Global control of development and antibiotic production by nutrient-responsive signalling pathways in Streptomyces

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Chapter II

The regulation of the secondary metabolism and development of *Streptomyces*

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1. The *Streptomyces* life-cycle

Streptomycetes are high-GC Gram-positive, soil-dwelling bacteria belonging to the phylum of actinobacteria. They have a morphologically complex life cycle (Figure 1), which starts with a spore that germinates to produce a branched mycelium consisting of long multinucleoid hyphae (Redington and Chater, 1997). When streptomycetes enter the developmental program, the vegetative mycelium (also known as substrate mycelium) is degraded following several rounds of autolysis (Manteca *et al*., 2005; Manteca & Sanchez, 2009). This autolytic degradation of the hyphae provides the nutrients for the newly formed aerial hyphae, that obtained their name for their ability to break through the moist soil surface and grow into the air. Eventually, the apical sites (tips) of the aerial hyphae differentiate to produce long chains of up to a hundred unigenomic spores (Flärdh & Buttner, 2009). Sporulation is a highly complex process of coordinated cell division and DNA segregation (reviewed in Jakimowicz & van Wezel, 2012).

Extensive studies of streptomycete morphology led to identification and characterization of two major classes of developmental mutants. The first class is that of the bald (*bld*) mutants, which are so called for their lack of the fluffy aerial mycelium, as they are locked in the vegetative growth phase (Merrick, 1976). The second class is that of the the white (*whi*) mutants, which produce aerial hyphae, but fail to form the grey-pigmented spores (Chater, 1972; Hopwood *et* al., 1970; reviewed in Flärdh & Buttner, 2009; Kelemen & Buttner, 1998).

Streptomycetes together with other members of the actinomycetales synthesize around 70% of all known antibiotics (Weber *et al*., 2003), as well as many other natural products such as immunosuppressants, insecticides and anti-tumor agents (Hopwood, 2007). The onset of morphological differentiation roughly coincides with that of chemical differentiation, *i.e*. the production of secondary metabolites (Bibb, 2005; Hopwood, 2007; van Wezel & McDowall, 2011).

Due to the rapid spread of drug-resistant infectious diseases a point of no return has been reached where novel antibiotics are an absolute necessity. However, it has become increasingly difficult to find novel classes of antibiotics with efficacy against multi-drug resistant pathogens such as MDR-TB (multi-drug resistant *Mycobacterium tuberculosis*), MRSA (methicillin-resistant *Staphylococcus aureus*) and the rapidly emerging MDR Gram-negative pathogens (*e.g. Pseudomonas*, *Klebsiella* and

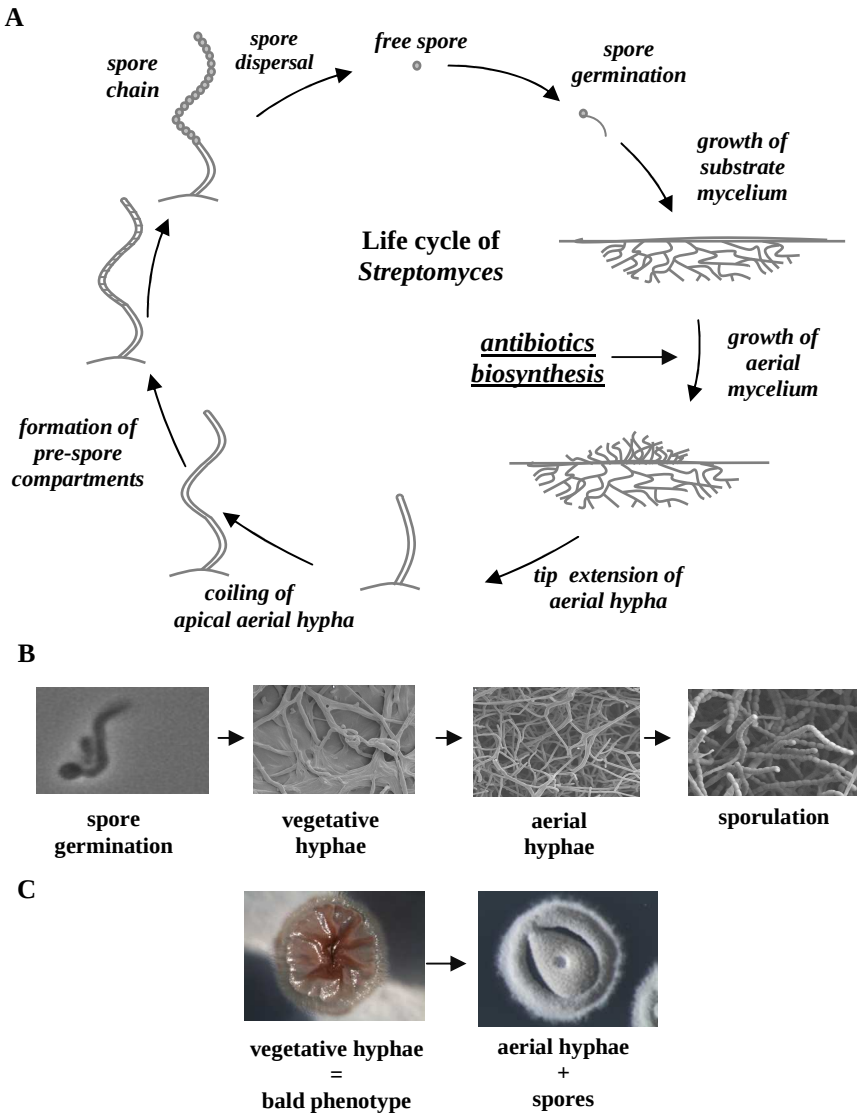

Figure 1. Life cycle of *Streptomyces coelicolor* A3(2). A) Schematic representation. B) Scanning electron micrographs showing *S. coelicolor* at different stages of life cycle. C) Stereo micrographs of single colonies either locked at vegetative state (bald phenotype) or displaying normal development with aerial mycelium and spores.

