^{32}P]ATP 6000Ci/mL and OptiKinase (USB) according to the manufacturer's instructions. 40 μg of RNA was hybridized to 1 pmol of either XdhA PE primer or XdhR PE labeled primer by heating reactions to 65° C for 20 min and the allowing them to cool to room temperature for 10 min. The annealed primer and RNA mixture was added to a reaction mixture containing 40 mM sodium pyrophosphate and 1 unit AMV Reverse Transcriptase (Promega) and incubated at 42° C for 30 min. A 305 bp fragment, generated by PCR using primers PE seq Forward and PE seq Reverse from chromosomal DNA, was used as template. This fragment was also used to generate the DNA sequence ladder with the fmol cycle DNA sequencing system (Promega) with 1 pmol 5' [γ - ^{32}P] 6000 Ci/mL labeled primer. Products from the DNA sequencing reactions were separated on a 6% denaturing polyacrylamide gel, and bands were visualized by autoradiography.

Xanthine dehydrogenase and aldehyde oxidoreductase assays. Cells were grown for 36 hrs at 30° C in YEME medium, harvested by centrifugation at 3500 g for 10 minutes and resuspended in extraction buffer (0.1M Tris-HCl pH 7.6, 10mM MgCl₂, 6mM 2-mercaptoethanol, 1mM phenylmethylsulphonyl fluoride). Extracts were obtained by first homogenization by sonication, followed by centrifugation at 12,000 g for 15min at 4° (Keiser, hopwood). XDH activity was measured spectroscopically as conversion of NAD⁺ to NADH at 340nm in 50 mM sodium pyrophosphate buffer (pH 8.5) containing 0.2 mM EDTA, 0.15 mM xanthine and 0.5 mM NAD⁺ (14). 20 μg of cell extract was added and

measurements were taken at 5, 10, 15 and 20 min intervals. AOR activity was determined by reduction of the electron acceptor 2,6-dichlorophenol-indophenol (DCPIP) 600 nm in 50 mM Tris-HCl buffer (pH 7.6) containing 35 μ M DCPIP, and 50 μ M acetylaldehyde (28). 20 μ g of cell extract was added and measurements were taken at 5, 10, 15 and 20 min intervals. XDH and AOR activities were normalized to glutamate dehydrogenase (GDH) activity. GDH activity was determined by the same method as XDH/AOR activity in 500 mM glycine/50 mM aminooxyacetic acid buffer (pH8.6) containing 5 mM NAD⁺ and 50 μ M xanthine (20). Protein concentrations were determined by Bradford assay (4).

Results

A deletion of the xdhR open reading frame results in a bld mutant phenotype. A mutation in *xdhR* was first identified by a transposon insertion into the SCO1135 open reading frame (25). The location of SCO1135 and the organization of the genes on the chromosome near it are shown in Figure 3.1. The *xdhR* open reading frame encodes a TetR family transcriptional regulator and is located directly adjacent to but in the opposite orientation of a gene cluster, *xdhA* (SCO1134), *xdhB* (SCO1133), and *xdhC* (SCO1132), predicted to encode a molybdopterin binding protein complex. The transposon mutant was completely defective in morphogenesis failing to make the aerial hyphae associated with the initiation of development but overproduced the blue pigment associated with the polyketide antibiotic actinorhodin. While the original mutant had a single copy of the transposon it also required arginine for growth on minimal medium. The arginine auxotrophy was presumed to be unrelated to the morphological phenotype (25). A deletion of the *xdhR* open reading frame was constructed using the targeted marker replacement method developed by Gust, et al. (9).

The deletion mutant, $\Delta xdhR::aac(3)IV$, had the same morphological phenotype as the insertion mutant but was able to grow on minimal medium suggesting that the arginine auxotrophy was most likely a second and unrelated mutation.

Scanning electron microscopy of the $\Delta xdhR::aac(3)IV$ mutant (Figure 3.2) grown on MYM sporulation medium revealed normal substrate mycelia but no evidence of aerial hyphae or spores. Unlike many *bld* mutants that are substantially delayed in morphogenesis but will eventually produce some spores, the $\Delta xdhR::aac(3)IV$ mutant never produces either aerial hyphae or spores even with prolonged growth on sporulation agar.

The morphogenic and antibiotic production phenotypes of the $\Delta xdhR::aac(3)IV$ mutant were not complemented with the wild type allele of *x dhR* introduced on a pSET plasmid integrated at the $\phi C31$ attachment site. Similar experiments with the original SE293 transposon insertion mutant also failed to show complementation by the wild type allele (25). To test whether the observed phenotypes were due to the *x dhR* mutation, cosmid 2STG38 was introduced into the $\Delta xdhR::aac(3)IV$ mutant and exconjugants containing a marker replacement of the mutation with the wild type allele were obtained. The resulting strain was wild type. This suggests that the *x dhR* mutation, in fact, caused the defects in morphogenic and antibiotic production and while it is not clear why the mutation is not complemented by a wild type copy of the gene, it raises the possibility that the *x dhR* gene product does not work *in trans*.

