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Wezel, G. van; McKenzie, N.; Nodwell, J.; Hopwood, D.A.

CHAPTER FIVE

Applying the Genetics of Secondary Metabolism in Model Actinomycetes to the Discovery of New Antibiotics

Gilles P. van Wezel,* Nancy L. McKenzie,† *and* Justin R. Nodwell†

Contents

Abstract

The actinomycetes, including in particular members of the filamentous genus *Streptomyces*, are the industrial source of a large number of bioactive small molecules employed as antibiotics and other drugs. They produce these molecules as part of their "secondary" or nonessential metabolism. The number and diversity of secondary metabolic pathways is enormous, with some estimates suggesting that this one genus can produce more than 100,000 distinct

* Molecular Genetics, Leiden Institute of Chemistry, Gorlaeus Laboratories, The Netherlands
† Michael G. DeGroote Centre for Infectious Disease Research, Department of Biochemistry and Biomedical Sciences, McMaster University, Hamilton, Ontario, Canada

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molecules. However, the discovery of new antimicrobials is hampered by the fact that many wild isolates fail to express all or sometimes any of their secondary metabolites under laboratory conditions. Furthermore, the use of previously successful screening strategies frequently results in the rediscovery of known molecules: the all-important novel structures have proven to be elusive. Mounting evidence suggests that streptomycetes possess many regulatory pathways that control the biosynthetic gene clusters for these secondary metabolic pathways and that cell metabolism plays a significant role in limiting or potentiating expression as well. In this article we explore the idea that manipulating metabolic conditions and regulatory pathways can "awaken" silent gene clusters and lead to the discovery of novel antimicrobial activities.

1. Introduction

The modern antibiotic era began with the discovery and large-scale application of penicillin and streptomycin to combat diseases such as septicemia and tuberculosis (Hopwood, 2007). The spectacular success of these and other antibiotics led to the belief that infectious diseases could be conquered. Correspondingly, due in part to a perceived lack of need, the discovery of new antibiotics declined steeply after the 1960s (Hopwood, 2007). Ironically, antibiotic resistance, which was discovered soon after the clinical introduction of penicillin and streptomycin, and which has accompanied the introduction of each new drug, has increased steadily ever since to the point where it now constitutes a significant threat to human health. Meanwhile, antibiotic discovery and research in most large pharmaceutical companies has all but ceased (Katz *et al.*, 2006; Payne *et al.*, 2007), with the result that in the past 40 years only two new chemical classes of antibiotics have been approved for clinical use: linezolid (an oxazolidinone) in 2000 and daptomycin (a cyclic lipopeptide) in 2003 (Wright, 2007). Resistance to these new classes of antibiotics has already emerged (Bersos *et al.*, 2004; Hayden *et al.*, 2005).

There are several impediments to antibiotic discovery, including the low return on investment (as compared to drugs for chronic diseases) and the rigorous regulatory environment governing the clinical testing of new antibiotics. However, it is likely that technical difficulties in identifying and developing truly novel and clinically useful antibiotics is the most significant factor (Bradley *et al.*, 2007; Katz *et al.*, 2006; Payne *et al.*, 2007). The success of previous screening efforts has created a situation in which conventional screening technologies tend to result in the repeated identification of known molecules. Estimates vary, but it is believed by some that if conventional technology were used it would be necessary to test millions of new isolates of environmental microbes to find a single new antimicrobial compound (Baltz, 2006). New thinking in this area is therefore a high priority (Grundmann *et al.*, 2006; Mukherjee *et al.*, 2004).

It is clear that novel strategies and technologies are required to avoid the rediscovery of known antibiotics and to increase the probability of finding new ones. One encouraging example is the recent discovery of platensimycin, produced by *Streptomyces platensis*, which represents a novel class of antibiotic that inhibits FabF, an enzyme involved in fatty acid biosynthesis—see Chapter 17 in Volume 459 (Wang *et al.*, 2006). Platensimycin was identified by screening 83,000 strains grown under different growth conditions against a *Staphylococcus aureus* strain in which antisense RNA against *fabF* was used to lower its gene expression (Wang *et al.*, 2006), thereby making the bacteria more sensitive to inhibitors of the targeted protein, and increasing the probability of finding a hit. Such occasional successes using novel approaches kept hopes alive to identify natural products from bacterial sources (Clardy *et al.*, 2006). New thinking on this old problem is what is required most.

2. Actinomycetes as Antibiotic Factories

Filamentous microorganisms are widely used as industrial producers of antibiotics and other medicinal agents (Bennett, 1998; Demain, 1991; Hopwood *et al.*, 1995). These organisms include the eukaryotic filamentous fungi (ascomycetes) and the prokaryotic actinomycetes (e.g., *Amycolatopsis, Nocardia, Thermobifida*, and *Streptomyces*). The market capitalization for antibiotics is greater than 30 billion US dollars per year. Approximately 30% of all antibiotics are produced by fungi (Hersbach *et al.*, 1984; van den Berg *et al.*, 2007, 2008; Wright, 1999) and 65% by filamentous actinomycetes, including mostly streptomycetes (Hopwood, 2007).