Enterobacter) (Baltz, 2008; Payne *et al*., 2007). Actinomycetes are prolific antibiotic producers, and full genome sequencing efforts have established that even the widely studied species are still relatively untapped sources of natural products, with some 20

gene clusters for secondary metabolites found in a single streptomycete (Challis & Hopwood, 2003). Sequencing of the numerous *Streptomyces* genomes, including *S. coelicolor, S. avermitilis, S. griseus, S. scabies,* and *S. clavuligerus* (Bentley *et al*., 2002; Ikeda *et al*., 2003; Ohnishi *et al*., 2008; Song *et al*., 2010) has revealed their high coding capacity to produce multiple secondary metabolites (Donadio *et al*., 2002; Nett *et al*., 2009). The genes encoding enzymes for the production of a secondary metabolite are typically organized in large gene clusters, often in association with one or more genes that regulate their transcription as well as genes encoding the resistance mechanism. Despite extensive studies, relatively little is known about the molecular mechanisms underlying the regulation of secondary metabolism.

In this chapter, current knowledge on the control and regulation of antibiotic production in *Streptomyces* is reviewed, with main focus on *S. coelicolor* A3(2), the model organism of the species. Approaches are also discussed that can be used to stimulate the production of antibiotics or trigger the production of so-called 'cryptic' or 'silent' antibiotics, which are not expressed under standard laboratory conditions.

2. Carbon catabolite repression (CCR) in bacteria

Regulatory mechanisms that control carbon source utilization in bacteria have a major impact on adaptation of the organism to often rapidly changing environmental conditions. Bacteria utilize a wide variety of carbon sources. These carbon sources are sometimes co-metabolized (Gunnewijk *et al*., 2001; Kim *et al*., 2009; Wendish *et al*., 2000), but bacteria also have extensive regulatory systems that ensure that preferred carbon sources in terms of yield of energy and ease of metabolism are preferentially used over less favorable nutrients. Efficient carbon source utilization is critical from the perspective of competition in the natural habitat. The control system that allows the assimilation of preferred carbon sources is known as carbon catabolite repression (CCR). CCR has been extensively studied in both Gram-positive and Gram-negative bacteria. In many organisms, CCR of catabolic genes is achieved by the combined activities of global and operon-specific regulatory mechanisms (Görke & Stülke, 2008). The phosphoenolpyruvate dependent phosphotransferases system (PTS) plays a major role in the regulation of CCR in many bacteria.

2.1 The phosphoenolpyruvate-dependent phosphotransferases system (PTS)

The first step in the metabolism of almost any carbohydrate is its internalization into the cell. Different transport mechanisms exist in bacteria for the uptake of carbohydrates: passive facilitated diffusion, active transport driven by electrochemical ion gradients or ATP, and group translocation. Sugar uptake by group translocation is unique for prokaryotes and requires a high-energy phosphate compound such as ATP or phosphoenolpyruvate (PEP) as energy source. A well-characterized example of group translocation is the phosphoenolpyruvate-dependent phosphotransferases system (PTS), which uses a protein phosphoryl transfer chain to transport and phosphorylate carbohydrates. The energy for their translocation is provided by the glycolytic intermediate phosphoenolpyruvate. PTSs consist of two general energy coupling phosphotransferases: enzyme I (EI, gene *ptsI*) and HPr (gene *ptsH*) and a diverse carbohydrate-specific transport complexes called Enzymes IIs (EII). The major role of the energy coupling proteins is phosphorylation of HPr at the expanse of PEP. Phosphorylated HPr (HPr-P) possesses the property to transfer its phosphate group to the EII complexes. The EIIs consist of three to four domains called IIA, IIB, IIC and if present IID and are responsible for carbohydrate transport across the cytoplasmic membrane and its subsequent phosphorylation (Postma *et al*., 1993; Saier & Reizer, 1992). The PTS proteins, apart of their role in sugar transport, are also involved in regulatory mechanisms (Postma *et al*., 1993).

The PTS and its involvement in the regulation of CCR have been studied in detail in the model organisms of Gram-negative and low-GC Gram-positive bacteria, *E. coli* and *B. subtilis*, respectively (Brückner & Titgemeyer, 2002; Görke & Stülke, 2008; Gunnewijk *et al*., 2001). Regulation of CCR in *E. coli* is controlled by modulation of the phosphorylation state of EIIA domain of the glucose transporter ($EIIA^{Glc}$). In the presence of a PTS substrate, for example glucose, and the low intracellular PEP to pyruvate ratio, the phosphate from $EIIA^{Glc}$ is transferred to the incoming PTS sugar. As a consequence, $EIIA^{Glc}$ exists primarily in its unphosphorylated form during active PTS transport. It then inhibits transport of non-PTS sugars (e.g. lactose, maltose, melibiose, raffinose) by direct binding to sugar permeases and prevents formation of the inducer molecules for less preferred carbon sources, such as allolactose for the induction of the lactose (*lac*) operon (Brückner & Titgemeyer, 2002; Hogema *et al*., 1998). Phosphorylated $EIIA^{Glc}$ activates

the membrane-bound enzyme adenylate cyclase (AC), which in turn leads to cyclic AMP (cAMP) synthesis by the enzyme adenylate cyclase. The resulting cAMP then interacts with its receptor protein CRP, and the cAMP-CRP complex activates the promoters of many catabolic genes and operons (Görke & Stülke, 2008). In *E. coli* not only enzyme IIA^{Glc} plays a central role in intracellular signal transduction. Additionally, dephosphorylated EI inhibits autophosphorylation of the sensor kinase ChiA of the bacterial chemotaxis machinery, decreasing the flow of phosphate to CheY and eliciting smooth, straight movement of the microorganism towards the attractant(s) (*e.g.* a PTS substrate) (Lux *et al.*, 1995). HPr mediates the control of glycogen storage in this organism. Unphosphorylated HPr allosterically activates glycogen phosphorylase (GlgP), resulting in the breakdown of glycogen, while phosphorylated HPr stimulates cAMP synthesis and expression of the anabolic glycogen operon glgCAP leading to glycogen accumulation (Seok *et al*., 1997).

In *B. subtilis*, the phosphocarrier protein HPr plays the central role in the regulation of CCR. The uptake of a preferred carbon source stimulates phosphorylation of HPr at Ser-46 by a bifunctional enzyme, the HPr kinase/phosphorylase (HprK/P). HPr kinase activity is stimulated by the increased concentration of fructose-1,6-bisphosphate (FBP), while HPr phosphorylase activity is triggered by the accumulation of inorganic phosphate in the cell (Jault *et al*., 2000; Mijakovic *et al*., 2002). Phosphorylation of HPr enables its interaction with the pleiotropic transcription factor CcpA (catabolite control protein A) and leads to binding of the CcpA-HPr(Ser-P) complex to operator sites of catabolic operons (Deutscher *et al*., 1995; Jones *et al*., 1997), causing their repression. Similarly to *E. coli*, chemotaxis in *B. subtilis* is also affected by the PTS. Deletion of *ptsH* prevents *B. subtilis* from moving towards most PTS carbohydrates. In contrast to *E. coli*, phosphorylated EI (EI-P) has the ability to inhibit *B. subtilis* CheA autophosphorylation, leading to smooth bacterial movement.