The x dhR gene product acts to represses its own transcription as well as transcription of an adjacent gene cluster that encodes a putative molybdopterin binding complex. To test whether a mutation in x dhR affected regulation of itself or the adjacent gene cluster, RT-PCR

reactions were performed using RNA isolated from wild type *S. coelicolor* and the $\Delta xdhR::aac(3)IV$ mutant that had been grown for 24, 36, 48 or 60 hrs on MYM sporulation medium. This solid medium supports morphological development of the wild type and the stages of development are clearly detectable. Cells harvested after 16 hours were growing vegetatively (no aerial mycelia present). Aerial mycelia began to appear between 24 and 36 hours and were abundant between 36 and 48 hours as was the blue pigment associated with actinorhodin production. The grey pigment associated with mature spores was evident at 60 hours.

As shown in Figure 3.3, transcript from *x dhA*, the first of a series of the genes adjacent to *x dhR*, was detected at low levels during growth phase and at higher levels as development proceeded with a peak in wild type cells coincident the aerial hyphae production.

Transcription of *x dhA* was constitutive in the $\Delta xdhR::aac(3)IV$ mutant. RT-PCR reactions were also performed using RNA isolated from wild type *S. coelicolor* and the $\Delta xdhR::aac(3)IV$ mutant to examine the effect of the mutant on its own expression. As shown in Figure 3.3, in the wild type strain, transcript from *x dhR* was similar to that of the adjacent cluster, present at low levels during growth phase and at higher levels as development proceeded with a peak in wild type cells coincident the aerial hyphae production. Transcription of *x dhR* was constitutive in the $\Delta xdhR::aac(3)IV$ mutant. The same RNA preparations used for this analysis were used for detection of the *hrdB* transcript, which served as a control for the level of RNA (Figure 3.3). The PCR products shown are samples taken during the exponential phase of the PCR reaction so that the amount of product is representative of quantitative differences in RNA level. These data suggest that the *x dhR*

gene product acts to repress, either directly or indirectly, transcription of itself as well as the genes in the adjacent gene cluster.

The xdhR gene product binds the intergenic region between xdhR and xdhA in vitro. To test whether the *xdhR* protein made direct contact with the *xdhR* and/or *xdhA* promoter regions, a His₆-XdhR protein was constructed and expressed in *E. coli* and used in a gel mobility shift assays with a DNA fragment containing the intergenic region between *xdhR* and *xdhA*. As shown in Figure 3.4, crude extracts from *E. coli* cells expressing the His₆-XdhR protein retarded the promoter-containing fragment, whereas extracts from uninduced cells did not. Furthermore, the amount of probe shifted in the retardation assay was reduced in the presence of unlabelled probe but not nonspecific DNA suggesting that binding of XdhR to the promoter-containing fragment was sequence or motif specific.

Primer extension analysis, Figure 3.5 Panel A, was used to identify apparent transcription start sites for *xdhR* and *xdhA*. The start site of *xdhR* maps to an adenine or guanine nucleotide located 19 or 20 nucleotides upstream of the annotated translational start codon. The sequences centered at -10 and -35 with respect to the apparent start site show little homology to known consensus sequences for RNA polymerase binding. The start site for *xdhA* maps to guanine or adenine located 18 or 19 nucleotides upstream of the annotated translational start site and like *xdhR* the -10 and -35 regions show little homology to known RNA polymerase binding sites. While the leader region of these predicted transcripts is relatively short, each contains a potential ribosome binding site, in fact, the putative ribosome binding site for *xdhR* was previously annotated in the genome sequence.

The xdhR mutant is defective in the expression of other developmental genes. The $\Delta xdhR::aac(3)IV$ mutation results in a *bld* phenotype. To test the effect of this mutation on the expression of other developmental genes, RT-PCR was performed on RNA isolated from wild type *S. coelicolor* M145, and the $\Delta xdhR::aac(3)IV$ mutant. Primers specific for *whiG*, *whiB*, *whiH*, *xdhA*, *xdhR* and *hrdB*, a constitutively expressed gene as a control for RNA, were used. While transcripts from *whiG*, *whiB*, and *whiH* were readily detected from the wild type strain no expression of these genes was detected in the $\Delta xdhR::aac(3)IV$ mutant. The *whiG*, *whiB*, and *whiH* genes play early roles in the cascade of *whi* gene expression but their expression is dependent on several *bld* genes and *xdhR* is clearly one of them.

The SCO1132-34 gene cluster likely encodes a xanthine dehydrogenase/aldehyde oxidoreductase enzyme complex. The predicted protein products of the *xdhA* (SCO1134), *xdhB* (SCO1133), *xdhC* (SCO1132) gene cluster show significant similarity to a molybdopterin binding protein in the aldehyde oxidoreductase (AOR)/xanthine dehydrogenase (XDH) family (19). *XdhC* shows 63% similarity (50% identity) to the molybdodenum binding subunit of *S. erythraea* XDH; *xdhB* shows 73% similarity (61% identity) to the FAD-binding subunit and *xdhA* shows 73% similarity (60% identity) to the [2FE-2S] binding subunit (<http://www.ncbi.nlm.nih.gov/BLAST/>). Molybdenum is often bound to a pterin cofactor and the synthesis of the molybdopterin cofactor has been shown to require more than twelve proteins that are highly conserved in all organisms (11). Molybdenum containing enzymes perform a variety of functions, but all involve oxidation or reduction reactions. These enzymes take advantage of the ability of molybdenum to exist in a variety of oxidation states under physiological conditions. This allows the enzyme to catalyze