Streptomyces coelicolor is an important model system for the study of antibiotic production and its regulation in actinomycetes. Before its complete genome sequence became available, four antibiotics had been identified, namely actinorhodin (Act; Rudd and Hopwood (1979)), undecylprodigiosin (Red; Feitelson *et al.* (1985)), the calcium-dependent antibiotic (CDA; Hopwood and Wright (1983)), and the plasmid-encoded methylenomycin (Mmy; Wright and Hopwood (1976)). Later, the complete genome sequences of *S. coelicolor* (Bentley *et al.*, 2002), *Streptomyces avermitilis* (Ikeda *et al.*, 2003), and *Streptomyces griseus* (Ohnishi *et al.*, 2008) revealed that their antibiotic-producing potential had been underestimated; each genome contains more than 20 sets of putative biosynthetic genes for secondary metabolites (Challis and Hopwood, 2003). For unknown reasons, many of these are not expressed under laboratory conditions. To take advantage of this untapped source of potentially valuable natural products, we need a better understanding of the genetic and environmental parameters, particularly metabolic inputs and secondary metabolite production.

Recently, examples have been presented of dormant biosynthetic clusters that could be induced by selective growth conditions, such as the enediyne-type PKS antibiotics in several actinomycetes (Zazopoulos *et al.*, 2003) (see Chapter 5 in Volume 459) or the *kas* (*cpk*) gene cluster in *S. coelicolor* (Rigali *et al.*, 2008). Understanding the control of cryptic antibiotic biosynthetic clusters is a major challenge and the potential benefits are enormous (Van Lanen and Shen, 2006; Wilkinson and Micklefield, 2007). It has been estimated that the genus *Streptomyces* alone is capable of producing >100,000 distinct antimicrobial compounds of which only ~3% have been identified and investigated so far (Watve *et al.*, 2001).

Antibiotic biosynthesis is mediated by large contiguous gene clusters ranging in size from a few to over 100 kb (Bentley *et al.*, 2002; Ikeda *et al.*, 2003; Ohnishi *et al.*, 2008). The actinorhodin, undecylprodigiosin, and CDA biosynthetic genes are clustered in distinct locations on the *S. coelicolor* chromosome. The 82-kb CDA cluster is the largest secondary metabolite cluster, consisting of at least 40 genes (*Streptomyces* locus accession numbers: SCO3210-3249). The actinorhodin and undecylprodigiosin biosynthetic clusters are smaller, each consisting of 22–23 genes (actinorhodin: SCO5072-5092, 22 kb; undecylprodigiosin: SCO5877-5899, 32 kb).

In this article, we outline the metabolic and regulatory factors that are important for antibiotic production and describe how these can be manipulated for the discovery of new molecules. The regulation of antibiotic biosynthesis has been most extensively investigated in two model organisms, *S. coelicolor* and *S. griseus*, but there is a dense literature describing important regulatory phenomena in many others (Baltz, 1998; Bate *et al.*, 2006; Cundliffe, 2006; Hopwood, 1989). We will argue that this information can be used in the search for new antibiotics: by understanding the interaction of regulatory pathways with nutrients and metabolites (including in particular carbon sources) we can create synthetic media that trigger the expression of otherwise cryptic secondary metabolic pathways. In parallel, by manipulating the regulators themselves, by overexpression, mutation, or both, we can bypass unknown road blocks for antibiotic biosynthesis and create constitutively overexpressing strains for novel small molecules.

3. Effects of Culture Conditions and Metabolism

3.1. Growth-dependent control mechanisms

Secondary metabolism, the dispensable metabolic pathways that produce antibiotics, usually occurs in a growth phase-dependent manner, peaking as cells approach or after they reach stationary phase. It is therefore influenced

by a wide variety of nutritional (carbon, nitrogen, and phosphate levels), growth-related (cell density, morphology), and physiological factors (cyclic AMP, GTP, and ppGpp levels) (Bibb, 2005; Champness, 2000).

3.2. Stringent control

One factor that modulates antibiotic production is guanosine tetraphosphate or ppGpp. Under conditions of amino acid limitation, the ribosome-associated RelA protein synthesizes ppGpp in response to uncharged tRNAs occupying the ribosomal A-site (Chakraburtty and Bibb, 1997). In turn, the 50S ribosomal L11 protein encoded by *rplI* (also known as *relC*) activates RelA and thus ppGpp synthesis (Ochi, 1990). Under nitrogen-limiting conditions ppGpp causes a dramatic switch in cellular physiology, activating the expression of genes involved in stationary phase processes, such as morphogenesis and secondary metabolite production (CDA and actinorhodin), while repressing genes involved in active growth (Hesketh *et al.*, 2007). The exact mechanism by which ppGpp exerts its effect remains to be determined but it is conceivable that ppGpp affects the affinity of RNA polymerase for promoters of genes associated with stationary phase processes (Hesketh *et al.*, 2007).

3.3. Phosphate-mediated control

Whereas ppGpp is required for antibiotic biosynthesis under nitrogen starvation, a ppGpp-independent signaling mechanism operates under phosphate-limited conditions (Chakraburtty and Bibb, 1997). Inorganic phosphate (P_i) inhibits antibiotic biosynthesis at concentrations as low as a few 100 μM, whereas lower levels of P_i trigger antibiotic biosynthesis (Martin, 2004). The PhoR/PhoP two-component signal transduction system senses and responds to low P_i levels and activates genes involved in phosphate metabolism, allowing growth of the bacteria at low P_i concentrations. Deletion of *phoP* results in increased actinorhodin and undecylprodigiosin production. In a *phoP* deletion mutant, the supply of P_i is reduced and the mutant is prematurely starved of P_i. This P_i starvation is thought to be responsible for triggering antibiotic biosynthesis in a ppGpp-independent manner (Sola-Landa *et al.*, 2003).