2.2 The PTS and CCR in *Streptomyces*

In contrast to Gram-negative and low-GC Gram-positive bacteria , CCR and PTS may not be directly linked in *Streptomyces*. Main knowledge of the PTS in streptomycetes comes from studies in *S. coelicolor* (Kamionka *et al*., 2002; Nothaft *et al*., 2003a; Nothaft *et al*. 2003b), while research was also conducted in a few other streptomycetes, including *S.*

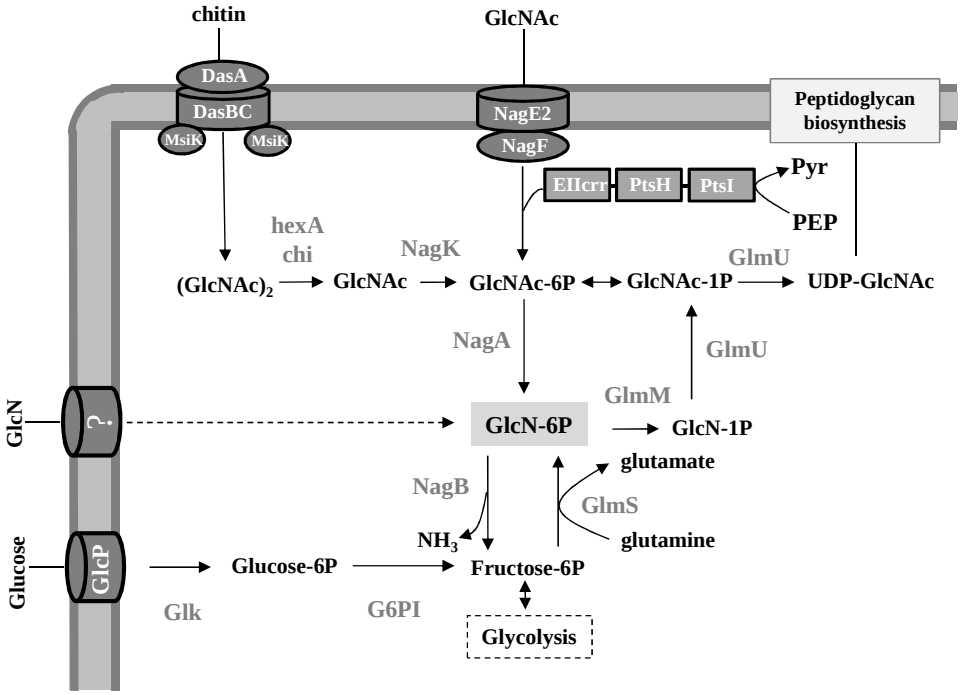

Figure 2. Glucose and GlcNAc transport and metabolism in *S. coelicolor* A3(2).

lividans, S. griseofuscus, S. olivaceoviridis (Titgemeyer *et al*., 1995; Wang *et al.*, 2002). Two of PTS-energy coupling proteins, enzyme I (EI; gene *ptsI*, SCO1390) and HPr (gene *ptsH*, SCO5841) are involved in the transport of fructose and *N*-acetylglucosamine (GlcNAc) in *S. coelicolor* (Nothaft *et al*., 2003a; Nothaft *et al*., 2003b; Titgemeyer *et al*., 1995). The carbohydrate-specific EIIABC permease complex is encoded by FruA (*fruA*, SCO3196) for fructose, while the components of the EIIABC complex for GlcNAc are encoded by $EIIA^{Crr}$ (*crr*, SCO1391), NagF (*nagF*, SCO2905) and NagE2 (*nagE2*, SCO2907), respectively. $EIIA^{Crr}$ is a homologue of $EIIA^{Glc}$ from *E. coli*. It transfers a phosphate from HPr to $EIIB^{GlcNAc}$ domain, which in turn phosphorylates the incoming GlcNAc via $EIIC^{GlcNAc}$ (Nothaft *et al*., 2010). Deletion of the global *pts* genes of *S. coelicolor* (*ptsH*, *ptsI* and *crr*) leads to vegetative arrest and consequently mutants have a bald phenotype (Nothaft *et al*., 2003a; Rigali *et al*., 2006). This observation reveals a direct link between carbon utilization and the control of development, but it is currently unknown how this is mediated. HPr is also required for fructose-dependent repression of glycerol kinase (Nothaft *et al*., 2003b; Parche, 2001), and $EIIA^{Crr}$ subsititutes the

regulatory function of $EIIA^{Glc}$ of *E. coli* by blocking transport of maltose, when present in its unphopshorylated form (Kamionka *et al*., 2002). However, and perhaps surprisingly, in contrast to the situation in other bacteria, deletion of *ptsH* has no effect on glucose repression of glycerol kinase, agarase or galactokinase (Butler *et al*., 1999; Nothaft *et al*., 2003b). Since the direct effect of both HPr and $EIIA^{Crr}$ on global CCR could not be demonstrated (Kamionka *et al*., 2002; Parche, 2001), it was concluded that the PTS does not play a general role in CCR and that there are additional mechanisms necessary to control carbon source regulation in *Streptomyces*.