redox-reactions that require the movement of one or two electrons (11). Molybdenum enzymes are grouped in three families, the xanthine oxidase/aldehyde oxidoreductase family, the sulfite oxidase family, and the DMSO reductase family. Like XDH and AOR proteins, the putative heterodimeric protein encoded by the *xdhABC* gene cluster is composed a [2Fe-2S] iron-sulfur binding domain, a flavin adenine dinucleotide (FAD) binding domain, and a molybdopterin binding/dimerization domain (17). The enzyme catalyzes the reactions that convert hypoxanthine into xanthine and then xanthine to uric acid.

To test whether this gene cluster in fact encoded a xanthine dehydrogenase complex, enzyme assays were performed on extracts from wild type and $\Delta xdhR::aac(3)IV$ mutant cells. XDH activity was determined spectrophotometrically by assaying conversion of NAD⁺ to NADH at 340 nm in the presence of xanthine. In the presence of xanthine, the rate of NAD⁺ to NADH increased 2.7 fold in $\Delta xdhR::aac(3)IV$ mutant strain as compared to wild type. Aldehyde oxidoreductase activity was assayed by reduction of DCPIP as measured by increased absorbance at 600 nm and there was no significant difference between the wild type and $\Delta xdhR::aac(3)IV$ mutant for this activity. Bradford assays were used to determine protein concentration of cell-free extracts. All enzyme activities were normalized to glutamate dehydrogenase activity.

Dicussion

Scanning electron microscopy of the $\Delta xdhR::aac(3)IV$ mutant showed that the mutation resulted in complete loss of morphological development (no aerial mycelium even after prolonged incubation on sporulation medium) while apparently increasing actinorhodin antibiotic production. RT-PCR analysis of transcripts from *xdhR* and *xdhABC*, the first gene

in an adjacent gene cluster, suggested that *xdhR* acts to repress its own transcription as well as transcription of *xdhABC*. Gel mobility shift experiments using His₆-XdhR protein and the *xdhR-xdhA* intergenic region showed that the XdhR protein binds this region directly *in vitro* and primer extension analysis identified apparent transcription start sites for *xdhR* and *xdhABC* within this region. Xanthine dehydrogenase assays showed that the level of enzyme activity was significantly increased in the *xdhR* mutant supporting the prediction from BLAST analysis that the *xdhABC* gene cluster encodes a xanthine monoxide dehydrogenase complex. RT-PCR analysis of transcription of other genes required for morphological development in *S. coelicolor* showed that *xdhR* is required for transcription of *whiG*, *whiB*, and *whiH*.

XdhR is a member of the TetR-family of transcriptional regulators and, like other members of this group is a DNA binding protein that acts to repress transcription. Xanthine monoxide dehydrogenase is a molybdo-flavoenzyme that participates in purine catabolism and catalyzes the conversion of hypoxanthine to xanthine and xanthine to uric acid. Xanthine dehydrogenase is a heterodimeric protein composed of two [2Fe-2S] cluster binding domains, a flavin-adenine (FAD) binding domain, and a domain for dimerization and binding of the MOCO molybdenum-cofactor (5). Electrons from the substrate are passed from the MOCO center to FAD by the two [2Fe-2S] clusters. Once electrons reach the FAD site, they are transferred either to molecular oxygen or to NAD⁺ to form NADH. In *E. coli* conversion of hypoxanthine to xanthine by xanthine dehydrogenase plays a role in the purine salvage pathway. Deletion mutants of *xdhA* were sensitive to exogenous adenine, a phenotype previously shown to be due to inefficient conversion of adenine to guanine due to decreased xanthine availability (18, 31).

Why should over-expression of xanthine monooxygenase result in loss of morphogenesis while increasing antibiotic production? The purine tetraphosphate, ppGpp, is produced under conditions of amino acid limitation by the activity of RelA (10) which phosphorylates GTP to ppGpp and pppGpp. These molecules have been implicated in the sensing of nutritional shifts in both *E. coli* and *S. coelicolor*. Interestingly, *S. coelicolor*, *relA* mutants are defective in antibiotic production and delayed in morphogenesis but the delay in morphogenesis occurs only under conditions of nitrogen limitation (26). Antibiotic production is restored by over-expression of *relA* suggesting that increased levels of ppGpp restore antibiotic production. *Streptomyces clavuligerus relA* mutants are defective in antibiotic production and are *bld* under all conditions (15). In *S. clavuligerus*, ppGpp synthesis is accompanied by depletion of the cellular GTP pool (15). If, as in *E. coli*, the xanthine dehydrogenase encoded by the *xdhABC* cluster is involved in purine salvage, under starvation conditions such as those that signal the initiation of morphogenesis and antibiotic production in *Streptomyces species*, induction of purine salvage might be a signal for the initiation of antibiotic production and morphological development. Inappropriate expression of this xanthine dehydrogenase might interfere with this signaling pathway and lead to a defect in the ability of the organism to appropriately sense or interpret starvation signals. Over expression of a XDH cluster could result in an abnormally high level of xanthine in the cells leading to an elevated level of GTP during the time when ppGpp synthesis is strongest. High levels of ppGpp synthesis that result in depletion of the intracellular GTP pool are normally enough to trigger morphological differentiation in *Streptomyces species* (15). A large GTP pool could cause an arrest in morphological development while at the same time increased ppGpp production could cause over production of actinorhodin.