Consistent with low levels of P_i triggering antibiotic production, disruption of another gene, *ppk*, involved in P_i metabolism also resulted in increased actinorhodin production and increased levels of transcription of the *act*II-ORF4, *redD*, and *cdaR* pathway-specific regulatory genes for actinorhodin, undecylprodigiosin, and CDA, respectively (Chouayekh and Virolle, 2002). Ppk catalyzes the reversible polymerization of the γ-phosphate of ATP into polyphosphate, a phosphate- and energy-storage polymer (Chouayekh and Virolle, 2002). *ppk* is optimally expressed under

P_i-limiting conditions and is positively regulated by the PhoR/PhoP two-component system (Ghorbel *et al.*, 2006). The exact mechanism by which P_i triggers antibiotic production remains unknown.

3.4. Interactions between metabolism and the DasR regulon

Antibiotics are synthesized by dedicated biosynthetic pathways, but the precursors and cofactors these pathways require are derived from primary metabolism, and high-level production of antibiotics draws heavily on the available building blocks. For example, polyketide biosynthesis requires high levels of acetyl-CoA, malonyl-CoA, and the reducing equivalents NADH and NADPH (Borodina *et al.*, 2005; Hutchinson and Fujii, 1995), placing a burden on the pentosephosphate pathway under production conditions. Interestingly, by targeting phosphofructokinase (Pfk), a key glycolytic enzyme that catalyses the conversion of fructose-6-P to fructose-1,6-biP, the groups of Dijkhuizen and Nielsen could enhance production of actinorhodin and undecylprodigiosin in *S. coelicolor* (Borodina *et al.*, 2008). It was demonstrated that deletion of *pfkA2* (SCO5426), encoding the major Pfk, resulted in increased carbon flux through the pentose phosphate pathway, primarily as a result of the accumulation of glucose 6-phosphate and fructose 6-phosphate. Excellent reviews on metabolic engineering of filamentous microorganisms and the impact of antibiotic production may be found elsewhere (e.g., (Heide *et al.*, 2008; Hershberger, 1996; Martin, 1998; Stephanopoulos, 2002; Thykaer and Nielsen, 2003)).

However, one direct link between primary and secondary metabolism is of particular relevance to this article. The C/N sources glutamate and *N*-acetylglucosamine are important carbon sources for streptomycetes, and growth studies show that they are preferred over glucose by *S. coelicolor* (Nothaft *et al.*, 2003; van Wezel *et al.*, 2006b). Both compounds have a high energy value and are just two metabolic steps away from fructose-6-P. Furthermore, glucosamine-6-P is the major precursor for cell-wall biosynthesis. Surprisingly, higher concentrations of *N*-acetylglucosamine (>5–10 m*M*) inhibit development and antibiotic production under rich growth conditions, while they activate them under poor growth conditions. This complex phenomenon is somehow mediated via the global regulator DasR, a GntR-family regulator whose regulon includes *N*-acetylglucosamine metabolism and transport as well as antibiotic production (Rigali *et al.*, 2006, 2008). DasR directly controls the promoter of *act*II-ORF4, the pathway-specific activator gene for actinorhodin biosynthesis, and transcription of all known chromosomally-encoded antibiotic biosynthetic clusters of *S. coelicolor* (*act, cda, red*, and the ''cryptic'' *kas* or *cpk* cluster) is enhanced in *dasR* mutants on minimal media (Rigali *et al.*, 2008; van Wezel *et al.*, 2006b). DasR repression is relieved during differentiation, when *N*-acetylglucosamine

accumulates and is converted to glucosamine-6-phosphate. This is due to direct binding of glucosamine-6-phosphate to DasR, which reduces the protein's affinity for DNA (Rigali *et al.*, 2006).

N-acetylglucosamine is the monomer of the abundant natural polymer chitin and an important constituent of the cell-wall peptidoglycan. Following chitin utilization or autolytic cell-wall degradation, *N*-acetylglucosamine can enter the cell via at least three different sugar transporters, namely as the monomer via the NagE2 permease (SCO2907), which is part of the PTS phosphotransferase system, or as the dimer chitobiose via DasABC (SCO5232-5234), or via the NgcEFG transporter (SCO6005-6007) (Nothaft *et al.*, 2003; Parche *et al.*, 2000; Saito *et al.*, 2007; Schlösser *et al.*, 1999). The PTS consists of several carbohydrate-specific permeases (designated Enzyme IIBC) and a global part consisting of Enzyme I (EI, encoded by *ptsI*), HPr (histidine protein, encoded by *ptsH*), and Enzyme IIA (EIIA, encoded by *crr*) (Parche *et al.*, 2000; Postma *et al.*, 1993; Titgemeyer *et al.*, 1995). EI, HPr, and EIIA form the phosphate-transfer system, using phosphoenolpyruvate as the energy source, resulting in phosphorylation of the incoming sugar. Interestingly, in *Streptomyces* the general PTS components play a much more global role in antibiotic production (and sporulation) than just the transport of *N*-acetylglucosamine. For example, deletion of *dasR*, but also of *dasA, ptsH, ptsI*, or *crr*, results in developmental arrest and—surprisingly—this is independent of the carbon source (Colson *et al.*, 2008; Rigali *et al.*, 2006). The details of this pleiotropic regulatory mechanism are unknown. Application of DasR for screening purposes is discussed below.

3.5. Morphology as determinant of productivity

Streptomycetes grow as a branched multicellular network of hyphae—the mycelium—in which cell division is relatively rare and does not lead to full cytokinesis (Flardh, 2003). A reproductive cell type, which on solid medium grows up into the air, leads to regularly spaced septation events, and the production of spores. Evidence suggests that the growth of the spore-forming cells occurs at the expense of substrate hyphal lysis. In this way, a single spore will eventually develop into a large multicellular network. Antibiotic production responds as much to morphology as it does to the specific medium conditions. For example, erythromycin production by *Saccharopolyspora erythraea* requires clumps that are at least 90 μm in diameter (Bushell, 1988; Wardell *et al.*, 2002). Thus, while fragmented growth is generally favorable in terms of growth rate and biomass accumulation, it can have a detrimental effect on antibiotic production.

