Signalling pathways that control development and antibiotic production in streptomyces

Urem, M.; Urem M.

CHAPTER II

INTERTWINING NUTRIENT-SENSORY NETWORKS AND THE CONTROL OF ANTIBIOTIC PRODUCTION IN *STREPTOMYCES*

Mia Urem, Magdalena A. Świątek-Połatyńska,
Sébastien Rigali and Gilles P. van Wezel

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Abstract

Actinobacteria are producers of a plethora of natural products of agricultural, biotechnological and clinical importance. In an era where mankind has to deal with rapidly spreading antimicrobial resistance, streptomycetes are of particular importance as producers of half of all antibiotics used in the clinic. Genome sequencing efforts revealed that their capacity as antibiotic producers has been underestimated, in particular as many biosynthetic pathways are silent under standard laboratory conditions. Here we review the global regulatory networks that control antibiotic production in streptomycetes, with emphasis on carbon- and aminosugar-related nutrient sensory pathways. Recent research has revealed intriguing connections between these regulons, and overlap and antagonism between the activities of among others the global regulatory proteins AtrA, DasR and Rok7B7 as well as GlnR (nitrogen control) and PhoP (phosphate control), are discussed. Finally, we provide ideas as to how these novel insights might help us to find ways to activate the transcription of silent biosynthetic gene clusters.

INTRODUCTION

Streptomycetes are filamentous soil bacteria that produce around 50% of all antibiotics in clinical use as well as a wide range of other natural products of medical, biotechnological or agricultural importance, including anti-cancer, anti-inflammatory and anti-fungal agents (Bérdy, 2005; Hopwood, 2007). The streptomycetes belong to the family *Streptomycetaceae* that are characterized by mycelial growth, with large GC-rich chromosomes that reflect the complexity of both their life cycle and rapidly changing environmental conditions (Labeda *et al*., 2012; Ludwig *et al*., 2012; Barka *et al*., 2016). Like other filamentous actinobacteria, streptomycetes are found in terrestrial and marine environments, where nutrient supplies are often heterogeneous or fluctuating rapidly. To meet such diverse challenges, they metabolize a wide range of carbon, nitrogen, phosphate and sulphur sources using extensive sensing and transport systems that monitor the nutritional status of the environment and assist in scavenging of a wide range of nutrients. Streptomycetes possess a large number of genes for polysaccharide hydrolases (Hodgson, 2000) which facilitate the degradation and subsequent metabolism of complex polysaccharides, a large number of carbohydrate transport systems, the majority of which are ATP-binding cassette (ABC) transporters (Bertram *et al*., 2004), and a staggering 700 regulatory proteins are encoded by the genome of the model actinomycete *Streptomyces coelicolor* (Bentley *et al*., 2002).

Under conditions of stress, such as nutrient depletion, streptomycetes enter a complex life cycle, resulting in sporulation (Fig. 1). Once dispersed, spores germinate and grow as a branched, vegetative mycelium that consists of long multinucleoid hyphae. These hyphae are compartmentalized into large segments by cross-walls, making streptomycetes a model system for bacterial multicellularity (Elliot *et al*., 2008; Claessen *et al*., 2014). When streptomycetes enter the developmental program, the vegetative mycelium is degraded and a programmed cell death (PCD) process (Miguelez *et al*., 1999, 2000; Manteca *et al*., 2005a) provides the nutrients for the newly formed aerial mycelium, which eventually produces chains of unigenomic spores (Flärdh and Buttner, 2009). Sporulation is a highly complex process of coordinated cell division and DNA segregation, involving actinomycete-specific regulatory proteins (Traag and van Wezel, 2008; Jakimowicz and van Wezel, 2012; McCormick and Flardh, 2012).

The onset of morphological differentiation roughly coincides with that of chemical differentiation, i.e. the production of secondary metabolites, also referred to as specialized metabolites or natural products (Fig. 1) (Bibb, 2005; van Wezel and McDowall, 2011). The model organism *S. coelicolor* produces a variety of antibiotics, including the red-pigmented prodiginines (Red) and the blue-pigmented actinorhodin (Act), the calcium-dependent antibiotic (Cda) and the plasmid-encoded methylenomycin (Mmy) (Bentley *et al*., 2002; Hopwood, 2006). Besides Act, Cda, Mmy and Red, the genome of *S. coelicolor* encodes the Cpk (cryptic polyketide) proteins for the biosynthesis of yet another antibiotic, recently elucidated as coelimycin P1 (Gomez-Escribano *et al*., 2012). Located within the biosynthetic gene clusters lie genes for pathway-specific transcriptional regulators, which then activate the production of the biosynthetic pathways. The extensively studied pathway-specific activators ActII-ORF4 (activates Act biosynthesis), CdaR (for Cda) and RedD (for Red) all belong to the SARP family of *Streptomyces* antibiotic regulatory proteins (Wietzorrek and Bibb, 1997). Once the pathway-specific activators are expressed, the biosynthetic gene clusters are activated with little downstream control (Bibb, 2005; van Wezel and McDowall, 2011). Indeed, when the *redD* transcriptional activator gene is put under the control of a different promoter, timing and/or localization of Red production is altered

accordingly (van Wezel *et al*., 2000a). However, intricate regulatory networks are present to govern the timing of antibiotic production and its linkage to cell growth, nutrient selection, development and environmental stresses. Here, we review the intertwining global regulatory networks that translate environmental signals and the bacterium's metabolic status to the control of the specialized metabolism of streptomycetes, using the well-studied *S. coelicolor* as the model organism, and with special attention to the complex role of aminosugars.