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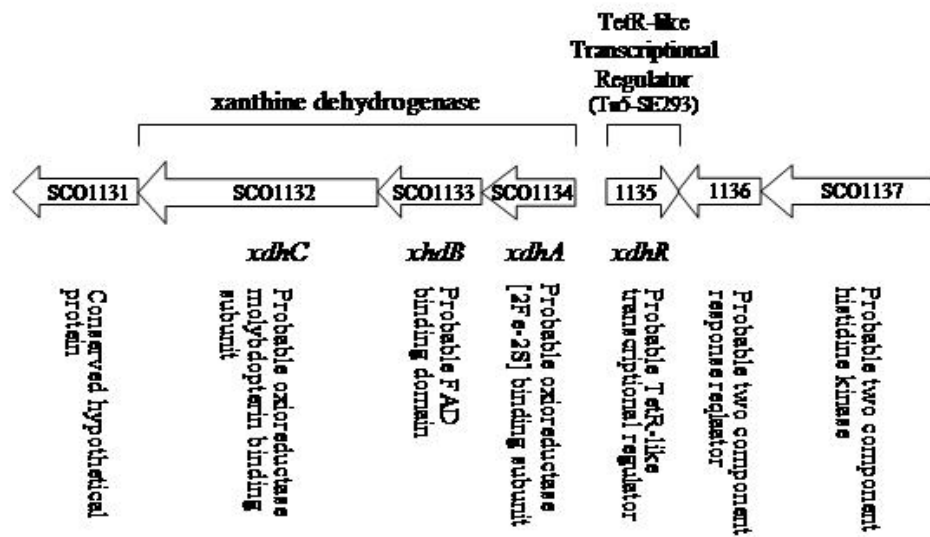


Figure 3.1. Organization of the *S. coelicolor* genome showing the *xdhR* open reading frame identified by transposon insertion and adjacent genes. Gene notations are based on the Sanger Centre Sequencing Projects (http://www.sanger.ac.uk/Projects/S_coelicolor).