This is not true for all antibiotics. Enhanced expression of the cell division activator protein SsgA induces fragmentation of *S. coelicolor* and, while this almost abolishes production of the polyketide antibiotic

actinorhodin, it brings about a >20-fold increase in undecylprodigiosin production during batch fermentation (Traag and van Wezel, 2008; van Wezel *et al.*, 2000a, 2006a). Similarly, the chloramphenicol producer, *Streptomyces venezuelae*, grows in an extremely fragmented way while efficiently producing antibiotics (Bewick *et al.*, 1976; Glazebrook *et al.*, 1990). Over-expression of the cell division protein FtsZ resulted in the formation of large and dense clumps (flocks), which effected the massive overproduction of actinorhodin in *Streptomyces lividans*, which hardly produces this antibiotic under normal growth conditions (van Wezel *et al.*, 2000b). Therefore, approaches to improve antibiotic production should include analyzing the effects of changes in the morphology of liquid-grown cultures, as these can have spectacular effects on productivity. As a rule of thumb, antibiotics that are produced during late exponential growth or earlier (like undecylprodigiosin) benefit from fragmented growth (Fig. 5.1), while those produced during late transition or stationary phase (like actinorhodin) are produced much more efficiently by clumps (van Wezel *et al.*, 2006a).

Figure 5.1 Overproduction of the cell division activator SsgA leads to an ~20-fold increase in the production of the red-pigmented antibiotic undecylprodigiosin during fermentation of *S. coelicolor*.

4. Molecular Genetic Factors that Regulate Antibiotic Production

Genetic experiments have revealed a diverse collection of regulatory proteins that control antibiotic production (Fig. 5.2); these fall naturally into three groups. The first includes pleiotropic regulators that influence both antibiotic production and sporulation. A well-understood example is *bldA,* which encodes the only tRNA that efficiently translates the UUA codon in *Streptomyces* (Lawlor *et al.*, 1987). *bldA* mutants fail to produce aerial hyphae or most of the antibiotics (Fernandez-Moreno *et al.*, 1991; White and Bibb, 1997). The second group includes pleiotropic regulators of several antibiotic biosynthetic pathways that have little or no effect on the sporulation pathway. An example is the *absA* operon, which encodes a two-component system that serves to repress antibiotic production in *S. coelicolor* and *S. griseus* (Aceti and Champness, 1998; Adamidis *et al.*, 1999; Anderson *et al.*, 1999, 2001; Brian *et al.*, 1996, 2001; Champness and Brian, 1998; Champness *et al.*, 1992; Ishizuka *et al.*, 1992; McKenzie and Nodwell, 2007; Ryding *et al.*, 2002; Sherman and Anderson, 2001; Sheeler *et al.*, 2005). The most specific regulators constitute a third group, the pathway-specific regulators.

4.1. Pathway-specific regulation

Located within the antibiotic biosynthetic clusters are genes encoding biosynthetic enzymes, resistance determinants, transporters, and pathway-specific regulators. There are several types of biosynthetic cluster-encoded,

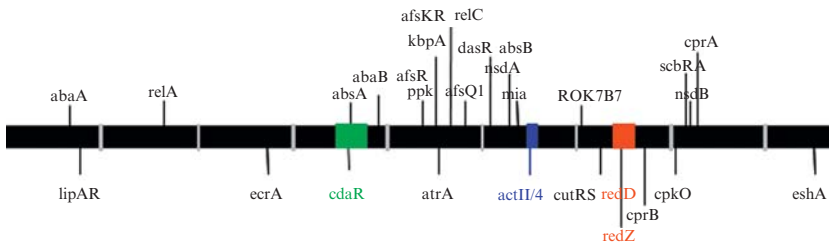

Figure 5.2 The 8,667,507-base pair linear chromosome of *S. coelicolor.* The locations of the CDA (cdaR), actinorhodin (actII/4), and undecylprodigiosin (redD/redZ) biosynthetic gene clusters and the antibiotic regulatory genes are depicted. The genes encoding the pleiotropic regulators are shown above the chromosome, and those encoding the pathway-specific regulators are below. Regulatory genes that are embedded within the biosynthetic clusters are colored. Vertical grey lines divide the chromosome into 1-Mb segments.

pathway-specific regulators, the best-characterized being the *Streptomyces* antibiotic regulatory proteins (SARPs), which are transcriptional activators.

SARPs typically bind to direct repeats that overlap the –35 regions of promoters controlling the expression of antibiotic biosynthetic genes (Wietzorrek *et al.*, 1997). Members of this family have a characteristic N-terminal OmpR-like winged helix-turn-helix DNA-binding motif (Wietzorrek *et al.*, 1997), followed by a bacterial transcriptional activation domain that may help to stabilize the DNA-bound dimer (Alderwick *et al.*, 2006). The function of the C-terminal half of these proteins remains unknown and is not required for DNA-binding specificity (Lee *et al.*, 2002; Sheldon *et al.*, 2002) or for the initiation of transcription *in vitro* (Tanaka *et al.*, 2007). SARPs activate transcription of some or all of the antibiotic biosynthetic genes in the cluster. The grouping of codirectional genes into operons controlled by a single promoter allows a SARP to activate many genes through just a few promoters. In *S. coelicolor, act*II-ORF4 and *redD* encode the SARPs for actinorhodin and undecylprodigiosin production, respectively, and are embedded within the biosynthetic cluster they regulate (Arias *et al.*, 1999; Takano *et al.*, 1992). These genes are essential for antibiotic biosynthesis: deletion of either of them abolished production of actinorhodin and undecylprodigiosin, respectively (Floriano and Bibb, 1996).

