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

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

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Chapter I

General introduction and outline: a preview to this thesis

Introduction

Streptomycetes are high-GC Gram-positive, soil-dwelling bacteria belonging to the phylum of actinobacteria. They have a complex life cycle, morphologically similar to that of filamentous fungi. The life cycle starts with a spore that germinates to produce a branched mycelium consisting of long multinucleoid hyphae. Under adverse conditions, such as nutrient limitation, streptomycetes enter the developmental program, whereby autolytic degradation of the vegetative mycelium provides the nutrients for the newly formed aerial mycelium. Eventually, the aerial hyphae produce chains of unigenomic spores. Actinomycetes produce a wide range of natural products, including antibiotics, immunosuppressants, insecticides and anti-tumor agents (Hopwood, 1999). These natural products are typically produced during later stages of the developmental programme. Streptomycetes are saprophytic microorganisms, and metabolize almost all carbon sources, such as sugars, alcohols, amino acids and fatty acids (Romano & Nickerson, 1958; Pridham & Gottlieb, 1948); these microorganisms have an important function in the soil as nutrient scavengers and in the degradation of naturally occurring polysaccharides, such as cellulose, chitin, mannan, starch, xylan, dextran and agar. Many of the enzymes involved in polysaccharide degradation find their way into industry (Ramachandra *et al.*, 1987; Bentley *et al.*, 2002; Hodgson, 2002; McCarthy, 1987). These properties make *Streptomyces* a very interesting and at the same time industrially highly relevant object for study.

Streptomyces coelicolor A3(2) is genetically the best characterized representative of the genus and serves as a model organism for the study of actinomycete biology and chemistry. Its genome contains around 7825 genes and harbours more than 20 secondary metabolite clusters. Some 12% of the entire genome encodes predicted regulatory proteins (Bentley *et al.*, 2002), which highlights the complexity of the regulatory networks that govern the transcriptional control of cell differentiation, secondary metabolites production and signalling cascades in streptomycetes.

Regulation of development and antibiotic biosynthesis

High level production of industrially important compounds, especially antibiotics, requires engineering of the primary metabolism (reviewed in **Chapter II**), and the link between primary and secondary metabolism is extensively studied (Gunnarsson *et al.*,

2004; van Wezel & McDowall, 2011; Borodina *et al.*, 2008; Maharjan *et al.*, 2010). In this thesis the involvement of primary metabolites (**Chapter III-IV**), of global transcriptional regulators (**Chapter V-VI**) and of extracellular signalling molecules (**Chapter VII**) in the control of antibiotic production and development, is discussed.

GlcNAc/GlcN control over development and antibiotic production (Chapter III-IV)

N-acetylglucosamine (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 colonies under rich growth conditions (feast) it promotes vegetative growth, thereby blocking developmental processes, while under poor nutritional conditions (famine) the accumulation of GlcNAc promotes development and antibiotic production (Rigali *et al.*, 2008; van Wezel *et al.*, 2009). In **Chapter III** the primary metabolism revolving around GlcNAc is analysed in more detail, as well as its role in the control of antibiotic production and development. GlcNAc is transported via the phosphoenolpyruvate-dependent phosphotransferase system (PTS) and enters the cell in its phosphorylated form as a GlcNAc-6P (reviewed in **Chapter II**). The enzymes responsible for the conversion of GlcNAc to the glycolytic intermediate fructose-6P (Fru-6P) are dissected in **Chapter III**. These include the GlcNAc-6P deacetylase NagA, which converts intracellular GlcNAc-6P to glucosamine-6P (GlcN-6P), and the GlcN-6P deaminase NagB, which convert GlcN-6P to Fru-6P, which is part of glycolysis. The discovery that *nagB* mutants fail to grow on medium supplemented with GlcNAc (**Chapter III**) or its deacetylated form GlcN (**Chapter IV**) - most probably due to intracellular accumulation of GlcN-6P - allowed a screen to find suppressor mutants, that are mutated in genes involved in GlcNAc metabolism. Analysis of these mutants was performed, revealing novel insight in GlcN(Ac) metabolism (**Chapters IV**).

As expected, one such suppressor mutation was identified in the metabolic gene *nagA* when *nagB* mutants were grown on GlcNAc. The fact that this mutation also alleviated the toxicity of GlcN was surprising though, suggesting involvement of NagA in the metabolism of GlcN. Additionally, new GlcNAc/GlcN-related genes were identified and characterized. Those were genes for a ROK-family protein (SCO1447) and a likely sugar isomerase (SCO4393), which were mutated in the *nagB* suppressors selected on GlcN and GlcNAc, respectively. This reveals new regulatory and metabolic genes

involved in aminosugar utilization, and since homologues of SCO4393 were identified in many bacteria, we may have identified a novel metabolic enzyme involved in central metabolism. **Chapter IV** also provides an overview summarizing our knowledge about aminosugar transport and metabolism in *Streptomyces* and presents new strategies which can be used to improve metabolic engineering strategies leading to an increased antibiotic production.

Control of development and antibiotic production via pleiotropic transcriptional regulators (Chapter V-VI)

The accumulation of *N*-acetylglucosamine as a result of autolytic degradation of the cell wall is a checkpoint for the onset of development and antibiotic production, which is mediated through the nutrient sensor DasR, which belongs to the GntR-family of transcriptional regulators. In **Chapter V** an integrated systems biology approach, including microarray and chip-on-chip analysis, was applied and revealed that DasR is a regulatory master switch for carbon utilization and antibiotic production. The change in the *in vivo* binding of DasR to its targets following the induction by GlcNAc was analyzed under different growth conditions, revealing two different classes of target genes; Class I genes contain a conserved *dre* (DasR-responsive element) and are also bound by DasR *in vitro*, while Class II genes lacking such an element and likely require an additional co-regulator for binding.

In **Chapter VI**, the possible involvement of seven of the ROK-family regulatory proteins in the control of development and antibiotic production in *S. coelicolor* is analyzed. Mutational analysis followed by phenotypic characterization of the respective mutants identified two possible novel pleiotropic transcriptional regulators, namely ROK7B7 (SCO6008) and ROK8G12 (SCO7543). Both mutants showed delay in the antibiotic production and morphological differentiation under various growth conditions. Microarray analysis revealed that over 700 genes were differentially expressed in the *rok7B7* mutant, including several developmental targets involved in the control of later stages of differentiation. Furthermore this strain also displayed enhanced carbon catabolite repression on MM with xylose, maltose and glucosamine. The surprisingly large overlap between the DasR and Rok7B7 response regulons suggests cross-talk between these pleiotropic regulators.

Control of antibiotic production by extracellular signalling (Chapter VII)

γ -butyrolactones are extracellular signalling molecules involved in the control of development and antibiotic production, and A-factor of *Streptomyces griseus* is the best studied example (Hara *et al.*, 2009; Tomono *et al.*, 2005; Horinouchi, 2002). In **Chapter VII** the extracellular proteomes of *S. griseus* B-2682 and its A-factor non-producing mutant called AFN were compared. The absence of A-factor resulted in the overproduction of stress-related and nutrient-scavenging proteins, and the deregulation of in particular many extracellular proteases suggests that A-factor plays a major role in protein reutilization. Despite its highly pleiotropic function in the control of the developmental programme, normal growth and development could be restored to the AFN mutant by the genetic expression or extracellular addition of the signalling protein Factor C from *Streptomyces flavofungini*. This suggests the existence of a yet unknown mechanism in quorum sensing-related signalling.

In the final chapter (**Chapter VIII**) the results presented in this thesis are summarized and the implications with respect to antibiotic production are discussed.

Chapter II

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

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1. The *Streptomyces* life-cycle
2. Carbon catabolite repression (CCR) in bacteria
 - 2.1 The phosphoenolpyruvate-dependent phosphotransferases system (PTS)
 - 2.2 The PTS and CCR in *Streptomyces*
 - 2.2.1 Control of carbon catabolite repression by Bld proteins
3. Carbon source regulation of antibiotic production in *Streptomyces*
 - 3.1 *N*-acetylglucosamine and the DasR regulon
4. Global transcriptional regulators involved in the control of antibiotic production in *S. coelicolor*
5. Engineering of primary metabolism for improved antibiotic production
6. Extracellular signalling molecules and the control of antibiotic production and development in *Streptomyces*
7. Concluding remarks

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

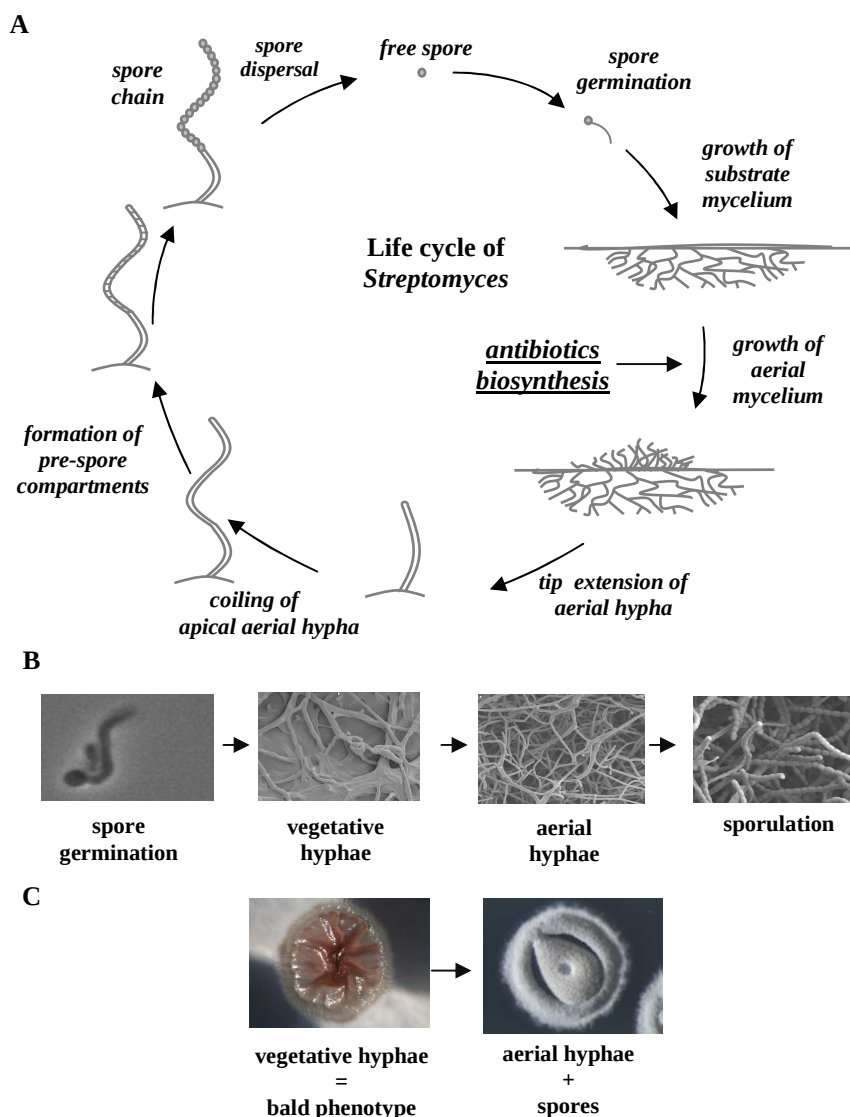


Figure 1. Life cycle of *Streptomyces coelicolor* A3(2). A) Schematic representation. B) Scanning electron micrographs showing *S. coelicolor* at different stages of life cycle. C) Stereo micrographs of single colonies either locked at vegetative state (bald phenotype) or displaying normal development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

regulatory function of EIIA^{Glc} of *E. coli* by blocking transport of maltose, when present in its unphosphorylated 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.

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. aureofaciens* 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-6P 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* (Nothhaft *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, QRFs 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)

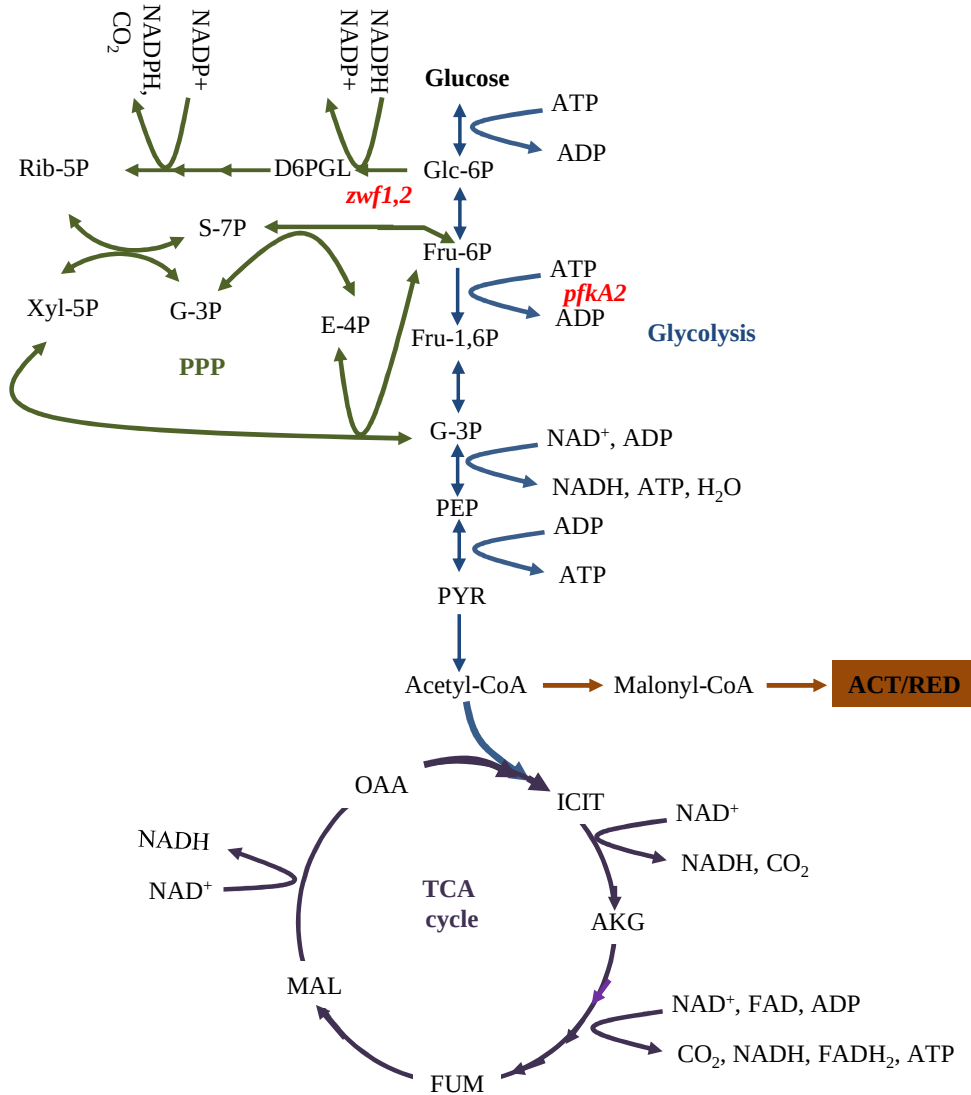


Figure 3. Overview of central carbon metabolism. The abbreviations used are as follows: *Glc-6P*, glucose 6-phosphate; *Fru-6P*, fructose 6-phosphate; *Fru-1,6P*, fructose 1,6-bisphosphate; *D6PGL*, glucono-1,5-lactone-6-phosphate; *Rib-5P*, ribose 5-phosphate; *Xyl-5P*, xylulose 5-phosphate; *S-7P*, 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-3R-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). σ BldN of *Streptomyces venezuelae* controls expression of chaplins and rodmins (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 too 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.

Chapter III

Functional analysis of the *N*-acetylglucosamine metabolic genes of *Streptomyces coelicolor* and role in the control of development and antibiotic production

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Abstract

N-acetylglucosamine, the monomer of chitin, is a favored carbon and nitrogen source for streptomycetes. Its intracellular catabolism requires the combined actions of the *N*-acetylglucosamine-6-phosphate (GlcNAc-6P) deacetylase NagA and the glucosamine-6-phosphate (GlcN-6P) deaminase/isomerase NagB. GlcNAc acts as a signalling molecule in the DasR-mediated nutrient sensing system, activating development and antibiotic production under poor growth conditions (*famine*), while blocking these processes under rich conditions (*feast*). In order to understand how a single nutrient can deliver opposite information according to the nutritional context, we carried out a mutational analysis of the *nag* metabolic genes *nagA*, *nagB* and *nagK*. Here we show that the *nag* genes are part of the DasR regulon in *S. coelicolor*, which explains their transcriptional induction by GlcNAc. Most likely as the result of the intracellular accumulation of GlcN-6P, *nagB* deletion mutants fail to grow in the presence of GlcNAc. This toxicity can be alleviated by the additional deletion of *nagA*. We recently showed that in *S. coelicolor*, GlcNAc is internalized as GlcNAc-6P via the phosphoenolpyruvate-dependent sugar phosphotransferase system PTS. Considering the relevance of GlcNAc for the control of antibiotic production, improved insight into GlcNAc metabolism in *Streptomyces* may provide new leads towards biotechnological applications.

INTRODUCTION

Streptomyces are Gram-positive, soil-dwelling mycelial bacteria that have a complex life cycle that starts with the germination of a spore, which then grows out to form a branched mycelium of vegetative hyphae. The multicellular mycelial life style of streptomyces makes these organisms unique among bacteria, whereby connected compartments are formed that are separated by cross-walls. When morphological differentiation is initiated a so-called aerial mycelium is produced, and the erected aerial hyphae eventually form chains of spores (Flårdh & Buttner, 2009; Redington & Chater, 1997). Streptomyces are prolific producers of natural products, producing some fifty percent of all known antibiotics as well as many anticancer, antifungal and immunosuppressant agents. The production of these so-called secondary metabolites is part of the developmental program, roughly coinciding with growth cessation and the onset of sporulation (Bibb, 2005; van Wezel & McDowall, 2011).

As saprophytic soil bacteria, streptomyces utilize naturally occurring polysaccharides such as chitin, xylan and cellulose as carbon sources. *N*-acetylglucosamine, the monomer of chitin, is a preferred carbon and nitrogen source for streptomyces, and the related metabolite glutamate is preferred over glucose (Nothaft *et al.*, 2003b; van Wezel *et al.*, 2006). Chitin is one of the most abundant polysaccharides on earth and found in among others the cuticles and shells of insects and crustaceans and within the cell wall of fungi, and to utilize this polysaccharide streptomyces have an extensive chitinolytic system (Cohen-Kupiec & Chet, 1998; Schrempf, 2001). *N*-acetylglucosamine (GlcNAc) is a substrate in *Streptomyces coelicolor* for transport via the phosphoenolpyruvate-dependent phosphotransferase system (PTS; reviewed in Brückner & Titgemeyer, 2002). During PTS-mediated carbon source uptake, a phosphoryl group is transferred from phosphoenolpyruvate to the general phosphotransferase enzyme I (EI; encoded by *ptsI*), from there to HPr (encoded by *ptsH*) and further onto Enzyme IIA (EIIA^{Crr}; encoded by *crr*) and finally Enzyme IIB (NagF) (Nothaft *et al.*, 2003b; Nothaft *et al.*, 2010). NagF phosphorylates incoming via the permease IIC (NagE2) GlcNAc to form *N*-acetylglucosamine-6-phosphate (GlcNAc-6P). Removal of any of the general PTS components EI, EIIA or HPr blocks development (Rigali *et al.*, 2006). An alternative route for the internalization of monomeric GlcNAc exists in *Streptomyces olivaceoviridis* and probably many other streptomyces, namely

via the ATP-binding cassette (ABC) transporter NgcEFG, which imports both *N*-acetylglucosamine and its disaccharide *N,N'*-diacetylchitobiose (GlcNAc)₂ with similar affinities (Wang *et al.*, 2002; Xiao *et al.*, 2002). (GlcNAc)₂ is also imported via the ABC sugar transporter DasABC (Colson *et al.*, 2008; Seo *et al.*, 2002).

We previously demonstrated that GlcNAc is an important signalling molecule for streptomycetes and a major decision point towards the onset of development and antibiotic production (Rigali *et al.*, 2006; Rigali *et al.*, 2008). When GlcNAc accumulates under rich growth conditions (*feast*) it promotes growth, thereby blocking developmental processes, while under poor nutritional conditions (*famine*) the accumulation of GlcNAc promotes development and antibiotic production (Rigali *et al.*, 2008; van Wezel *et al.*, 2009). Under famine conditions, a complete signalling pathway was proposed from GlcNAc uptake to the onset of antibiotic production, whereby the pleiotropic GntR-family transcriptional regulator DasR controls the genes for GlcNAc transport and metabolism and for antibiotic synthesis (Rigali *et al.*, 2006; Rigali *et al.*, 2008). Glucosamine-6-phosphate (GlcN-6P) is a key molecule in this signalling cascade, and acts as an allosteric effector preventing DasR DNA-binding ability. This results in the loss of transcriptional repression of specific activator genes for antibiotic production, including *actII*-ORF4, encoding the pathway-specific activator for actinorhodin (Act) production, and *redZ*, for the response regulator RedZ that activates prodiginine (Red) production. DasR also represses the *cpk* gene cluster for the cryptic polyketide Cpk, but this effect is probably indirect (van Wezel & McDowall, 2011). Under feast conditions, the molecular mechanism of how GlcNAc blocks sporulation and antibiotic production is still unknown. Non-phosphorylation of key developmental proteins by global PTS components (EI, HPr, and EIIA^{Ctr}) upon GlcNAc sensing is so far the only realistic hypothesis proposed (Colson *et al.*, 2008; Rigali *et al.*, 2006).

As can be gleaned from extensive studies of aminosugar metabolism in other bacteria, the signalling molecule GlcN-6P is produced from *N*-acetylglucosamine-6-phosphate by a GlcNAc-6P deacetylase (NagA), and is then converted by a GlcN-6P deaminase (NagB) to fructose-6-phosphate (Fru-6P), which enters the glycolytic pathway (Alvarez-Anorve *et al.*, 2005; Plumbridge & Vimr, 1999; Vogler & Lengeler, 1989). To better understand the aminosugar-mediated signalling pathway in streptomycetes, dissection of GlcNAc metabolism in these organisms is required. In this work we analyze

the GlcNAc-metabolic pathway and specifically block one or more steps of GlcNAc utilization via directed deletion mutants. This allows us to provide a first assessment as to how the flux of GlcNAc influences the global regulatory routes that control *Streptomyces* development and antibiotic production.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Bacterial strains and plasmids are listed in Table 1 and Supplemental Table S1, respectively. *Escherichia coli* JM109 (Sambrook *et al.*, 1989) and ET12567 (Kieser *et al.*, 2000) were used for routine cloning procedures and for extracting non-methylated DNA, respectively. *Streptomyces coelicolor* A3(2) M145 was the parent for all mutants described in this work. All media and routine *Streptomyces* techniques are described in the *Streptomyces* manual (Kieser *et al.*, 2000). Cells of *E. coli* were grown in Luria–Bertani broth (LB) at 37°C. Phenotypic characterization of *Streptomyces* mutants was carried out on minimal media (MM) and on R5- (-glucose) agar plates and in liquid NMMP or MM cultures (Kieser *et al.*, 2000) with 1% of carbon sources as indicated. SFM (soy flour mannitol) agar plates were used to prepare spore suspensions.

Table 1. Strains of *Streptomyces coelicolor* A3(2) described in this study.

Strain	Genotype ^a	Reference
M145	prototrophic SCP1 ⁺ SCP2 ⁺	(Kieser <i>et al.</i> , 2000)
GAM1	M145 <i>nagA::aacC4</i> , Apr ^R	this work
GAM2	M145 <i>nagB::aacC4</i> , Apr ^R	this work
GAM3	M145 <i>nagK::aacC4</i> , Apr ^R	this work
GAM4	M145 Δ <i>nagA</i> ^{IFD}	this work
GAM5	M145 Δ <i>nagB</i> ^{IFD}	this work
GAM6	M145 Δ <i>nagK</i> ^{IFD}	this work
GAM7	M145 Δ <i>nagKA::aacC4</i> , Apr ^R	this work
GAM8	M145 Δ <i>nagKA</i> ^{IFD}	this work
GAM9	M145 Δ <i>nagB</i> ^{IFD} <i>nagA::aacC4</i>	this work
GAM10	M145 Δ <i>nagKA</i> ^{IFD} <i>nagB::aacC4</i>	this work
GAM12	GAM4 + pGAM5	this work
GAM13	GAM5 + pGAM6	this work

^a IFD, in-frame deletion mutant; Apr^R, apramycin resistant.

Plasmids and constructs

All constructs described in this work are listed in Supplemental Table S1 and oligonucleotides in Supplemental Table S2.