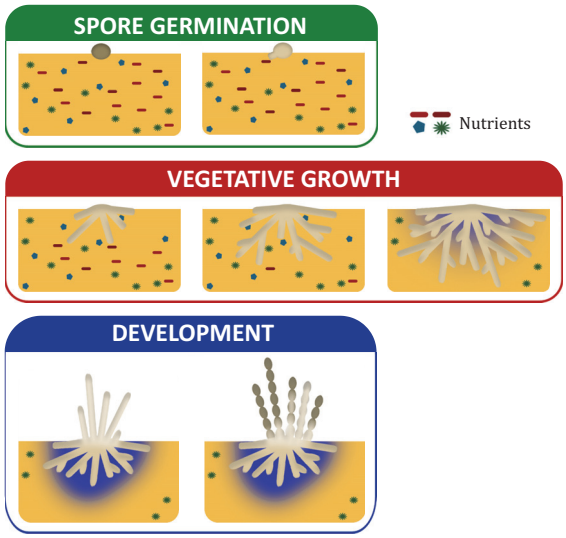

FIGURE 1. The *Streptomyces* life cycle.
Streptomycetes form a branched mycelium that grows into the soil/media to scavenge nutrients. When nutrients become depleted, complex sensory and regulatory networks relay the environmental cues into responses. This leads to the production of antibiotics and other secondary metabolites as well as the development of aerial mycelia which eventually form spores as a means of escape from unfavourable conditions through dispersal. (Top) Germination: a spore germinates once favourable (nutrient-rich) conditions are sensed; (Middle) Vegetative growth: when nutritional content of the media is high, growth is promoted through branching and elongation of the hyphae via apical growth. Favourable nutrients (red) are depleted, and once other nutrients are also depleted, the developmental program is initiated which is accompanied by the production of antibiotics (blue, last panel); (Bottom) Development: to escape the nutrient-depleted environment, streptomycetes form erected aerial hyphae at the expense of the underlying substrate mycelium. At this time antibiotics (blue) are also produced. The aerial hyphae septate and are ultimately converted into chains of spores.

CARBON CATABOLITE REPRESSION AND THE CONTROL OF ANTIBIOTIC PRODUCTION

The most extensively studied nutrient sensory and control system in bacteria is without any doubt carbon catabolite repression (CCR), also known as glucose repression. Efficient carbon source utilisation is critical from the perspective of competition in the natural habitat. CCR of catabolic genes is often achieved by the combined activities of global and operon-specific regulatory mechanisms, including inducer exclusion (Gorke and Stülke, 2008). The phosphoenolpyruvate-dependent phosphotransferase system (PTS) plays a major role in the regulation of CCR in many microorganisms (Gunnewijk *et al*., 2001; Brückner and Titgemeyer, 2002; Gorke and Stülke, 2008). The PTS transport system is specific to prokaryotes and uses a protein phosphoryl transfer chain, with phosphoenolpyruvate (PEP) as phosphoryl donor, to transport and phosphorylate specific carbohydrates. The system consists of the general energy-coupling phosphotransferase Enzyme I (EI) and phosphocarrier protein HPr in combination with diverse, carbohydrate-specific transport complexes called Enzyme II (EII) (Saier and Reizer, 1992; Postma *et al*., 1993). In *Streptomyces*, deletion of the general *pts* genes (*ptsH*, *ptsI* and *crr* which encode HPr, EI

and EIIA, respectively), leads to vegetative arrest and consequently mutants have a non-sporulating (bald) phenotype (Nothaft *et al*., 2003a; Rigali *et al*., 2006). This phenomenon is most likely associated with altered production of the siderophore desferrioxiamine, which is essential for proper development on especially glucose-containing media (Yamanaka *et al*., 2005; Traxler *et al*., 2012; Lambert *et al*., 2014). This observation reveals a direct link between carbon utilization, iron homeostasis and the control of development, but it is currently unknown how this is mediated.

In both *Escherichia coli* and *Bacillus subtilis*, the PTS is critical for the regulation of CCR, however, the mode of regulation differs greatly. In *E. coli*, CCR is controlled via modulation of the phosphorylation state of the glucose transporter ($EIIA^{Glc}$). $EIIA^{Glc}$ exists primarily in its non-phosphorylated state during active PTS-mediated glucose transport and inhibits the transport of non-PTS sugars (e.g. lactose, maltose, melibiose, raffinose), resulting in inducer exclusion for many sugar operons (Hogema *et al*., 1998; Brückner and Titgemeyer, 2002). Conversely, in the absence of glucose, $EIIA^{Glc}$ remains phosphorylated and activates the membrane-bound adenylate cyclase, leading to the accumulation of cyclic AMP (cAMP). This then forms a complex with its receptor protein CRP, which activates the transcription of many catabolic genes and operons (Gorke and Stülke, 2008). In *B. subtilis*, the phosphocarrier protein HPr plays the central role in the regulation of CCR. HPr is phosphorylated by the HPr kinase/phosphorylase (HprK/P), with HprK/P kinase activity stimulated by fructose-1,6-bisphosphate, while HprK/P phosphorylase activity is triggered by the accumulation of inorganic phosphate (Jault *et al*., 2000; Mijakovic *et al*., 2002). Phosphorylation of HPr triggers its interaction with the pleiotropic transcription factor CcpA (catabolite control protein A), which then exerts global repression of sugar catabolic operons (Deutscher *et al*., 1995; Jones *et al*., 1997).