Transcription of *act*II-ORF4 is growth-phase-dependent and reaches a maximum during the transition from exponential to stationary phase growth (Gramajo *et al.*, 1993). Overexpression of *act*II-ORF4 in *S. coelicolor* resulted in overproduction of actinorhodin that began during exponential growth (Gramajo *et al.*, 1993). While no direct DNA-binding studies have been done with RedD, microarray analysis revealed that most of the genes involved in the biosynthesis of undecylprodigiosin are RedD-dependent and activated by this SARP (Huang *et al.*, 2001, 2005). Transcription of *redD* increases dramatically during late exponential growth and maximal levels of *redD* transcripts must be present to initiate transcription of the biosynthetic genes (Takano *et al.*, 1992). As with *act*II-ORF4, overexpression of *redD* resulted in elevated levels of undecylprodigiosin and synthesis started earlier during exponential growth (Takano *et al.*, 1992). *redD* itself is *trans*-activated by a second pathway-specific activator inside the *red* cluster, the response regulator-like RedZ (Guthrie *et al.*, 1998; White and Bibb, 1997).

Other SARPs in *S. coelicolor* include CdaR, the proposed activator of the biosynthetic genes for the calcium-dependent antibiotic, a relative of daptomycin (Huang *et al.*, 2005; Khanin *et al.*, 2007; Ryding *et al.*, 2002). Two others are encoded by *cpkO* (formally known as *kasO*) and *cpkN*, which are embedded in a cryptic type I polyketide synthase (*cpk*) gene cluster (SCO6229-6288) (Pawlik *et al.*, 2007; Takano *et al.*, 2005). (The identity of the final product of the cluster is unknown; Takano *et al.* (2005)). *cpkN* has not been characterized but it has been shown that expression of the cryptic polyketide cluster depends on *cpkO* (Takano *et al.*, 2005).

4.2. Pleiotropic regulation

There are at least fifteen pleiotropic regulators of antibiotic biosynthesis in *S. coelicolor*. Predictably, many of these are putative or demonstrated transcription factors, although others are less well understood.

AfsR exhibits sequence similarity to the SARP proteins and controls actinorhodin, undecylprodigiosin, and CDA (Floriano and Bibb, 1996). AfsR contains two extra domains: a central ATPase domain and a C-terminal tetratricopeptide repeat (TPR) domain presumed to associate with other regulatory proteins (Tanaka *et al.*, 2007). Unlike other SARPs that directly activate transcription of the antibiotic biosynthetic genes, AfsR activates *afsS*, encoding a small protein that stimulates transcription of the SARPs *act*II-ORF4 and *redD* by an as yet unidentified mechanism (Floriano and Bibb, 1996; Lee *et al.*, 2002). The ATPase domain of AfsR is essential for *afsS* transcriptional activation and may serve to stimulate open complex formation with RNA polymerase (Lee *et al.*, 2002). AfsR may activate other gene(s) as well—its over-expression has been shown to stimulate antibiotic production independently of *afsS* in addition to the more familiar *afsS*–dependent mechanism (Lee *et al.*, 2002). Importantly, AfsR is subject to phosphorylation by the serine/threonine kinase AfsK, where phosphorylation stimulates its affinity for DNA and modulates its ATPase activity (Lee *et al.*, 2002). AfsK is localized to the inner side of the membrane and autophosphorylates in response to an unknown signal (Matsumoto *et al.*, 1994). In turn, AfsK is modulated by KbpA, an AfsK-binding protein that accumulates after antibiotic production has begun and is believed to prevent unlimited antibiotic production (Umeyama and Horinouchi, 2001). Thus, *afsK, kbpA, afsR*, and *afsS* genes constitute a linear signal transduction system that activates antibiotic production in *S. coelicolor*. Disruption of *afsK, afsR*, or *afsS* reduces antibiotic production, while disruption of *kbpA* enhances it (Floriano and Bibb, 1996; Matsumoto *et al.*, 1994; Umeyama and Horinouchi, 2001).

The *absA* locus encodes a two-component system consisting of a sensor kinase, AbsA1, and the response regulator AbsA2 (Aceti and Champness, 1998; Adamidis *et al.*, 1999; Anderson *et al.*, 1999, 2001; Brian *et al.*, 1996, 2001; Champness and Brian, 1998; Champness *et al.*, 1992; Ishizuka *et al.*, 1992; McKenzie and Nodwell, 2007; Ryding *et al.*, 2002; Sherman and Anderson, 2001; Sheeler *et al.*, 2005). Importantly, these genes appear to serve primarily as negative regulators of antibiotic production. The AbsA1 protein can both phosphorylate AbsA2 and dephosphorylate AbsA2∼P (Sheeler *et al.*, 2005) and, since mutations that impair kinase activity stimulate antibiotic production while those that impair phosphatase activity inhibit it, the current model posits that AbsA2∼P is a repressor of antibiotic biosynthetic gene expression. Indeed, it has been shown that AbsA2∼P can interact directly with *actII-ORF4, redZ*, and *CDAR* (McKenzie and Nodwell, 2007).

A second two-component system, *absAQ1/2*, may also control antibiotic production in *S. coelicolor* (Ishizuka *et al.*, 1992). Unlike, the *absA* locus, the *afsQ1/2* system genes are highly conserved in other streptomycetes (McKenzie and Nodwell, unpublished).