Constructs for creating gene-replacement and in-frame deletion mutants

The strategy for creating knock-out mutants is based on the unstable multi-copy vector

pWHM3 (Vara *et al.*, 1989) as described previously (van Wezel *et al.*, 2005). For the deletion of *nagA*, the -1365/+6 and +1133/+2484 regions relative to the start of *nagA* were amplified by PCR, using primer pairs nagALF-1365 and nagALR+6 and nagARF+1133 and nagARR+2484 respectively. Fragments were cloned into pWHM3, and the engineered *Xba*I site was used for insertion of the apramycin resistance cassette *aacC4* flanked by *loxP* sites between the flanking regions. The presence of the *loxP* recognition sites allows the efficient removal of the apramycin resistance cassette following the introduction of a plasmid pUWLcre expressing the Cre recombinase (Fedoryshyn *et al.*, 2008; Khodakaramian *et al.*, 2006). The constructed plasmid was called pGAM1. Using essentially the same strategy as for pGAM1, we constructed the knock-out plasmids pGAM2 and pGAM3 for the single gene replacement of *nagB*, *nagK* respectively. pGAM2, contains the -1185/+6 and +770/+1918 regions relative to the start of *nagB*, and pGAM3 the -1450/+6 and +963/+2570 regions relative to the start of *nagK*. Plasmid pGAM4 for the in-frame deletion of *nagKA* contains the upstream -1450/+6 region of *nagK* and downstream +1133/+2484 region of *nagA*, which were amplified by PCR, using primer pairs nagKLF-1450 and nagKLR+6 and nagARF+1133 and nagARR+2484, respectively. The *nagAB* (GAM9) double mutant was constructed by replacing *nagA* by the apramycin cassette in the *nagB*^{IFD} mutant GAM5. GAM8 was used as the parent for *nagKAB* triple mutant GAM10, following replacement of *nagB* by the apramycin resistance cassette.

To analyse correctness of the mutants, PCRs were done on mycelium from liquid-grown MM cultures, with oligonucleotides pairs nagA_FOR-198 and nagA_REV+1417 for *nagA* and nagB_FOR-336 and nagB_REV+1098 for *nagB*.

For complementation of the *nag* mutants the low copy vector pHJL401 (Larson and Hershberger, 1986) harboring the corresponding *nag* gene and promoter region (nt positions -454/+796 relative to the start of *nagB*; fragment -512/+2262 relative to the start of *nagK* were used to complement *nagK* and *nagA*) was used.

Antibiotic production

Quantification of prodiginines and actinorhodin was performed as described (Kieser *et al.*, 2000), and corrected for biomass (wet weight). Values were normalized against the parental strain M145 (set to 100%). Cultures were grown on R2YE agar plates overlaid

with cellophane discs to allow separation of biomass and spent agar.

Production and purification of DasR

Production and purification of His₆-tagged DasR was based on the protocol described previously (Colson *et al.*, 2007; Mahr *et al.*, 2000) with several modifications. *Escherichia coli* BL21 Rosetta (DE3) cells carrying pFT240 (Rigali *et al.*, 2004) were grown at 37°C in 250 ml LB medium containing 100 µg/ml of ampicillin until the culture reached an OD₆₀₀ of 0.6. Production of His₆-tagged DasR was induced by addition of 1 mM isopropyl thio-β-d-galactoside (IPTG) during 3h. Cells were collected by centrifugation, and ruptured by sonication in lysis buffer (100 mM phosphate buffer pH 8 ; 250 mM NaCl ; 30 mM imidazole ; 0.1% Triton). Soluble proteins were loaded onto a pre-equilibrated Ni²⁺-NTA-agarose column (5 ml bed volume). His₆-tagged DasR eluted at around 200 mM imidazole and fractions containing the pure protein were pooled and dialysed overnight at 4°C against EMSA buffer (see below).

Electromobility gel shift assays

EMSAs on double-stranded oligonucleotides were performed using Cy5-labelled *dre* probes (0.25 µM final concentration) and His₆-tagged DasR (between 1.75 and 2.5 µM final concentration) in a total reaction volume of 50 µl. All reactions were carried out in EMSA buffer (10 mM Tris-Cl pH 7.5, 1mM DTT, 0.25 mM CaCl₂, 0.5 mM MgCl₂, 50 mM KCl, and 2% glycerol) containing a 100-fold excess of non-specific DNA (salmon sperm DNA). After 15 min of incubation at room temperature, reaction mixtures were loaded into a 1% (w/v) agarose gel. Bound and unbound probes were separated by gel electrophoresis at room temperature and fluorescent DNA was visualised using a Typhoon Trio+ Variable Mode Imager. Oligonucleotides (see Table S2) used to generate 40 bp double-stranded DNA probes for EMSAs were taken from the upstream regions of *dasA* (*dre^{dasA}*), *nagB* (*dre^{nagB}*), and the *nagKA* operon (*dre^{nagKA}*). The *cis*-acting element OP1 of the gene for the *Bacillus licheniformis* β-lactamase repressor BlaI was used as negative control.

RNA isolation and RT-PCR

For transcript analysis, RNA was isolated from liquid cultures of *S. coelicolor* M145 or

the *dasR* mutant. Mycelium was grown in NMMP and induced by addition of 50 mM GlcNAc at OD~0.2. Samples were taken before induction (t = 0') and 30 min after induction (t = 30'). RNA isolation and semi-quantitative RT-PCR was carried out twice as described previously (Birko et al., 2009). 200 ng of RNA was used for each reaction (concentration was assessed using a Nanodrop® spectrophotometer). Quantification was done using a Bio-Rad FX molecular imager with Quantity One software (Bio- Rad). Oligonucleotides used for RT-PCR are presented in Supplemental Table S2.

Stereo microscopy

Strains were grown on MM and R5- agar plates supplemented either with mannitol or GlcNAc for five days. The mutants were imaged using a Zeiss Lumar V12 stereomicroscope.

Computer analyses

DNA and protein databank searches were performed using the BLAST server of the National Center for Biotechnology Information at the National Institute of Health, Bethesda, MD, USA (<http://www.ncbi.nlm.nih.gov>) and the *S. coelicolor* genome page services (<http://strepdb.streptomyces.org.uk/>). Alignments were generated with CLUSTALW available at <http://www.ebi.ac.uk/clustalw>. Regulon predictions were performed using PREDetector (Hiard *et al.*, 2007). The general information about amino sugar metabolic pathway of *S. coelicolor* was found in the KEGG database (http://www.genome.jp/kegg-bin/show_pathway?map00520a).

RESULTS

In silico* identification of the GlcNAc utilization genes in *S. coelicolor

The genetic environment of the *nag* metabolic genes on the *S. coelicolor* chromosome and the predicted gene products are presented in Fig. 1A. The *nagK* (SCO4285) and *nagA* (SCO4284) genes have overlapping start and stop codons, and encode *N*-acetylglucosamine kinase NagK and *N*-acetylglucosamine-6P deacetylase NagA, respectively. The NagA and NagK proteins show 34.5% and 37.2% amino acid sequence identity to the corresponding enzymes in *E. coli*, respectively. Downstream of the *nagKA* operon lies SCO4283, for a phosphofructokinase-like sugar kinase, and upstream lies SCO4286, which encodes an ABC-type sugar binding protein of unknown function. The *nagB* gene for glucosamine-6P-deaminase (SCO5236) lies immediately downstream of the *dasABCD* gene cluster involved in (GlcNAc)₂ uptake and degradation (Colson *et al.*, 2008; Saito *et al.*, 2007), and upstream of SCO5237 for a likely 3-ketoacyl-(acyl-carrier-protein) reductase. NagB shows 44.6% amino acid sequence identity with NagB from *E. coli*. The GlcNAc-related metabolic pathway of *S. coelicolor*, based on the KEGG database is shown in Fig. 1B.

Expression of *nagA* and *nagB* is induced by GlcNAc and repressed by DasR

Prior to assessing the effect of the inactivation of the *nag* genes on the GlcNAc-mediated signalling pathway in *S. coelicolor*, we first analyzed the GlcNAc-dependent control of the *nagA*, *nagB* and *nagK* genes. DasR is a global regulator of primary metabolism and antibiotic production, and is known to control among others the chitinolytic system in *S. coelicolor* (Colson *et al.*, 2007; Nazari *et al.*, 2011), as well as *nagB* (Rigali *et al.*, 2006). The latter has a DasR-responsive element or *dre* at position -68 upstream of *nagB* (tGTGGTtTAGACCAaT, *dre*^{*nagB*}), which has a 13 nt match to the *dre* consensus sequence (AGTGGTCTAGACCACT, *dre*^{*cons*}; (Rigali *et al.*, 2006)). Suggestively, the program PREDetector (Hiard *et al.*, 2007) also identified a *dre* element (AGaGGTCTAGtCCACT, *dre*^{*nagKA*}) at position -83 upstream of the *nagKA* operon that matches 14 out 16 nt to the *dre* consensus sequence. Furthermore, the computational prediction of the DasR regulon in five other streptomycetes revealed identical or similar *dre*-like sequences upstream of the respective *nagKA* and *nagB* orthologs, and the very high conservation suggests that these sites are true functional *cis*-acting elements. In

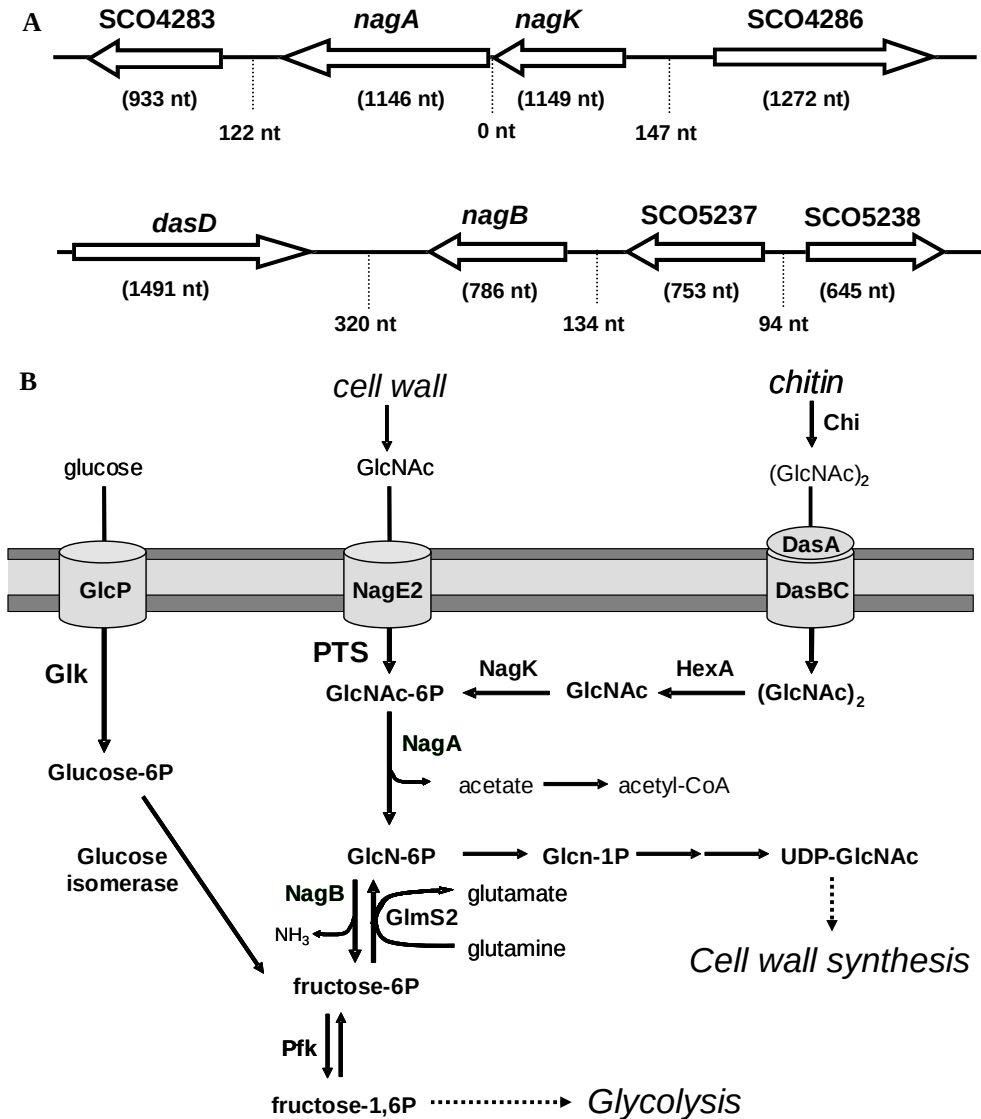


Figure 1. Genetic environment of the *nag* metabolic genes and GlcNAc-related primary metabolism.

A. Map of the genomic regions around the aminosugar utilization genes. *nagB* (SCO5236) is a single transcription unit, while *nagK* (SCO4285) and *nagA* (SCO4284) together form the *nagKA* operon. ORFs are indicated by open arrows. Lengths of the genes (in brackets below the genes) and intergenic regions (dotted line) are indicated. **B.** Aminosugar metabolism in *S. coelicolor* and connecting pathways. Annotation based on KEGG and experimental evidence. Arrows show direction of metabolism, and the responsible enzymes are depicted next to them. It is unknown whether GlcN-6P can be metabolized directly to GlcNAc-6P. Names and functions of the enzymes and the reactions they catalyze are listed in Table 2.

Table 2. Genes involved in GlcNAc transport and metabolism in *S. coelicolor* A3(2).

Name	Gene	Predicted function ^a
<i>crr</i>	SCO1390	Global PTS component EIIA ^{Crr}
<i>gdhA</i>	SCO4683	Glutamate dehydrogenase (EC 1.4.1.4) Glu + H ₂ O + NADP ⁺ $\rightleftharpoons$ 2-OG + NH ₃ + NADPH + H ⁺
<i>glmM</i>	SCO4736	Phosphoglucosamine mutase (EC 5.4.2.10) GlcN-1P $\rightleftharpoons$ GlcN-6P
<i>glmS1</i>	SCO4740	glucosamine-fructose-6-phosphate aminotransferase (EC 2.6.1.16) Gln + Fru-6P $\rightleftharpoons$ Glu + GlcN-6P
<i>glmS2</i>	SCO2789	glucosamine-fructose-6-phosphate aminotransferase (EC 2.6.1.16) Gln + Fru-6P $\rightleftharpoons$ Glu + GlcN-6P
<i>glmU</i>	SCO3122	bifunctional N-acetylglucosamine-1-phosphate uridylyltransferase/ glucosamine-1-phosphate acetyltransferase (EC 2.7.7.23) UTP + GlcNAc-1P $\rightleftharpoons$ 2P _i + UDP-GlcNAc (EC 2.3.1.157) Ac-CoA + GlcN-1P $\rightleftharpoons$ CoA + GlcNAc-1P
<i>hexA</i>	SCO2758, SCO2786, SCO2943, SCO4860	beta-N-acetylglucosaminidase (EC 3.2.1.52) beta-N-acetylhexosaminidase (EC 3.2.1.52) sugar hydrolase (EC 3.2.1.52) secreted hydrolase (EC 3.2.1.52) (GlcNAc) ₂ + H ₂ O $\rightleftharpoons$ 2 GlcNAc
<i>nagA</i>	SCO4284	N-acetylglucosamine-6-phosphate deacetylase (EC 3.5.1.25); GlcNAc-6P + H ₂ O $\rightleftharpoons$ GlcN-6P + OAc-
<i>nagB</i>	SCO5236	Glucosamine phosphate isomerase (EC 3.5.99.6); GlcN-6P + H ₂ O $\rightleftharpoons$ Fru-6P + NH ₃
<i>nagE2</i>	SCO2907	PTS ^{GlcNAc} transmembrane component EIIC
<i>nagF</i>	SCO2905	PTS ^{GlcNAc} transmembrane component EIIB
<i>nagK</i>	SCO4285	N-acetylglucosamine kinase (EC 2.7.1.59) GlcNAc + ATP $\rightleftharpoons$ GlcNAc-6P + ADP
<i>murA</i>	SCO2949	UDP-N-acetylglucosamine 1-carboxyvinyltransferase (EC 2.5.1.7) UDP-N-acetylglucosamine transferase (EC 2.5.1.7)
<i>(murA2)</i>	SCO5998	PEP + UDP-GlcNAc $\rightleftharpoons$ uaccg + OP
<i>murB</i>	SCO4643	UDP-N-acetylenolpyruvoylglucosamine reductase (EC 1.1.1.158) UNAM + NADP ⁺ $\rightleftharpoons$ uaccg + NADPH + H ⁺
<i>murQ</i>	SCO4307	N-acetylmuramic acid-6-phosphate etherase (EC 4.2.-.-) MurNAc-6P + H ₂ O $\rightleftharpoons$ GlcNAc-6P + Lactate
<i>ptsH</i>	SCO5841	Global PTS component HPr
<i>ptsI</i>	SCO1391	Global PTS component EI

^a Abbreviations of metabolites: OAc⁻, acetate; AcCoA, acetyl coenzyme A; ADP, Adenosine Di-Phosphate; ATP, Adenosine Tri-Phosphate; (GlcNAc)₂, chitobiose; 2P_i, diphosphate; chitobiose, N,N'-diacetylchitobiose; Fru, fructose; Fru-6-P, fructose-6-phosphate; GlcNAc, N-acetyl-D-glucosamine; GlcNAc-1P, N-acetyl glucosamine 1-phosphate; GlcNAc-6P, N-acetyl glucosamine-D-6-phosphate GlcN, glucosamine; GlcN-1P, Glucosamine 1-phosphate; GlcN-6P, Glucosamine 6-phosphate; Gln, glutamine; Glu, glutamate; MurNAc-6P, N-Acetylmuramic acid 6-phosphate; NADP, nicotinamide adenine dinucleotide phosphate; NADPH, nicotinamide adenine dinucleotide phosphate (reduced form); OP, Orthophosphate; 2-OG, oxoglutarate; PEP, phosphoenolpyruvate; UDP-GlcNAc, Uridine diphosphate N-acetyl D-glucosamine; uaccg, UDP-N-acetyl -3-O-(1-carboxyvinyl)-D-glucosamine; UNAM, UDP-N-acetylmuramate.

order to validate our *in silico* predictions, electromobility gel shift assays (EMSAs) were performed *in vitro* using His₆-tagged DasR and a short double-stranded oligonucleotide centered on *dre*^{nagKA} and *dre*^{nagB}. Positive specific DasR-*dre* interactions were observed for both *dre* sequences as well as for another positive control (the *dre* upstream of *dasA*), while the negative control (the *cis*-acting element of *blaI* of *Bacillus licheniformis*) was not bound by DasR (Fig. 2A).

If the *nag* genes are repressed by DasR, it would follow that their expression should be at least partially independent of induction by GlcNAc in a *dasR* null mutant. To test this hypothesis, *S. coelicolor* M145 and its Δ *dasR* null mutant were grown in liquid NMMP medium until OD₆₀₀~0.2 followed by the addition of 25 mM GlcNAc. Mycelia were harvested for RNA preparation immediately before and 30 min after GlcNAc addition. Semi-quantitative RT-PCR on total RNA samples from the wild-type strain *S. coelicolor* M145 showed an induction of *nagA*, *nagK*, and *nagB* transcript levels by two-, three-, and five-fold, respectively following the addition of GlcNAc, while transcripts of the control *rspI* (for the ribosomal protein S9) were readily identified before and after induction and did not show significant induction (Fig. 2BC). In contrast, expression of the *nag* genes was already high in the *dasR* null mutant before induction, with similar transcript levels before and after the addition of GlcNAc (Fig. 2BC). This is consistent with induction of the *nagA*, *nagB* and *nagK* genes by GlcNAc and repression of these genes by DasR. The induction by GlcNAc most likely acts via the allosteric inactivation of DasR by the GlcNAc-derived metabolite GlcN-6P (Rigali *et al.*, 2006; Rigali *et al.*, 2008).

***nagA* and *nagB* gene-replacement and in-frame deletion mutants**

To study the effect of the accumulation of GlcNAc-related metabolites on growth, development and antibiotic production, null mutants were constructed for *nagA* and *nagB*. For this, we first replaced the entire coding region of *nagA* or *nagB* by the apramycin cassette (*aacC4*) flanked by *loxP* sequences and then removed the cassette to generate the respective in-frame deletion (IFD) mutants (see Materials and Methods section). For each experiment five transformants exhibiting the desired double-crossover phenotype (Apra^R Thio^S) were selected and verified by PCR as described in the Materials and Methods section. The mutants were designated GAM1 (*nagA*::*aacC4*), and GAM2

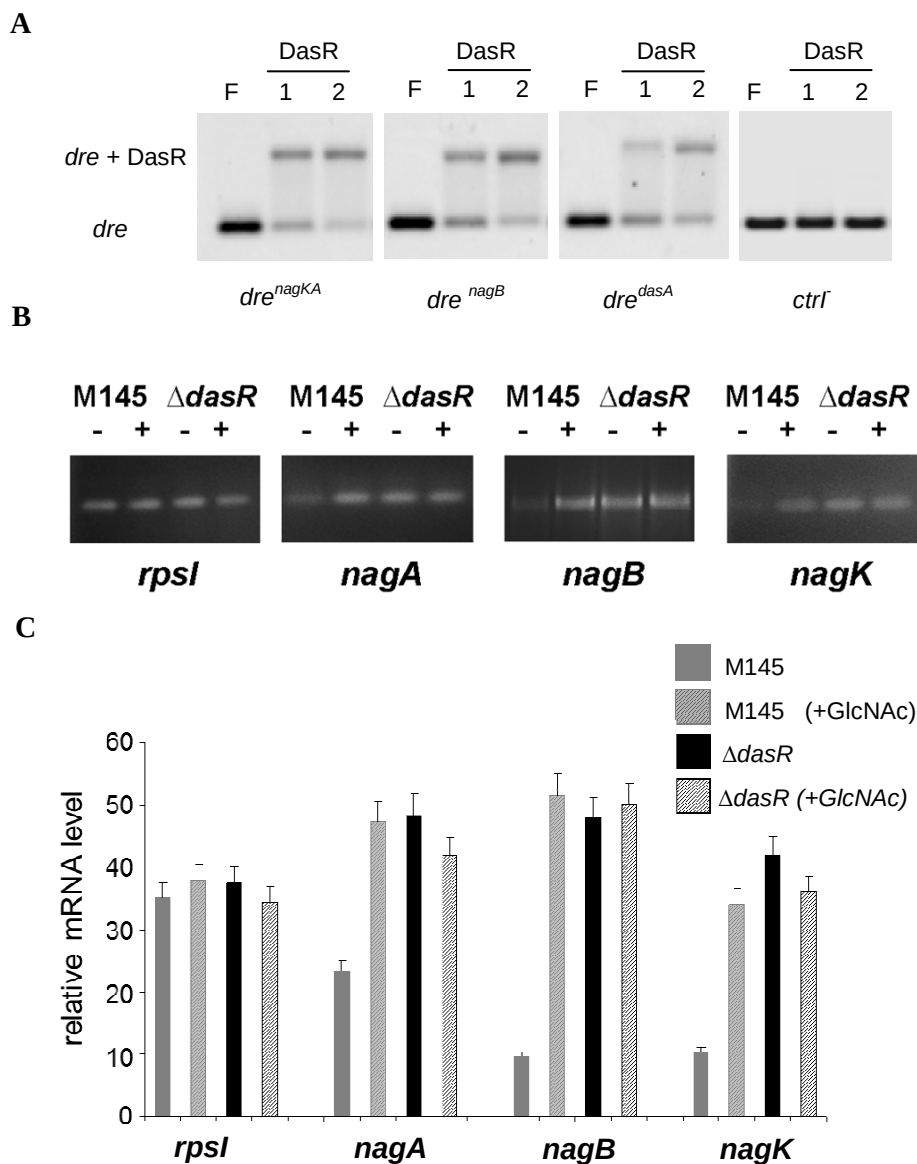


Figure 2. Direct transcriptional control of *nagKA* and *nagB* by DasR.

A. DasR binding to predicted *dre* upstream of *nagKA* and *nagB*. EMSAs were performed with pure DasR-His₆ and a 40 bp Cy5 double-stranded oligonucleotide probe centred on the DasR-responsive elements (*dre*) in the promoter regions of *nagKA* (*dre^{nagKA}*), *nagB* (*dre^{nagB}*), and *dasA* (*dre^{dasA}*, positive control), with short flanking sequences. The *cis*-acting element of *blaI* of *Bacillus licheniformis* was used as negative control (*ctrl*). **fp**, free probe (0.25 μ M of double stranded Cy5 labelled probe); **1** and **2**, DasR/*dre* binding reactions with 1.75 and 2.5 μ M of DasR-His₆, respectively; ***dre***, DasR responsive element.