In contrast to most bacteria, deletion of the general *pts* genes has no effect on glucose repression in *Streptomyces* (Butler *et al*., 1999; Nothaft *et al*., 2003b) and the cAMP-CRP complex, though globally impacting antibiotic production, has no role in CCR (Derouaux *et al*., 2004a; Derouaux *et al*., 2004b; Gao *et al*., 2012). Surprisingly, in streptomycetes, glucose is not transported via the PTS but via the Major Facilitator Superfamily (MFS) permease GlcP (van Wezel *et al*., 2005; Perez-Redondo *et al*., 2010; Romero *et al*., 2015) (see Fig. 2). This may have been an evolutionary adaptation to facilitate the key role of glucose kinase (also known as glucokinase) in CCR in these bacteria and at the same time prioritize mixed C- and N-sources. Indeed, *N*-acetylglucosamine (GlcNAc) and glutamate are highly preferred substrates for streptomycetes; in cultures with both glucose and glutamate, *S. coelicolor* consumes all the glutamate before glucose is metabolized (van Wezel *et al*., 2006a; Romero-Rodríguez *et al*., 2016). The surprising observation that the PTS does not play a general role in CCR may be due to the critical role GlcNAc plays in central metabolism and the control of development and antibiotic production (Nothaft *et al*., 2003a; Rigali *et al*., 2006), as discussed in the next section, and therefore alternative systems have been put in place to take over the control of carbon source regulation. Still, like in other bacteria, glucose exhibits CCR over other the utilisation of other carbon sources, such as glycerol, arabinose, fructose and galactose.

Glucose kinase (Glk, encoded by the *glkA* gene) performs the first step in glycolysis by phosphorylating glucose to glucose-6P (Fig. 2). A member of the ROK family of proteins (Repressors, ORFs and Kinases [Titgemeyer *et al*., 1994]), Glk also plays a predominant role in CCR in *S. coelicolor* (Angell *et al*., 1992; Angell *et al*., 1994) and probably also in *Streptomyces peucetius* (Guzman *et al*., 2005). While genome sequence comparison suggests that it is very likely that Glk also fulfils a dominant role in CCR in other streptomycetes, this concept awaits

experimental analysis. Deletion or mutation of *glkA* results in loss of glucose utilization as well as glucose repression of catabolite-controlled genes, including those for the utilization of agar (*dagA*), glycerol (*glyCABX)*, galactose (*galP*), maltose (*malEFG*) and chitin (*chi*) (Angell *et al*., 1992; Angell *et al*., 1994; Hindle and Smith, 1994; van Wezel *et al*., 1997; Saito *et al*., 2000). The enzymatic activity of Glk strongly depends on the carbon source and growth phase, with high activity of Glk in glucose-grown cultures and low activity in cultures grown on the non-repressing carbon source mannitol, whereby Glk activity is likely modulated through metabolite-dependent activation and/or post-translational modification of the enzyme (van Wezel *et al*., 2007). CCR also represses the production of antibiotics (Sanchez *et al*., 2010). In the soil, sugars are closely monitored and the presence of high-energy nutrients promotes growth and suppresses developmental processes as well as antibiotic production, therefore many antibiotics are subject to CCR (Hostalek, 1980; Sanchez *et al*., 2010). Examples include the production of chloramphenicol by *Streptomyces venezuelae* (Bhatnagar *et al*., 1988), cephamycin by *Streptomyces clavuligerus* (Cortes *et al*., 1986), erythromycin by *Saccharopolyspora erythraea* (Escalante *et al*., 1982) and streptomycin by *Streptomyces griseus* (Demain and Inamine, 1970).