Glucose kinase (Glk), a member of the ROK family of proteins (Repressors, ORFs and Kinases), is central to CCR in streptomycetes (Angell *et al*., 1994; Angell *et al*., 1992; van Wezel *et al*., 2007). In *S. coelicolor* glucose is not transported via a permease associated with the PEP-dependent PTS, but instead imported via MFS (Major Facilitator Superfamily) permease GlcP (van Wezel *et al*., 2005) (Figure 2). The incoming glucose is phosphorylated by glucose kinase (Glk, encoded by *glkA*) in an ATP-dependent manner. 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 (*gylCABX)*, galactose (*galP*), maltose (*malEFG*) or chitin (*chi*) (Angell *et al*., 1994; Angell *et al*., 1992; Kwakman & Postma, 1994; Saito *et al*., 2000; van Wezel *et al*., 1997). Comparison of cultures growing in the presence of the non-repressing carbon source mannitol or the repressing carbon source glucose revealed that Glk is expressed constitutively under both growth conditions, but Glk activity assays and Western blot analysis showed that enzyme activity varies depending on the carbon source and growth phase, with high activity in glucose-grown cultures, and low activity in cultures grown on mannitol. Additionally, two isoforms of Glk were detected, with a second isoform predominant in glucose-grown cultures and directly correlating to activity of the Glk enzyme. These observations suggested that Glk activity is modulated through metabolite-dependent activation and/or posttranslational modification of the enzyme (van Wezel *et al*., 2007). Recently, it has been shown that glucose kinase of *S. griseus* SgGlkA undergoes different conformational changes in the presence of various substrates (Miyazono *et al*., 2012). Because glucose kinase lacks a DNA-binding motif, direct control of gene expression is highly unlikely. Therefore, the conformational changes in the presence of multiple substrates could be a mechanism by which glucose kinase

transmits the existence of repressing carbon source to other proteins which regulate the CCR in *Streptomyces*. Additionally, there are reports suggesting that glucose kinase most likely functions in concert with other proteins, which determine its catalytic and regulatory role (Angell *et al*., 1994; Gagnat *et al*., 1997; Guzman *et al*., 2005). A landmark experiment was the demonstration that complementation of the *S. coelicolor glkA* mutant by introduction of an orthologous *glkA* gene from *Zymomonas mobilis* restored glucose utilization and glycolysis, but not glucose repression (Angell *et al*., 1994). This showed that the flux of carbon did not play a decisive role in glucose repression. SCO2127, the gene located upstream of *glkA*, is involved in stimulating transcription of *glkA* and perhaps the glucose transporter gene *glcP* (Guzman *et al*., 2005), and subsequently improves GlkA activity and glucose transport (Angell *et al*., 1994; Guzman *et al*., 2005). Disruption of the *sblA* gene of *S. lividans* led to relief of glucose repression of the α-amylase gene *aml*, suggesting that SblA plays a direct role in CCR (Chouayekh *et al*., 2007; Gagnat *et al*., 1999). SblA shares significant sequence similarities with various phosphatases that acts on small phosphorylated molecules. It is believed that SblA is involved in the degradation of an internal inducer of *aml* expression or of a precursor of such an inducer (Gagnat *et al*., 1999). Thus, while interesting new data have come to light, the exact mechanism by which Glk governs CCR still remains to be elucidated.

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2.2.1 Control of carbon catabolite repression by Bld proteins

Involvement of developmental proteins in control of CCR was suggested after observation that mutants of some *bld* genes are insensitive to CCR (Champness, 1988). Like most *bld* mutants, *bldB* null mutants of *S. coelicolor* are unable to produce an aerial mycelium nor antibiotics on different carbon sources. This shows the linkage between carbon utilization and the transition from vegetative to aerial growth and antibiotic production. The *bld* mutants *bldA*, *bldG*, *bldH* are also blocked in aerial mycelium formation during growth in presence of glucose or cellobiose (a dimer of glucose) (Champness, 1988). However, repression of morphological differentiation is relieved for many *bld* mutants when they are grown on non-repressing carbon sources, like arabinose, galactose, glycerol, mannitol or maltose (Mobley *et al*., 1982). Thus, the majority of *bld* mutants exhibit carbon-source dependent differentiation. Interestingly, the additional

deletion of *glkA* allowed *bldA* mutants to sporulate on media containing both mannitol and glucose, providing direct evidence for the role of Glk-dependent CCR in the control of morphogenesis (van Wezel & McDowall, 2011).

3. Carbon source regulation of antibiotic production in *Streptomyces*

The availability and particular source of carbon has a major impact on antibiotic production and other secondary metabolites (*e.g.* industrially important enzymes). Glucose is a preferred carbon source and as such effects strong carbon catabolite repression (see above), and also represses the biosynthesis of structurally diverse antibiotics, including β-lactams, aminoglycosides, and macrocyclic polyketides, in different *Streptomyces* species (Ruiz *et al*., 2010; Sanchez *et al*., 2010). In *Streptomyces lividans* glucose inhibits actinorhodin production by repressing the *afsR2* gene, which encodes a global regulatory protein involved in the stimulation of secondary metabolite biosynthesis in diverse *Streptomyces* species (Kim *et al*., 2001). In *S. coelicolor*, AfsR2 binds to a putative secreted solute binding protein encoded by SCO6569. Overexpression of this newly characterized protein in *S. coelicolor* significantly reduced actinorhodin production, while gene disruption lead to accelerated antibiotic production (Lee *et al*., 2009). This suggests that SCO6569 is an AfsR2-dependent down-regulator of actinorhodin production in *S. coelicolor*. Multiple reports show that not only glucose, but also other carbohydrates, such as glycerol, maltose, xylose, fructose and sucrose affect antibiotic production in *Streptomyces* (Demain & Fang, 1995; Sanchez *et al*., 2010). For instance, glycerol represses actinomycin production in *S. parvulus* by decreasing enzymatic activity of hydroxykynureninase, an enzyme involved in antibiotic biosynthesis (Troost *et al*., 1980); mannose and glucose negatively affect streptomycin production in *S. aurofaciens* by acting on mannosidostreptomycinase (Demain & Inamine, 1970). Therefore, it is important to find the optimal carbon source in terms of allowing the efficient production of industrially important metabolites.