B. Induction of *nag* transcription by GlcNAc and control by DasR. Semi-quantitative RT-PCR was carried out on RNA isolated from the *dasR* null mutant or its parent *S. coelicolor* M145, grown in liquid mineral media (NMMP + mannitol) cultures immediately prior to (-) or 30 min after (+) the addition of 25 mM GlcNAc. The constitutive expression of *nagA*, *nagK* and *nagB* in *dasR* null mutants and their enhanced transcript levels in wild-type cells 30 min after induction, shows that the *nag* genes are repressed by DasR and induced by *N*-acetylglucosamine.

(*nagB::aacC4*) respectively. Following removal of the *aacC4* cassette by the Cre recombinase, in-frame deletion (IFD) mutants were obtained for *nagA* (designated GAM4) and *nagB* (GAM5). For all experiments both the apramycin resistant and the IFD mutants were analyzed. Considering that there were no phenotypic differences between them in terms of antibiotic production and development, from this point onwards we will only describe the results obtained for the IFD mutants unless stated otherwise. The *nagAB* double mutant GAM9 was created by replacing *nagA* by the apramycin cassette in the *nagB*^{IFD} mutant GAM5.

Effect of GlcNAc on growth of the *nagA* and *nagB* mutants in liquid-grown cultures

The addition of GlcNAc is expected to result in the accumulation of GlcNAc-6P in *nagA* mutants, and of GlcN-6P in *nagB* mutants (Fig. 1B). Growth of *nagB* mutants was analyzed in liquid mineral medium (NMMP) and compared to that of the parental strain *S. coelicolor* M145. While there was little difference in terms of growth on mannitol, *nagB* mutants failed to grow in the presence of GlcNAc. This is in line with the observed toxicity of the NagB substrate GlcN-6P in other bacteria (Bernheim & Dobrogosz, 1970; Plumbridge, 2009; White, 1968). Addition of mannitol did not restore growth, strongly suggesting that rather than the inability to utilize GlcNAc, it is toxicity of a GlcNAc-derived metabolite that prevents growth of *S. coelicolor* in the absence of *nagB* (Fig. 3A). To analyze if the sensitivity to GlcNAc prevails throughout growth or only occurs during the earliest growth phases (*i.e.* germination and very early vegetative growth), we monitored growth in NMMP supplemented with mannitol (1% w/v), followed by the addition of 25 mM GlcNAc at an OD₆₀₀ of 0.2 (early log phase) (Fig. 3B). Surprisingly, under these conditions the mutant strain had a similar growth rate as the parent, indicating that GlcNAc toxicity is alleviated once a certain amount of biomass has accumulated. To rule out an effect specifically on spore germination, spores of *S. coelicolor* M145 and its *nagB* null mutant GAM5 were allowed to germinate for 8h in

2xYT at 30°C and used as inoculum of liquid mineral media cultures. Despite the obvious emergence of germ tubes as well as many young vegetative hyphae, as assessed by phase-contrast microscopy, the germinated *nagB* mutant spores still failed to grow in the presence of GlcNAc (Fig. 3C). This illustrates that in the absence of the GlcN-6P deaminase activity of NagB, GlcNAc is toxic to young vegetative hyphae, but that this is alleviated once a threshold amount of biomass has been produced.

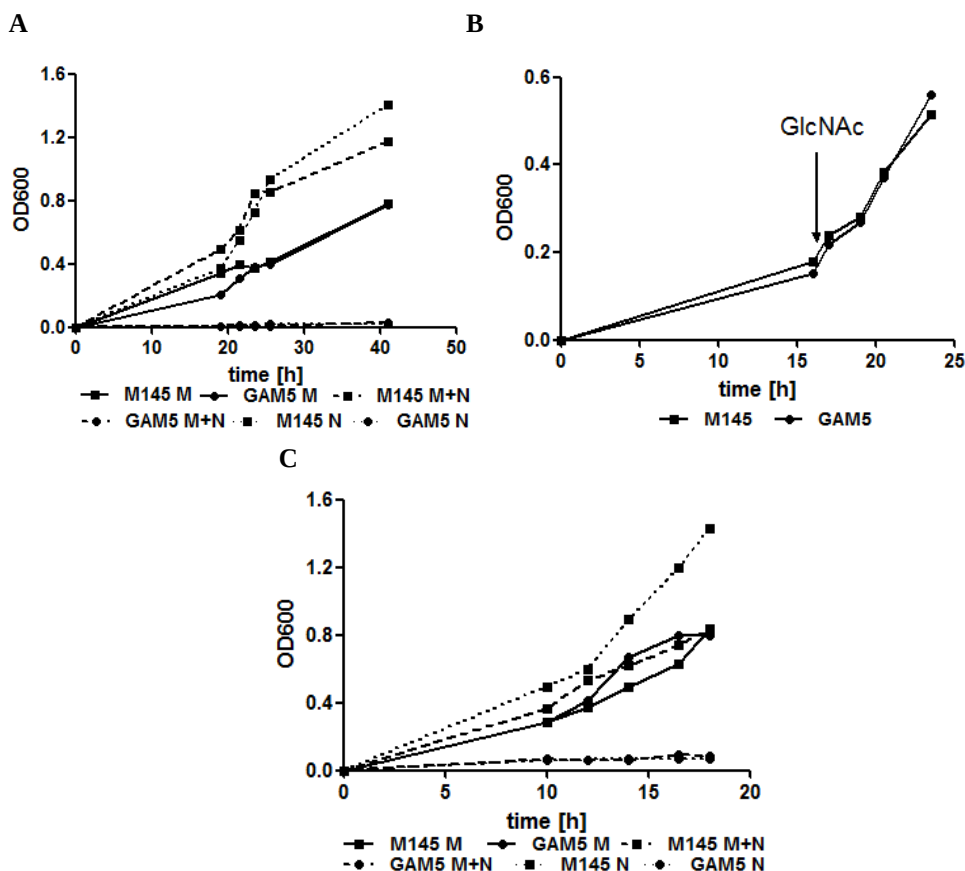


Figure 3. Effect of GlcNAc on growth of the *nagB* mutant.

A. Effect of GlcNAc on growth of *S. coelicolor* A3(2) M145 (■) and its *nagB* null mutant GAM5 (●). Strains were grown in liquid MM supplemented with either mannitol (50 mM) (M, solid line), GlcNAc (25 mM) (N, dashed line) or mannitol + GlcNAc (M+N, dotted line). **B.** Effect of GlcNAc induction on growth of *S. coelicolor* A3(2) M145 (■) and its *nagB* null mutant GAM5 (●). Strains were grown in liquid NMMP supplemented with mannitol (1% w/v), followed by the addition of 25 mM GlcNAc to the cultures at an OD₆₀₀ ~ 0.2. The addition of GlcNAc is indicated with an arrow. **C.** Same as (A), but now with spores that were pregerminated for 8 h in 2xYT at 30°C.

The *nagA* mutant GAM4 grew with a similar doubling time ($T_d \sim 3$ h) as the parent M145 in liquid-grown MM cultures in the presence of mannitol, but it reproducibly reached higher biomass levels (Fig. 4). Conversely, in MM with GlcNAc the doubling time of *nagA* mutants was much lower ($T_d \sim 5$ h) as compared to the parent for M145 ($T_d \sim 2$ h). This suggests that the likely accumulation of GlcNAc-6P impairs growth of *nagA* mutants, but the effect is not as dramatic as the accumulation of GlcN-6P in *nagB* mutants.

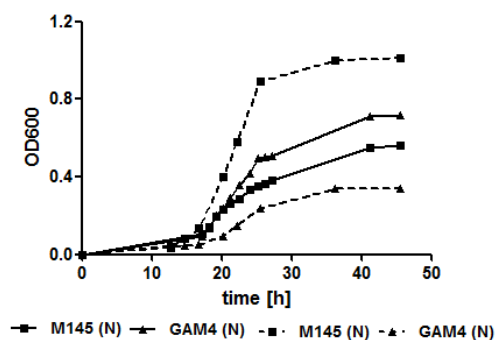


Figure 4. Effect of GlcNAc on growth of the *nagA* mutant.

Effect of GlcNAc on growth of *S. coelicolor* A3(2) M145 (■) and its *nagA* null mutant GAM4 (●). Strains were grown in liquid NMMP supplemented with either mannitol (50 mM) (M, solid line) or *N*-acetylglucosamine (25 mM) (N, dashed line). Doubling times were derived from the linear part of the growth curves.

Effect of GlcNAc on development and antibiotic production of the *nagA* and *nagB* mutants in solid-grown cultures

The *nagA* and *nagB* mutants and the *nagAB* double mutant were similar to that of the parental strain in terms of colony morphology when grown on SFM, R5⁻ or MM agar plates supplemented with glucose, fructose, galactose, glycerol, maltose, mannitol, mannose, or xylose as the sole carbon source (Supplemental Fig. S1). However, major differences were observed when GlcNAc was added to the media. The toxicity of GlcNAc to *nagB* mutants in liquid-grown cultures was also observed on solid media, with *nagB* null mutants failing to grow on MM agar plates supplemented with 25 mM GlcNAc (Fig. 5A), while very poor growth was observed on R5 agar plates with GlcNAc (Fig. 5B). *nagA* mutants showed normal growth and development on MM agar plates with mannitol, while GlcNAc resulted in impaired growth, although some aerial hyphae were

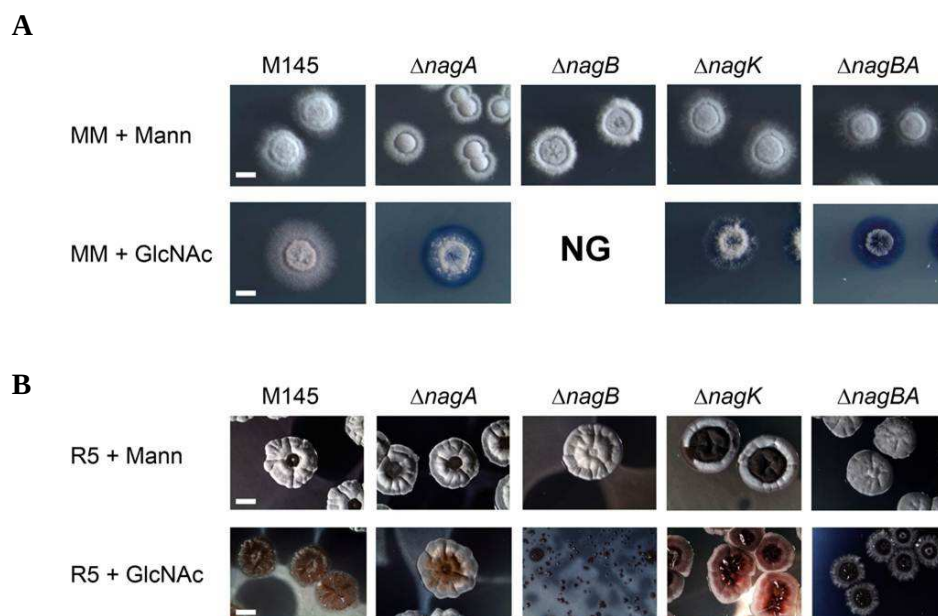


Figure 5. Phenotypic analysis of the *nag* mutants. Phenotypes of *S. coelicolor* M145 and its *nagA*, *nagB*, *nagK*, *nagAB* mutants (designated GAM4, GAM5, GAM6 and GAM9, respectively) were analyzed by stereomicroscopy. Colonies were grown for five days on MM (**A**) or R5 (**B**) agar plates, either with mannitol (top) or GlcNAc (bottom). For phenotypes of the strains on other media see Supplemental Fig. S1. Note that *nagB* mutants fail to grow (NG) on MM supplemented with GlcNAc, while on R5 with GlcNAc *nagB* mutants only produced a very thin layer of biomass. For complementation of the *nagA* and *nagB* mutants see Fig. S3.

formed. Interestingly, while GlcNAc was highly toxic to *nagB* null mutants on R5 agar plates supplemented with GlcNAc, the *nagA* null mutant GAM4 was able to enter development under these conditions (Fig. 5B), a phenotype that is similar to that of mutants lacking the GlcNAc transporter gene *nagE2* (Nothhaft *et al.*, 2010). In line with the idea that GlcN-6P accumulation causes toxicity to *nagB* null mutants grown on GlcNAc, the double mutant GAM9 (M145 $\Delta nagAB$) had a phenotype that was very similar to that of *nagA* mutants, with impaired growth on MM with GlcNAc and normal development on R5 agar plates with GlcNAc (Fig. 5). This is most likely due to the fact that the absence of *nagA* prevents the accumulation of GlcN-6P and thus toxicity of GlcNAc to *nagB* mutants.

The ability of the mutants to produce the pigmented antibiotics prodigine (Red) and actinorhodin (Act) was assessed in detail on R2YE agar plates. Since Red

production is switched on earlier than Act production (Bibb, 2005), antibiotic production was measured at different time points, namely after 42 h of growth (for Red production just prior Act biosynthesis) and after 48 h and 120 h for early and late Act production, respectively. Deletion of *nagA* only caused a minor reduction of Red production (about 25%) while deletion of *nagB* resulted in a 2.5-fold increase of prodiginine biosynthesis (Fig. 6A). Both deletions had a more drastic effect on Act production resulting in earlier and higher production with a 7- and 25-fold increase of Act biosynthesis after 48 h of growth for *nagA* and *nagB* mutants, respectively (Fig. 6B). After 120 h of growth all mutants showed similar excess of Act biosynthesis compared to the parental strain *S. coelicolor* M145 (increase of around 13-fold; Fig. S2). In contrast, *nagB* null mutant GAM5 formed extremely small colonies, with around 40% reduction of Red production and complete inhibition of extracellular Act production in the presence of GlcNAc (Fig. 6). After prolonged incubation (120 h) *nagB* null mutant GAM5 accumulated large amounts of intracellular Act on R5 supplemented with GlcNAc (Fig. S2).

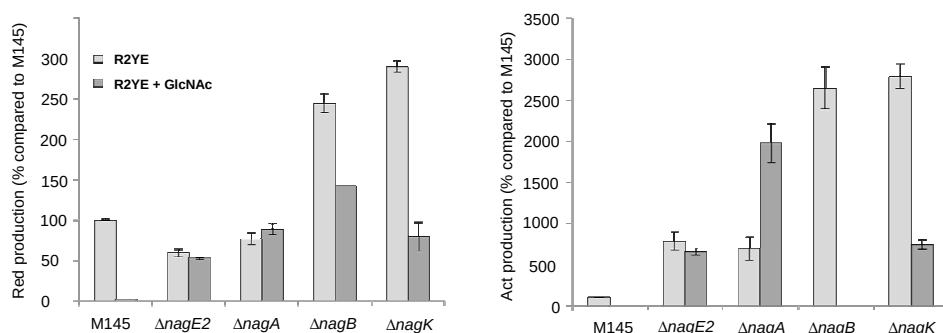


Figure 6. Quantification of antibiotic production. Production of prodiginines (top) or actinorhodin (bottom) was quantified relative to the production by the parental strain *S. coelicolor* M145 (which was set to 100%). Cultures were grown for 42 h (Red) or 48 h (Act) on R2YE agar plates with (dark bars) or without (light bars) GlcNAc. For 120 h see Fig. S2.

In a control experiment, the *nagA* and *nagB* gene replacement and IFD mutants were complemented with the low-copy vector pHJL401 (Larson & Hershberger, 1986) harboring the corresponding *nag* gene and its native promoter. *nagA* mutants GAM1 and GAM4 were complemented with pGAM5 (pHJL401/*nagKA*) and *nagB* mutants GAM2 and GAM5 with pGAM6 (pHJL401/*nagB*). The *nagKA* operon was used for complementation of *nagA* mutants to allow expression from the operon promoter.

Growth on MM and R5 agar plates with and without GlcNAc demonstrated that the complemented mutants had normal growth and GlcNAc sensing, underlining that the defects in growth and development in response to GlcNAc were indeed specifically due to the deletion of the respective *nag* genes (Fig. S3). Specifically, toxicity of GlcNAc to *nagB* mutants was relieved on both media, and GlcNAc sensing restored to both *nagA* and *nagB* mutants (*e.g.* bald phenotype and blocked antibiotic production on R5 with GlcNAc).

Effect of the deletion of *nagK*

The sugar kinase NagK catalyzes the phosphorylation of GlcNAc to GlcNAc-6P and the chromosomal deletion of *nagK* was performed as described previously for *nagA* and *nagB* mutants (see Materials and Methods section for details). We previously showed that transport of GlcNAc is mediated via the PTS in *S. coelicolor* (Nothaft *et al.*, 2010). If GlcNAc is exclusively transported via the PTS, the only internal source of GlcNAc is derived from the cell wall dismantling during programmed cell death (PCD) or chitin degradation providing chitooligosaccharides ((GlcNAc)_n, *e.g.* (GlcNAc)₂ and (GlcNAc)₃; Fig. 1B). When grown on rich medium agar plates the *nagK* mutant presented a 3- and 27-fold increased Red and Act production compared to the parental strain M145. Interestingly, on R2YE agar plates with GlcNAc the *nagK* mutant showed partial loss of the GlcNAc repressing effect that we could quantify to about 75% of the wild-type GlcNAc-mediated repression. This suggests that GlcNAc may be internalized in a PTS-independent manner under these growth conditions. As expected, the additional deletion of *nagK* did not significantly alter the morphology of the *nagA*, *nagB* or *nagAB* mutants on any of the media conditions tested (data not shown).

DISCUSSION

The aminosugar *N*-acetylglucosamine (GlcNAc) is a major carbon and nitrogen source for *Streptomyces*, which contributes to both cell-wall synthesis and primary metabolism, but also plays an important role as developmental signalling molecule (Nothaft *et al.*, 2003; Rigali *et al.*, 2008). The signalling cascade from nutrient sensing to development and antibiotic production is a highly complex process, based on cooperative interaction between signalling molecules, a phosphorylation state of the PTS, global and pathway-

specific regulators and their effector molecules. We previously demonstrated that GlcNAc acts as a signalling molecule that controls the onset of development and antibiotic production (Rigali *et al.*, 2006; Rigali *et al.*, 2008). Higher concentrations of GlcNAc outside the cell lock *Streptomyces coelicolor* in the vegetative growth phase under rich growth conditions (*feast*), but activate development and antibiotic production under poor conditions (*famine*). This principle has been applied successfully to enhance the expression of antibiotic biosynthetic gene clusters, including the clusters for Act, Red and the cryptic polyketide Cpk (Rigali *et al.*, 2008). The GntR regulator DasR acts as a responsive nutrient sensor, which has a pleiotropic regulon and among others represses *nag* metabolic genes, chitinolytic genes and the pathway-specific activator genes for antibiotic production (Colson *et al.*, 2007; Nazari *et al.*, 2011; Rigali *et al.*, 2006; Rigali *et al.*, 2008). Since the repressing activity of DasR is relieved allosterically by the effector molecule GlcN-6P (Rigali *et al.*, 2006), it follows that controlling the flux of GlcNAc may be a promising approach for metabolic engineering relating to improved screening of novel antimicrobial compounds.

From analysis of aminosugar metabolism and connected pathways (Fig. 1B) it follows that during growth on GlcNAc, *nagA* null mutants likely accumulate GlcNAc-6P and *nagB* null mutants likely accumulate GlcN-6P. Indeed, in line with the requirement of NagA for the production of the signalling molecule GlcN-6P, *nagA* null mutants had lost GlcNAc sensing. This underpins the current model of a direct signalling pathway from external GlcNAc to the accumulation of GlcN-6P, which then acts as allosteric effector of the developmental regulator DasR. However, when grown on GlcNAc, *nagA* null mutants likely accumulate large amounts of GlcNAc-6P, which in *E. coli* has two important regulatory roles, namely as allosteric activator for NagB (Calcagno *et al.*, 1984) and as effector molecule for NagC, the repressor of the *nag* regulon. In *E. coli*, inactivation of *nagA* (and hence accumulation of GlcNAc-6P) thus evokes derepression of the *nag* regulon (Plumbridge, 2009; Plumbridge, 1991). An additional effect is cell lysis, suggesting moderate toxicity of the accumulation of GlcNAc-6P to *E. coli* (White, 1968). Similar observations were made for *nagA* mutants in *Staphylococcus aureus* or *Gluconacetobacter xylinus*, which showed strongly reduced growth rates in the presence of GlcNAc (Komatsuzawa *et al.*, 2004; Yadav *et al.*, 2011). Indeed, we also found growth reduction for *nagA* null mutants of *S. coelicolor* in liquid minimal media with

GlcNAc, and the negative effect of GlcNAc on growth of the *nagA* mutant was shown by microscopy.

The accumulation of GlcN-6P is toxic to *S. coelicolor*, as demonstrated by the inability of *nagB* mutants to grow on GlcNAc. This toxicity was relieved by the additional deletion of *nagA* or the complete *nagKA* operon (Δ *nagABK*), and could also be complemented by re-introducing *nagB* on a low-copy vector. Interestingly, exponentially growing cells of *nagB* mutants had overcome the sensitivity to GlcNAc, as addition of GlcNAc during early logarithmic growth ($OD_{600} \sim 0.2$) did not lead to growth inhibition. In contrast, prolonged germination of the spores, which also resulted in the formation of many young vegetative hyphae, did not relieve the sensitivity. This strongly suggests that in particular young vegetative hyphae are sensitive to the accumulation of GlcN-6P, perhaps because alternative ways to reduce the GlcN-6P pool are not yet active enough. GlcN-6P occupies a central position between cell-wall synthesis and glycolysis, and besides being converted to Fru-6P by NagB, it is also incorporated into murein following its conversion to UDP-GlcNAc by the action of GlmM (phosphoglucosamine mutase) and GlmU (N-acetylglucosamine-1-phosphate uridyltransferase) (Fig. 1B) (Jolly *et al.*, 1997; Mengin-Lecreulx & van Heijenoort, 1994). In the absence of NagB, the major route to deplete GlcN-6P is incorporation into murein. Cell-wall synthesis exclusively takes place at the apical sites (Flårdh, 2003; Gray *et al.*, 1990) and with very few growing tips during early growth there may not be sufficient cell-wall synthesis to convert sufficient amounts of GlcN-6P, with as result the net accumulation of large amounts of this toxic metabolite. An alternative explanation would be lower enzymatic activity of GlmM during early growth. In *E. coli* GlmM is activated by phosphorylation, either by autophosphorylation or by the Ser/Thr kinase StkP (Jolly *et al.*, 1999; Jolly *et al.*, 2000). It is yet unknown how GlmM is activated in streptomycetes.

Preliminary work investigating the role of the GlcNAc kinase NagK revealed that the addition of GlcNAc has a less profound effect on development and antibiotic production in rich media when the *nagK* gene is deleted. The effect of NagK on the DasR-mediated GlcNAc nutrient-sensing system suggests the accumulation of its substrate GlcNAc. GlcNAc is internalized via the PTS in *S. coelicolor* (Nothaft *et al.*, 2003; Nothaft *et al.*, 2010), the existence of another import system that may be active under specific growth conditions cannot be ruled out. One candidate is perhaps the ABC

transporter encoded by SCO6005-6007 in *S. coelicolor*, which is orthologous to the GlcNAc/chitobiose transport operon *ngcEFG* in *Streptomyces olivaceoviridis*, although the sequence similarity is low (the various gene products share 30-40% aa identity). Alternatively, the accumulated GlcNAc-6P (which is a labile molecule) may be hydrolyzed to GlcNAc, which is not toxic. In both cases, NagK would be required to produce GlcNAc-6P in order to allow formation of the DasR effector molecule GlcN-6P.

Interestingly, when challenged with GlcNAc, we readily obtained suppressor mutants of the *nagB* null mutant. This is similar to observations in other bacterial systems, where spontaneous second-site mutations relieved the toxicity of accumulated sugar phosphates in *nagB* mutants (Plumbridge, 2009; White, 1968). Interestingly, when the *nagB* mutant spores were plated at high density onto MM agar plates containing GlcNAc, some colonies emerged with a frequency of around $1:10^5$, suggesting they had undergone spontaneous suppressor mutations to render the colonies insensitive to the toxicity of GlcNAc. Likely candidates for suppressor mutations include the GlcNAc-specific transporter genes *nagE2* and *nagF* and the metabolic genes *nagA* and *nagK*, but analysis of 16 independent suppressor mutants did not reveal changes in these genes or their promoter regions. We are currently analyzing the genomes of a series of the obtained suppressor mutants, and following re-creating the mutations in *nagB* mutants this will reveal which genes have undergone mutations, which will shed important new light on the metabolism of GlcNAc in *Streptomyces*.