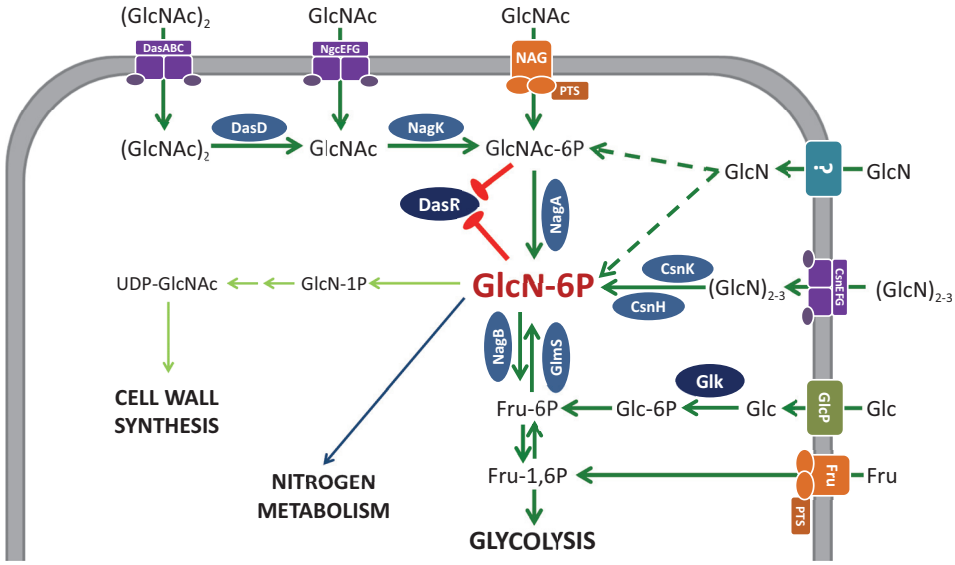

FIGURE 2. Carbohydrate metabolic pathways in *S. coelicolor.*
The primary metabolism of *S. coelicolor* is shown for *N*-acetylglucosamine (GlcNAc), *N*-*N*′-diacetylchitobiose $(GlcNAc)_2$, chitosan-derived oligosaccharides $(GlcN)_{2\text{-}3}$, glucose (Glc) and fructose (Fru) in addition to proposed pathway(s) for glucosamine (GlcN). Glucosamine 6-phosphate (GlcN-6P, red) is a central metabolite that stands at the crossroads of aminosugar metabolism, glycolysis, nitrogen metabolism and cell-wall synthesis. It also plays an important role in signalling of antibiotic production and development by modulating the activity of the global repressor DasR. GlcN-6P is obtained from internalization of monomers or oligomers of GlcN or GlcNAc and metabolism via CsnK and CsnH and via NagA, or in a reverse reaction by GlmS from glycolytic precursors. $(GlcNAc)_2$ is hydrolysed by DasD to GlcNAc and subsequently phosphorylated by NagK to GlcNAc-6P. A hypothetical metabolic route for GlcN via the GlcNAc pathway is presented, though no transporter or metabolic enzymes have so far been identified (Świątek et al., 2012a; Viens et al., 2015). Internalized glucose is phosphorylated by glucose kinase (Glk), which is key to carbon catabolite repression in *S. coelicolor*. ABC transporters are presented in purple, PTS transporters in orange, putative GlcN transporter in turquoise and the glucose MFS permease GlcP in green. Primary metabolic routes are represented by green arrows, with unknown routes indicated by dotted arrows. The cell-wall biosynthetic reactions are shown as light green arrows and nitrogen metabolism by a blue arrow.

In *Streptomyces lividans*, glucose inhibits actinorhodin production by repressing the *afsR2* gene, which encodes a global regulatory protein involved in the activation of specialized metabolite biosynthesis in diverse *Streptomyces* species (Kim *et al*., 2001). In *S. coelicolor*, the homologue of AfsR2, AfsS, binds to a putative secreted solute binding protein encoded by SCO6569. Overexpression of this newly characterized protein significantly reduced actinorhodin production, while gene disruption led to accelerated antibiotic production (Lee *et al*., 2009). This suggests that SCO6569 is an AfsS-dependent down-regulator of actinorhodin production in *S. coelicolor*. The flux of carbon through glycolysis also influences antibiotic production. Deletion of *pfkA2*, encoding one of the three phosphofructokinases in *S. coelicolor*, leads to enhanced production of the pigmented antibiotics actinorhodin and undecylprodigiosin (Borodina *et al*., 2008). Genome-scale metabolic simulations suggested that decreased phosphofructokinase activity leads to an increase in the flux through the pentose phosphate pathway, which then stimulates the flux towards pigmented antibiotics (Borodina *et al*., 2008).

AMINOSUGAR METABOLISM IN STREPTOMYCETES

Aminosugars are not only important nutrients but also play a key role as signalling molecules in streptomycetes, with *N*-acetylglucosamine (GlcNAc) involved in the activation of the onset of development and antibiotic production under poor nutritional conditions (Rigali *et al*., 2008). A metabolic intermediate of aminosugar metabolism, glucosamine-6-phosphate (GlcN-6P) is a central molecule that stands at the cross-roads of many metabolic pathways, including glycolysis, cell-wall synthesis, glutamine and glutamate metabolism (Durand *et al*., 2008). After the internalization and phosphorylation of GlcNAc, *N*-acetylglucosamine-6-phosphate (GlcNAc-6P) is deacetylated to GlcN-6P by the *N*-acetylglucosamine-6-phosphate deacetylase NagA. The resulting GlcN-6P can then be deaminated to fructose-6P by GlcN-6P deaminase NagB or go to other metabolic routes (Bates and Pasternak, 1965; Midelfort and Rose, 1977; Plumbridge, 2015). See Fig. 2 for an overview.

GlcNAc is the monomer of chitin, which is one of the most abundant polysaccharides on earth and a preferred substrate for streptomycetes, acting as both a source of carbon and nitrogen. Together with *N*-acetylmuramic acid, GlcNAc also forms the strands of peptidoglycan (PG), which make up the bacterial cell wall (Terrak *et al*., 1999). Transport and subsequent internalization of GlcNAc has been studied in many bacteria and in most, uptake of GlcNAc occurs via the PTS (Bouma and Roseman, 1996; Reizer *et al*., 1999; Alice *et al*., 2003; Komatsuzawa *et al*., 2004; Nothaft *et al*., 2010; Liao *et al*., 2014b; Plumbridge, 2015), but alternative uptake systems have been described (Xiao *et al*., 2002; Saito *et al*., 2007; Eisenbeis *et al*., 2008; Boulanger *et al*., 2010; Liao *et al*., 2014b). In *E. coli*, GlcNAc is transported by NagE, while the related aminosugar glucosamine (GlcN) is transported via the non-specific hexose transporter ManXYZ (Jones-Mortimer and Kornberg, 1980; Postma *et al*., 1996). *B. subtilis* transports GlcNAc via NagP, while GamP and the glucose transporter PtsG both transport GlcN (Reizer *et al*., 1999; Bertram *et al*., 2011; Gaugué *et al*., 2013). Similar systems also exist in streptomycetes (Titgemeyer *et al*., 1995; Wang *et al*., 2002). As mentioned above, glucose is not transported by the PTS in streptomycetes, but instead the PTS is biased for GlcNAc and fructose utilization (Kamionka *et al*., 2002; Nothaft *et al*., 2003a; Nothaft *et al*., 2003b; Nothaft *et al*., 2010). GlcNAc is imported via the EIIABC complex, consisting of $EIIA^{Crr}$ (Crr), $EIIB^{GlcNAc}$ (NagF) and $EIIC^{GlcNAc}$ (NagE2), while it is unclear how monomeric GlcN is internalized (Fig. 2). The utilization of GlcN, which originates from chitosan (an *N*-deacetylated derivative of chitin), appears equally complex as that of GlcNAc, involving multiple (unidentified) uptake and regulatory systems, in