3.1 *N*-acetylglucosamine and the DasR regulon

N-acetylglucosamine (GlcNAc) is the monomer of chitin and together with *N*-acetylmuramic acid forms the basic structure of bacterial peptidoglycan (Terrak *et al*., 1999). It is also an excellent carbon and nitrogen source for bacterial growth. The

mechanism of GlcNAc transport and subsequent internalization has been studied in many different bacteria, namely *E. coli* (White, 1970), *B. subtilis* (Mobley *et al*., 1982; Reizer *et al*., 1999), *Bacillus sphaericus* (Alice *et al*., 2003), *Staphylococcus aureus* (Komatsuzawa *et al*., 2004), *Vibrio furnissii* (Bouma & Roseman, 1996), *Caulobacter crescentus* (Eisenbeis *et al*., 2008), *Klebsiella pneumonia* (Vogler & Lengeler, 1989), *Gluconacetobacter xylinus* (Yadav *et al*., 2011), and *Xanthomonas campestris* (Boulanger *et al*., 2010). In most bacteria uptake of GlcNAc occurs via a PTS component, such as NagE in *E. coli* (White, 1970), NagP in *B. subtilis* (Mobley *et al.*, 1982; Reizer *et al*., 1999) and NagE2 in *S. coelicolor* (Nothaft *et al*., 2010). Alternative GlcNAc uptake systems have been identified. For example, in *Xanthomonas campestris* GlcNAc uptake occurs via the NagP, belonging to the Major Facilitator Superfamily transporters (Boulanger *et al*., 2010). In *Streptomyces olivaceoviridis*, besides via the PTS (Wang *et al*., 2002), *N*-acetylglucosamine is also transported via the ABC-type transporter NgcEFG (Xiao *et al*., 2002). NgcEFG imports both *N*-acetylglucosamine and its disaccharide *N,N'*-diacetylchitobiose $(GlcNAc)_2$ with similar affinities (Xiao *et al*., 2002). Alternatively, chitobiose can be transported via the DasABC transporter in streptomycetes (Saito *et al*., 2007; Colson *et al*., 2008).

The first reaction in the GlcNAc (PTS internalized) metabolic pathway is deacetylation of the intracellular *N*-acetylglucosamine-6-phosphate (GlcNAc-6P) to glucosamine-6-phosphate (GlcN-6P) by NagA (GlcNAc-6P deacetylase). GlcN-6P is a central molecule that stands at the cross-roads of many metabolic pathways, including glycolysis, cell-wall synthesis (see below), glutamine and glutamate metabolism (see KEGG database; http://www.genome.jp/kegg-bin/show_pathway?org_name=sco&mapno=00520). GlcN-6P is deaminated and isomerized by NagB (GlcN-6P deaminase) to fructose-6-phosphate (Fru-6P), which enters the glycolytic pathway (Alvarez-Anorve *et al.*, 2005). It can also be incorporated into murein following its conversion to uridine diphosphate-*N*-acetylglucosamine (UDP-GlcNAc) by the subsequent action of GlmM (phosphoglucosamine mutase) and GlmU (GlcN-1P acetyltransferase) (Durand *et al*., 2008) (Figure 2). During growth on carbon sources other than aminosugars, GlcN6-P can be produced from Fru-6P by GlcN-6P synthase (*glmS*) (Figure 2).

GlcNAc is an important signalling molecule for the onset of development and

antibiotic production of streptomycetes (Rigali *et al*., 2006; Rigali *et al*., 2008). When GlcNAc accumulates around *Streptomyces* colonies under rich growth conditions (so-called *feast*) it promotes vegetative growth, thereby blocking developmental processes and antibiotic production, while under poor nutritional conditions (*famine*) the accumulation of GlcNAc promotes development and antibiotic production (Rigali *et al*., 2008; van Wezel *et al*., 2009). A complete signalling cascade was proposed from GlcNAc sensing and uptake to the onset of antibiotic production in *S. coelicolor*. Following its internalization and phosphorylation by the PTS, GlcNAc-6P is converted by NagA to GlcN-6P (Figure 2). GlcN-6-P then acts as an allosteric effector of the global transcriptional regulator DasR (deficient in aerial mycelium and spore formation), thus preventing its DNA binding (Rigali *et al*., 2006 and Chapter IV). DasR belongs to the GntR-family of transcriptional regulators; it represses antibiotic production by direct binding to the promoter region of *actII-ORF4*, encoding the pathway-specific activator for actinorhodin (Act) production, and *redZ*, for the response regulator RedZ that activates prodiginine (Red) production (Rigali *et al*., 2008). Additionally, DasR directly represses *pts* and *nag* genes (Rigali *et al*., 2006; Rigali *et al*., 2008).

The sequences of other actinomycetes genomes have been scanned for sites matching the consensus binding site for DasR (*dre*, for DasR responsive element). A range of putative targets relating to secondary metabolism was identified, suggesting that DasR may control the production of many important clinical drugs, such as clavulanic acid, chloramphenicol and the glycopeptide antibiotics daptomycin and teichoplanin (van Wezel & McDowall, 2011). Whether or not these genes are indeed all controlled (*i.e.* repressed) by DasR remains to be elucidated, but it is rather likely that at least some of these are true DasR targets. In terms of evolutionary conservation, binding sites for DasR have been predicted for *S. avermitilis* in the promoter regions of three quarters of the orthologues of genes thought to be regulated directly by DasR in *S. coelicolor* (Rigali & van Wezel, unpublished data). Considering its pleiotropic function and wide distribution in actinomycetes, manipulating the DasR regulon could be applied in strategies to enhance antibiotic production. Addition of high concentrations of GlcNAc will lead to the accumulation of GlcN-6P, the effector molecule for DasR, changing its DNA binding capacity and resulting in the global de-repression of antibiotic production. 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.

4. Global transcriptional regulators involved in the control of antibiotic production in *S. coelicolor*

The *S. coelicolor* genome sequence revealed the complexity of regulatory networks that govern the transcriptional control over the crucial cellular processes such as cell growth, morphological differentiation and antibiotic production. Transcriptional regulators responsible for the control of antibiotic production, including *actII*-ORF4 and *redD*, are among others located within the biosynthetic gene clusters themselves. The global antibiotic regulatory genes (not pathway-specific) are more difficult to identify among more than 700 possible regulatory genes present in the *S. coelicolor* genome sequence. However, a number of proteins, including DasR, have a pleiotropic role in the control of development and antibiotic production. One such regulator is the TetR-family transcriptional regulator encoded by SCO1712. Disruption of this gene enhanced antibiotic production through the activation of pathway-specific regulators (Lee *et al*., 2010). Interestingly, further stimulation of antibiotic production was observed when besides SCO1712 also *wblA*, a repressor of antibiotic production (Fowler-Goldsworthy *et al*., 2011) was deleted (Lee *et al*., 2010).