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Chapter IV

Engineering of *N*-acetylglucosamine metabolism for improved antibiotic production in *Streptomyces coelicolor* A3(2) and an unsuspected role of NagA in glucosamine metabolism

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ABSTRACT

N-acetylglucosamine, the monomer of chitin and constituent of bacterial peptidoglycan, is a preferred carbon and nitrogen source for streptomycetes. Recent studies have revealed new functions of GlcNAc in nutrient signalling of bacteria. Exposure to GlcNAc activates development and antibiotic production of *Streptomyces coelicolor* under poor growth conditions (famine) and blocks these processes under rich conditions (feast). Glucosamine-6-phosphate (GlcN-6P) is a key molecule in this signalling pathway and acts as an allosteric effector of a pleiotropic transcriptional repressor DasR, the regulon of which includes the GlcNAc metabolic enzymes *N*-acetylglucosamine-6-phosphate (GlcNAc-6P) deacetylase NagA and GlcN-6P deaminase (NagB). Intracellular accumulation of GlcNAc-6P and GlcN-6P enhanced production of the pigmented antibiotic actinorhodin. When the *nagB* mutant was challenged with GlcNAc or GlcN, spontaneous second-site mutations that relieved the toxicity of the accumulated sugar phosphates were obtained. Surprisingly, deletion of *nagA* also relieved toxicity of GlcN, indicating novel linkage between the GlcN and GlcNAc utilization pathways. The strongly enhanced antibiotic production observed for many suppressor mutants shows the potential of the modulation of GlcNAc and GlcN metabolism as a metabolic engineering tool towards the improvement of antibiotic productivity or even the discovery of novel compounds.

Introduction

The alarming emergence of multiply antibiotic-resistant pathogens coupled with the lack of effective antimicrobials has revealed an urgent requirement for discovery of new antibiotics. Streptomycetes are Gram(+), soil-dwelling bacteria that have a complex life cycle that starts with the germination of a spore, which then grows out to form a branched vegetative mycelium. When morphological differentiation is initiated, a so-called aerial mycelium is produced, followed by the formation of long chains of unigenomic spores (Chater & Losick, 1997; Flårdh & Buttner, 2009). Streptomycetes are best known for their ability to produce a broad range of secondary metabolites, including around 50% of all known antibiotics, as well as many antifungal, anticancer and immunosuppressant agents (Hopwood, 2007). The control of antibiotic production is complex, with typically many different transcriptional regulators dictating the expression of a single gene cluster, allowing the producing organism to correctly time biosynthesis, for example in response to growth phase or nutrient availability (Sanchez *et al.*, 2010; van Wezel & McDowall, 2011).

As saprophytic soil bacteria, streptomycetes are able to grow on a wide range of organic compounds due to production of numerous extracellular enzymes, such as amylases, cellulases, chitinases, proteases and lipases. Since organic matter is also a predominant source of nutrition for other microorganisms, bacteria had to develop mechanism of predation leading to elimination of competing organisms. It is suggested that in natural habitat of bacteria, this mechanisms is stimulated by production of antibiotics leading to thwart of growth of competing organisms (Hopwood, 2007).

Sequencing of the genomes of many *Streptomyces* and other actinomycetes revealed that most likely all filamentous actinomycetes possess a large arsenal of secondary metabolites, and only part of these are produced under standard laboratory conditions (Challis & Hopwood, 2003). Therefore, novel strategies not only for the improvement of antibiotic production, but also to wake up sleeping (also referred to as silent or cryptic) biosynthetic clusters are required to harness this potential. Current methods and technological advances regarding the discovery of new antibiotics have been reviewed elsewhere (Baltz, 2008; Martin & Liras, 2010; van Wezel *et al.*, 2009). Such approaches include engineering primary metabolism and regulatory cascades controlling antibiotic production (Butler *et al.*, 2002; Chen *et al.*, 2010; Martin & Liras,

2010).

Role of GlcNAc in control of development and antibiotic production

N-acetylglucosamine (GlcNAc), the monomer of chitin and constituent of peptidoglycan, and the related amino acid glutamate are preferred nutrients as they serve as highly favourable carbon and nitrogen sources for streptomycetes (Nothaft *et al.*, 2010; van Wezel *et al.*, 2006). Besides as nutrient, GlcNAc also serves as a signalling molecule in streptomycetes. Accumulation of GlcNAc around the colonies at higher concentrations controls both morphological differentiation (sporulation) and antibiotic production; under poor nutritional conditions (famine) the accumulation of GlcNAc promotes development and antibiotic production, while under rich growth conditions (feast) these processes are inhibited (Rigali *et al.*, 2008). We previously proposed a complete signalling cascade from GlcNAc uptake to the onset of antibiotic production under famine conditions. This pathway is transmitted via the master regulator DasR, whose regulon includes genes for GlcNAc transport and metabolism as well as antibiotic production. A key molecule in this signalling cascade is GlcN-6P, which acts as an allosteric effector of DasR and therefore prevents its DNA binding ability (Rigali *et al.*, 2006). This results in the relief of transcriptional repression of the pathway-specific regulatory genes for antibiotic production, including *actII*-ORF4 and *redZ*, the pathway-specific activator genes for actinorhodin and prodiginine biosynthesis, respectively. DasR also represses the *cpk* gene cluster for the cryptic polyketide Cpk, although this interaction may be mediated via the control of *scbA* and *scbR* (van Wezel & McDowall, 2011), which encode the synthetase for the γ -butyrolactone Scb1 and the Scb1-responsive transcriptional activator for Cpk biosynthesis, respectively (D'Alia *et al.*, 2011; Hsiao *et al.*, 2007). The molecular mechanism of GlcNAc signalling under rich nutritional conditions, which results in the blockage of sporulation and antibiotic production, is still unknown.

***N*-acetylglucosamine metabolism**

In *S. coelicolor*, the model organism of species, GlcNAc is transported via the phosphoenolpyruvate-dependent phosphotransferase system (PTS) (Brückner & Titgemeyer, 2002; Nothaft *et al.*, 2003a). During PTS-mediated carbon source uptake, a phosphoryl group is transferred from phosphoenolpyruvate (PEP) to the general

phosphotransferase enzyme I (EI; encoded by *ptsI*), from there to HPr (encoded by *ptsH*), and then further onto enzyme IIA (EIIA^{Crr}; encoded by *crr*) and enzyme IIB (e.g. NagF) (Nothhaft *et al.*, 2003b; Nothhaft *et al.*, 2010). NagF phosphorylates incoming via the permease IIC (NagE2) GlcNAc to form *N*-acetylglucosamine-6-phosphate (GlcNAc-6P) (Fig. 1). Null mutants that have been constructed for any of the global PTS components (EI, EIIA, or HPr) locks streptomycetes in the vegetative growth phase (Rigali *et al.*, 2006).

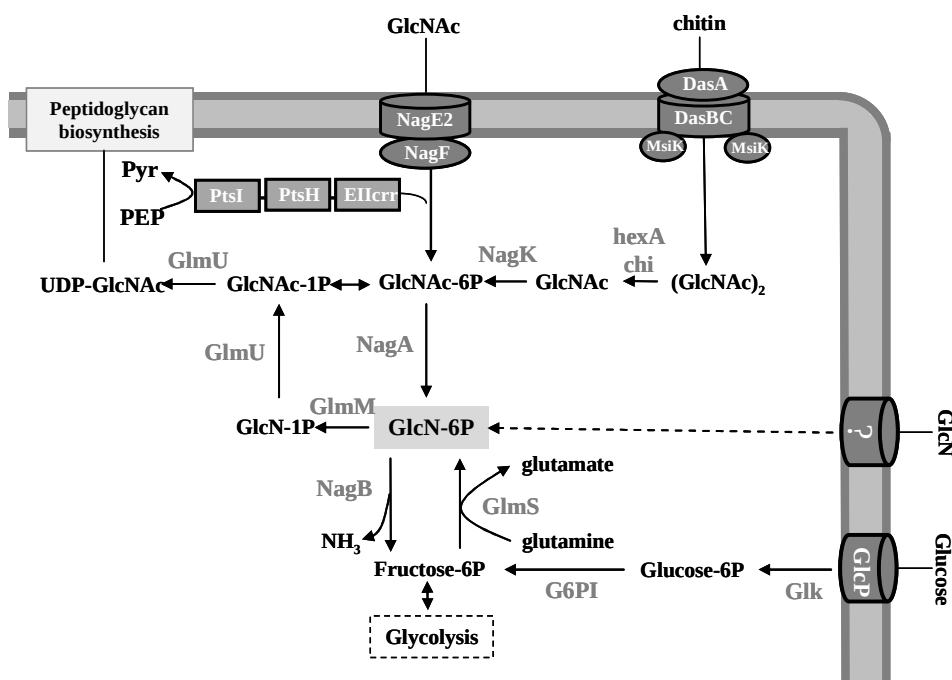


Figure 1. Glucose and aminosugar metabolism in *S. coelicolor*, with connecting pathways. Annotation is based on the KEGG database (http://www.genome.jp/kegg-bin/show_pathway?org_name=sco&mapno=00520) and experimental evidence. Arrows shows the direction of metabolism, and the responsible enzymes are presented in bold face. See text for further details.

Recently we characterized the metabolic enzymes in *S. coelicolor*, namely GlcNAc-6P deacetylase (NagA; SCO4284), GlcN-6P deaminase (NagB; SCO5236) and GlcNAc kinase (NagK; SCO4285) (Świątek *et al.*, 2012). Similarly to the GlcNAc utilization pathway of other bacteria, intracellular GlcNAc-6P is deacetylated by NagA, which results in glucosamine-6-phosphate (GlcN-6P), the molecule which occupies a central position in the pathways towards cell-wall synthesis and glycolysis. GlcN-6P is

converted by NagB to fructose-6-phosphate (Fru-6P), which enters the glycolytic pathway (Plumbridge & Vimr, 1999; Vogler & Lengeler, 1989), but is also incorporated into murein following its conversion to UDP-GlcNAc by the action of GlmM (phosphoglucosamine mutase) and GlmU (*N*-acetylglucosamine-1-phosphate uridyltransferase) (Fig. 1). During growth on other carbon sources than aminosugars, GlcN-6P can be produced from Fru-6P by GlcN-6P synthase (*glmS*) (Fig. 1). NagK catalyzes the phosphorylation of intracellular GlcNAc to GlcNAc-6P (Fig. 1). In *S. coelicolor*, monomeric GlcNAc is transported via the PTS, resulting in intracellular GlcNAc-6P (Nothaft *et al.*, 2010). Intracellular unphosphorylated GlcNAc is most likely internalized as *N*-*N*'-diacetylchitobiose ((GlcNAc)₂) and/or other chito-oligosaccharides ((GlcNAc)_n), such as those derived from chitin via the chitinolytic system.

Interestingly, some *Streptomyces* species, including *S. bingchenggensis*, *S. cattleya*, and *S. violaceusniger*, do not have a *nagB* orthologue. Genome mining revealed the presence of an open reading frame, which appears to be a hybrid of *nagB/glmS2* and shows similarity to the *nagB*-II genes present in Gram-positive bacteria. These NagB-IIs contain the C-terminal domain of GlmS, but display only the catabolic activity of NagB (Teplyakov *et al.*, 1999; Yang *et al.*, 2006). The fact that these three streptomycetes also contain the PTS genes for internalization and phosphorylation of GlcNAc, including *nagE2* (SBI_06686, SCATT_19300, Strvi_8260) and *nagF* (SBI_06687, SCATT_19290, Strvi_8259), as well as *nagA* (SBI_04956, SCAT_2457, Strvi_8764) for the subsequent deacetylation of GlcNAc-6P to GlcN-6P, strongly suggests that indeed these organisms can catabolize GlcNAc using the NagB/GlmS2 hybrid enzyme instead of the canonical NagB.

Engineering of aminosugar metabolism as an approach to enhance antibiotic production

Better understanding of GlcNAc-mediated signalling in streptomycetes requires the creation of multiple mutants, single and in combination, to block metabolic routes so as to force the accumulation of specific metabolites. Thus new insight is obtained with regard to the flux of GlcNAc and related metabolites, and how this influences the global regulatory routes that control *Streptomyces* development and antibiotic production. In the absence of *nagA*, addition of GlcNAc results in the accumulation of GlcNAc-6P, while in

nagB mutants GlcN-6P accumulates (Fig. 1). *nagA* deletion mutants showed strongly enhanced production of the blue-pigmented antibiotic actinorhodin in the presence of GlcNAc, on both rich (R2YE) and poor (MM) solid media (Świątek *et al.*, 2012). The likely accumulation of GlcN-6P in *nagB* mutants led to a strong increase in Act production on R2YE agar plates supplemented with GlcNAc, while the mutant failed to grow on MM agar plates supplemented with either GlcNAc or its deacetylated form, glucosamine (GlcN) (Świątek *et al.*, 2012 and Fig. 2). Toxicity of GlcN-6P was reported previously in *Escherichia coli* (Bernheim & Dobrogosz, 1970; Plumbridge, 2009; White, 1968). In line with the idea that the accumulation of GlcN-6P was the cause of the toxicity, *nagAB* double mutants (which do not accumulate GlcN-6P for the lack of NagA activity; Fig. 1) were able to grow on GlcNAc, but the enhanced antibiotic production remained (Fig. 2). However, we could not immediately explain the surprising observation that also the toxicity of GlcN to *nagB* mutants was relieved by additional deletion of *nagA*, as involvement of NagA in GlcN metabolism was not anticipated (Fig. 2). This sheds new light on GlcN metabolism and suggests direct involvement of NagA in, or linkage between, both pathways.

When *nagB* mutant spores were plated at high density onto MM agar plates containing either GlcNAc or GlcN, suppressor mutants were readily obtained, with a frequency of around $1:10^5$, which corresponds to expected single mutations or indels (insertions or deletions). This is a logical consequence of the fact that single mutations in for example *nagA* or any of the transporter genes should suffice to circumvent the accumulation of GlcN-6P as the likely cause for the observed toxicity. Reproducibly at least three different phenotypes were obtained for suppressor mutants allowing growth in the presence of GlcNAc restreaked on MM+GlcNAc, while suppressor mutants with restored growth in the presence of GlcN had similar phenotypes on MM+GlcN. This suggests that suppressor mutations may have arisen in different genes.

Fifteen suppressor mutants obtained on GlcNAc, designated SMA1-SMA15 (Suppressor Mutants isolated on GlcNAc) and five independent suppressor mutants obtained on GlcN-containing media, SMG1-SMG5 (Suppressor Mutant isolated on GlcN), were re-streaked on MM agar plates supplemented with mannitol, glucose, GlcN or GlcNAc. All strains developed normally on MM agar plates supplemented with mannitol (Fig. 3). However, three different phenotypes were observed for *nagB*

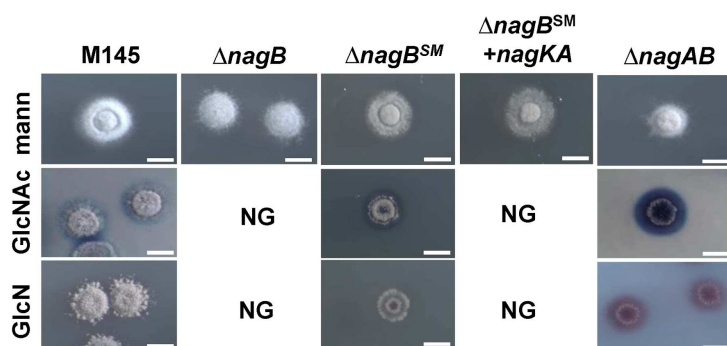


Figure 2. Phenotypic analysis of *nag* mutants. Stereomicrographs showing the phenotypes of *S. coelicolor* M145, its *nagB* and *nagAB* mutants, *nagB* suppressor mutant SMG4 (obtained on GlcN) that has a deletion in *nagA*, and the complemented suppressor mutant expressing *nagKA*. Strains were grown for 5 days on MM agar plates supplemented with mannitol, GlcN or GlcNAc. Note that *nagB* mutants and complemented *nagB* suppressor mutants fail to grow (NG) on either aminosugar, while growth was restored to the *nagAB* double mutant and the *nagB* suppressor mutants, and also led to enhanced antibiotic overproduction.

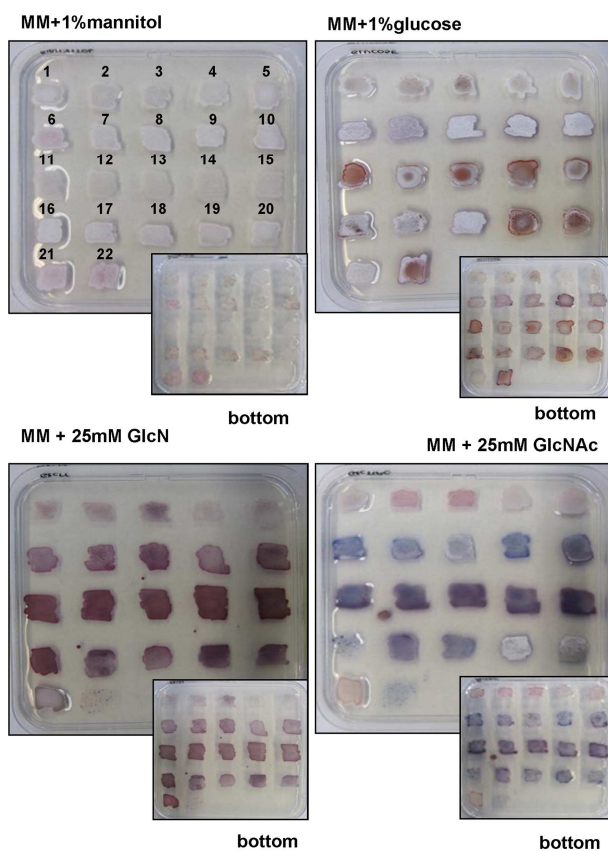


Figure 3. Analysis of antibiotic production by *nagB* suppressor mutants. Phenotypes of *nagB* suppressor mutants originally selected on GlcNAc (1-15; corresponding to SMA1-SMA15) or GlcN (16-20; corresponding to SMG1-SMG5). The parental strain M145 (21) and the *nagB* mutant (22) were used as controls. Strains were grown on MM agar plates supplemented with mannitol, glucose, GlcN or GlcNAc. Note that the *nagB* mutant failed to grow on MM supplemented with either aminosugar. Both front and reverse (inserts) of the agar plates are shown.

suppressor mutants obtained in the presence of GlcNAc (Fig. 3 and Fig. 4). The first class of GlcNAc-selected *nagB* suppressor mutants displayed decreased antibiotic production but normal development (SMA1; SMA4-SMA5). The two other classes showed enhanced antibiotic production and either blocked (SMA2-SMA3; SMA6-SMA7; SMA9; SMA11-SMA15) or normal development (SMA8 and SMA10) (Fig. 3 and Fig. 4).

What happens when GlcN-obtained suppressor mutants are grown in the presence of GlcNAc or *vice versa*, when GlcNAc-obtained mutants are grown on GlcN-containing media? All suppressor mutants except one, were able to grow on both aminosugars. However, GlcN-obtained *nagB* suppressor SMG1 is of particular interest, as it fails to grow on GlcNAc. This suggests that the second-site mutation specifically relieved the toxicity of GlcN and not of GlcNAc. Therefore, elucidation of this mutation would be of great interest for our understanding of GlcN(Ac) metabolism. A likely candidate is a mutation in the GlcN transport system, which so far has not been elucidated. All other SMG strains grown on MM agar plates supplemented with GlcNAc overproduced antibiotics, and for SMG2 and SMG3 development was blocked (Fig. 3). Like for SMA suppressors, SMG mutants displayed three distinct phenotypes under this condition. All GlcNAc-selected *nagB* suppressor mutants showed impaired development when grown on MM with GlcN, while some also displayed reduced antibiotic production (SMA1-SMA10) as compared to the wild-type strain (Fig. 3 and Fig. 4).

Interestingly, we also observed a strong effect of the inactivation of *nagB* and the subsequent suppressor mutants on antibiotic production on media not supplemented with GlcNAc or GlcN (Fig. 3). The *nagB* mutant and most of the suppressor mutants obtained from either GlcNAc or GlcN showed enhanced antibiotic production and strong delay in aerial mycelium formation on MM agar plates supplemented with glucose. Glucose is transported to the cell via the GlcP permease and enters glycolysis after subsequent phosphorylation by glucose kinase (Glk), respectively (van Wezel *et al.*, 2005) (Fig. 1). Glucose-6P can enter glycolysis or be converted in two steps into GlcN-6P, mediated via glucose-6P isomerase and GlmS (Fig. 1). Accumulation of part of the

glucose as GlcN-6P may explain the effect of glucose on antibiotic production in the mutants.

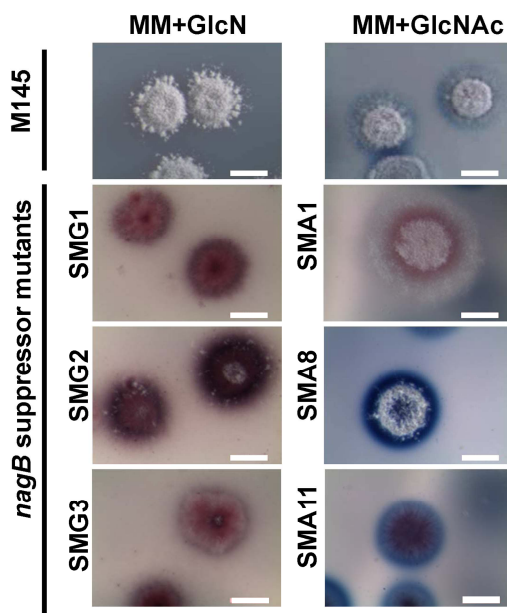


Figure 4. Phenotypic diversity of *nagB* suppressor mutants. Stereomicrographs illustrating the phenotypes of *S. coelicolor* M145 and representatives of three different classes of SMA mutants (SMA1, SMA8, SMA11) and of SMG mutants (SMG1-SMG3) grown on MM agar plates supplemented with GlcN (M145 and SMG mutants; left) or GlcNAc (M145 and SMA mutants; right). Colonies were grown for 5 days. Note that the majority of *nagB* suppressor mutants overproduce antibiotics.

Identification second-site mutations relieving GlcN and GlcNAc toxicity to *nagB* mutants

To identify the second-site mutations the complete genome analysis of four independent suppressor mutants selected either on GlcN (called SMG1 and SMG4) or GlcNAc (SMA1 and SMA11) was performed. Whole-genome sequencing revealed many changes relative to the reference sequence. Most of these were identical mutations occurring in all mutants, and were therefore not considered further. The unique changes only occurring in one of the mutants are shown in Table 1. Since still quite a few different candidate mutations were identified in all of the mutants, we focused our initial attention to genes that were functionally related to primary metabolism, sugar transport or regulatory genes. The mutations discussed below were all verified by PCR followed

by DNA sequencing.

Table 1. Mutations identified by next-generation genome sequencing in the independent *nagB* suppressor mutants SMG1, SMG4 (obtained on GlcN), SMA1 and SMA11 (obtained on GlcNAc).

Single Nucleotide Polymorphism (SNP)							
SMG1	SMG4	SMA1	SMA11	Function	aa changes	nt position*	
		SCO1859		putative aminotransferase	p.Val255Leu	763 (G->C)	
	SCO2672				p.Ala718Gly	2153 (C->G)	
	SCO2975				p.Gly1055Arg	3163 (G->C)	
SCO3554				putative integral membrane protein	p.Gln117Lys	349 (C->A)	
			SCO4393	conserved hypothetical protein	p.Asp179Asn	535 (G->A)	
	SCO4476				p.Ala434Gly	1301 (C->G)	
			SCO4931	putative membrane protein	p.Ala346Pro	1036 (G->C)	
		SCO5235		DasD	p.Ala490Ser	1468 (G->T)	
SCO6167				proline rich protein (putative membrane protein)	p.His182Pro	545 (A->C)	
Deletion-Insertion Polymorphism (DIP)							
SMG1	SMG4	SMA1	SMA11	Function	aa changes	nt position*	
SCO1447				ROK-family transcriptional regulatory protein	p.Arg9fs	26 ⁱ	
	SCO4284			N-acetylglucosamine-6-phosphate deacetylase	p.Thr87fs	261- 676 ^d	

'fs' – frame shift; '*' – nucleotide position relative to the start codon; 'i' – insertion; 'd' – deletion; 'G, A, T, C' – guanine, adenine, thymine, cytosine.

Only those changes relative to the parent M145 are shown that were not found in all strains, and that gave rise to a change in the predicted protein sequence of the respective genes.