addition to the *csnR-K* operon responsible for the import and utilization of GlcN dimers and chito-oligosaccharides (Dubeau *et al*., 2011; Viens *et al*., 2015). A suppressor screen, making use of the lethality of GlcN and GlcNAc to *S. coelicolor nagB* mutants, directly or indirectly due to the accumulation of GlcN-6P, showed that a lot of questions remain on how GlcN(Ac) is metabolized in streptomycetes (Świątek *et al*., 2012a; Świątek *et al*., 2012b). As expected, spontaneous suppressor mutants that survive on GlcNAc include those mutated in *nagA*, which is required for the formation of GlcN-6P. Surprisingly, GlcN toxicity is also relieved by mutations in *nagA*. This suggests that GlcN may be exclusively metabolized via the GlcNAc metabolic route (Świątek *et al*., 2012b) (Fig. 2). Indeed, deletion of *nagK*, which encodes GlcNAc kinase that phosphorylates intracellular GlcNAc, also relieves GlcN toxicity to *nagB* mutants. Importantly, suppressor mutants were identified that could not be correlated to any of the known *nag* (GlcNAc metabolic pathway) genes and these are currently being elucidated by genome sequencing. Furthermore, deleting the *nag* metabolic genes has significant and growth medium-dependent effects on antibiotic production by *S. coelicolor*, with some mutants overproducing the pigmented antibiotic actinorhodin (Świątek *et al*., 2012a; Świątek *et al*., 2012b). For one, DNA binding activity of the pleiotropic antibiotic repressor DasR (which is discussed in detail below) is likely influenced by binding of various (amino-)sugars, including GlcN-6P and GlcNAc-6P (Rigali *et al*., 2006; Rigali *et al*., 2008; Liao *et al*., 2015b; Fillenberg *et al*., 2016) and different phosphorylated C6-sugars generated during glycolysis (Świątek-Połatyńska *et al*., 2015; Tenconi *et al*., 2015). Control of antibiotic production may therefore be influenced by the metabolic balance of these molecules. Approaches for rational strain engineering based on interference of the metabolic flux of amino sugars may, therefore, be a useful strategy. This concept awaits further experimental testing.

GlcN-6P stands at the crossroads of C- and N- metabolism and developmental pathways

In streptomycetes, GlcN-6P plays many key roles including a central role in the control of antibiotic production, thereby directly connecting the control of primary metabolism to that of secondary metabolism. GlcN-6P, together with GlcNAc-6P, acts as an allosteric effector of the nutrient sensory regulator DasR (Figs 2 and 3). The crystal structure of DasR in *S. coelicolor* and its distant orthologue NagR of *Bacillus subtilis* in complex with GlcN(Ac)−6P have been elucidated (Fillenberg *et al*., 2015; Fillenberg *et al*., 2016). DasR derives its name from the adjacent *dasABC* operon involved in *N*-*N*′-diacetylchitobiose [$(GlcNAc)_2$] metabolism but is also important for development, with *das* standing for deficient in aerial mycelium and spore formation (Seo *et al*., 2002; Colson *et al*., 2008). DasR is a GntR-family repressor with a pleiotropic role in the regulation of primary metabolism, development and antibiotic production. Extensive analysis of the DasR regulon showed that DasR directly controls the *pts, nag* and *chi* genes (for the chitinolytic system) and represses antibiotic production by direct binding to the promoter regions of the pathway-specific regulatory genes for all antibiotic biosynthetic gene clusters in *S. coelicolor* (Rigali *et al*., 2006; Rigali *et al*., 2008; Nazari *et al*., 2012; Świątek-Połatyńska *et al*., 2015). A similar pleiotropic role of DasR has also been reported in the erythromycin producer *Saccharopolyspora erythraea* (Liao *et al*., 2014b; Liao *et al*., 2015b). Recently, it was also shown that DasR indirectly represses the biosynthesis of iron-chelating siderophores through the direct control of the iron-homeostasis regulator *dmdR1* in *S. coelicolor* (Craig *et al*., 2012; Lambert *et al*., 2014).

The activity of DasR and its response to GlcN-6P and GlcNAc-6P levels depends on environmental conditions; growth on high concentrations of GlcNAc under famine

conditions (i.e. on minimal media) results in the global de-repression of its targets and enhanced antibiotic production and development while on rich media, GlcNAc represses antibiotic and development (Rigali *et al*., 2006; Rigali *et al*., 2008; van Wezel *et al*., 2009). This phenomenon reflects conditions of *feast* or *famine*: under rich growth conditions (feast) streptomycetes likely interpret GlcNAc as derived from chitin and hence abundance of nutrients, while under poor growth conditions (famine) it would be seen as a by-product of hydrolysis of peptidoglycan and hence its own cell death. Abundance of nutrients will promote growth and postpone development, while the initiation of cell death requires sporulation and antibiotic production (Fig. 1).