Amazingly, a large number of transcriptional regulators directly control the pathway-specific activator gene *actII*-ORF4 in *S. coelicolor*. The reason for this complexity is poorly understood, and strongly suggests that ActII-ORF4 (and actinorhodin itself?) plays a more important role in the *S. coelicolor* life cycle than so far anticipated. AtrA is another TetR-family transcriptional regulator involved in the control of antibiotic biosynthesis. Disruption of *atrA* led to reduced actinorhodin (Act) production, whereas its overexpression increased Act production in wild-type *S. coelicolor* (Towle, 2007; Uguru *et al*., 2005). AtrA directly controls transcription of *actII*-ORF4 and multiple other targets (Boomsma, 2008). Additionally, it was suggested that AtrA may adjust acetyl-CoA metabolism, which supplies precursors for polyketides, as well as stimulating production of the Act biosynthetic genes (van Wezel & McDowall, 2011). Interestingly, AtrA controls both the initial and final steps of the proposed DasR-mediated signalling pathway (proposed in Rigali *et al*., 2008), namely internalization of

the signal (GlcNAc) via 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). Therefore, AtrA and DasR have antagonizing activities in *S. coelicolor*.

Another example is AfsR, a member of the SARP (*Streptomyces* antibiotic regulatory protein) family, which also includes ActII-ORF4 and RedD. Phosphorylated AfsR binds to the promoter of *afsS*, encoding a transcription factor, and thereby activates its transcription. AfsS then stimulates the transcription of *actII*-ORF4 and *redD*, the gene products of which in turn activate the actinorhodin and undecylprodigiosin gene clusters, respectively. AbsA is a two-component signal transduction system, comprised of the sensor kinase AbsA1 and the response regulator AbsA2. AbsA2 acts as a negative regulator of antibiotic production, repressing transcription of *actII*-ORF4, *cdaR* (encoding an activator of CDA biosynthesis) and *redZ*, when present in phosphorylated form (AbsA2~P) (McKenzie & Nodwell, 2007). In the absence of AbsA2~P antibiotics are produced earlier and more abundantly (Anderson *et al*., 2001). The pleiotropic effect of the AbsA system found its implication in the screening actinomycetes for new antibacterials. Alleles of *absA1* that enhance production of antibiotics in *S. coelicolor* have been identified and shown to encode a sensory kinase with reduced ability to phosphorylate AbsA2. Introduction of the *absA1* alleles into *S. flavopersicus* led to the activation of pulvomycin production, a metabolite which previously was not attributed to this species (McKenzie *et al*., 2010). Another antibiotic-related two-component system in *Streptomyces* is CutRS, which represses actinorhodin production (Chang *et al*., 1996), although the molecular mechanism of this repression is yet unknown.

Two recently characterized LAL regulators (Large ATP-binding regulators of the LuxR family), encoded by SCO0877 and SCO7173, were shown to positively control actinorhodin biosynthesis in *S. coelicolor* (Guerra *et al*., 2012). Disruption of either gene led to decreased actinorhodin production (Guerra *et al.*, 2012). Yet another regulator controlling *actII*-ORF4 is the ROK-family (Regulators, ORFs and Kinases) protein ROK7B7 (SCO6008) in *S. coelicolor*. The protein shows 48% amino acid identity to a protein encoded by *rep*, a gene isolated from a metagenomic library from uncultivated actinomycetes, which accelerated sporulation and enhanced antibiotic production in *Streptomyces lividans* (Martinez *et al*., 2005). ROK7B7 controls expression of the

adjacent xylose transport operon, SCO6009-SCO6011 (Chapter VI). Furthermore, as shown by a DNA affinity capture assay, ROK7B7 may bind directly to promoters of *actII* -ORF4 and *redD*, suggesting direct control of antibiotic production (Park *et al.*, 2009).

The pleiotropic role of multiple transcriptional regulators makes them promising tools for drug discovery strategies. Already, engineering of regulatory mechanisms that control the biosynthesis of antibiotics is a commonly used approach to increase the production of valuable fermentation products.

5. Engineering of primary metabolism for improved antibiotic production

Over the last two decades the application of metabolic engineering for the improvement of antibiotic production remarkably increased. Secondary metabolites are synthesized in dedicated biosynthetic routes, but precursors and co-factors are derived from primary metabolism. Therefore antibiotic production can be improved by, *e.g.*, (a) increasing the flux of biosynthetic pathways; (b) increasing precursors and cofactors supply; (c) reducing formation of repressing product; or (d) manipulating regulatory elements, as discussed above.

Most antibiotic biosynthetic pathways involve reductive steps, with NADPH as the cofactor (Butler *et al.*, 2002). The majority of NADPH is produced by the activity of two enzymes of the pentose phosphate pathways (PPP), glucose-6-phosphate dehydrogenase (G6PDH) (Zwf) and 6-phosphogluconate dehydrogenase (6PGDH) (Figure 3). Deletion of two genes encoding isozymes of G6PDH, *zwf1* and *zwf2*, led to decrease in Zwf activity and in consequence also Act and Red production in *S. lividans* growing in the presence of glucose. Surprisingly, when only one of these genes was deleted, Act and Red production increased. It was suggested that the presumed lower flux of carbon through the PPP in each of the *zwf* mutants allowed more efficient utilization of glucose via glycolysis, resulting in improved antibiotic production (Figure 3) (Butler *et al.*, 2002). The major precursor for polyketide synthesis, acetyl-CoA, is a conversion product of pyruvate, an end product of glycolysis (Figure 3).

Subsequent studies demonstrated the effect of decreased carbon-flow through glycolysis on secondary metabolite production. Deletion of *pfkA2* (SCO5426), encoding a key enzyme in glycolysis, phosphofructokinase (Figure 3), led to improved production of the pigmented antibiotics actinorhodin and undecylprodigiosin in *S. coelicolor* A3(2)