As mentioned above suppressor mutant SMG1 could grow on GlcN but not on GlcNAc (Fig. 3), which suggests that the mutation only relieves toxicity to the former non-acetylated aminosugar. A deletion-insertion polymorphism (DIP) analysis of the SMG1 genome revealed a frame-shift insertion of a single cytosine at nt position 26 in SCO1447, encoding a ROK-family protein. The ROK proteins are widespread among bacteria and many of them are involved in the control of aminosugar and/or sugar transport and utilization (Deutscher *et al.*, 2006; Plumbridge, 2001; Chapter VI). Upstream of SCO1447 lies the divergently transcribed SCO1448, which encodes a probable transport protein containing a Major Facilitator Superfamily (MFS) domain. Examination of intergenic region between SCO1447 and SCO1448 identified the 29 bp inverted repeat GTAAACTATCAGGGAGCCTGCCTGATAGATAG, which is also found associated with these genes in all other streptomycete genomes analysed (Fig. 5). We anticipate that it is a possible binding site for the SCO1447 gene product. Considering that only a single element is found on genome, suggesting that if indeed SCO1447 binds there, SCO1448 may be its only direct target. Following this logic, since SMG1 only relieves toxicity of GlcN, we anticipate that SCO1448 may encode a GlcN transporter, and that its expression depends on SCO1447. This is currently being

investigated.



Figure 5. Highly conserved inverted repeat identified in the intragenic region between SCO1447 and SCO1448 and corresponding genes in other streptomycetes. Sequence motif was generated by using MEME.

SMG4 contained an out-of-frame deletion of 416 bp in *nagA* (nt positions 261-676). This result confirms our previous observation, that the toxicity of GlcN to *nagB* mutants can be relieved by additional deletion of *nagA* (Fig. 2). *nagAB* double mutants (which do not accumulate GlcN-6P for the lack of NagA activity) grew well regardless of whether they were challenged with GlcN or GlcNAc (Świątek *et al.*, 2012). Moreover, the mutants displayed enhanced antibiotic production (Fig. 2).

SMA1 has a G→T substitution at nt position 1468 in *dasD* (SCO5235), resulting in an Ala490Ser change in the protein. DasD is a predicted β -*N*-acetylglucosaminidase, which is a highly specific exoglycosidase that catalyzes the hydrolysis of terminal, non-reducing β -*N*-acetylglucosamine residues from oligosaccharides. Upstream of *dasD* lies the *dasA-dasBC* gene cluster for an oligo-GlcNAc-binding protein (DasA) and chitobiose permease (DasBC), respectively. Whereas *dasA* and *dasBC* are transcribed individually, *dasD* is probably co-transcribed with *dasBC* (Colson *et al.*, 2008; Saito *et al.*, 2007). It is anticipated that DasD might serve for hydrolysis of chitobiose [(GlcNAc)₂], which is taken up via the DasABC system (Colson *et al.*, 2008; Saito *et al.*, 2007). Downstream of *dasD* lies transcribed in the opposite direction *nagB*.

Suppressor mutant SMA11 has a G→A substitution at nt position 535 in SCO4393, resulting in a Asp179Asn change in the SCO4393 gene product. SCO4393 encodes a highly conserved protein across *Streptomyces* species. It contains a SIS (Sugar ISomerases) domain characteristic of phosphosugar isomerases and phosphosugar binding proteins. The best known examples of SIS domain containing proteins are bacterial helix-turn-helix (HTH) transcriptional regulators belonging to the RpiR family. The RpiR regulators control the expression of genes encoding sugar metabolic enzymes. This control is modulated through direct interaction of the domain with substrates of regulated metabolic enzymes. It is unlikely that the SCO4393 product is a transcriptional regulator, since it lacks a clear DNA binding motif, and we expect it may have an

enzymatic function. Glucosamine-6P synthetase (GlmS) is an example of a metabolic enzyme containing a SIS domain. Excitingly, gene synteny analysis revealed that SCO4393 is linked to *nagA* in many organisms. In Gram-negative alpha-proteobacteria the orthologs of SCO4393 are clustered with *nagA* and *nagB*, while in firmicutes they are adjacent to *pts* genes (Fig. 6). These results highlight the likely involvement of the SCO4393 gene product in the aminosugar utilisation, and strongly suggest that indeed the Asp179Asn change in SCO4393 may be the cause of the suppressor phenotype.

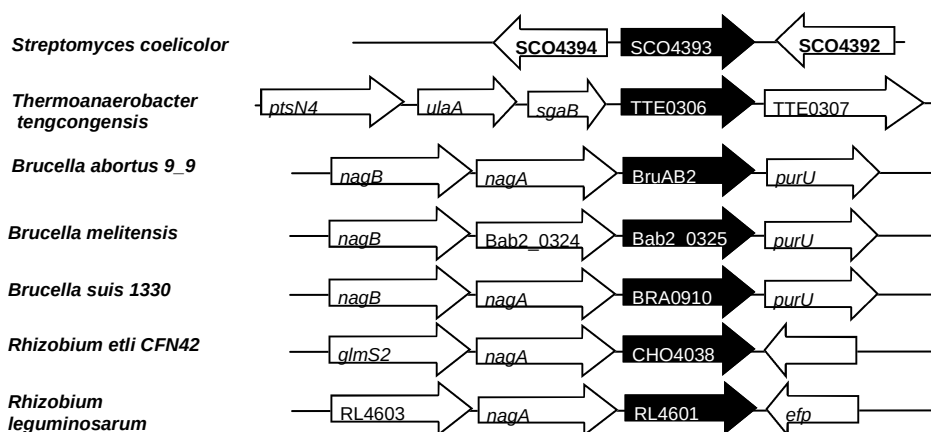


Figure 6. Gene synteny of the region centered on SCO4393. The gene synteny of each SCO4393-encoded ortholog is shown with ortholog in black. See text for further details.

In a control experiment, each of these four *nagB* suppressor mutants was complemented with the low-copy-number vector pHJL401 (Larson & Hersherberger, 1986) harboring a wild-type copy of the putative target genes sustaining the suppressor mutation transcribed from either the native promoter or - in case a promoter was not identified in the upstream region - from the *ermE** promoter. For the latter we used the plasmid pHJL401-SuperP, which contains the *ermE** promoter and the ribosome binding site from the *tuf1* gene, which allows very efficient translational initiation (Vijgenboom *et al.*, 1994). SMG1 was complemented with pGAM30 (pHJL401/SCO1447), SMG4 with pGAM5 (pHJL401/*nagKA*), SMA1 with pGAM31 (pHJL401-SuperP/*dasD*) and SMA11 with pGAM32 (pHJL401/SCO4393). The sensitivity of all strains to aminosugars was tested on MM agar plates. Importantly, in all cases indeed the complemented suppressor mutants failed to grow on amino sugars, strongly suggesting that these specific mutations

were the cause of the restored growth (Fig. 7).

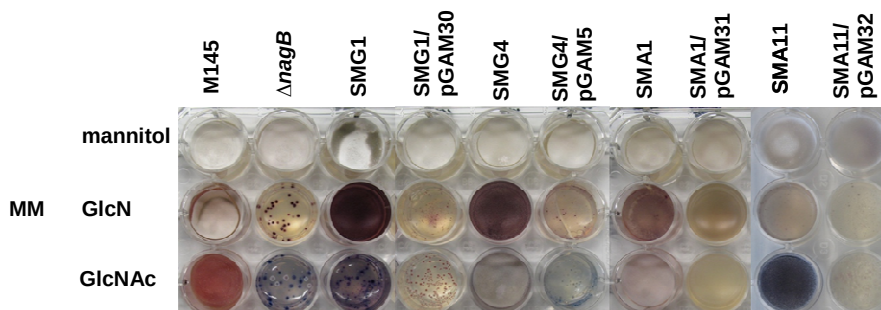


Figure 7. Complementation of *nagB* suppressor mutants. Phenotypes of *S. coelicolor* M145, the *nagB* mutant, *nagB* suppressor mutants SMG1, SMG4, SMA1, SMA11 and the complemented suppressor mutants expressing respectively SCO1447, *nagA*, *dasD* and SCO4393 are shown. Strains were grown for 5 days on MM agar plates supplemented with mannitol, GlcN or GlcNAc. Note that *nagB* mutants and complemented *nagB* suppressor mutants fail to grow (NG) on either aminosugar.

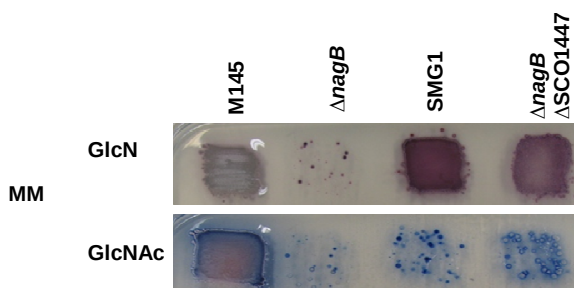


Figure 8. Phenotypic analysis of the double *nagB*-SCO1447 mutant. Patches of *S. coelicolor* M145 and its derivatives on MM agar plates with either GlcN (top) or GlcNAc (bottom) as the sole carbon source are shown. Note that *nagB* mutants, SMG1 and double *nagB*-SCO1447 mutants do not grow on GlcNAc, whereas growth of the last two is restored on GlcN. Patches were grown for 5 days at 30°C.

To know if more suppressor mutants contained the same second-site mutations, fifteen additional suppressor mutants obtained on GlcN and nineteen suppressor mutants obtained on GlcNAc were transformed with all complementation constructs. Two of the GlcN-selected suppressor mutants regained sensitivity to this aminosugar upon introduction of a construct expressing SCO1447, while on MM agar plates supplemented with GlcNAc, 13 of the GlcNAc-selected suppressor mutants regained sensitivity to GlcNAc following introduction of pGAM32 harbouring SCO4393, while none of the tested strain was sensitive to GlcNAc upon transformation with plasmid pGAM31 harbouring *dasD*. 10 GlcN-selected and three GlcNAc-selected *nagB* suppressor mutants

lost their ability to grow in the presence of either aminosugar, when complemented with a wild-type copy of the *nagA* gene.

The high frequency of second-site *nagA* mutations relieving GlcN toxicity of *nagB* mutants strongly suggests that NagA is somehow involved in the conversion of internalized GlcN into the toxic compound GlcN-6P, or a derivative thereof, thus revealing a novel and unexpected intersection between the GlcN and GlcNAc utilization pathways in *S. coelicolor*. Up to date there are no reports presenting the involvement of the enzyme NagA in GlcN metabolism, nor is there any evidence of conversion of GlcN to the NagA substrate GlcNAc-6P (see KEGG database; http://www.genome.jp/kegg-bin/show_pathway?org_name=sco&mapno=00520). This presents one example of the high relevance of the suppressor mutants, and closer analysis of GlcN and GlcNAc metabolism is currently in progress.

In contrast to SMG4, SMG1 harboring the second site mutation in SCO1447 could grow only in the presence of GlcN (Fig. 3 and Fig. 7). In a control experiment, SCO1447 was deleted from the *nagB* mutant and subsequently growth of the obtained strain was tested on both aminosugar. Again, the double mutant was able to grow on GlcN, but not GlcNAc (Fig. 8). Therefore SCO1447 is specifically involved in the control of GlcN utilization, perhaps by governing control the transporter gene SCO1448, which may transport GlcN. However, disruption of SCO1448 did not restore growth of *nagB* mutant on GlcN (data not shown).

Thus, the identification of second-site mutations in *nagB* mutants restoring growth on GlcNAc and/or GlcN has allowed us to identify novel candidate genes that are somehow involved in aminosugar metabolism in streptomycetes. These molecules play an important role in central metabolism and cell-wall biosynthesis and accumulation of GlcN(Ac)-derived metabolites, which among others serve as a signal for the onset of antibiotic production. Better understanding of aminosugar metabolism in streptomycetes may provide alternative tools for the improvement of antibiotic productivity as well as the possible activation and discovery of new antibacterial compounds.

Acknowledgements

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Chapter V

System-wide *in vivo* analysis of the regulon for the nutrient sensor DasR in *Streptomyces coelicolor* reveals its global role in controlling metabolism and development

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submitted for publication

ABSTRACT

Streptomyces produce a wealth of natural products, including antibiotics, anti-cancer agents and immunosuppressants. *Streptomyces* genomes likely harbour many sleeping antibiotic gene clusters, and to allow exploitation of these hidden treasures, we need to understand the global mechanisms that control their expression. Here we show by an integrated systems level analysis that the nutrient sensor DasR is a master regulator of primary and secondary metabolism and development, with extensive primary and secondary response regulons. DasR binding *in vivo* is relieved by extracellular addition of *N*-acetylglucosamine. Two classes of direct targets were identified, namely those associated with a DasR-responsive element (*dre*) that were also bound *in vitro* (Class I) and those not associated with a *dre*, likely requiring a co-repressor for binding (Class II). In line with the function of DasR as a master regulator, additional specific induction is typically required for the induction of specific regulons. The control of early developmental regulatory genes such as *bldD*, *bldK*, *bldN*, *crp* and *wblA* suggests that DasR is one of the earliest acting regulatory networks in the *Streptomyces* life-cycle.

INTRODUCTION

The ability of bacteria to adapt to a wide range of nutritional environments requires intricate regulatory systems to ensure that gene expression connects to changes in the key routes of primary metabolism. The flux of carbon and nitrogen is thereby typically controlled by a number of global regulators, each with a larger number of locally acting regulators in their response regulon. The global regulators ensure that the flux of essential metabolites is closely monitored and tuned (Martinez-Antonio & Collado-Vides, 2003; Sonenshein, 2007). Streptomycetes are soil-bound bacteria and adaptation to the rapidly changing conditions in this habitat is particularly challenging and hence likely requires a network of sensory systems. Indeed, the *Streptomyces coelicolor* genome encodes 45 ECF (extracellular functions) sigma factors and some 70 two-component regulatory systems (Hutchings *et al.*, 2004). As producers of over half of the known antibiotics, streptomycetes are a paradigm of secondary metabolite-producing microorganisms (Chater & Losick, 1997; Hopwood, 2007). Typically, antibiotic biosynthetic gene clusters are activated by a pathway-specific regulator within or adjacent to the cluster, and in a growth-phase dependent manner (van Wezel & McDowall, 2011). A pivotal question to address is how the primary environmental signals are relayed to global carbon and nitrogen control and further to the control of development and antibiotic production. The elucidation of the *S. coelicolor* genome sequence - the first from an antibiotic-producing actinomycete (Bentley *et al.*, 2002) – was a landmark event, and showed that the antibiotic-producing potential of actinomycetes had been underestimated. Indeed, actinomycete genomes typically contain some 20 sets of putative biosynthetic genes for secondary metabolites, and it is yet unclear how many specific biologically active antibiotics (Challis & Hopwood, 2003). Many of these clusters are not expressed under typical laboratory conditions of rapid growth on nutrient-rich media, often referred to as sleeping or cryptic antibiotic clusters. This may explain why they have been missed in the massive screening efforts performed by Big Pharma (Cooper & Shlaes, 2011; Payne *et al.*, 2007). Many antibiotic biosynthetic gene clusters are regulated in subtle ways so as to allow the organisms to adapt to life in the soil, with its varying physical, chemical and biological stresses, and the challenge is now to exploit this untapped source of potentially valuable natural products (Baltz, 2008; Hopwood, 2007).

To allow activation and screening of sleeping antibiotic biosynthetic clusters,

detailed insight is required into the linkages between environmental (nutritional) signals and secondary metabolite production. Carbon source utilization is a major determining factor in the metabolic control of antibiotic production (Sanchez *et al.*, 2010; van Wezel & McDowall, 2011). The major control system for carbon utilization in bacteria is carbon catabolite repression (CCR). In the model organisms *Escherichia coli* and *Bacillus subtilis*, CCR is mediated via the phosphoenolpyruvate-dependent phosphotransferase system (PTS; reviewed in Brückner & Titgemeyer, 2002). In streptomycetes, the PTS does not seem to play a major role in carbon control and CCR is mediated through glucose kinase, although the mechanism is still unknown (Angell *et al.*, 1994; van Wezel *et al.*, 2007). Additionally, glucose is not internalised via the PTS but rather via the major facilitator GlcP permease (van Wezel *et al.*, 2005). In streptomycetes the PTS fulfills a dual role, and is involved in both *N*-acetylglucosamine (GlcNAc) uptake (Nothaft *et al.*, 2003; Nothaft *et al.*, 2010) as well as in the switch from normal growth to development (Rigali *et al.*, 2006; Rigali *et al.*, 2008). This switch is controlled by the extracellular level of GlcNAc, accumulation of which causes developmental arrest and blockage of antibiotic production in *S. coelicolor* (Rigali *et al.*, 2006). GlcNAc is the monomer of the abundant natural polymer chitin - after cellulose the second most abundant carbohydrate on earth - and also a major constituent of cell-wall peptidoglycan. GlcNAc is a privileged C- and N-source for streptomycetes, and the related metabolite glutamate is even preferred as a carbon source over glucose by *S. coelicolor* (van Wezel *et al.*, 2006). The GlcNAc regulon is controlled by the GntR-regulator DasR (Rigali *et al.*, 2006; Rigali *et al.*, 2004; Świątek *et al.*, 2012), and DNA binding activity is inhibited *in vitro* by glucosamine-6-phosphate (Rigali *et al.*, 2006), a metabolic product derived from GlcNAc. DasR was identified as a nutrient sensory protein that is not only essential for development of streptomycetes, but also directly controls antibiotic production. *In silico* and *in vitro* evidence revealed likely direct repression of the pathway-specific activator genes for *actII*-ORF4 and *redZ* for actinorhodin and prodiginine production, respectively (Rigali *et al.*, 2008). The effect of GlcNAc on *S. coelicolor* depends on the specific culture conditions. On rich media, addition of GlcNAc blocks development and antibiotic production, whereas on minimal media with agar as the sole carbon source it accelerates these processes. These different media are considered to reflect the states of feast or famine, respectively, in the natural environment.

Here we describe a system-wide analysis of the DasR regulon and show that it plays a key role in the control of genes necessary for primary metabolism and antibiotic production, as well as for control of early growth and development. The study also reveals how DasR binding varies with culturing conditions, with relieve of *in vivo* binding following the induction by GlcNAc.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Escherichia coli JM109 (Sambrook, 1989) and ET12567 (Kieser *et al.*, 2000) were used for routine cloning procedures and for extracting non-methylated DNA, respectively. All media and routine *Streptomyces* techniques are described in the *Streptomyces* manual (Kieser *et al.*, 2000). Cells of *E. coli* were grown in Luria–Bertani broth (LB) at 37°C. SFM (mannitol soya flour) agar plates were used to prepare spore suspensions.

Strains were cultivated on MM agar plates with mannitol (1% w/v) (covered with cellophane discs) or in liquid R5 or NMMP supplemented with mannitol (1% w/v). For induction experiments, liquid cultures were grown at 30°C until mid-logarithmic phase (OD₆₀₀ approximately 0.4 for NMMP cultures and 0.8 for R5 cultures), followed by the addition of GlcNAc (50 mM). For the ChIP-on-chip studies on MM mannitol solid medium mycelium was harvested after 24 h and 54 h.

Plasmids and constructs

For the ChIP-on-chip studies a recombinant *dasR* gene was synthesised that allows production of DasR containing a C-terminal triple FLAG-tag epitope (GenScript, La Jolla, USA). The DNA fragment contained the *dasR* gene along with 306 bp promoter region, with the stop codon of the gene replaced by the sequence 5'-ATGGACTACAAGGACCACGACGGCGACTACAAGGACCACGACATCGACTACAAGGACGACGACGACAAGTAGACCAGAGCCCGCTCACCCGGCCCCAGATTGCGGTTGAAGTCC-3' (stop codon underlined). In this way, the DasR protein was extended with the amino acid sequence MDYKDHDGDYKDHDIDYKDDDDK. The fragment was cloned into the integrative vector pSET152 (Bierman *et al.*, 1992). This resulted in vector pGAM29, which expresses DasR-3xFLAG for use in ChIP-on-chip experiments.

Chromatin immunoprecipitation, gDNA labeling and array hybridizations

S. coelicolor M145 containing the integrative vector pGAM29 expressing 3xFLAG-tagged DasR and the *dasR* null mutant GAM29 were grown in duplicate (see *Bacterial strains and growth conditions*). Cultures were treated with formaldehyde (final

concentration 1%, 10 min, 30°C) in order to crosslink proteins to DNA. Subsequently glycine (final concentration of 0.5M) was added to quench the formaldehyde and cultures were incubated for further 5 minutes at 30°C. Mycelium was harvested by centrifugation, washed twice with 1xPBS and stored at -70°C. Chromatin isolation, labeling and hybridization were performed as described previously (Bucca *et al.*, 2003; Bucca *et al.*, 2009). Biological replicates of DasR-3FLAG-IP chromatin were labeled with either Cy3 or Cy5 –dCTP in a dye swap experimental design and co-hybridised with the mock ‘no-antibody’ IP chromatin on 4 ´ 44K whole genome *Streptomyces* arrays manufactured by Agilent Technologies (Bucca *et al.*, 2009).

RNA isolation, cDNA synthesis and labeling

Total RNA was purified using the Kirby-mix protocol (Kieser *et al.*, 2000). DNaseI treatment was used to fully remove any traces of DNA. Before use, the RNA preparations were checked for their quality and integrity on the Agilent 2100 Bioanalyzer (Agilent Technologies). cDNA synthesis and labeling was performed as previously described (Bucca *et al.*, 2003; Bucca *et al.*, 2009).

Microarray expression data and ChIP-on-chip data analysis

For the time-course gene expression studies *S. coelicolor* M145 and *dasR* mutant were cultivated on MM mannitol agar plates overlaid with cellophane discs. Samples were taken at 24 h, 30 h, 36 h, 42 h and 54 h. Two biological replicates were analysed for all samples and for each strain. The filtered data sets for the gene expression experiments were analysed using Rank Products analysis (Breitling *et al.*, 2004) via the Rank-Prod package in R (version 2.5.0) (Hong *et al.*, 2006) using the web-based implementation, RankProdIt Laing E, Smith CP. (2010) 'RankProdIt: A web-interactive Rank Products analysis tool.' *BMC Res Notes*, 3 221). Differentially expressed genes were identified as having a pfp (probability of false prediction) value (Breitling *et al.*, 2004) less than or equal to 0.15, equal to a false discovery rate of approximately 15%. Some additional genes were also identified that demonstrated a >1.5 fold change in expression between wild-type and the *dasR* mutant. ChIP-on-chip data, allowing identification of genes/regions directly controlled by DasR were analysed as described previously (Bucca *et al.*, 2009).

RESULTS

Predicted DasR regulon and growth conditions

Previous bioinformatic predictions using the PREDetector algorithm (Hiard *et al.*, 2007) revealed some 200 sequences that conformed to the consensus binding site for DasR (*dre*, for DasR responsive element), namely the palindromic 16 bp consensus sequence A (G/C)TGGTCTAGACCA(G/C)T. These sequences are found primarily associated with genes related to primary metabolism, sugar transport and extracellular polysaccharide hydrolysis, as well as antibiotic production (Craig *et al.*, 2012). The transcriptomic analysis of the mutant of the pleiotropic *dasR* regulatory gene, with impact on primary metabolism, secondary metabolite production and sporulation, requires a well-defined experimental set up. Major changes were previously observed in the *dasR* mutant in terms of antibiotic production on either MM agar plates with only agar as the carbon source, or on complex media (Rigali *et al.*, 2008). However, growth is poor on MM with agar and *dasR* null mutants fail to sporulate on rich media, and strains in different phases of the life cycle can not be properly compared (den Hengst *et al.*, 2010). We therefore used MM agar plates with mannitol for comparison of wild-type and *dasR* mutant, as strains display roughly similar development under these growth conditions (Rigali *et al.*, 2006). RNA was isolated from mycelia harvested after 24 h, 30 h, 36 h, 42 h or 54 h of growth, corresponding to vegetative growth, early aerial growth, aerial growth, early sporulation and sporulation, respectively.

Transcriptomic analysis

Microarray analysis revealed some 1200 genes that were significantly differentially expressed in the *dasR* null mutant of *S. coelicolor* relative to the parent M145, and the transcription of over 400 genes was more than two-fold up- or down-regulated at one or more time points in the *dasR* mutant (across two biological replicates). These genes were considered as significantly affected by the deletion of *dasR* (Table S1); of these, some 100 genes showed more than three-fold and 26 genes showed more than five-fold change in transcription relative to the parental strain. Classes of genes that were overrepresented are those related to: primary metabolism; protein biosynthesis; development and secondary metabolism; SapB, chaplins, rodmins and other small peptides; transposases

and DNA recombination.