Key in the sensing system is probably that chitin is metabolized and internalized as $(GlcNAc)_2$, which is imported via the ABC-transporter system DasABC-MsiK, while GlcNAc is imported via the PTS (Nothaft *et al*., 2003a; Saito *et al*., 2007; Saito *et al*., 2008; Nothaft *et al*., 2010). This GlcNAc disaccharide, $(GlcNAc)_2$, hydrolysed from chitin, induces the expression of chitinase genes as well as DasABC transporter of chitobiose in *S. coelicolor* (Saito *et al*., 2007). In *Streptomyces olivaceoviridis*, the *ngcEFG* operon encodes an ABC transporter that imports *N*-acetylglucosamine and $(GlcNAc)_2$ with similar affinities (Xiao *et al*., 2002; Saito and Schrempf, 2004). A homologue of this system exists in *S. coelicolor*, which might also import monomers and/or dimers of GlcNAc under certain conditions. After uptake, $(GlcNAc)_2$ is cleaved by the *N*-acetyl-β-d-glucosaminidase DasD into monomers of GlcNAc (Saito *et al*., 2013) which are then phosphorylated by the NagK kinase and GlcNAc-6P is fed into the GlcNAc pathway described above (Fig. 2). The precise role of these transporters in nutrient sensing is not yet well understood, such as why deletion of either any of the *pts* genes or of *dasA* (but not *dasBC*) blocks development, even in the absence of the molecules they transport (Seo *et al*., 2002; Rigali *et al*., 2006; Colson *et al*., 2008). In addition to GlcN-6P and GlcNAc-6P, other metabolites also modulate the DasR response regulon, including high concentrations of phosphate (organic or inorganic) which enhance binding of DasR to its recognition site *in vitro* (Świątek-Połatyńska *et al*., 2015; Tenconi *et al*., 2015). This suggests that the metabolic status of the cell determines the selectivity of DasR for its recognition site and thus the expression of its regulon.

CROSS-TALK BETWEEN ATRA, DASR AND ROK7B7

Regulatory complexity in *S. coelicolor* is governed by the cooperative or antagonistic action of various global regulators such as AtrA, DasR and Rok7B7 (Fig. 3), which are controlled in a growth phase-dependent manner and by the nutritional status of the cell. Like DasR, the TetR-family transcriptional regulator AtrA is highly conserved in streptomycetes, and AtrA is required for actinorhodin production in *S. coelicolor* and streptomycin production in *Streptomyces griseus*, by directly controlling the pathway-specific activator genes *actII-*ORF4 and *strR*, respectively (Uguru *et al*., 2005; Hong *et al*., 2007). AtrA affects multiple regulatory pathways, including those that control sporulation (Nothaft *et al*., 2010; Kim *et al*., 2015). Interestingly, AtrA controls both the initial and final steps of the proposed DasR-mediated signalling pathway, namely the internalization of the signal (GlcNAc) via the activation of the transporter gene *nagE2*, and activation of the biosynthetic cluster for actinorhodin production via the transcriptional activation of *actII-ORF4* (Nothaft *et al*., 2010). In this way, AtrA and DasR have antagonizing activities in *S. coelicolor* (Fig. 3).

The ROK-family protein Rok7B7 takes up an interesting position in the regulatory network as it connects the control of antibiotic production and CCR. Rok7B7 shares 48% amino acid identity to a protein encoded by *rep*, a gene isolated from a metagenomic library that accelerates sporulation and antibiotic production in *Streptomyces lividans* (Martinez *et*

al., 2005). Rok7B7 controls the expression of the adjacent xylose transport operon *xylFGH* and in the absence of Rok7B7, *S. coelicolor* grows very well on xylose, which normally is not used efficiently as a carbon source (Świątek *et al*., 2013). Mutants lacking *rok7B7* also show delayed development and deregulated antibiotic production, as well as altered CCR. Indeed, Rok7B7 activates Act production but represses the biosynthesis of Red and Cda (Park *et al*., 2009). A DNA affinity capture assay suggested that Rok7B7 can bind directly to promoters of *actII*-ORF4 and *redD* (Park *et al*., 2009), but a binding site has so far not been identified and it is therefore still a mystery how Rok7B7 controls its regulon (Park *et al*., 2009; Świątek *et al*., 2013). Like AtrA, Rok7B7 also activates primary and secondary metabolism through control of the GlcNAc *pts* (specifically *nagE2*) and *actII*-ORF4 (Fig. 3) (Park *et al*., 2009; van Wezel and McDowall, 2011). It thus appears that there is direct competition between DasR-mediated repression and activation by Rok7B7 (and AtrA) of key metabolic and antibiotic regulatory genes (Fig. 3).

Proteomic analysis of *S. coelicolor* and the *S. coelicolor glkA* deletion mutant revealed that glucose activates Rok7B7 and XylFGH in a Glk- and CCR-independent manner (Gubbens *et al*., 2012) and this was also observed in a recent transcriptomic analysis (Romero-Rodríguez *et al*., 2016). Interestingly, DasR and Rok7B7 both repress the expression of Glk (Świątek *et al*., 2013; Świątek-Połatyńska *et al*., 2015), while Glk represses Rok7B7 (Fig. 3) (Gubbens *et al*., 2012). Conversely, deletion of *rok7B7* results in a loss of CCR, which directly implicates Rok7B7 in CCR (Gubbens *et al*., 2012; Romero-Rodríguez *et al*., 2016). The upregulation of Rok7B7 in glucose-grown cultures may be explained as feedback control to try to achieve CCR in the absence of Glk activity. Since Glk expression is constitutive and Glk is activated posttranslationally (van Wezel *et al*., 2007; Romero-Rodríguez *et al*., 2016), direct transcriptional control of *glkA* by Rok7B7 is unlikely. The ligand for Rok7B7 is unknown, although its phylogenetic linkage to xylose utilization suggests that it is mediated by a C5 carbon.