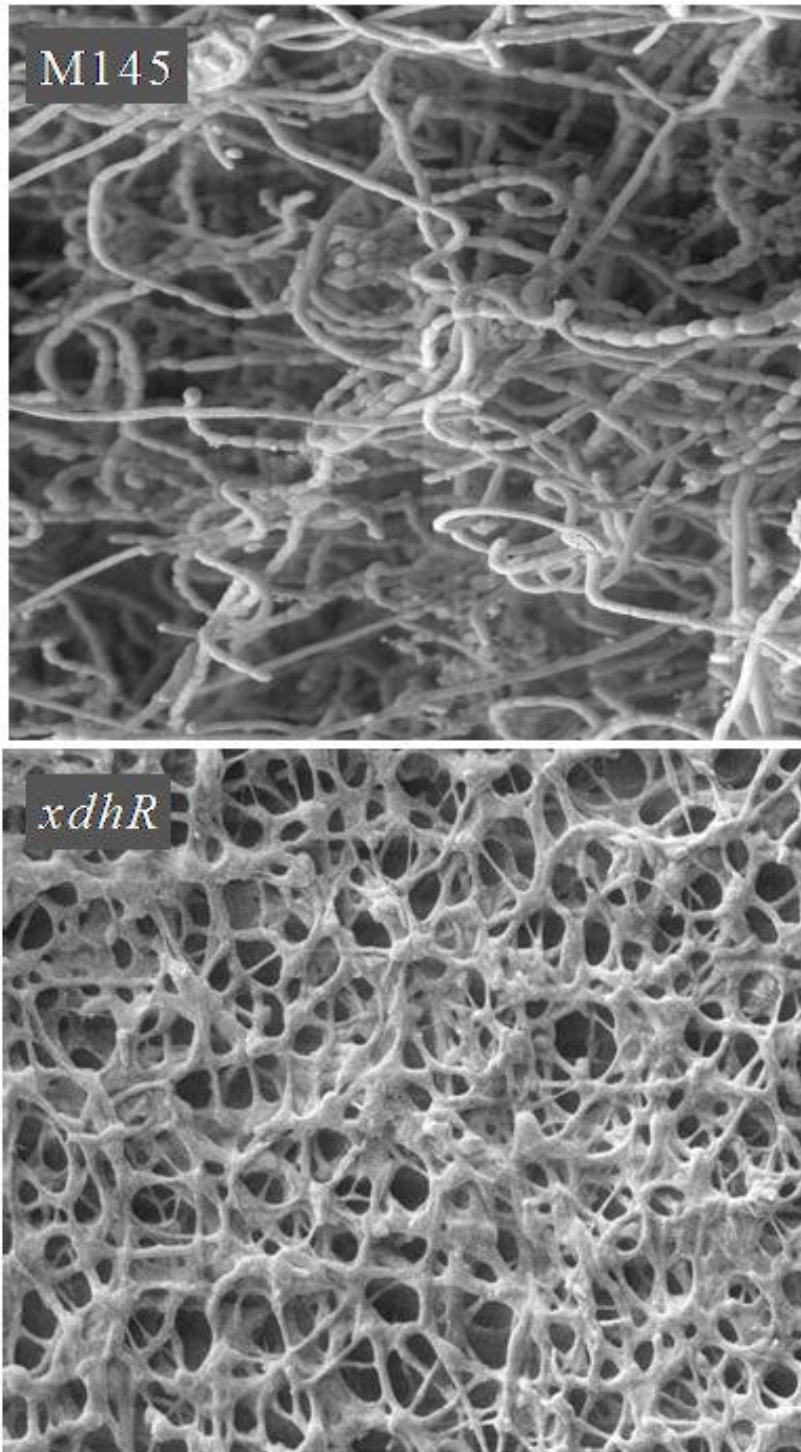


Figure 3.2. Scanning electron micrographs of wild type *S. coelicolor* (M145) and the *xdhR* mutant ($\Delta xdhR::aac(3)IV$) at X2,000 magnification. Both strains were grown for 5 days at 30° C on MYM agar medium.

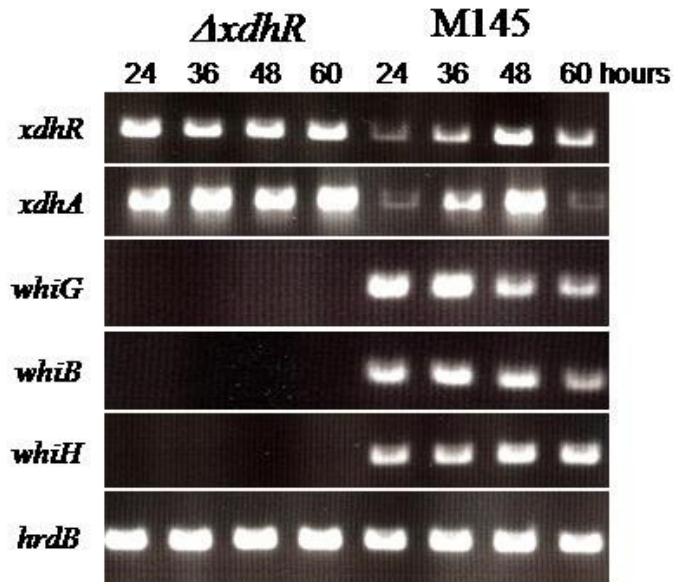


Figure 3.3. RT-PCR analysis of transcripts from various morphological mutants in wild type *S. coelicolor* (M145) and the *xdhR* deletion mutant ($\Delta xdhR::aac(3)IV$). *hrdB* was used as a control for RNA.

Labeled <i>xdhA</i> / <i>xdhR</i> promoter DNA	+	+	+	+	+	+	+	+
Uninduced crude extract	-	+	+	-	-	-	-	-
Induced crude extract	-	-	+	-	-	-	-	-
Purified His ₆ -XdhR protein	-	-	-	5µg	10µg	20µg	5µg	5µg
Unlabeled <i>xdhA</i> / <i>xdhR</i> promoter DNA	-	-	-	-	-	+	+	-
100x excess non-specific DNA	-	-	-	-	-	-	-	+
	1	2	3	4	5	6	7	8

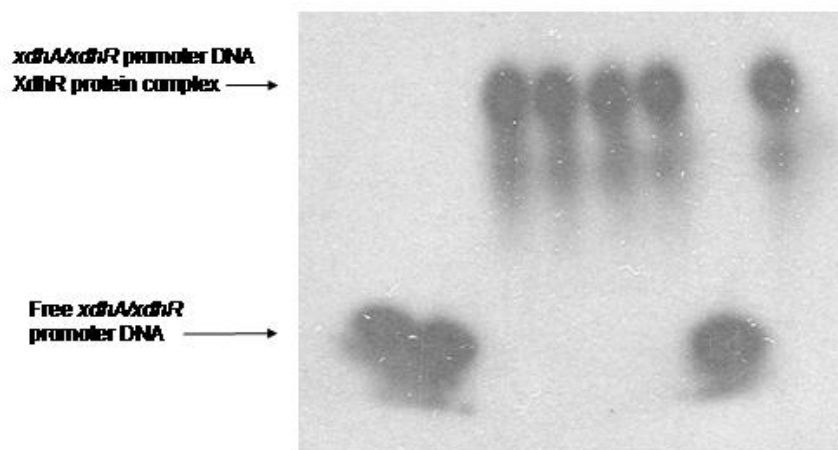


Figure 3.4. Gel mobility shift assays using a DNA fragment containing the intergenic region between *xdhR* and *xdhA* and the His₆-XdhR protein. Lane 1, labeled fragment; lane 2, crude extract from uninduced cells containing the His₆-XdhR construction; lane 3, crude extract from induced cells containing the His₆-XdhR construction; lanes 4 to 6, labeled fragment 5 µg, 10 µg, or 20 µg of purified His₆-XdhR protein; lane 7, labelled fragment with 100 ng unlabeled promoter-containing fragment and 5 µg purified His₆-XdhR protein; lane 9, labeled fragment with 100 µg sonicated salmon sperm DNA and 5 µg purified His₆-XdhR protein.