Primary metabolism and protein biosynthesis

Genes of the DasR core regulon (*nag*, *pts*) were up-regulated in the *dasR* mutant and mostly at all time points, including *nagE2*, *nagF*, *ptsH* (encoding PTS components); *nagB*, *nagK* (encoding GlcNAc metabolic enzymes); *glmS1* and *glmS2* for glucosamine-fructose-6-phosphate aminotransferase (which has the opposite catalytic activity as NagB); and *dasA-dasBC* (for the chitobiose transporter (Colson *et al.*, 2008; Saito *et al.*, 2007) (Fig. 1A). Most of these genes are direct targets, and hence repressed by DasR (Colson *et al.*, 2007; Rigali *et al.*, 2008; Świątek *et al.*, 2012). Additionally, several genes involved in central metabolism, such as glycolysis, the TCA cycle, gluconeogenesis and pyruvate metabolism as well as for biosynthetic pathways for amino acids, nucleotides and fatty acids were up-regulated in the *dasR* mutant (Supplemental Table S1). Strong targets were ABC transporters, in particular the branched-chain amino acid transporter operon SCO2008-SCO2012, the SCO6005-SCO6007 operon (Fig. 1A) that is orthologous to the *ngcEFG* chitobiose/GlcNAc transport operon in *S. olivaceoviridis* (Wang *et al.*, 2002) and the oligopeptide transporter operon SCO5476-SCO5480 (Supplemental Table S1). Despite the strong binding of DasR to the genes belonging to the chitinolytic system (see below), their transcription was similarly low in both wild-type and *dasR* mutant cells. The lack of enhanced expression of the *chi* genes is most likely explained by the lack of inducer that is required to activate their transcription.

The widespread deregulation of protein biosynthetic genes is noteworthy, in particular almost all genes for ribosomal proteins, as well as genes for translation initiation factors IF-1 and IF-3 and elongation factors EF-G, EF-P, EF-Ts, EF-Tu1 and EF-Tu3, as well as the chaperonins GroES, GroEL1 and GroEL2. Transcription of all of the genes belonging to the larger ribosomal protein operons S10 (SCO4701-4711), L14 (*spc*; SCO4712-4722), S12 (*str*; SCO4659-4662) and S13 (SCO4727-4730) responded in the same way to the deletion of *dasR*, with up-regulation during early growth (24 h) and sporulation (54 h), while the genes were down-regulated around 30 h (change from vegetative to aerial growth). No major changes were found at 36 and 42 h (aerial growth) (Supplemental Fig. S1A).

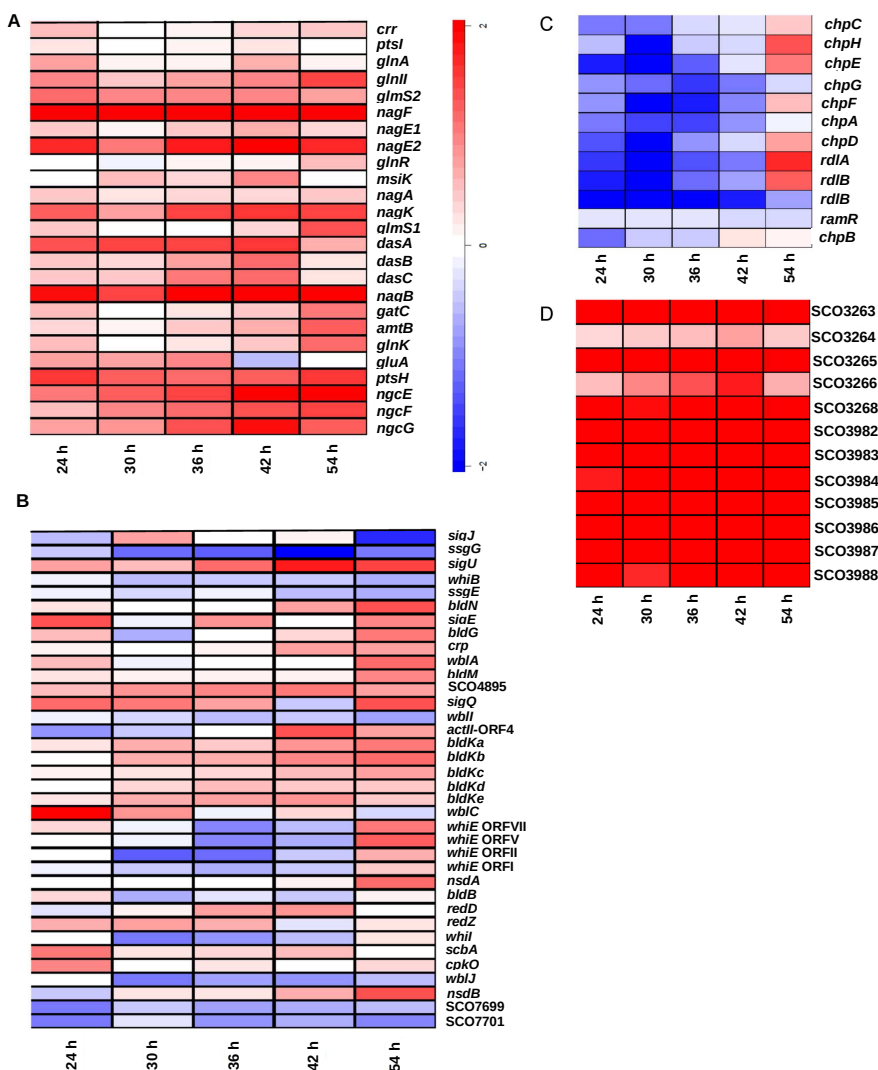


Figure 1. Selected categories of genes differentially expressed in the *dasR* null mutant. Important categories of genes whose transcription was altered more than two-fold in microarray experiments comparing the *dasR* mutant GAM29 relative to its parent *S. coelicolor* M145 are shown; these are (A) genes for amino acid and (amino)-sugar transport and metabolism; (B) genes related to development and antibiotic production, (C) genes for chaperones, rodlin, and SapB, and (D) the SCO3263-3268 and SCO3982-3988 operons for small peptides of unknown function. RNA was isolated from *S. coelicolor* M145 or its *dasR* mutant GAM29 cultivated on MM mannitol agar plates after 24 h (vegetative growth), 30 h (vegetative/aerial growth), 36 h (aerial growth), 42 h (aerial growth/sporulation) and 54 h (sporulation). A heat map is provided as reference for the fold changes in log scale ($\log_2(\text{mutant/wild-type})$). Note that many genes shown a larger than four-fold change in transcription. For exact values refer to Supplemental Table S1.

Secondary metabolism

Transcription of the biosynthetic gene clusters for actinorhodin (SCO5070-SCO5091) and for prodiginine was enhanced in the *dasR* mutant, in particular around 42 h (Supplemental Table S1). Interestingly, *scbA* (SCO6266), which encodes the γ -butyrolactone synthase ScbA implicated in the control of the antibiotic clusters *act*, *red* and *cpk* (encoding a cryptic modular type I polyketides synthases) in *S. coelicolor* (Hsiao *et al.*, 2007), was up-regulated at 24 h (Fig. 1B). *cpkO* (SCO6280), the transcriptional activator gene of the *cpk* cluster, was also up-regulated at 24 h, and this is followed by enhanced expression of the *cpk* cluster in the next time point (30 h). Conversely, four genes (SCO3218, SCO3222, SCO3224, SCO3244) belonging to the *cda* cluster for calcium-dependent antibiotic CDA were down-regulated, in particular at 24 h (Fig. 1B and Supplemental Table S1).

Transcription of the two-component system genes *afsQ1-Q2* (SCO4906-07) was also enhanced. In *S. coelicolor*, AfsQ activates antibiotic production on MM with glutamate as sole nitrogen source (Shu *et al.*, 2009). Other antibiotic-related genes that were up-regulated in the *dasR* mutant include *rfsA* (SCO4677), an *abaA*-like regulatory gene which controls *afsQ* and represses antibiotic production and morphological differentiation (Choi *et al.*, 2010; Kim *et al.*, 2008), *nsdA* (SCO5582), a negative regulator of antibiotic production and morphological differentiation in *S. coelicolor* (Li *et al.*, 2006) and its paralogue *nsdB* (SCO7252; Zhang *et al.*, 2007).

Finally, the deoxysugar gene cluster SCO0381-SCO0401 was up-regulated in the *dasR* mutant (Supplemental Table S1). SCO0381-SCO0401 shows significant similarity to the *ste* gene cluster (*Streptomyces eps*) of *Streptomyces* sp. 139 required for Ebosin biosynthesis. Ebosin is an exopolysaccharide (EPS) which shows anti-rheumatic arthritis activity *in vivo* (Wang *et al.*, 2003).

Development

The DasR regulon includes a surprisingly large number of regulators, with 58 regulatory genes significantly up- or down-regulated in the mutant at one or more time points. In line with the notion that DasR acts in particular as a switch from vegetative to aerial growth, specifically early developmental genes were up-regulated in the *dasR* mutant at later time points, namely *bldK*, *bldM*, *bldN*, *crp* and *wblA* (Fig. 1B), while expression of

sporulation genes (*e.g. whi, ssg*) was left unchanged. The pentacistronic *bldK* operon encodes an oligopeptide transporter required for early stages of development (Nodwell & Losick, 1998; Nodwell *et al.*, 1996), *bldN* encodes a developmentally regulated σ factor, σ^{BldN} , required for aerial mycelium formation, which in turn is required for the transcription of the response regulator gene *bldM* (Bibb *et al.*, 2000), *crp* is involved in the control of spore germination (Derouaux *et al.*, 2004; Piette *et al.*, 2005), and the WhiB-like protein WblA represses antibiotic production and may also be involved in the transition from early aerial hyphal cells to the subapical stem and apical compartments that precede sporulation (Fowler-Goldsworthy *et al.*, 2011). Some of the most highly up-regulated genes were *wblC* (SCO5190) and its flanking genes SCO5189 and SCO5191, and *mtrA* (SCO3013), the homologue of which controls proliferation of *Mycobacterium tuberculosis* (Fol *et al.*, 2006). Expression of *ssgG* (SCO2924), a homologue of *ssgB* whose gene product recruits FtsZ during sporulation (Willemse *et al.*, 2011), was down-regulated in the *dasR* mutant (Fig. 1B).

Strikingly, *chpABCDEFGH*, *rdlAB*, and *ramS*, encoding chaplins, rodlinins and SapB, respectively, were massively down-regulated during early time points. The chaplins and rodlinins are hydrophobic ‘coat’ proteins that assemble into a rodlet layer that provides surface hydrophobicity to aerial hyphae and spores, thus allowing them to break through the moist soil surface (Claessen *et al.*, 2003; Claessen *et al.*, 2006; Wösten & Willey, 2000); the spore-associated protein SapB is a lantibiotic-type signalling peptide required for the onset of development (Willey *et al.*, 1991). Several of the genes featured in the top 10 of most strongly down-regulated genes, particularly during early time points (Fig. 1C), strongly suggesting their direct or indirect dependence on DasR. The absence of *dre* elements associated with the genes, as well as lack of direct binding in ChIP-on-chip experiments (see below) strongly suggests that a transcriptional regulator for these genes is deregulated in the *dasR* null mutant.

Transcription of two other gene clusters encoding small (60-100 aa) secreted proteins and a GntR regulator, was also strongly repressed, with transcription of the SCO3982-SCO3988 cluster so strongly enhanced that it suggests that the cluster is silenced by DasR (Fig. 1D). Transcription of the paralogous gene cluster SCO3263-3268 (with SCO3982 similar to SCO3268 and so on) was also strongly repressed by DasR (Fig. 1D). SCO3983 contains a TTA codon suggesting it is subjected to translational

control by the tRNA BldA, which effects developmental control of gene expression (Lawlor *et al.*, 1987). The function of the gene clusters is yet unknown, but their massive up-regulation in the *dasR* mutant is intriguing.

Identification of the direct DasR binding sites *in vivo* by ChIP-on-chip

In order to discriminate between genes directly or indirectly regulated by DasR and to obtain detailed biological insight into the genome-wide distribution of DasR binding sites *in vivo*, we conducted ChIP-on-chip analysis using mycelia grown on MM agar plates with mannitol as the sole carbon source. For this, the binding profiles of wild-type *S. coelicolor* M145 carrying the integrative vector pGAM29, which expresses C-terminally 3xFLAG-tagged DasR, and the *dasR* null mutant GAM29 (control) were directly compared. For each strain two biological replicates were analysed. Samples were collected at 24 h and 54 h, which for the parental strain corresponded to vegetative growth and full development (aerial hyphae and spores), respectively. The *dasR* null mutant also sporulated under these conditions, and growth rates were essentially the same for the two strains. DasR showed statistically relevant binding to 76 sites after 24 h and to 265 sites after 54 h (Supplemental Table S2). However, using a stringent threshold of 0.5 ($\log_2$ (wild-type with *dasR*-flag antibody/no-antibody)/(*dasR* mutant antibody/no-antibody)) to identify significantly enriched probes (following a t-test as detailed in Bucca *et al.*, 2009), and setting a minimum of two adjacent probes significantly enriched, this list was reduced to 22 and 57 sites that had their promoter proximal regions bound with high probability in the 24 h and 54 h old samples, respectively (Table 1). The distribution of the *in-silico* predicted and experimentally validated DasR-binding *dre* elements is presented schematically in Fig. 4A and Fig. 4B, respectively. It should be noted at this point that we also performed eight additional ChIP-on-chip experiments on liquid-grown MM and R5 cultures, to analyse the effect of GlcNAc on *in vivo* binding (see below). Taken together, this provided very good validation for the *in vivo* binding of DasR, with up to eight independent verifications for the target genes (Table 1).

Table 1. Summary of ChIP-on-chip experiments and validation by EMSAs (+, binding; -, no binding)

Gene ID or nearest gene	Name	24h	54h	Induction		IDs #	EMSA	dre (cis)-element			
				MM	R5			Sequence 5'→3'	position "	score ^	
Primary metabolism											
SCO1390*-SCO1391* SCO1429* ^{54h}	<i>crr-ptsI</i>	+	+	+	+	8	+	tgtggtctagacctct	-130	17.14	
	<i>chiD</i>	+	+	-	+	4	+	actggtctagtcctcc aatggtccgaaccatt	-96 -118	12.17 7.45	
SCO1444*	<i>chil</i>	+	+	-	-	2	+	actggtctagtcctct attggtccatacctat	-53 -75	16.23 10.22	
SCO2503* ^{54h}	<i>chiJ</i>	+	+	-	+	4		aaaggtctggaccaca	-78	12.37	
								cttggctcagacctct	-99	10.78	
								tctggaccacagcact	-73	7.29	
SCO2833*	<i>chb</i>	+	+	+	+	6		acatgtccataccaaa gcaggtctagaccaag	-110 -70	9.34 8.67	
SCO2906	<i>nagF</i>	+	+	-	+	6		actggtctacaccagt atcggtctgcaccagt caaggtgtagacctct	-41 718 1287	17.52 8.37 12.27	
SCO2907	<i>nagE2</i>	+	+	-	+	4		acaggtctacaccact agtgggttagaccacc caaggtgtagacctct	-49 -32 -236	16.57 14.37 12.27	
SCO4284-SCO4285	<i>nagKA</i>	+	+	+	+	7	+	agaggtctagtccact ggtggtgtagacctta	-83 -63	16.64 9.64	
SCO5003-SCO5004* ^{54h}	<i>chiA</i>	+	+	+	+	4		ggtggtccagaccaat ggtggtccagaccaat	-77 -258	10.43 10.43	
SCO5230		+	+	+	+	5		tctggtctagtcctgg	-118	9.87	
SCO5231-5232*	<i>dasR</i>	+	+	+	+	5	+	cttggctagtccata tctggtctagtcctgg	-150 755	9.05 9.87	
	<i>dasA</i>						+	actggtctacaccatt cttggctagtccata	-106 -322	17.13 9.05	
SCO5236	<i>nagB</i>	+	-	-	+	3	+	tgtggttagaccaat	-68	17.51	
SCO5376	<i>chiC</i>	+	-	-	-	1		ataggtctggaccaat aaaggtctggaccata	-109 -88	11.92 9.23	
SCO5423	<i>pyk2</i>	-	-	+	-	4					
SCO5673*	<i>chiB</i>	+	+	+	+	7		attggtctggacaaa	-63	10.69	
SCO5841	<i>ptsH</i>	+	+	-	-	2	+	agtgtctagaccagt tctgtctagaccagt	-51 -66	18.11 16.25	
SCO6004-SCO6005* ^{54h}	<i>ngcE</i>	-	+	+	+	7		agtggactatacctgt agtggactatacctgt	-244 -334	16.05 16.05	
SCO6012-SCO6013	<i>chiH</i>	+	-	-	+	2	+	aatggctctggaccaga atgggactagaccaat	-111 -127	12 10.22	
								aatggctctggaccaga atgggactagaccaat	-274 -258	12 10.22	
SCO6032-33		+	+	-	+	4		cttggctagtccatt cttggctagtccatt	-154 -278	10 10	
SCO6300		+	-	-	+	2		ataggtctagacaaaa agaggtctagacaaaa	-131 -116	13.72 13.48	
SCO6344-45		+	-	-	+	2		taaggtctagacctgc gtaggtctagacctgc	-133 -153	9.6 8.13	
		<i>chi</i>	+	-	-	+	2	+	taaggtctagacctgc gtaggtctagacctgc	-114 -94	9.6 8.13
SCO7225* ^{54h}	<i>chi</i>	+	+	-	+	3	+	tcaggtctagacctgt cctgtctagaccaat tatggtctagacctga	-34 -168 -55	15.28 13.38 13.25	
SCO7335 ^c	<i>pep1B</i>	-	+	-	-	1					

Development and secondary metabolism										
SCO3230 ^c	<i>cdaPSI</i>	-	+	+	+	5				
SCO3231 ^{c,*54h}	<i>cdaPSII</i>	-	+	+	+	5				
SCO5085	<i>actII-4</i>	-	-	+	+	4	+	tgttgagtaggcctgt	-59	10.49
SCO5877	<i>redD</i>	-	-	+	+	4		actgctggagaccggt	612	7.15
SCO5881 ^c	<i>redZ</i>	-	-	+	+	4	+	agtgtttccacctca	-201	12.44
SCO6273 ^c	<i>cpkC</i>	-	+	+	-	4		acatgcgtaatcaact	-13	9.2
SCO6274 ^c	<i>cpkB</i>	-	+	+	-	4				
SCO6275 ^{c,*54h}	<i>cpkA</i>	-	+	-	-	1				
SCO1276	<i>sigJ</i>	-	+							
SCO1488-89 ^{*54h}	<i>pyrR</i>	-	+	-	+	5				
	<i>bldD</i>									
SCO3323 ^{*54h}	<i>bldN</i>	-	+	-	-	1				
Translation										
SCO2504-05	<i>glyS</i>	-	+	-	-	1		agtggctgcacctgg	515	10.75
SCO3092		+	+	-	+	5				
SCO3679		+	+	-	-	2		tgtgtctagccaat	-314	15.32
SCO4092		-	-	-	+	4				
SCO5239		+	+	-	-	2	+	agtggctagtcacaca	-335	16.23
SCO5550 ^d		+	+	+	+	8	+	actgggtctaaaccaca	-18	17.27
SCO4123 ^d		-	+	+	+	6				
SCO1792 ^d		-	+	+	+	6				
SCO1390		-	+	+	+	6				
SCO3019 ^d		-	+	+	+	6				
SCO5746		-	+	+	+	6				
SCO3334		-	+	+	+	6				
Transposases and DNA topology										
SCO0020 ^c		-	+	-	-	1				
SCO0098-SCO0099		-	-	+	-	2				
SCO0567		-	-	+	-	3				
SCO1471		-	-	+	-	1				
SCO1603		-	-	+	-	3				
SCO3466		-	-	+	+	5				
SCO3467		-	-	+	-	3				
SCO3468		-	+	-	-	1				
SCO3490 ^c		-	+	-	-	1		aatcgtaagacctgt	-118	8.75
SCO4344		-	+	-	-	1				
SCO4698 ^c		-	+	-	-	1				
SCO4699 ^c		-	+	+	+	4		agaggctgacaccggt	2130	9.17
SCO6400 ^c		-	+	+	+	6				
SCO6627 ^c	<i>pglX</i>	-	+	-	-	1		cacgggtgtagacatca	841	7.41
SCO6910-SCO6911		-	-	+	+	5				
SCO7798 ^c		-	+	-	-	1				
Other										
SCO1606		-	-	+	+	5				
SCO2078		-	+	+	+	6				
SCO3262 ^c		-	+	+	+	4		acaggaggacaccatt	-118	8.53
SCO5190	<i>wblC</i>	-	-	+	-	4				
SCO7056		+	+	+	+	3		attgggtctaaaccagc	-79	15.65
SCO4646	<i>secE</i>	-	-	-	+	4				
SCO4067	<i>dnaZ</i>	-	-	-	+	3				

* one probe; * 54 h one probe at 54 h; c coding; d downstream

Total nr of independent ChIP-chip experiments showing DasR binding

^ matrix score indicating the statistical relevance of the predicted *dre* element relative to the consensus sequence (only scores >7 were considered)

" position indicates spacing between the *dre* element and start of the gene

The nag/chi regulon and validation of the ChIP-on-chip data

Strong signals for DasR binding were found in the upstream regions of many genes that belong to the *nag* regulon, the *pts* genes and the chitinolytic system, which can be described as the core regulon for DasR, and coincide with bioinformatically predicted *dre* elements with generally very high scores (Table 1). These include the upstream regions for the *nag* metabolic genes *nagKA* and *nagB*, the *pts* genes *nagF*, *nagE2*, *ptsH*, *crr-ptsI*, and members of the chitinolytic system, namely *chiA*, *chiB*, *chiC*, *chiD*, *chiH*, *chiI*, *chiJ*, SCO6032 (for β -N-acetylhexosaminidase), SCO6300 (for a secreted β -N-acetyl-glucosaminidase), SCO2833, SCO6345, the SCO6005-6007 operon, *dasA* and *dasR* itself (Fig. 2A and Supplemental Fig. S2). It should be noted that *crr-ptsI*, *chiI*, *chiB*, SCO2833, the SCO6005-6007 operon promoter and *dasR* only had one probe significantly enriched, but most of these were also bound in the induction experiments (Table 1). Validation for the biological relevance of these ChIP-on-chip data is provided by the fact that direct binding of DasR to many genes of the *nag* and *chi* regulons was already demonstrated by electrophoretic shift assays (EMSAs), namely for *nagKA* and *nagB*, *crr*, *ptsI*, *ptsH*, *nagF* and *nagE2* and for the chitinase genes *chiD*, *chiH*, *chiI*, SCO6345 and SCO7225 (Colson *et al.*, 2007; Rigali *et al.*, 2006; Świątek *et al.*, 2012). Importantly, the genomic distribution of the observed DasR binding was different between the cultures harvested at 24 h and 54 h. All of the genes discussed above except SCO6005-6007 were bound by DasR at 24 h, but after 54 h no significant binding was found for *chiC*, *crr-ptsI*, *chiH*, SCO6300, SCO6345 and SCO7225. Conversely, binding to the promoter region of the SCO6005-6007 operon was observed at the later point.

Direct control of antibiotic production by DasR

DasR bound exclusively after 54 h to probes corresponding to genes involved in development and antibiotic production. Binding was observed to the intragenic regions of three biosynthetic genes (SCO6273-6275) of the *cpk* gene cluster for the cryptic type I polyketide synthase (Cpk) and of *cdaPSI* and *cdaPSII* (SCO3230 and SCO3231) encoding the peptide synthetases I and II for the synthesis of calcium-dependent antibiotic (CDA). Additionally, in liquid-grown cultures binding of DasR was detected in the promoter proximal region of *actII-ORF4* and *redD*, *redZ*, encoding the pathway-specific activator genes for Act and Red biosynthetic clusters, respectively (see Table 1,

Fig. 3 and below). *dre* elements were identified *in silico* for *actII*-ORF4, *redZ*, *cpkC*. These data candidate DasR as a pleiotropic regulator of antibiotic production.