The *mia* sequence (for multicopy inhibition of antibiotic synthesis) eliminated production of actinorhodin, undecylprodigiosin, and CDA when expressed at high copy number (Champness *et al.*, 1992). It has been suggested that the *mia* locus and at least one other newly cloned gene cluster may encode small RNAs that modulate antibiotic biosynthetic gene expression. Consistent with this, deletion of *absB*, which encodes an RNase III protein, abolishes antibiotic production (Aceti and Champness, 1998; Adamidis and Champness, 1992; Chang *et al.*, 2005; Price *et al.*, 1999; Sello and Buttner, 2008).

The *abaA* locus, another poorly characterized pleiotropic regulator, is now believed to consist of at least five open reading frames involved primarily in the control of CDA production but potentially also of other antibiotics (Fernandez-Moreno *et al.*, 1992). One of the *abaA* gene products is a putative transcription factor and a second is a protein similar in sequence to BldB, a dimeric protein of unknown function that is required both for the production of antibiotics and for the sporulation pathway (Eccleston *et al.*, 2002, 2006; Harasym *et al.*, 1990; Pope *et al.*, 1998). The other three *abaA* gene products are proteins of unknown function (Hart *et al.*, unpublished observations).

While the number of around 25 genes involved in the control of antibiotic production appears rather large for a single organism, several new regulators have just been uncovered, and the impact of others has been underestimated. As an example of the latter, the TetR-like regulator AtrA was shown to ''specifically'' activate the biosynthesis of actinorhodin and, apparently, no other antibiotic (Uguru *et al.*, 2005). This activation involves a direct interaction with the *actII*-ORF4 promoter sequence. However, AtrA occurs in all streptomycetes, its DNA binding domain is extremely well conserved (>90% amino acid identity), and an AtrA orthologue activates streptomycin production in *S. griseus* (Hirano *et al.*, 2008), which is compelling phylogenetic evidence against a specific function in *S. coelicolor*. Indeed, evidence is accumulating that AtrA controls a more diverse regulon including metabolic genes, and recent DNA binding experiments revealed binding sites in the *cda* gene cluster, suggesting a pleiotropic (activating?) function for AtrA in the control of antibiotic production (K. McDowall, Pers. Comm.). Furthermore, induced expression of *atrA* can bypass the effects of hyper-repressive alleles of pleiotropic regulators such as *absA1* (McKenzie and Nodwell, unpublished results).

With increasing genomics efforts the landscape is expected to change further. This is exemplified by the discovery of *nsdA* and *nsdB*, which negatively control antibiotic production in *S. coelicolor*: disruption of these

genes results in strongly enhanced actinorhodin production (Li *et al.*, 2006; Zhang *et al.*, 2007). Like, AfsR, the predicted gene products have a TPR domain associated with mediating protein–protein interactions, but their mode of action is unknown.

An interesting approach towards the discovery of novel antibiotic control proteins is metagenomics. In this approach, a library is constructed of genomic DNA isolated from a certain ecological niche, and introduced into a potential expression host such as *S. coelicolor*. A novel activator of antibiotic production was identified in this way from a soil-derived DNA library (Martinez *et al.*, 2005). This gene had previously been identified as *ngcR* in *S. olivaceoviridis* or *rok7B7* (SCO6008) in *S. coelicolor*. The ROK-family regulator it encodes was identified as the transcriptional activator of the *N*-acetylglucosamine and chitobiose transporter NgcEFG in *S. olivaceoviridis* (Schlösser *et al.*, 1999; Xiao *et al.*, 2002) and it also activates PTS and Act production in *S. coelicolor* (Nothaft, 2004). By affecting *N*-acetylglucosamine transport, NgcR also influences the highly pleiotropic DasR regulon, as the metabolic derivative glucosamine-6P modulates its DNA binding activity. Evidence is accumulating that ROK7B7/NgcR is the mirror image of DasR in terms of its regulon, both of which affect hundreds of genes and many antibiotic biosynthetic gene clusters (Swiatek and van Wezel, unpublished).

5. Applications for New Antibiotic Screening Technologies

At present there are many gaps in our understanding of the connectivity between the regulators, pleiotropic, or specific, that have been discovered in *S. coelicolor* and, as argued above, it is likely that many are still to be uncovered even in this single organism. How these regulators interact with signaling molecules and metabolic cues is for the most part completely unknown. One recurring theme is that regulators encoded outside the biosynthetic gene clusters often act by stimulating the expression of the activator genes in the clusters. As this field progresses, it will be interesting to learn whether there are other mechanisms for pleiotropic or pathway-specific control. An intriguing possibility, for example, is enhancing the accumulation of antibiotic precursor molecules.

Regardless of the gaps in our knowledge, it is clear that the genes and culture effects we know about can be exploited in the discovery of new antibiotics. While there are clearly big differences between the core regulatory phenomena in different streptomycetes, frequently these differences involve shuffling the order or manner in which individual proteins act (Chater and Horinouchi, 2003). It is likely, therefore, that what is known

in *S. coelicolor* and other model streptomycetes can be used to manipulate many streptomycetes to increase the chances of discovering new molecules.