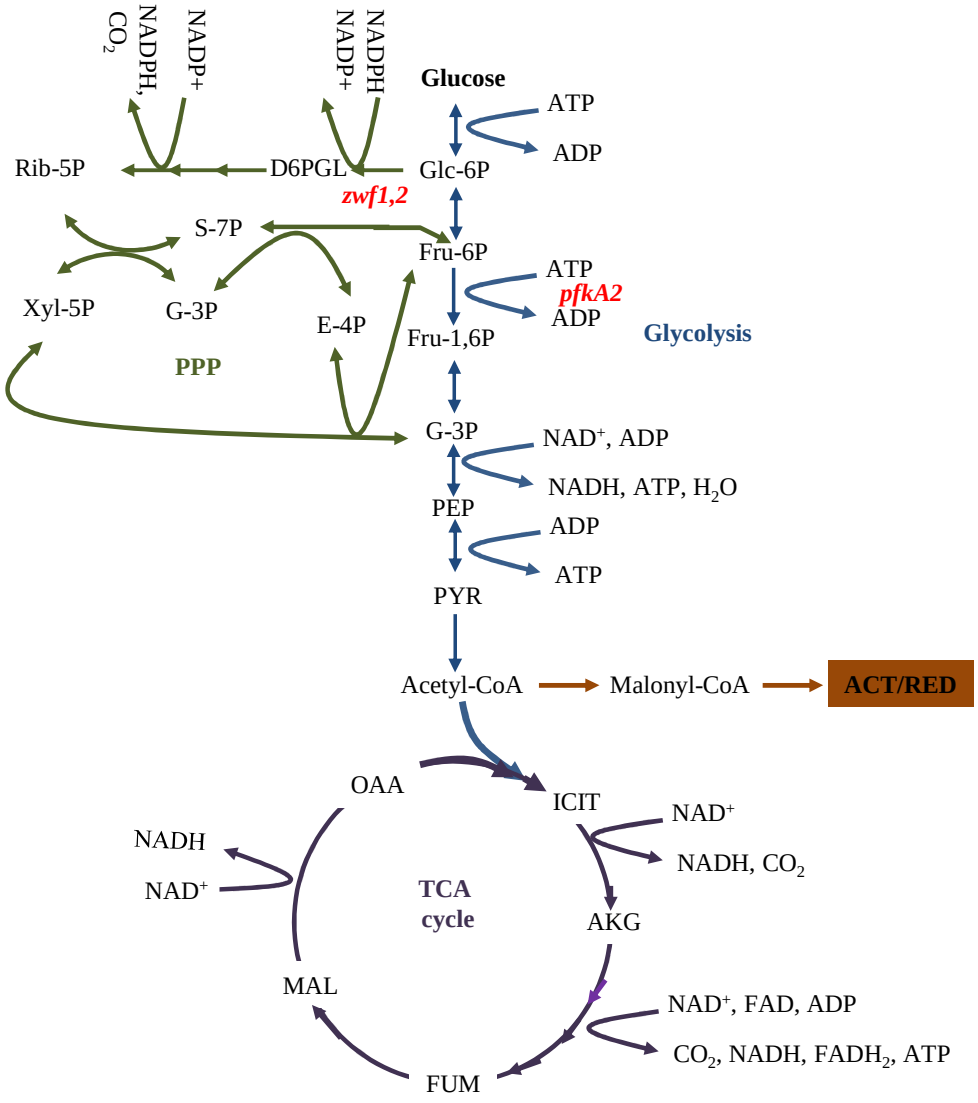

Figure 3. Overview of central carbon metabolism. The abbreviations used are as follows: *Glc-6P*, glucose 6-phosphate; *Fru-6P*, fructose 6-phosphate; *Fru-1,6P*, fructose 1,6-bisphosphate; *D6PGL*, glucono-1,5-lacton-6-phosphate; *Rib-5P*, ribose 5-phosphate; *Xyl-5P*, xylulose 5-phosphate; *S-7P*, sedoheptulose 7-phosphate; *E-4P*, erythrose 4-phosphate; *G-3P*, glyceraldehyde 3-phosphate; *PEP*, phosphoenolpyruvate; *PYR*, pyruvate; *ACT*, actinorhodin; *RED*, undecylprodigiosin; *ICIT*, isocitrate; *AKG*, α-ketoglutarate; *FUM*, fumarate; MAL – malate; *OAA*, oxaloacetate; *zwf 1,2* - isozymes of glucose-6-P dehydrogenase; *pfkA2* - phosphofructokinase.

(Borodina *et al.*, 2008). Based on genome-scale metabolic simulations, it was suggested that decreased phosphofructokinase activity leads to an increase in pentose phosphate

pathway flux, which in turn stimulates the flux towards pigmented antibiotics (Figure 3) (Borodina *et al.*, 2008). These demonstrate the usefulness of metabolic engineering approaches to steer the flux of building blocks such as to achieve higher level production of secondary metabolites.

6. Extracellular signalling molecules and the control of antibiotic production and development in *Streptomyces*

In streptomycetes, a quorum-sensing mechanism mediated by γ-butyrolactones and their cognate receptors is known to trigger secondary metabolism and morphological differentiation (Takano, 2006). γ-butyrolactones are typically produced in very small quantities, which impedes their identification and structural elucidation. The most studied γ-butyrolactone is A-factor (2-isocapryloyl-3*R*-hydroxymethyl-γ-butyrolactone) of *S. griseus*, which controls both aerial mycelium formation and production of all the secondary metabolites, including streptomycin (Horinouchi, 2002). There has been a great interest in understanding the molecular mechanisms of A-factor biosynthesis and the A-factor-governed signalling cascade. The complete biosynthetic pathway for A-factor has been elucidated (Kato *et al.*, 2007) and a model for the A-factor-dependent signalling pathway leading to the onset of development and streptomycin production in S. *griseus* has been proposed (Ohinishi *et al.*, 2005). The key enzyme for A-factor biosynthesis is AfsA. Deletion of *afsA* led to the loss of γ-butyrolactone production, while its introduction into non-producing *Streptomyces* strains resulted in A-factor production (Kato *et al.*, 2007). A-factor gradually accumulates in the cell in a growth-dependent manner, and when its concentration reaches a critical level it binds to its receptor protein ArpA. This results in dissociation of ArpA as repressor from its DNA targets, and allowing their transcription. In particular, when no A-factor is available ArpA represses the highly pleiotropic regulatory gene *adpA*. The AdpA protein directly activates various genes required for both morphological differentiation and secondary metabolism, including *strR*, encoding a pathway-specific activator of the streptomycin biosynthetic gene cluster, and *adsA*, encoding an extracytoplasmic function (ECF) sigma factor of RNA polymerase essential for aerial mycelium formation (Ohinishi *et al.*, 2005). The *adsA* orthologue of *S. coelicolor*, *bldN*, is also required for aerial mycelium formation. It directly controls *bldM* (Bibb *et al*., 2000) which encodes a response regulator, probably