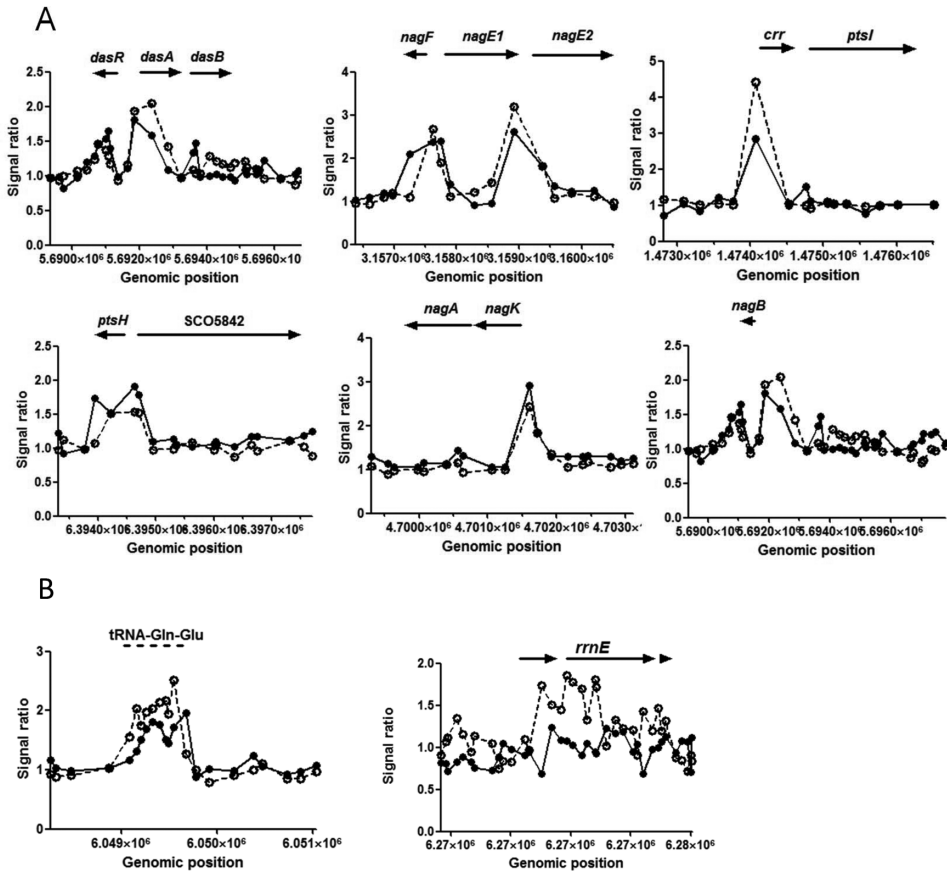


Figure 2. Selected DasR targets identified in ChIP-on-chip time-course experiments. Peaks indicate the presence of DasR binding sites in the regions near (A) the *dasR*, *dasABC*, *nag* and *pts* genes (B) the gene cluster specifying tRNA^{Gln/Glu} and the rRNA operon *rrnE*. Plots present DasR binding at 24 h (solid line, closed circles) and 54 h (dashed line, open circles). The arrows indicate the orientation of the target genes. Note that the y-axis (signal ratio) varies between the plots.

Genes for noncoding and stable RNAs

Interestingly, DasR binding was observed for many transfer-RNA (tRNA), ribosomal RNA (rRNA) and noncoding RNA (ncRNA) genes. As for ncRNAs, binding was observed to *dre* elements upstream of *scr3092* (Swiercz *et al.*, 2008) and *scr5239* (Vockenhuber & Suess, 2012) (Table 1). *scr5239* decreases the production of the

antibiotic actinorhodin, and represses expression of the extracellular agarase gene *dagA* (SCO3471) at the post-transcriptional level (Vockenhuber & Suess, 2012). Additionally, DasR likely controls the expression of several tRNA genes, as suggested by binding to multiple sequences associated with highly conserved *dre* elements - and in several independent ChIP-on-chip experiments - within the five-membered tRNA^{Gln/Glu} gene cluster, to a gene for tRNA^{Gly} upstream of SO3679, to tRNA^{Met} and to the tRNA^{Glu/Asp/Phe} gene cluster upstream of SCO4092. Additionally, after 54 h of growth on MM agar plates, DasR binding was observed upstream of SCO2504 (*glyS*) encoding a glycyl-tRNA synthetase (Table 1). Among the most highly enriched probes were those corresponding to the rRNA operons *rrnA*, *rrnB*, *rrnC*, *rrnD*, *rrnE* and *rrnF* (Table 1 and Fig. 2B), with many probes in the operons bound by DasR. The expression of genes specifying stable RNAs was not detected due to the design of the microarrays (biased for mRNA).

Other targets

Microarray analysis showed massive up-regulation of all genes in the cluster SCO3982-3988, which encode small secreted proteins of unknown function. This gene cluster is most likely directly controlled by DasR, because the GntR regulatory gene SCO3986, the likely regulator of this cluster, was identified as a DasR target in the ChIP-on-chip experiments, both in surface-grown cultures and in liquid-grown cultures (see Table S3 and below).

A surprising number of binding events were detected upstream of or inside transposase genes, namely SCO0020, SCO3468, SCO3469, SCO3490 (and many sites in the genomic region between SCO3468-3490 at 54 h), SCO4344, SCO4698, SCO6400, SCO7798. Several of these genes exist in multiple copies on the *S. coelicolor* genome. Additionally, other DNA recombination related gene such as *pglX* (SCO6627) encoding a putative adenine-specific DNA methyltransferase that is part of a phage integration system was identified as a DasR target. Another genomic region that is overrepresented in terms of the number of binding sites is that between SCO6373-6401 (11 binding sites after 54 h), but most were represented by a single probe and did not result in changes in expression as judged by the microarray data.

Visualization of GlcNAc-mediated induction of the DasR regulon *in vivo*

We previously showed that glucosamine-6P (GlcN-6P) acts as an effector molecule that prevents DasR DNA-binding *in vitro* (Rigali *et al.*, 2006). This and the identification of *dre* elements upstream of all genes for GlcNAc internalization and utilization and the control of antibiotic production, suggested a direct signalling cascade from external nutrient availability to the control of secondary metabolism (Rigali *et al.*, 2006). To visualise and manipulate this putative signalling cascade *in vivo*, we monitored the global response of the DasR regulon to the extracellular addition of GlcNAc. For this purpose, mycelia of *S. coelicolor* M145 harbouring pGAM29 and the *dasR* mutant were each grown in liquid NMMP mannitol or R5 cultures, and cells were induced by the addition of 50 mM GlcNAc. Samples were collected prior to (T_0) and 30 (T_1), 60 (T_2), or 120 min (T_3) after GlcNAc addition, and DasR-enriched chromatin was analysed as described in the Materials and Methods section.

The ChIP-on-chip experiments revealed 54 and 69 sites that were determined as bound by DasR with high statistical relevance in MM- and R5-grown mycelia prior to GlcNAc induction, respectively (Supplemental Table S3). In line with the experiment performed on mycelia from surface-grown cultures, at T_0 DasR was bound to sites belonging to its core regulon *pts*, *nag* and *chi* (Supplemental Fig. S3). Immediately after GlcNAc addition DasR dissociated from the target sites, providing direct evidence that indeed DasR responds to the addition of GlcNAc *in vivo*, thereby reducing its affinity for its target DNA as hypothesised previously (Rigali *et al.*, 2006). Binding was also observed for many transposase-related genes, with dissociation of DasR following addition of GlcNAc. This also connects to possible involvement of DasR in the control of genome rearrangement (see Discussion).

After the addition of GlcNAc, DasR dissociated from the target sites upstream of genes that are part of the deduced GlcNAc-dependent signalling cascade, namely (i) genes for GlcNAc transport and phosphorylation by the PTS (*crr-ptsI* and *nagE2*); (ii) upstream of the transcription unit that includes *nagA* which is necessary to provide the DasR effector GlcN-6P, and (iii) upstream of *actII-ORF4* and of *redD* and *redZ*, encoding the pathway-specific activator genes for Act and Red biosynthetic clusters, respectively (Fig. 3). Likewise, there was binding in the intragenic region of *cdaPSI* and *cdaPSII* (SCO3230-3231) for CDA peptide synthetases I and II, and for *cpkBC* (for Cpk

polyketide synthases; (Pawlik *et al.*, 2007)).

In MM-grown cultures virtually all DasR binding events were relieved following the addition of GlcNAc. In contrast, in nutrient-rich R5 cultures, 29 cases were found where DasR binding was not relieved by the addition of GlcNAc, 15 of which showing enhanced binding. Examples of constant binding include, besides *pyrR-bldD*, the upstream regions of the tRNA genes/operons tRNA^{Glu/Asp/Phe}, tRNA^{Gln/Glu} and tRNA^{Met}, and *secE* for the preprotein translocase subunit SecE. Examples of enhanced binding of DasR include the upstream regions of *dnaZ* for DNA polymerase II subunit gamma, of *cydA* for cytochrome oxidase subunit I, of the cell division-related gene SCO2078 and of the ncRNA scr3092.

Finally, DasR binding was also observed for *atrA*, and this binding is relieved by the addition of GlcNAc. AtrA is required for actinorhodin production via the direct activation of *actII-ORF4* (Uguru *et al.*, 2005), and also activates the GlcNAc transporter gene *nagE2*, while DasR does the opposite. In the DasR-mediated GlcNAc signalling cascade from nutrient to antibiotic production, internalization via the *nagE2* product is the first step, while control of pathway-specific regulators is the last step. Thus, AtrA and DasR antagonise each other in terms of their influence of this signalling cascade (Nothhaft *et al.*, 2010).

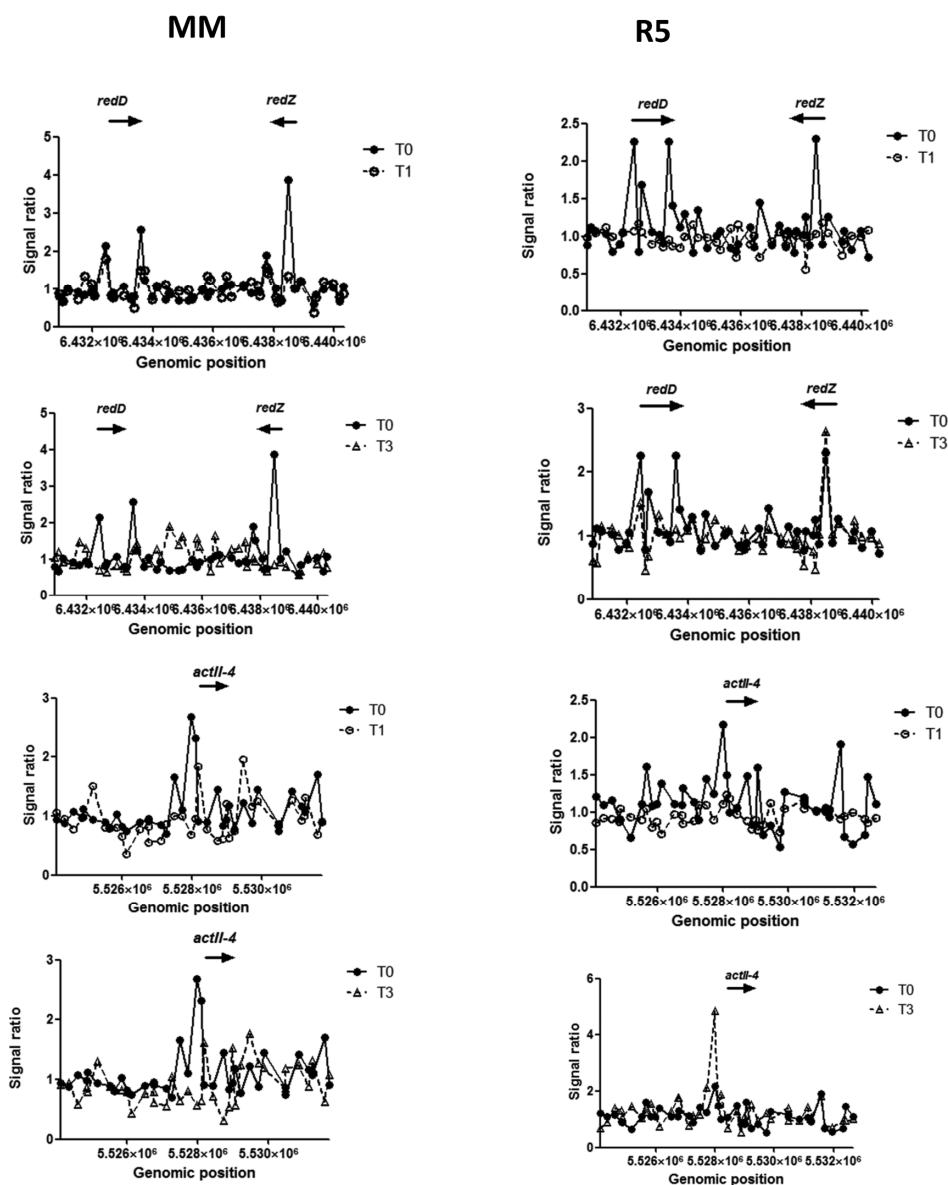


Figure 3. Chip-on-chip data for selected DasR targets detected in the GlcNAc induction experiment. Samples were collected prior to (T_0 , closed circles) and 30 (T_1 , open circles), 60 (T_2) or 120 min (T_3 , open triangle) after GlcNAc addition. Plots indicate change in affinity of DasR for the promoter regions of the pathway-specific activator genes for the biosynthesis of actinorhodin (*actII-ORF4*) and prodiginine (*redD*, *redZ*) upon addition of GlcNAc (25 mM) to liquid-grown MM (left panel) or R5 (right panel) cultures. Arrows indicate the orientation of the targeted genes. Note that the y-axis (signal ratio) varies between the plots.

A

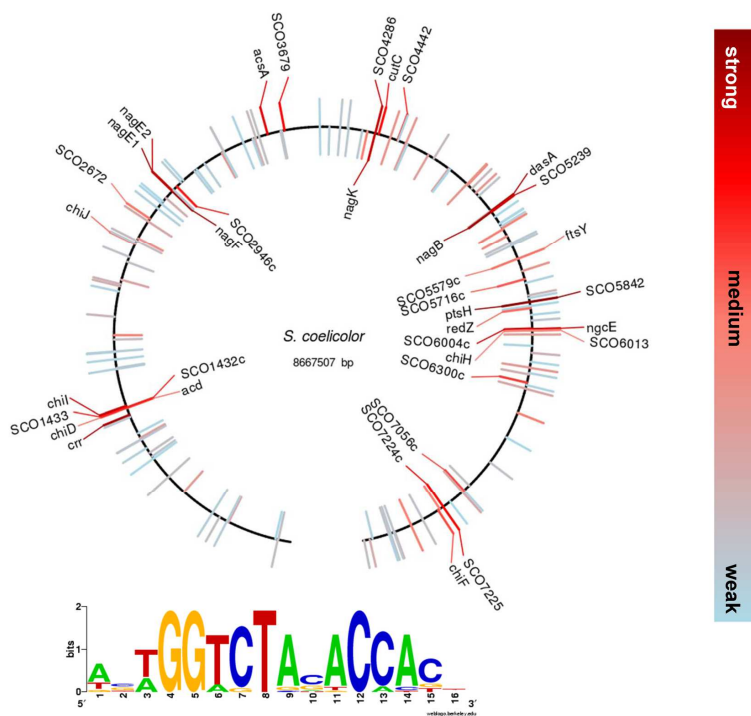
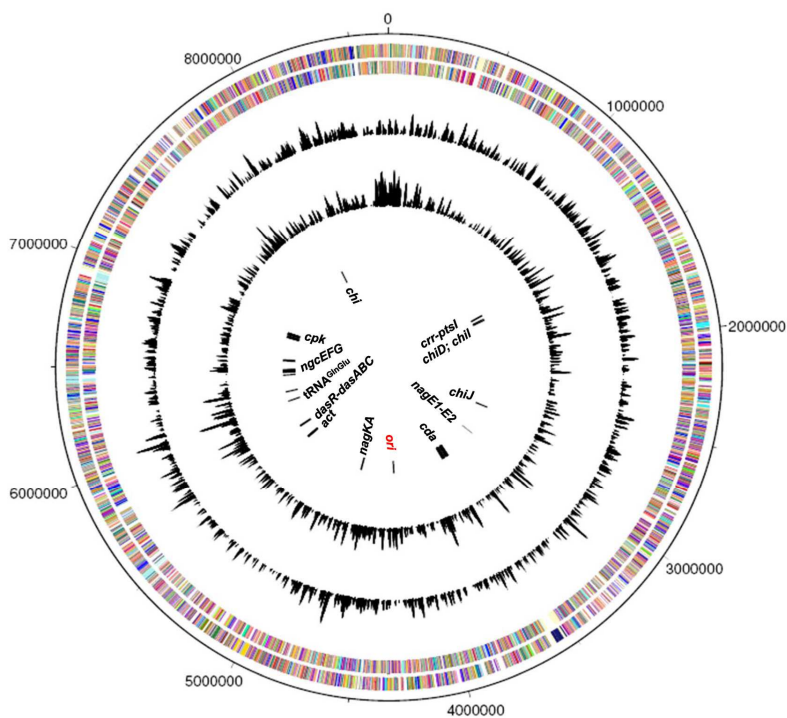
**B**

Figure 4. Circular representation of the DasR regulon. (A) Distribution of the DasR responsive elements (*dre*) along the *S. coelicolor* genome based on *in silico* analysis (Rigali *et al.*, 2008). The heat map indicates the matrix score (Hiard *et al.*, 2007) of each *dre* element relative to the consensus sequence. (B) Distribution of DasR-binding sites based on time-course ChIP-on-chip experiment. From the outside inwards, circles 1 and 2 show the position of genes transcribed in a clockwise and anticlockwise direction, respectively (for colour-coding see Table S4); circles 3 and 4 show distribution of the signal to noise ratios (log2 ratios of DasR_wt_Ab/NoAb vs DasR_mutant_Ab/NoAb) for all probes that passed filtering at 24 h and 54 h, respectively; circle 5 shows some key DasR target genes.

DISCUSSION

The DasR regulon consists of two classes of target genes

Streptomycetes usually live in an environmental context of poor nitrogen and rich carbon availability (Hodgson, 2000). In this respect, GlcNAc is a very particular energy source for these bacteria, as it can be used as both a nitrogen and a carbon source. We previously showed that DasR acts as a nutrient sensory protein that is required for development and antibiotic production under nutrient rich conditions, and that DasR binding is relieved *in vitro* by glucosamine-6P (Rigali *et al.*, 2006; Rigali *et al.*, 2008). *In silico* predictions indicate widespread distribution of the *cis*-acting DasR-responsive element (*dre*) with the consensus sequence A(G/C)TGGTCTAGACCA(G/C)T. The core of the DasR regulon is formed by genes related to amino sugar metabolism, namely the *nag* and *pts* genes for GlcNAc metabolism and transport, respectively, and the *chi* regulon involved in the catabolism of polymers of GlcNAc. Since Fru-6-P, acetate and ammonia are end products of GlcNAc catabolism, the DasR core regulon is immediately connected to major metabolic pathways such as glycolysis, nucleic acids, nitrogen, and fatty acid metabolism, the tricarboxylic acid (TCA) cycle and cell-wall biosynthesis.

Functional categories of genes that are associated with *dre* elements are besides those related to *N*-acetylglucosamine metabolism and transport and polysaccharide hydrolases, also several genes for ncRNAs and tRNAs. There is an apparently perfect correlation between the presence of a *dre* motif and DasR binding *in vitro* and *in vivo*, and indeed we have never seen a predicted *dre* with a high matrix score that is not bound by DasR by itself *in vitro*. However, many binding events were identified *in vivo* that are not associated with a canonical *dre* element (Table 1). Some of these were identified as

significantly enriched probes in as many as eight biologically independent ChIP-on-chip experiments, providing a very strong case that these are indeed *bona fide* binding sites. These are referred to as Class II targets. Genome-wide analysis of the regulons of several other globally acting bacterial transcription factors show similar duality, with binding to both their cognate (consensus) target sequences as to non-canonical sites *in vivo*, even though the latter binding sites show no similarity to the consensus sequence nor are they bound *in vitro*. Examples include LexA (Wade *et al.*, 2005) and FNR (Grainger *et al.*, 2007) in *E. coli* and CtrA in *Caulobacter crescentus* (Laub *et al.*, 2002). For *Bacillus subtilis* Spo0A, some 15% of the total number of binding sites was not bound *in vitro* (Molle *et al.*, 2003). AdpA of *Streptomyces griseus* is a recent example of a master regulator in streptomycetes with a significant portion of genes without recognisable binding site (Higo *et al.*, 2012). It is likely that binding by these regulators to non-canonical sites requires additional factors, such as cooperative interaction with other proteins and/or changes DNA conformation. An example of cooperative binding that is required for target recognition is that for CRP in *E. coli*, which binds to a noncanonical site in the *melAB* promoter in concert with MelR associated with adjacent target sites (Wade *et al.*, 2001).

Interestingly, the conventional Class I targets are generally bound better, or - in a stochastic binding model - more frequently than the unconventional Class II targets, as the most enriched probes in the ChIP-on-chip experiments were typically those for Class I targets. This is similar to the situation observed for LexA in *E. coli*, where unconventional targets were also not bound by LexA *in vitro*, and bound less tightly *in vivo* (Wade *et al.*, 2005). A proteomics-based approach is currently being employed to elucidate how DasR binds to Class II targets *in vivo*, with particular focus on identifying possible protein partners that bind cooperatively with DasR.

The DasR regulon and multi-level control of its target genes

The core regulon of the DasR homologue NagR in *Bacillus* also revolves around GlcNAc metabolism, but in contrast to *dre* elements, the NagR-responsive elements are found scarcely (up to three sites per genome; (Bertram *et al.*, 2011)). Bioinformatics analysis suggests that similar small DasR regulons are found in other low G+C Gram-positive bacteria such as *Streptococcus* and *Listeria* (not shown). Our work shows that

streptomycetes have adopted the GlcNAc regulatory system to build a much more intricate system that controls a wide range of genes. Indeed, with transcription of more than 1200 genes significantly differentially expressed in the *dasR* null mutant of *S. coelicolor* (i.e. 15% of the genome) and transcription of over 400 genes altered more than two-fold, DasR qualifies as a master regulator. The regulon incorporates more than 50 transcriptional regulatory genes, suggesting an extensive secondary response regulon. Global transcriptional regulators provide an additional layer of control on top of the control mediated by specific activators and/or repressors (Martinez-Antonio & Collado-Vides, 2003). Therefore, an ‘all or nothing’ change in transcription as has been observed for mutants lacking specific regulatory genes such as *gylR* (glycerol regulon), *malR* (maltose regulon), *redD* (activator for prodigine biosynthesis), or *actII-ORF4* (activating actinorhodin biosynthesis) was not anticipated for the *dasR* regulon. The specific environmental conditions are an important parameter for the response of the regulon, and additional control systems operate, such as substrate induction for induction of sugar utilization operons, or growth phase-dependent control of antibiotic production. The additional requirement of substrate induction is exemplified by the fact that despite DasR binding to almost all of the genes of the *chi* regulon, transcription of the genes was not notably changed in *dasR* mutant cells due to the lack of chitin as inducer.

Antibiotic production is extensively controlled, so as to ensure correct growth phase-dependent expression. Taking *actII-ORF4* as an example, this gene is controlled by some 10 transcriptional regulators. Besides DasR these include among others ActII-ORF4, AfsR, AtrA, SCO6008 and the stringent response mediated through ppGpp during growth cessation (Chakraborty & Bibb, 1997; Floriano & Bibb, 1996; Gramajo *et al.*, 1993; Uguru *et al.*, 2005; reviewed in van Wezel & McDowall, 2011). DasR directly controls the pathway-specific activator genes *actII-ORF4* and *redZ*, for Act and Red biosynthesis, respectively (Rigali *et al.*, 2008 and ChIP-on-chip data presented here), as well as genes for Cpk and CDA biosynthesis, and this control is relieved by the addition of GlcNAc. This again indicates that the nutrient sensor DasR act as a typical pleiotropic regulator, which adds higher order control of gene expression to allow streptomycetes to respond to changing nutritional conditions. The small changes in the microarray experiments are due to the choice of media, necessary to allow proper comparison between wild-type and mutant, which requires similar (and synchronized) growth and

development; however, the major changes under feast (rich media) and famine (MM agar) have been well documented (Rigali *et al.*, 2008).

***In vivo* response of DasR to the induction by GlcNAc**

We previously showed that glucosamine-6P (GlcN-6P) acts as an effector of DasR *in vitro* (Rigali *et al.*, 2006). This is not unexpected, as GlcN-6P stands at the crossroads of (GlcNAc)_n degradation, GlcNAc transport and intracellular metabolism, glycolysis, nitrogen and lipid metabolism, as well as peptidoglycan synthesis (Altermann & Klaenhammer, 2005; Mao *et al.*, 2005), and many genes of these pathways are subject to control by DasR. ChIP-on-chip analysis of MM- and R5-grown cultures before and after the addition of GlcNAc revealed the response of DasR to this inducer *in vivo*. The relief of DasR binding to its core regulon following the addition of GlcNAc to cultures provides *in vivo* evidence that the DasR regulon is indeed triggered by GlcNAc-derived metabolites. In terms of antibiotic production, the same relief of binding was seen for the pathway-specific activator genes *redD* and *redZ* for prodiginine production, and *actII-ORF4* and *atrA* for actinorhodin production, as well as biosynthetic genes for the cryptic polyketide Cpk and the calcium-dependent antibiotic CDA. This suggests that the activation of antibiotic production by GlcNAc is directly mediated through DasR in *S. coelicolor*.