BLAST searches (Altschul *et al.*, 1990; Gish and States, 1993) against eight fully sequenced *Streptomyces* genomes, (*S. avermitilis* (Ikeda *et al.*, 2003), *Streptomyces clavuligerus* (Accession EDY48520), *S. coelicolor* (Bentley *et al.*, 2002), *S. griseus* (Ohnishi *et al.*, 2008), *Streptomyces pristinaespiralis* (Accession EDY64088), *S. scabies* (http://www.sanger.ac.uk/projects/S_scabies), *Streptomyces sviceus* (Accession EDY54226), and *Streptomyces* species MG1 (Accession EDX24495)) using the known *S. coelicolor* regulators as bait identified putative orthologues of AbsB, AfsQ1/2, AfsR, AtrA, DasR, ROK7B7/NgcR, and Mia in all eight species. These genes exhibit >60% sequence similarity and tend to preserve chromosomal location and organization. Putative orthologues can also be found for AbsA, AbaA, AfsK, and other regulators, though not in all streptomycetes. Orthologues for the γ-butyrolactone-binding protein ScbR were also found in all streptomycetes. The sequence conservation in these regulators is lower in the C-terminal domains than in the N-terminus, consistent with the fact that they interact with distinct signaling molecules.

5.1. Heterologous overexpression and mutant alleles

A straightforward strategy would be to simply induce the constitutive expression of a pleiotropic activator from *S. coelicolor* in a streptomycete that, through conventional screening, shows little or no antimicrobial activity. Genes could be expressed in single copy from a strong promoter such as the *ermE* promoter or the stronger *ermE** promoter (Bibb *et al.*, 1994; Schmitt-John and Engels 1992), or the thiostrepton-inducible *tipA* promoter (Takano *et al.*, 1995) on an integrating vector such as pSET152 (Bierman *et al.*, 1992). Alternatively, genes with their own promoters could be expressed at high copy number using multicopy vectors derived from pIJ101, of which pIJ486 (Ward *et al.*, 1986) is the most frequently used due to its high stability and copy number. Introduction of these cloned genes into nonproducing streptomycetes could lead to the discovery of new antibiotics. Before turning to individual genes, it is worth pointing out that metagenomics approaches, such as the one successfully used to identify ROK7B7/NgcR as an antibiotic activator (Martinez *et al.*, 2005), are very promising. As discussed, many *Streptomyces* antibiotic regulators are rather well conserved in actinomycetes, and it is quite possible that regulators from other actinomycetes may induce antibiotic production in streptomycetes.

This approach of overexpressing a single antibiotic control-related gene is most logical for activator genes such as *afsR*, but are there ways that repressors could also be employed to trigger antibiotic production in nonproducing strains? One approach is to take advantage of the more complicated genetics of some systems. For example, several alleles of the

absA1 sensor kinase have been discovered that cause overproduction of antibiotics in *S. coelicolor*, It is agreed that these act by eliminating the protein's capacity to phosphorylate AbsA2 but leaving its ability to dephosphorylate AbsA2∼P (Anderson *et al.*, 2001; McKenzie and Nodwell, 2007; Ryding *et al.*, 2002; Sheeler *et al.*, 2005). This is important because such alleles are dominant over wild-type alleles (McKenzie and Nodwell, unpublished), causing enhanced antibiotic production even when the normal repressing allele is present. These genetic observations have been employed to explore the possible application of *absA1* to antibiotic discovery. As shown in Fig. 5.3, introduction of two different phosphorylation-defective, dephosphorylation-competent alleles of *absA1* from *S. coelicolor* into *S. lividans* results in a dramatic stimulation of production of the blue-pigmented antibiotic actinorhodin (McKenzie and Nodwell, unpublished). This trait has been extended to screens of 10 environmental *Streptomyces* isolates and nine well-characterized producers of known antibiotics. Screens of the engineered strains against panels of model prokaryotes and pathogenic strains such as *S. aureus* demonstrated the activation of new antimicrobial activities in at least six of the engineered strains. Two of these turned out to be known antibiotics (streptomycin and blasticidin S), but at least one of them appears to be novel (McKenzie*et al.*, unpublished observations). In addition to demonstrating the potential of this approach, these observations show how important it is that we acquire a clearer understanding of how these pleiotropic regulators work.

To date perhaps the most successful application of this approach is demonstrated by efforts to enhance antibiotic biosynthesis by manipulating the DasR regulon. The DasR protein and regulatory network is highly conserved in streptomycetes, with around 75% of the DasR-binding (*dre*) sites predicted in *S. coelicolor* also found upstream of the orthologous genes in

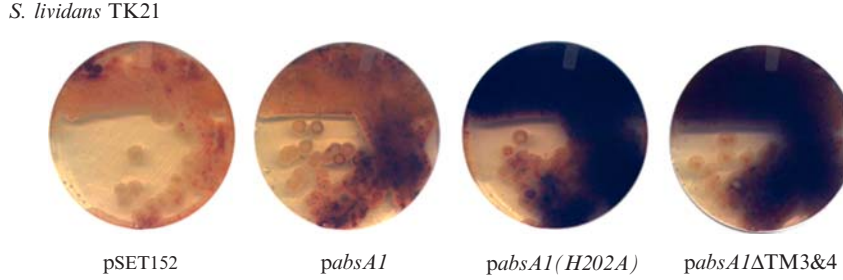

Figure 5.3 Activation of actinorhodin production by alleles of *absA1.* Mutants of the *S. coelicolor absA1* gene that encode proteins lacking AbsA2 kinase activity but having AbsA2∼P phosphatase activity enhance production of the blue-pigmented antibiotic actinorhodin when expressed in *S. lividans.* The strains growing on each plate are *S. lividans* TK21 bearing a control vector (pSET152) or vectors expressing wild type *absA1*, or antibiotic activating alleles *absA1(H202A)* and *absA1(delTM3 and 4).*

S. avermitilis (Rigali, Titgemeyer and van Wezel, unpublished data and (van Wezel *et al.*, 2006b). We suggest, therefore, that the *dasR* regulon is likely to be central to the production of natural products in other actinomycetes. Indeed, a scan of available genome sequences in the databases highlights a range of putative targets, suggesting that DasR may control many important clinical drugs, such as clavulanic acid, chloramphenicol, and the glycopeptide antibiotics daptomycin and teichoplanin (Table 5.1). Whether or not these genes are indeed controlled (repressed) by DasR remains to be elucidated.