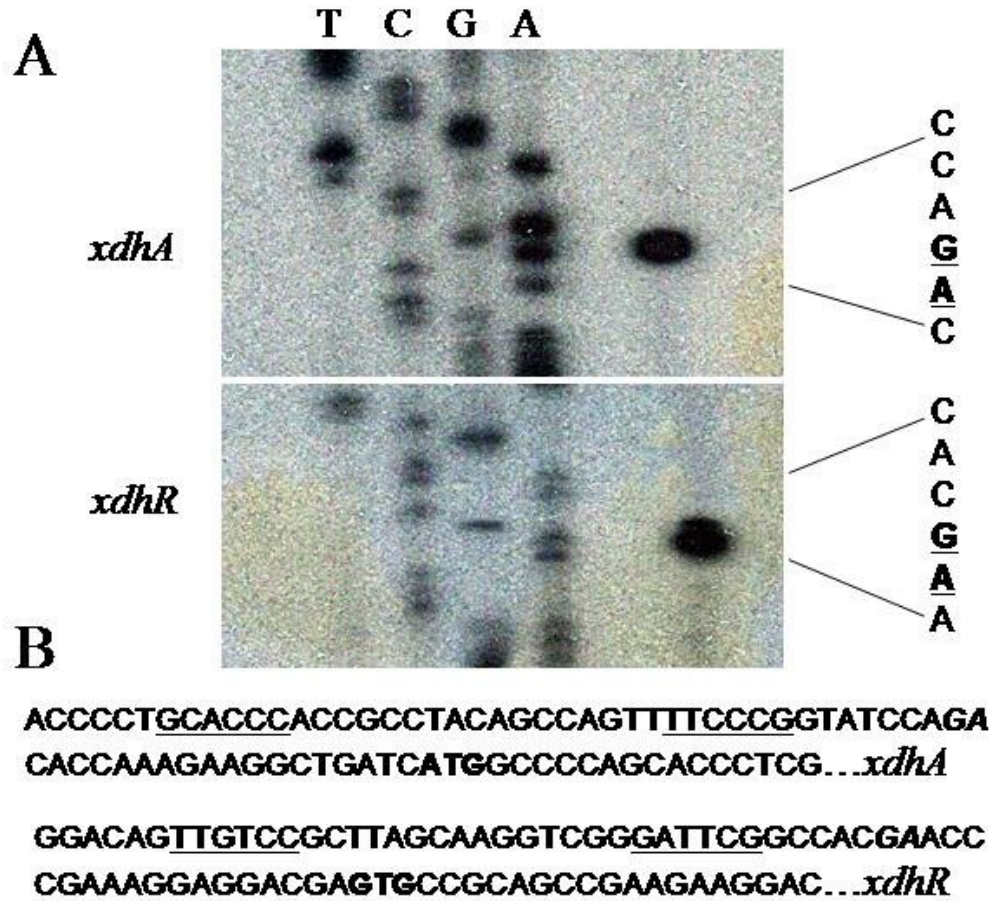


Figure 3.5. Panel A. Primer extension analysis of transcripts originating upstream of *xdhR* and *xdhA*. Panel B. DNA sequence upstream of the apparent transcription start sites (indicated in bold face type italics) of *xdhR* and *xdhA*. Potential RNA polymerase recognition sequences are underlined. The annotated translation starts are indicated by bold face type with no italics.