Linking nitrogen and phosphate control: GlnR and PhoP

A novel linkage between C- and N-metabolism was established recently, when it was shown that GlnR not only controls nitrogen metabolism but also the uptake and metabolism of carbon (Liao *et al*., 2015a). *S. coelicolor* can use a wide range of nitrogen sources, including ammonia, nitrate (Wang and Zhao, 2009), nitrite, urea (Tiffert *et al*., 2008), amino sugars and amino acids (Reuther and Wohlleben, 2007). Utilization of different nitrogen sources is controlled by the orphan response regulator GlnR (Fig. 3), whose gene expression is nitrogen dependent (Tiffert *et al*., 2008). In *Saccharopolyspora erythraea*, most ABC transporters are under control of GlnR and its disruption lead to impaired growth on sugars including maltose, mannitol, mannose, sorbitol and trehalose (Liao *et al*., 2015a) and in their transcriptional analysis of CCR in *S. coelicolor*, Romero-Rodríguez and colleagues also observed upregulation of GlnR by glucose (Romero-Rodríguez *et al*., 2016). Under control of GlnR, actinomycetes are able to induce carbohydrate uptake and metabolism when nitrogen, which is essential for the synthesis of proteins, co-factors and specialized metabolites, is limited. Interestingly, it was shown that the three genes for citrate synthase are all controlled by several global nutrient sensory regulators including GlnR and DasR, but also the cAMP receptor protein, CRP (Liao *et al*., 2014a). CRP controls early processes during growth in *Streptomyces* (Derouaux *et al*., 2004b; Piette *et al*., 2005) and acts as a global regulator of Act, Cda and Red production and it was suggested that it coordinates precursor flux from primary to secondary metabolism (Gao *et al*., 2012).

Besides the linkage with CRP and DasR, there is also significant cross-talk between

GlnR and the global phosphate regulator PhoP (Fig. 3), which is part of a two-component system. Phosphate plays an important role in the control of antibiotic production, with many antibiotics repressed by phosphate (reviewed in [Martin, 2004; Martin and Liras, 2010]). PhoP and GlnR together control antibiotic production by monitoring the metabolic status of phosphate and nitrogen in *Streptomyces* (Santos-Beneit *et al*., 2009; Santos-Beneit *et al*., 2012). Transcriptomic and biochemical data show that PhoP also controls expression of *glnR*, though it is unlikely that GlnR controls *phoP* directly. PhoP also competes with GlnR for the promoter regions of genes for nitrogen metabolism and this PhoP-mediated control of nitrogen metabolism may help balancing the cellular P/N equilibrium (Sola-Landa *et al*., 2013). Expression of antibiotic biosynthetic gene clusters is upregulated in response to low phosphate (Rodríguez-García *et al*., 2007; Nieselt *et al*., 2010; Allenby *et al*., 2012), as well as by ammonium limitation (Fink *et al*., 2002; Reuther and Wohlleben, 2007; Lewis *et al*., 2011), though ChIP-on-chip studies indicated that this is not through direct binding of pathway-specific antibiotic activators promoters. Instead, PhoP binds upstream of genes which encode for other regulatory proteins that control antibiotic gene clusters, including *afsS* and *atrA* (Allenby *et al*., 2012). The *afsS* gene encodes a small protein which activates antibiotic production in various streptomycetes, in a yet unknown manner (Martin *et al*., 2011; Santos-Beneit *et al*., 2011).

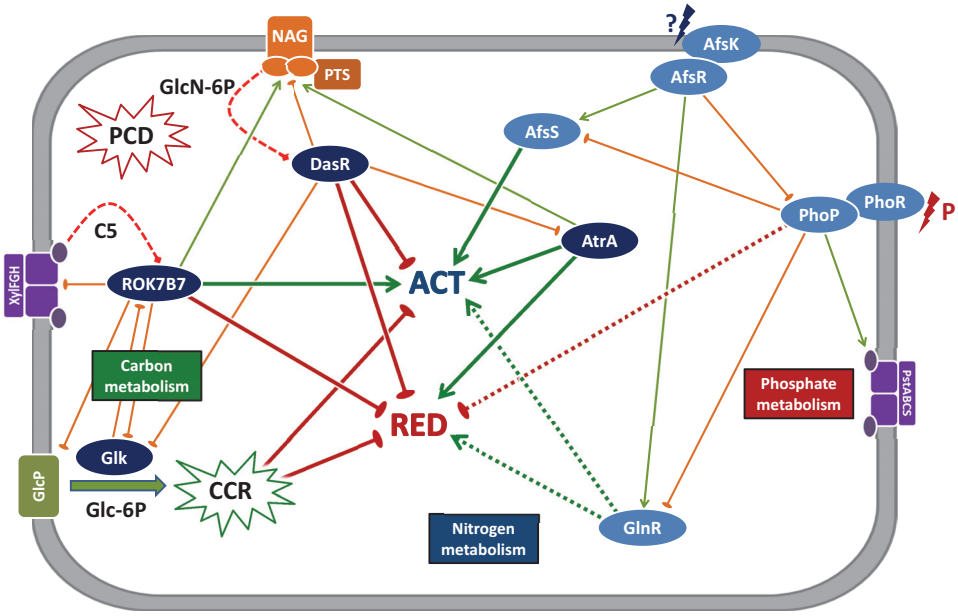

FIGURE 3. Intertwining nutrient regulatory networks that control antibiotic production in *S. coelicolor*.
Global regulatory networks translate environmental signals and the cell's metabolic status to secondary metabolic responses. Only regulatory networks controlling the biosynthetic genes for actinorhodin (Act) and prodiginines (Red) are shown. The biosynthesis of antibiotics is under the control of specific transcriptional regulators, situated within the biosynthetic clusters, which in turn are under global control. Carbon control proteins central to this review presented as dark blue ovals, other regulators light blue. Dotted lines indicate uncertainty of control (direct or indirect). Activation of antibiotic production is shown as thick green arrows and repression by thick red lines with an ellipse. In addition to regulatory control of antibiotic production, global transcriptional regulators also control enzymes involved in metabolic pathways as well as other global transcriptional regulator. This is represented by thin lines; positive control by green arrows, negative control by orange lines with an ellipse. CCR, carbon catabolite repression; PCD, programmed cell death. Dashed red lines indicate inhibition by ligands (Rok7B7 by C5 sugars (C5); DasR by GlcN-6P). For transporters see Fig. 2.