Finding a tool to manipulate the activity of DasR should therefore allow control of the expression of many industrially and medically relevant compounds (antibiotics, anti-tumor agents, agricultural compounds, and industrial enzymes) from the outside rather than by genetic engineering. As discussed above, the DNA-binding activity of DasR is inhibited by

Table 5.1 Selected antibiotic-related genes predicted or known (italics) to be controlled by DasR

Secondary metabolite	*Streptomyces*	Target gene(s)	Function
Clavulanic acid	*S. clavuligerus*	*pcbR*	PBP; β-lactam resistance
Actinorhodin (Act)	*S. coelicolor*	actII-ORF4	Pathway-specific activator
Undecylprodigiosin (Red)	*S. coelicolor*	redZ	Pathway-specific activator
Kas (cryptic antibiotic)	*S. coelicolor*	*kasO* (*cpkO*)	Pathway-specific activator
Calcium-dependent antibiotic (CDA)	*S. coelicolor*	*cdaR*	Pathway-specific activator
Valanimycin	*S. viridifaciens*	*vlmM*	Valanimycin transferase
Daptomycin	*S. filamentosus*	*dptABC*	Peptide synthetase 1, 2 and 3
Novobiocin	*S. spheroides*	*novH*	Peptide synthase
Actinomycin	*S. anulatus*	*acmC*	Peptide synthase III
Teichoplanin A47934	*S. toyocaensis*	*staQ*	Transcriptional regulator
Chloramphenicol	*S. venezuelae*	*papABD*	p-aminobenzoic acid synthase

Note that all known chromosomally-encoded antibiotics of *S. coelicolor* are controlled by DasR.

glucosamine-6-phosphate, a metabolic derivative of *N*-acetylglucosamine (Rigali *et al.*, 2006). Indeed, addition of *N*-acetylglucosamine to growth media at 5–10 m*M* results in a *dasR* mutant phenocopy, namely lack of development and antibiotic production under rich growth conditions and accelerated development and enhanced antibiotic production under poor growth conditions (van Wezel *et al.*, 2006b; Rigali *et al.*, 2008). In some cases, this had a spectacular effect on the production of antibiotics, as illustrated by *Streptomyces hygroscopicus* and *S. clavuligerus* (Fig. 5.4).

Obvious targets for this approach are cryptic clusters, which are not expressed under normal growth conditions and therefore have not yet been identified by routine activity-based screening assays. At least for the *cpk* cluster it was established that expression of these clusters is induced by *N*-acetylglucosamine, and the majority of the streptomycetes tested produced more antibiotics in the presence of *N*-acetylglucosamine in the growth medium (Rigali *et al.*, 2008). This is one example of novel approaches that may be employed to boost the potential of novel screening procedures.

6. Future Prospects

While there is a wealth of basic knowledge of the regulatory mechanisms governing antibiotic production, it is clear that the exploitation of this information is in its infancy. Indeed, at least one class of regulatory gene, the

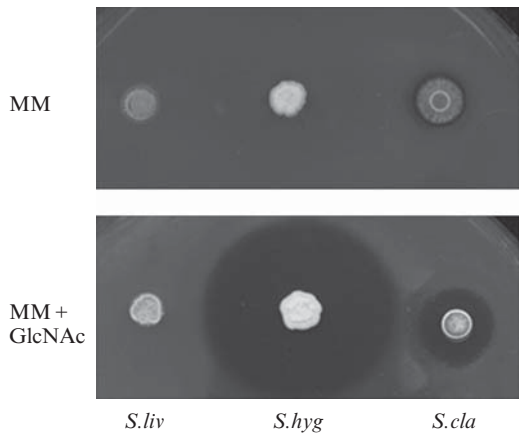

Figure 5.4 Effect of added *N*-acetyl glucosamine on antibiotic production by streptomycetes. *S. lividans* (*S.liv*; nonproducing control), *S. hygroscopicus* (*S.hyg*), and *S. clavuligerus* (*S. cla*) were grown on plates of minimal medium (MM) with added GlcNAc (bottom) or without (top). *Bacillus subtilis* was used as indicator strain. Halos in the indicator lawn demonstrate strong increase of antibiotic production by the streptomycetes.

small RNAs, appears to be missing from our collection. This is likely to change in the near future, however, as at least one group has evidence for control of antibiotic production via small RNA (Hindra and Elliot, McMaster University, Personal Communication). This is important because such genes might be particularly good candidates for heterologous regulation in less well-characterized strains.

Intriguing possibilities for future endeavors include manipulation of multiple regulatory pathways or exploring the effects of culture conditions on engineered strains. Indeed, one principal difference between the *Streptomyces* habitat (the soil) and the laboratory is the degree of crowding. Perhaps by mimicking the undoubtedly complex interspecific signaling events that occur in nature, it will be possible to trigger the expression of otherwise cryptic biosynthetic gene clusters.

Finally, it is clear that these approaches need to be brought to bear on more challenging target pathogenic microorganisms. Screens of engineered streptomycetes against pathogens exhibiting high levels of antibiotic resistance are clearly a high priority. If the rational engineering of streptomycete genomes yields new antimicrobial activities, screening against pathogens that lack sensitivity to known antibiotics could be a way of avoiding the rediscovery of known drugs.

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