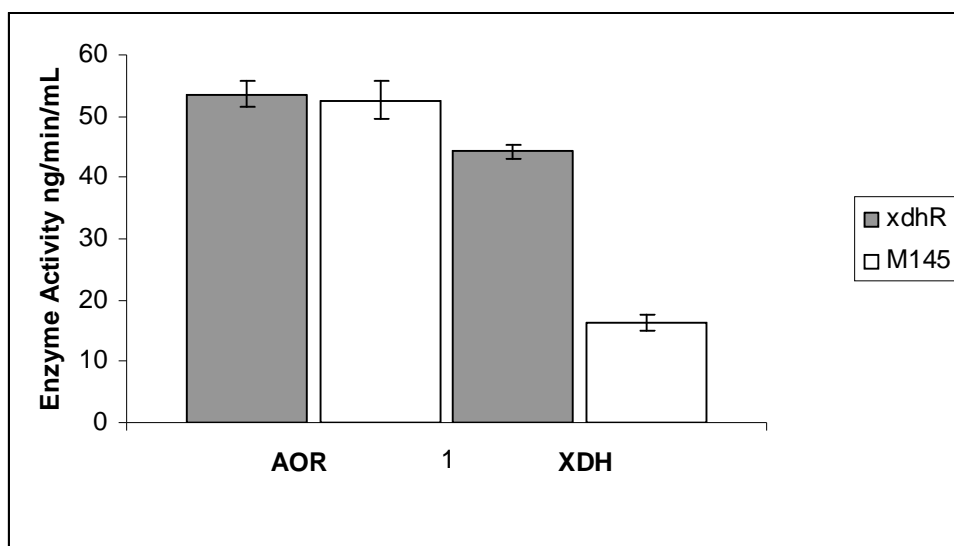
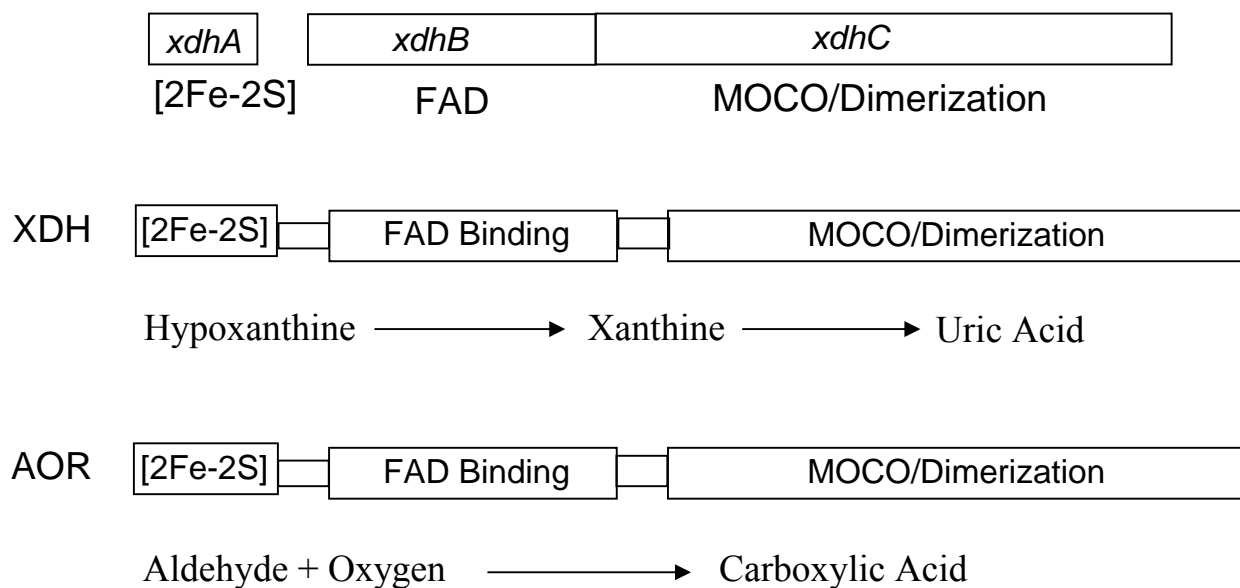


Figure 3.6. Panel A. Comparison of the predicted products of *xdhABC* with xanthine dehydrogenase (XDH) and aldehyde oxidoreductase (AOR). Panel B. Enzyme assays using cell extracts from either the wild type (M145) and the *xdhR* deletion mutant ($\Delta xdhR::aac(3)IV$). AOR activity was determined by reduction of 2,6-dichlorophenol-indophenol (DCPIP). XDH activity was measured spectroscopically as conversion of NAD⁺ to NADH at 340nm.

Chapter 4

Discussion

The primary objective of this work was to characterize two newly identified transcriptional regulators and determine what roles they played in regulation of morphological development and antibiotic production in *Streptomyces coelicolor*. I determined by mutation that the GntR-like transcriptional regulator encoded by *agl3R* was required for proper morphogenesis and development. The *agl3R* mutant exhibited a *whi* phenotype and failed to produce actinorhodin. Scanning electron microscopy confirmed the lack of spores that is characteristic of *whi* mutants. I further determined by S1 nuclease mapping that the phenotype was a result of loss of repression of the adjacent gene cluster, *agl3EFG*, which encodes an ABC transporter. Repression was shown to be due to direct interaction between Agl3R and the intergenic region between *agl3EFG* and *agl3R* by gel retardation assay with purified His-6-Agl3R protein. Using *xylE* fusions to the *agl3EFG* promoter, it was shown that transcription was induced in the presence of α -glucoside sugars, especially melibiose. RT-PCR reactions showed that inactivation of the *agl3R* gene did not significantly effect the transcription of the early *whi* genes, *whiB*, *whiG*, and *whiH* (2). As expected from the phenotype, transcripts from the late *whi* genes, *whiE* and *sigF* (2), were undetectable by RT-PCR. This lead me to the conclusion that *agl3R* most likely does not participate in the previously proposed WhiG-dependent cascade for morphogenesis (4). The phenotype of the *agl3R* mutant appears to be linked to the improper over expression of Agl3EFG. One explanation for this is that when the transporter is expressed at a time when its preferred substrates are not present, it imports a similar yet less ideal molecule. The idea that a *S. coelicolor* ABC transporter imports multiple molecules is not novel. The *blcK* gene encodes an ABC transporter that imports a morphogen required for initiation of the *blcD*

cascade that ultimately leads to SapB production and erection of aerial hyphae (6). The transporter encoded by the *bldK* gene also transports the drug bialophos and mutants are resistant to the drug (7). Taking the case of *bldK* in combination with the observation that Agl3EFG lacks stringent specificity, I postulated that the phenotype could be due to improper importation of a negative developmental signal.