The AfsK serine/threonine kinase and its cognate response regulator AfsR also control antibiotic production via modulation of *afsS* (Lee *et al*., 2002). In addition to competition for the *afsS* promoter, PhoP and AfsR also cross-regulate expression of *glnR*, the phosphate transporter gene *pstS* and *phoPR*, which encodes response regulator regulator PhoP and its cognate sensory kinase, the phosphate limitation sensor PhoR (Santos-Beneit *et al*., 2009; Santos-Beneit *et al*., 2012). The response regulator AfsQ1 and cognate sensory kinase AfsQ2 act as conditional (positive) pleiotropic regulators of phosphate transporter PtsS as well as development and antibiotic production (Wang *et al*., 2013), whereby the AfsQ1 binding site upstream of the Red biosynthetic genes overlaps with the site recognized by DasR.

The role of inorganic phosphate (Pi) in antibiotic production involves another direct target gene of PhoP, *ppk*, which encodes an enzyme that acts as an adenosyl diphosphate kinase (ADPK), regenerating ATP under conditions of Pi limitation (Chouayekh and Virolle, 2002; Ghorbel *et al*., 2006a; Ghorbel *et al*., 2006b). By regenerating ATP from ADP and polyphosphates, *ppk* plays a key role in maintaining the energetic homeostasis of the cell. Its deletion in *S. lividans* results in enhanced actinorhodin production in the glucose-rich and Pi limited medium R2YE (Chouayekh and Virolle, 2002). The increased Act production is attributed to increased degradation of lipid storage vesicles, containing mainly triacylglycerols (TAG), in order to restore the energetic balance caused by the ATP deficiency in the *ppk* mutant (Le Marechal *et al*., 2013). TAG degradation generates fatty acids and thus ultimately acetyl-CoA, which is among others a precursor for polyketide biosynthesis. The total TAG content in *S. coelicolor* and the amount of lipid vesicles are much lower than in *S. lividans*, suggesting higher degradation of storage lipids and thus higher accumulation of the precursor acetyl-CoA in this strong Act producer (Le Marechal *et al*., 2013).

Finally, phosphorylated sugars also inhibit antibiotic production in streptomycetes. This effect is mediated by the phosphate moiety rather than the sugar moiety of the extracellular phosphor-sugars as the inactivation of *phoP* and *ppk* prevents and enhances, respectively, their utilization as nutrient sources and their inhibitory effect on antibiotic production (Tenconi *et al*., 2012).

PERSPECTIVE: APPLICATION FOR ANTIBIOTIC DISCOVERY

Despite the phenomenal potential of actinomycetes as antibiotic producers, the antibiotic pipelines have nearly dried out. This is particularly due to replication, in other words, high-throughput screening efforts result in finding the same molecules over and over again, rather than identifying compounds with novel chemical structures and bioactivities (Payne *et al*., 2007; Cooper and Shlaes, 2011; Lewis, 2013; Kolter and van Wezel, 2016). However, genome sequencing established that even widely studied species are relatively untapped sources of natural products (Bentley *et al*., 2002; Ikeda *et al*., 2003; Ohnishi *et al*., 2008; Cruz-Morales *et al*., 2013) and the wealth of genome sequence information that is currently being fed into the databases will reveal tens to hundreds of thousands of biosynthetic gene clusters. Undoubtedly, only a fraction of these have successfully been induced under laboratory conditions and a critical step is to identify the nutritional and ecological triggers and cues that allow the activation of these silent biosynthetic gene clusters (Zhu *et al*., 2014a; Rutledge and Challis, 2015).

Understanding the regulatory networks that control biosynthetic gene clusters is of critical importance, with the induction by GlcNAc of the DasR-repressed *cpk* operon, which specifies the cryptic polyketide antibiotic Cpk, as an example of how such information can be harnessed to directly activate silent gene clusters (Rigali *et al*., 2008). Similarly, knowledge

gained from unravelling the cellobiose utilization regulon controlled by CebR in *Streptomyces scabies* was recently applied to induce the expression of the biosynthetic gene cluster for the herbicide thaxtomin (Francis *et al*., 2015). Scanning the genomes of actinomycetes for sites matching the consensus binding site for DasR revealed many putative target genes that relate to secondary metabolism, suggesting that DasR may control the production of a wide variety of specialized metabolites, including clinical drugs such as clavulanic acid, chloramphenicol, daptomycine and teichoplanin (van Wezel *et al*., 2006b). Indeed, the addition of GlcNAc to minimal media often elicits the production of antibiotics, identifying novel compounds (Zhu *et al*., 2014b). Bettering our understanding of the nutrient sensory regulatory networks and the way they are controlled by the metabolic status of the cell will also improve our fundamental understanding of the control of antibiotic production. Once more regulatory networks are unravelled, and the corresponding regulatory elements are mapped to the biosynthetic gene clusters, specific nutrient-mediated activation of natural products will become more and more routine, thereby providing new impetus to drug-discovery efforts.

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