active in early and late stages of development (Molle and Buttner, 2000). σBldN of *Streptomyces venezuelae* controls expression of chaplins and rodlins (Bibb *et al*., 2012), the major components of the hydrophobic sheath that coats the aerial hyphae and spores in *Streptomyces* (Bibb *et al*., 2012). Recently, the Sgr3394 protein was identified as an indirect target of AdpA (Chi *et al.*, 2011). Sgr3394 is produced only by A-factor-producing *S. griseus*, but not by an A-factor deficient mutant (Chi *et al*., 2011). Sgr3394 gene expression may therefore be controlled indirectly by AdpA via a protein positioned downstream of the AdpA-regulatory cascade (Chi *et al*., 2011). Interestingly, overexpression of the Sgr3394 protein in *S. lividans* and *S. coelicolor* also enhanced antibiotic production and had an inhibiting effect on its morphological differentiation, suggesting it has a more general function in the control of developmental processes in *Streptomyces*. New insight into the regulatory role of A-factor was gained after proteome analysis of the spontaneous A-factor non-producing mutant (AFN) of *S. griseus*. Overexpression of several nutrient-scavenging (ABC transporter solute-binding proteins) and stress response proteins was found in this strain (Birko *et al*., 2007 and Chapter VII). Time-course transcriptional analysis showed that these enhanced protein levels were in part due to enhanced gene expression (Chapter VII). Additionally, the differential expression of genes encoding nutrient-scavenging proteins was observed at the specific time-point corresponding to lysis of vegetative mycelium, considered as programmed cell death (PCD). The overproduction of nutrient-scavenging proteins in AFN may be a response to compensate for the reduced availability of nutrients (Chapter VII). Furthermore, identified classes of differentially expressed targets between AFN and the wild-type suggest that A-factor might be involved in the control of protein degradation during PCD (see General Discussion).

Factor C is a signalling protein isolated from the culture fluid of *S. griseus* 45H (Biro *et al*., 1980), which was recently shown to be *Streptomyces flavofungini* (Kiss *et al*., 2008). Interestingly, introduction of *facC* into a spontaneous A-factor-deficient bald mutant of *S. griseus*, AFN (discussed above), restored its aerial mycelium formation and sporulation (Biro *et al*., 2000). Proteome analysis revealed that in the *facC* transformant the production of several secreted proteins that belong to the A-factor regulon were restored (Birko *et al*., 2007 and Chapter VII). Subsequent HPLC-MS/MS analysis indicated that this was due to restoration of A-factor production in the transformant

(Birko *et al*., 2007). The molecular basis of this exciting phenomenon is a subject of current study.

The orthologues of AfsA and ArpA proteins are designated ScbA and ScbR in *S. coelicolor* (Takano *et al*., 2001). In contrast to A-factor in *S. griseus*, which is required for development and antibiotic production, the γ-butyrolactone SCB1 (*S. coelicolor* butanolide 1) produced by ScbA has a far less profound impact on development and antibiotic production in *S. coelicolor* (Takano *et al*., 2001). In fact, its main target appears to be the biosynthetic gene cluster for the production of the yellow-pigmented secondary metabolite yCPK (Gottelt *et al*., 2010). In contrast to the corresponding *afsA* mutants in *S. griseus*, deletion of *scbA* in *S. coelicolor* did not have a major effect on antibiotic production, and allowed precocious synthesis of both Act and Red (Takano *et al*., 2001). The effect of the *scbA* disruption on Act and Red production may be an indirect consequence of reducing production of yCPK and thus the competition for precursors (Gottelt *et al*., 2010). The yellow pigment (yCPK) and another secondary metabolite called abCPK (antibiotic *coelicolor* polyketide), which shows antibacterial activity, are two distinct products of the cryptic type I polyketide synthase (*cpk*) gene cluster (Gottelt *et al*., 2010). Preliminary results show that the antibacterial intermediate abCPK is converted into the yellow pigment yCPK by the enzyme ScF (Gottelt *et al*., 2010). Similarly to the *scbA* deletion, disruption of *scbR* led to blockage of SCB1 biosynthesis, but its effect on antibiotic production was the opposite, with delayed Act and Red production (Takano *et al*., 2001), and fully blocked yCPK synthesis (Gottelt *et al*., 2010). ScbR binds to the *scbRA* intergenic region and upstream of *cpkO* (SCO6280, initially called *kasO*), encoding a regulator of the *cpk* biosynthetic cluster (Takano *et al*., 2001; Takano *et al*., 2005). The ScbR-mediated control of *scbR, cpkO and scbA* was abolished upon SCB1 addition, confirming the role of ScbR as the SCB1 receptor protein (Takano *et al*., 2001; Takano *et al.,* 2005). Based on all obtained results it has been anticipated that ScbR negatively controls expression of its own gene and *cpkO*, whereas acts as a transcriptional activator of *scbA* (Takano *et al.*, 2001; Takano *et al.*, 2005).

The ScbR homolog ScbR2 (SCO6286) does not bind to SCB1, hence was designed as "pseudo" γ-butyrolactone receptor (Xu *et al.*, 2010). However, it binds to Act and Red as ligands, causing derepression of *cpkO* (Xu *et al*., 2010). Moreover, in contrast to ScbR, ScbR2 negatively controls the biosynthesis of SCB1 by directly repressing the

transcription of *scbA* (Wang *et al.*, 2011). Disruption of *scbR2* led to prolonged CPK synthesis (Gottelt *et al.*, 2010), whereas Act and Red production was impaired (Wang *et al.*, 2011). It is suggested that these changes in antibiotic production levels are due to SCB1 overproduction in the *scbR2* mutant (Wang *et al.*, 2011). Taken together, ScbR2 is part of ScbRA regulatory system. Despite some parallels in the regulation of secondary metabolites production by γ-butyrolactones and their associated proteins in *S. coelicolor* and *S. griseus*, the overall differences are to big to propose some common mechanism of action.

7. Concluding remarks

It is now clear that multiple regulatory mechanisms control antibiotic production and development in *Streptomyces*. The study of each of these mechanisms has revealed innovative strategies not only for the improvement of productivity, but also towards 'awakening' of the production of antibiotics whose expression is very low or not detectable under routine laboratory (*i.e.* screening) conditions. The importance of new antimicrobials discovery is given by the relentless emergence of pathogens which are resistant to existing antibiotics. The recent development of genome-wide approaches combined with molecular engineering strategies should allow for more efficient exploitation of the rich arsenal of natural products encoded by actinomycete genomes. Hopefully, this is the start of a new era of novel antimicrobials discovery.