In order to further characterize the role of Agl3EFG in carbohydrate transport and morphogenesis a logical first step would be to overexpress the gene cluster in a wild type background using an inducible promoter. The cluster could be placed downstream of the *tipA* inducible promoter and inserted into the genome using the phiC31 attachment site. It would be expected that this insertion would mimic the phenotype displayed by the *agl3R* mutant. While experimentation showed that transcription of *agl3EFG* increased during growth on melibiose, this tells us little about the actual preferred substrate. A pull down assay could be used to determine what molecules bind to the substrate binding protein of Agl3EFG. A simple experimental design involves isolation of a His-tagged version Agl3E followed by exposure to either used media or several carbohydrates. Substrate binding proteins generally display a high affinity for their targets, and hydrolysis of ATP is required for release. Once the substrate is bound, the protein can be eluted from the column and a determination of the substrate can be made. In this case there is a potential for identification of multiple substrates with the hopes of finding a signaling molecule responsible for the *whi* phenotype displayed by the *agl3R* mutant.

The second regulatory gene that I characterized, *xhdR*, encodes a TetR-like transcriptional regulator. The *xhdR* mutant displayed a *bld* phenotype and overproduced the antibiotic actinorhodin. Scanning electron microscopy confirmed the terminal *bld* phenotype

as evidenced by complete lack of aerial hyphae. Using S1 nuclease mapping, I determined that XdhR acts to repress the adjacent molybdopterin binding cluster, *xdhABC*. Gel retardation assays showed that XdhR bound specifically to the intergenic region between *xdhR* and *xdhABC*. XdhABC showed similarity to xanthine dehydrogenase (XDH) family members. I performed XDH activity assays and determined that activity increased in the *xdhR* mutant that lacks proper transcriptional repression of *agl3ABC*. XDH enzymes catalyze the conversion of hypoxanthine to xanthine and xanthine to uric acid, and have been indicated to act in purine salvage pathways (1, 9). One of the products of the purine salvage pathway is GTP, a molecule that has been shown to regulate morphogenesis in *S. coelicolor* (3). During times of nitrogen stress, GTP is converted into (p)ppGpp by RelA. In *S. coelicolor*, the levels of (p)ppGpp and GTP act as signals for development and secondary metabolite production (3). An increase in (p)ppGpp by phosphorylation of GTP by RelA has been shown to be required for antibiotic production, but not for initiation of development under normal growth conditions (5, 8). However, a decrease in the GTP pool is sufficient to trigger differentiation in *S. coelicolor* (5, 8). I suggested that overexpression of an enzyme exhibiting XDH activity may act to artificially inflate the GTP pool allowing for increased levels of (p)ppGpp that are not accompanied by a decrease in GTP to the level necessary to trigger morphogenic development. If this were the case, it would help to explain the uncoupling of morphogenesis and antibiotic production seen in the *xdhR* mutant.

The role of *xdhR* in morphogenesis and antibiotic production can be expanded upon by assaying the levels of GTP and (p)ppGpp in the *xdhR* mutant cells. This would tell if the assumptions made about the relationship between increased XDH activity and the levels of key starvation signals have any merit. It would be interesting to overexpress a truncated,

ribosome-independent version of RelA in the *xdhR* to determine whether increased conversion of GTP to (p)ppGpp can compensate for an inflated GTP pool. The expected result would be to at least partially rescue the *bld* phenotype. It has been shown that accumulation of (p)ppGpp is a signal for antibiotic production and a decrease in the level of GTP is a signal for morphogenesis. The drug decoyinine might be able to be used to further characterize the proposed disconnect. Decoyinine is an inhibitor of GMP synthetase, and addition of the drug to media should lower the intracellular level of GTP without increasing the levels of (p)ppGpp. Based on the previous assumption of how overexpression of XDH might affect morphogenesis, growth on media containing decoyinine would be expected to rescue the *bld* phenotype of the *xdhR* mutant. With some simple experimentation a solid connection could be made between XdhEFG and the developmental signaling pathways of *S. coelicolor*. The overall best way to characterize the role of XdhR in morphogenesis and antibiotic production is to use microarray analysis to compare global transcript levels between the *xdhR* mutant and wild type. This would show exactly which genes are affected by deletion of *xdhR*.

Regulation of development and antibiotic production in *S. coelicolor* is a complicated interconnection of many events. These processes have been studied for over forty years, and new genes are still being characterized that affect morphogenesis and secondary metabolite production. While the initial reason for starting this work was to further elucidate the known *bld* and *whi* cascades for development, it is hardly surprising that the genes characterized in this study do not fit neatly into either pathway. If you look at the established cascades themselves there are very few direct interactions that have been determined. As studies progress it is more common for genes required for development to fall outside of the

cascades than to fit into them. Although the initial goal of the study was not met exactly, the results generated do say something about how morphogenesis and antibiotic production are regulated. Taken together, the conclusions from this work help to strengthen the evidence that at the base of regulation is a response to nutritional stress.

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