DasR and translational control

An important control system that has hitherto not been identified in streptomycetes but which must almost inevitably be in place is that of the control of ribosomal proteins and stable RNA operons (specifying tRNA and rRNA). In *E. coli*, stable RNA promoters are regulated by the trans-acting proteins FIS (Factor for Inversion Stimulation), which activates transcription during logarithmic growth (Nilsson *et al.*, 1990) and LRP which - assisted by H-NS - forms a repressive nucleoprotein structure (Pul *et al.*, 2007). The mechanisms for ribosome feedback regulation and growth rate-dependent control of stable RNA synthesis in streptomycetes are poorly understood. The ChIP-on-chip data demonstrate binding of DasR to at least five tRNA genes or operons when *S. coelicolor* was grown in liquid R5 cultures. This binding was significantly enhanced after addition

of GlcNAc. Strong binding of DasR was observed to a *dre* element with one of the highest matrix scores upstream of the third gene of a five-members tRNA operon consisting of three identical genes specifying tRNA-Glu for the CUC codon and two specifying tRNA-Gln for the CUG codon. By leaving the first two tRNA genes (for tRNA-Gln and tRNA-Glu, respectively) untouched but controlling the expression of the other (identical) copies, DasR likely controls the abundance of these tRNAs. Many independent ChIP-on-chip experiments revealed DasR binding to many probes inside the rRNA (*rrn*) operons, suggesting that DasR may also control the expression of rRNAs (Table 1 and Table S3).

Besides stable RNAs, DasR also controls the expression of non-coding RNAs. In at least two independent ChIP-on-chip experiments DasR binding was associated with the upstream regions of the ncRNAs *scr3092* and *scr5239*. The latter is preceded by a highly conserved *dre* element (Table 1), and deletion of *dasR* results in a four-fold increase of expression of *scr5239* (not shown). The current generation of high-density *S. coelicolor* microarrays is primarily directed at identifying mRNAs, but better insight in the location of ncRNAs will soon be obtained from RNA seq experiments, so that we can obtain better insight into how many are controlled by DasR, and how.

Microarray analysis further revealed that the expression of nearly all the ribosomal protein genes was affected in the *dasR* mutant. Since with the exception of *rpmG* these genes were not identified as direct targets, these changes in expression either reflect control by a DasR-dependent regulator, or general differences in growth between the parental strain and its *dasR* null mutant. The possible role of DasR in the control of stable RNA and perhaps also ribosomal protein genes requires further investigation.

Connection of major developmental regulons

The phenotype of the *dasR* null mutant as well as extracellular complementation assays strongly suggests it is blocked in a very early stage of the life cycle, and *dasR* is probably one of the *bld* mutants acting earliest in the life cycle (Rigali *et al.*, 2006; Rigali *et al.*, 2008). Our data indicate that DasR exerts this early developmental control by targeting the expression of a number of key regulatory genes for the control of early developmental processes, including *bldKc*, *bldM*, *bldN*, *wblA*, *wblC* and *crp*. Of these,

bldKc, *bldN* and *wblC* were identified as direct targets by ChIP-on-chip analysis, forming Class II targets as none of them is preceded by a *dre* element. In turn, *bldN* gene product σ^{bldN} is required for transcription of the *chp*, *rdl* and *ramS* genes for the surfactants chaplins, rodmins and SapB, respectively (Bibb *et al.*, 2012). The increase in *bldN* transcription corresponds to an increase in *chp*, *rdl* and *ramS* expression during sporulation, but at earlier time points all genes were strongly down-regulated. The DasR-controlled regulatory gene responsible for this phenomenon has not yet been identified.

The *bldD* gene also emerged as a possible direct target for DasR, with binding to the *bldD* promoter region verified independently in samples obtained after 54 h of growth on MM agar plates as well as from liquid-grown R5 cultures, although no *dre* element could be identified upstream of *bldD*. The transcription of *bldD* was not changed in the *dasR* deletion mutant, but the sporulation block of most *bld* mutants is by-passed on minimal media with mannitol, suggesting their function is limited under these conditions. Considering the scale at which DasR and BldD control gene expression in streptomycetes, there is surprisingly little overlap between the two regulatory networks. Regulators of later sporulation events do not feature among the direct DasR targets, while, conversely, the primary BldD regulon contains a plethora of such developmental regulatory genes (den Hengst *et al.*, 2010), suggesting that these two proteins act in different time domains. The level of cross-talk or hierarchy between these two important regulatory networks deserves closer investigation.

In conclusion, our works establishes DasR as a master regulator that links nutritional control to antibiotic production and development (Fig. 5), and the exogenous addition of GlcNAc relieves binding by DasR to many of its binding sites. This supports the notion that accumulation of GlcNAc around *Streptomyces* colonies affects development via the direct interference with binding of the nutrient sensor DasR. The canonical binding of DasR to Class I targets is observed both *in vivo* and *in vitro*, while the unconventional binding to Class II targets presumably requires cooperative binding with another protein, which has hitherto not been identified. Alternatively, these sites may require conformational changes in the DNA, or even a combination of the two. DasR mediates a higher order level of control, and relieve or induction of binding to its target sites does not necessarily result in an immediate change in gene expression. For that, the environmental conditions are a very important determining factor - such as the presence

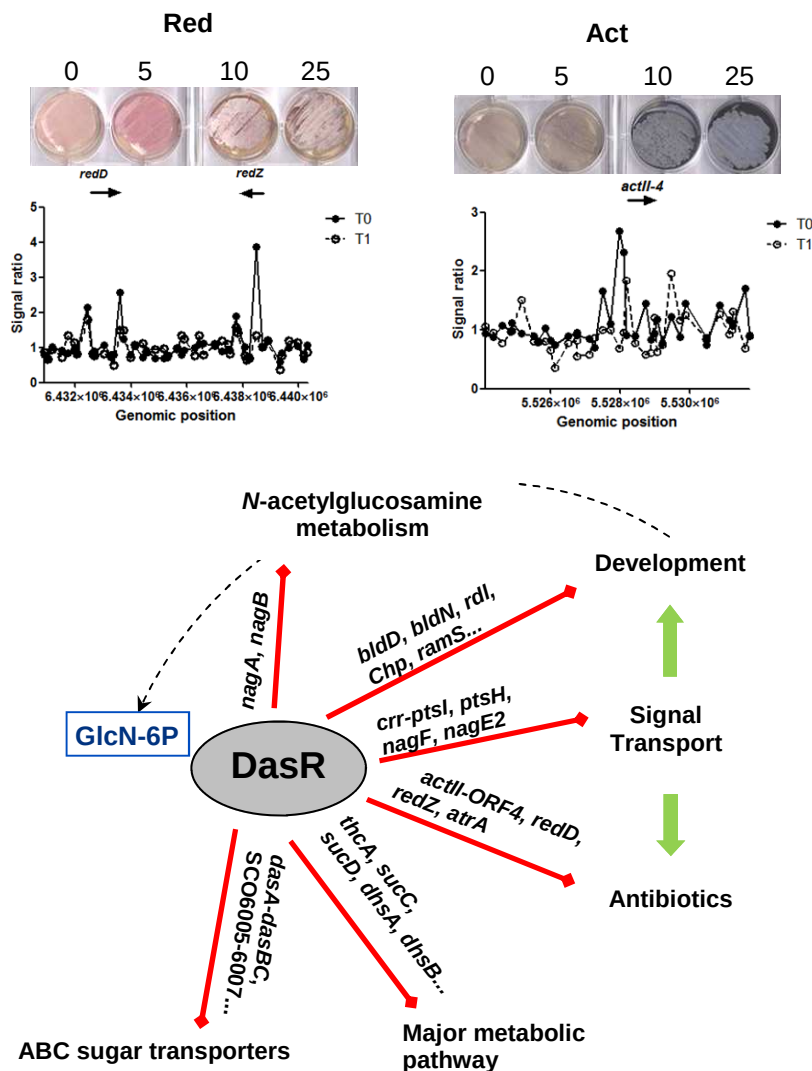


Figure 5. Model of the DasR regulon and its induction by N-acetylglucosamine. **Top:** induction of development (grey pigment) and Red (left; red pigment) or Act (right; blue pigment) production on MM agar plates with increasing concentrations of N-acetylglucosamine; the *S. coelicolor actII-ORF4* (M510) and *redD* (M511) mutants (Floriano & Bibb, 1996) were used to demonstrate the production of only the red-pigmented undecylprodigiosin or the blue-pigmented actinorhodin, respectively. **Middle,** relief of DasR binding *in vivo* after the addition of GlcNAc, as shown by ChIP-on-chip (refer to Fig. 3). T₀ (closed circles) and T₁ (open circles), time points before and 30 min after the addition of 25 mM GlcNAc; respectively. **Bottom,** summary of the DasR-controlled processes and major components of its regulon. Red lines indicate direct binding and transcriptional repression of the targeted genes by DasR. Depression of the DasR regulon in response to the accumulation of GlcN-6P results in activation (green lines) of among others development and antibiotic production.

of chitin for induction of *chi* gene expression - and further experimental data need to be obtained from genome-wide studies under different growth conditions. For example, in approaches to wake up sleeping antibiotic genes, relieve of DasR may well be an important step, but better understanding of the triggers that must be in place to effectively activate their biosynthetic gene clusters should aid in their discovery and exploitation.

Acknowledgements

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Chapter VI

Characterization of *rok* sugar regulatory genes in *Streptomyces coelicolor* and pleiotropic role of *rok7B7* in the carbon source-dependent control of development and antibiotic production

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Manuscript in preparation

ABSTRACT

Members of the ROK protein family include transcriptional regulators and kinases that relate to the control of sugar metabolism. In the search of new pleiotropic regulators, we performed *in silico* analysis of seven ROK proteins of *S. coelicolor* A3(2), followed by mutational and phenotypic analysis. Deletion of the transcriptional regulatory genes *rok7B7* (SCO6008) and *rok8G12* (SCO7543) revealed pleiotropic changes in growth and development, including delayed antibiotic production and morphological differentiation. Microarray analysis revealed that over 700 genes were differentially expressed in the *rok7B7* mutant. Expression of the xylose transport operon *xylFGH* which lies downstream of *rok7B7* was strongly up-regulated, while that of the upstream located transport operon SCO6005-6007 was not affected. The *rok7B7* mutant also displayed enhanced carbon catabolite repression on MM with xylose, maltose, and glucosamine. Transcriptional studies show up-regulation of several developmental targets in the *rok7B7* mutant, including *sigJ* and *sigF* genes, which encode sigma factors involved in the control of later stages of differentiation, and genes for the chaplin, rodlin and SapB surfactant proteins. Thus, we present novel pleiotropically acting transcriptional regulators that link the control of primary metabolism to that of secondary metabolism and morphogenesis in *Streptomyces*.

INTRODUCTION

Streptomyces are high G+C soil bacteria, well known as the major producers of antibiotics and natural products. Under nutrient limited conditions they undergo complex morphological changes, which results in formation of aerial mycelium and chains of spores. The developmental programme coincides with production of antibiotics (Hopwood, 1999). Numerous *Streptomyces* genomes have now been sequenced, the first of which was *Streptomyces coelicolor* A3(2), the model organism for the genus (Bentley *et al.*, 2002). The *S. coelicolor* genome contains around 7825 genes and more than 20 secondary metabolite gene clusters. Over 12% of the genome encodes predicted regulatory proteins, underlining the complexity of the regulatory networks that govern the transcriptional control of cell differentiation, secondary metabolites production and signalling cascades.

Carbon source regulation is a major factor in the control of morphological differentiation and secondary metabolism (recently reviewed in Sanchez *et al.*, 2010; van Wezel and McDowall, 2011; van Wezel *et al.*, 2009). Carbon catabolite repression (CCR) allows bacteria to utilise a preferred carbon source (such as glucose) when they are exposed to more than one carbohydrate. In Gram-negative bacteria and low-G+C Gram-positive bacteria such as *Escherichia coli* and *Bacillus subtilis*, the phosphoenolpyruvate-dependent phosphotransferase system (PTS) plays a major role in carbon regulation (Brückner and Titgemeyer, 2002; Deutscher, 2008). During PTS-mediated carbon source internalization, a phosphoryl group is transferred from the glycolytic intermediate phosphoenolpyruvate, via the PTS enzymes phosphotransferase enzyme I (EI), the histidine phosphor-carrier protein (HPr), and Enzyme IIA (EIIA) onto the incoming carbon source. Most PTS-mediated CCR mechanisms respond to the phosphorylation state of the PTS components, which is controlled by nutritional conditions and metabolic state of the cell. The PTS-mediated transport triggers the cAMP-receptor protein (Crp) in Gram-negative bacteria, and the catabolite control protein CcpA in low-GC Gram-positive bacteria (Brückner and Titgemeyer, 2002). In *Streptomyces*, glucose transport is not mediated via the PTS, but via the MFS transporter GlcP (van Wezel *et al.*, 2005), and in terms of global regulation streptomycetes lack a CcpA orthologue, while Crp is present but does not play a role in CCR (Piette *et al.*, 2005). Therefore, an alternative control mechanism must be in place for CCR.

One of these mechanisms revolves around glucose kinase, mutation of which results in loss of catabolite repression (Angell *et al.*, 1994; Angell *et al.*, 1992). Glucose kinase is a member of the ROK family of regulatory proteins (Repressors, QRFs and Kinases). The members of this family possess a highly conserved helix-turn-helix DNA binding domain (HTH), an ATP-binding motif (Walker A motif) as well as a cysteine-rich region with the consensus sequence CnCGnnCGnE. In *E. coli* two proteins belonging to this family, NagC and Mlc, are involved in regulation of GlcNAc and glucose-PTS, respectively (Plumbridge, 1995; Nam *et al.*, 2001). An interesting observation was made for the *rok*-family gene *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). Sequencing of the *S. coelicolor* genome revealed that it possesses 25 genes encoding ROK-family proteins, of which eight contain a DNA binding motif.

In this study we perform mutational analysis of seven of the *rok* genes of *S. coelicolor* in search of new pleiotropic regulatory genes that may play a role in carbon-dependent control of antibiotic production. Deletion of either ROK7B7 (SCO6008) or ROK8G12 (SCO7543) resulted in major changes in antibiotic production and morphological differentiation under many growth conditions. Detailed phenotypic and microarray analysis of the *rok7B7* null mutant established a relationship between ROK7B7 and CCR, depending on the nutritional status of the environment.

MATERIAL AND METHODS

Bacterial strains and growth conditions

Bacterial strains and plasmids are listed in Table 1 and Supplemental Table S1, respectively. *Escherichia coli* JM109 (Sambrook *et al.*, 1989) and ET12567 (Kieser *et al.*, 2000) were used for routine cloning procedures and for extracting non-methylated DNA, respectively. *Streptomyces coelicolor* A3(2) M145 was the parent for all mutants described in this work. All media and routine *Streptomyces* techniques are described in the *Streptomyces* manual (Kieser *et al.*, 2000). Cells of *E. coli* were grown in Luria–Bertani broth (LB) at 37°C. Phenotypic characterization of *Streptomyces* mutants was carried out on minimal media (MM) and on R2YE- (-glucose) agar plates (Kieser *et al.*, 2000) with 1% of carbon sources as indicated. SFM (soy flour mannitol) agar plates were used to prepare spore suspensions.

Table 1. Strains of *Streptomyces coelicolor* A3(2) described in this study

Strain	Genotype	Reference
M145	prototrophic SCP1 ⁺ SCP2 ⁺	Kieser <i>et al.</i> , 2000
GAM21	M145 SCO0794::aacC4, Apr ^R	This work
GAM22	M145 SCO1060::aacC4, Apr ^R	This work
GAM23	M145 SCO2846::aacC4, Apr ^R	This work
GAM24	M145 SCO6008::aacC4, Apr ^R	This work
GAM25	M145 SCO6566::aacC4, Apr ^R	This work
GAM26	M145 SCO6600::aacC4, Apr ^R	This work
GAM27	M145 SCO7543::aacC4, Apr ^R	This work
GAM30	M145 ΔSCO0794 ^D	This work
GAM31	M145 ΔSCO1060 ^D	This work
GAM32	M145 ΔSCO2846 ^D	This work
GAM33	M145 ΔSCO6008 ^D	This work
GAM34	M145 ΔSCO6566 ^D	This work
GAM35	M145 ΔSCO6600 ^D	This work
GAM36	M145 ΔSCO7543 ^D	This work

Plasmids and constructs

All constructs described in this work are listed in Supplemental Table S1 and oligonucleotides in Supplemental Table S2.

Constructs for gene-replacement and in-frame deletion mutants

The strategy for creating knock-out mutants is based on the unstable multi-copy vector

pWHM3 (Vara *et al.*, 1989) as described previously (van Wezel *et al.*, 2005). For each knock-out construct roughly 1.5 kb of upstream and downstream region of the respective genes were amplified by PCR from the *S. coelicolor* M145 genome and inserted into pWHM3 (for the exact location of the oligonucleotides see Table S2). Fragments were cloned into pWHM3, and the engineered *Xba*I site was used for insertion of the apramycin resistance cassette *aacC4* flanked by *loxP* sites between the flanking regions. The presence of the *loxP* recognition sites allows the efficient removal of the apramycin resistance cassette following the introduction of a plasmid pUWLcre expressing the Cre recombinase (Fedoryshyn *et al.*, 2008; Khodakaramian *et al.*, 2006). We constructed the knock-out plasmids pGAM8 to pGAM14 for the single gene replacement of SCO0794, SCO1060, SCO2846, SCO6008, SCO6566, SCO6600, SCO7543, respectively. To analyse correctness of the mutants, PCRs were done on mycelium from liquid-grown MM cultures, with oligonucleotide pairs as given in Table S2.

Antibiotic assays

Prior to chemical extraction of actinorhodin (Act) and prodiginines (Red) samples were frozen overnight at -80°C. After defrosting, samples were subjected to extraction with 90% methanol pH2.0 (2 h, shaking). The extracts were analyzed by non-quantitative colorimetric assay (red color corresponds to either Red or mixture of Act and Red, while blue color either to γ -Act or mixture of γ -Act and Red). To measure the Act production the extracted samples were alkalized by adding KOH (blue color - mixture of mostly γ -Act and Act; green - mixture of Act and Red; yellow/orange - mostly Red).

RNA isolation and DNA microarray analysis and validation

For transcript analysis, total RNA was isolated from surface-grown mycelia of *S. coelicolor* M145 and its *rok7B7* null mutant, cultivated on MM agar plates with mannitol (1% w/v) as the sole carbon source (plates were covered with cellophane discs). Two independent biological replicate experiments were performed. Samples were taken at 14h (early vegetative growth), 24 h (vegetative growth), 30 h (early aerial growth), 36 h (aerial growth), 42 h (early sporulation) and 54 h (sporulation). Total RNA was purified using the Kirby-mix protocol (Kieser *et al.*, 2000). DNaseI treatment was used to fully remove any traces of DNA. Before use, the RNA samples were checked for integrity on

an Agilent 2100 Bioanalyzer (Agilent Technologies). cDNA preparation and labeling was performed as previously described (Bucca *et al.*, 1997; Bucca *et al.*, 2009). The filtered data sets were analyzed using rank products analysis (Breitling *et al.*, 2004) via the Rank-Prod package in R (version 2.5.0) (Hong *et al.*, 2006). Differentially expressed genes were identified as demonstrating a >1.5 fold change in expression between the parental strain M145 (wild-type) and the *rok7B7* mutant. To verify the output from the microarray analysis, semi-quantitative RT-PCR analysis was carried out as described previously (Birko *et al.*, 2009). For each RT-PCR reaction 200ng of RNA was used (concentration was assessed using a Nanodrop® spectrophotometer). Oligonucleotides used for RT-PCR are presented in Supplemental Table S2.

RESULTS

The *rok*-family genes in *S. coelicolor*

In total 25 genes encoding proteins with a ROK-family signature are found on the *S. coelicolor* genome (Bentley *et al.*, 2002). These include glucose kinase (SCO2126), which catalyses the phosphorylation of glucose and plays a key role in carbon catabolite repression (Angell *et al.*, 1992), and NagK (SCO4285), the *N*-acetylglucosamine kinase (Świątek *et al.*, 2012). A phylogram is presented in Figure 1. Introduction of *rep*, a *rok*-family regulatory gene from an uncultured actinomycete, accelerated sporulation and enhances antibiotic production in *S. lividans* (Martinez *et al.*, 2005). BlastP analysis of the ROK-family proteins of *S. coelicolor* proteins with the Rep protein sequence as input revealed that SCO6008 is the nearest orthologue of Rep in *S. coelicolor*, with 48% amino acid identity, while the next strongest hits show 30-35% amino acid identity (Table 2). However, the gene synteny of *rep* does not correspond to that of SCO6008, nor to any of the other *rok* genes. To analyse the possible role of the ROK-family proteins of *S. coelicolor* with a NagC-type DNA binding domain, we performed a mutational analysis of seven *rok* genes, including the three with highest similarity to *rep*. The selected genes were SCO0794, SCO1060, SCO2846, SCO6008, SCO6566, SCO6600 and SCO7543, and according to the nomenclature suggested previously (Nothaft, 2003), these were designated *rokF43*, *rokG22*, *rokE20*, *rok7B7*, *rok4B5*, *rok8A6* and *rok8G12*, respectively; the numbers refer to the respective cosmids of the ordered *S. coelicolor* cosmid library (Redenbach *et al.*, 1996) on which the genes were located, namely SCF43, SCG22, SCE20, SC7B7, SC3F9, SC8A6 and SC8G12.

Gene context and synteny for the *rok* genes

The genetic context of the *rok* genes studied in this work is presented in Figure 2. Suggestively, five out of seven genes are located in the direct proximity of one or more ABC transporter systems. *rok7B7* (SCO6008) is highly conserved in streptomycetes and is flanked by two sugar transport operons, namely SCO6005-6007 (upstream) and the xylose transporter operon *xylFGH* (SCO6009-6011; downstream), with its gene synteny highly conserved in streptomycetes. *Rok7B7* shares 92% amino acid identity with *NgcR* of *Streptomyces olivaceoviridis*, which controls the upstream-located *N*-

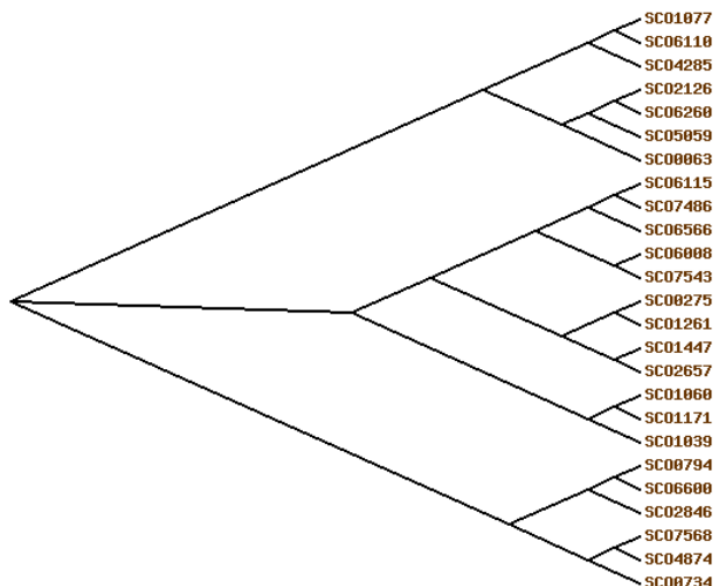


Figure 1. Phylogeny of the predicted ROK family proteins of *S. coelicolor* M145. The phylogram was based on ClustalW analysis on the Genebee website (<http://www.genebee.msu.su/clustal/basic.html>). ROK proteins/genes studied in this work are marked by asterisks.

Table 2. ROK regulators

Protein	Gene	length[aa]	% of amino acid [aa] identity to NagC of <i>E. coli</i>	% of amino acid [aa] identity to Rep protein, obtained from DNA soil library
ROKF43	SCO0794	429	28%	28%
ROKG22	SCO1060	413	27%	35%
ROKE20	SCO2846	406	29%	29%
ROK7B7	SCO6008	403	30%	48%
ROK3F9	SCO6566	584	28%	33%
ROK8A6	SCO6600	441	26%	30%
ROK8G12	SCO7543	424	27%	34%

The amino acid identities of *S. coelicolor* A3(2) ROK proteins to NagC of *E. coli* and Rep protein, isolated from the soil DNA library are presented. Protein lengths [aa] are listed in, the aa identities are denoted in [%].

acetylglucosamine and chitobiose ABC transporter operon *ngcEFG* (Xiao *et al.*, 2002). However, in *S. coelicolor* N-acetylglucosamine is transported via the PTS, with NagE2 and NagF as the membrane components EIIC and EIIB, respectively (Nothaft *et al.*, 2010), while chitobiose is transported via DasABC (Colson *et al.*, 2008). Since SCO6005-6007 also shows limited sequence similarity to NgcEFG (around 35% aa identity), it likely transports a molecule other than N-acetylglucosamine. Interestingly, *rok3F9* (SCO6566) is also connected to the utilization of C5 sugars. The gene is located

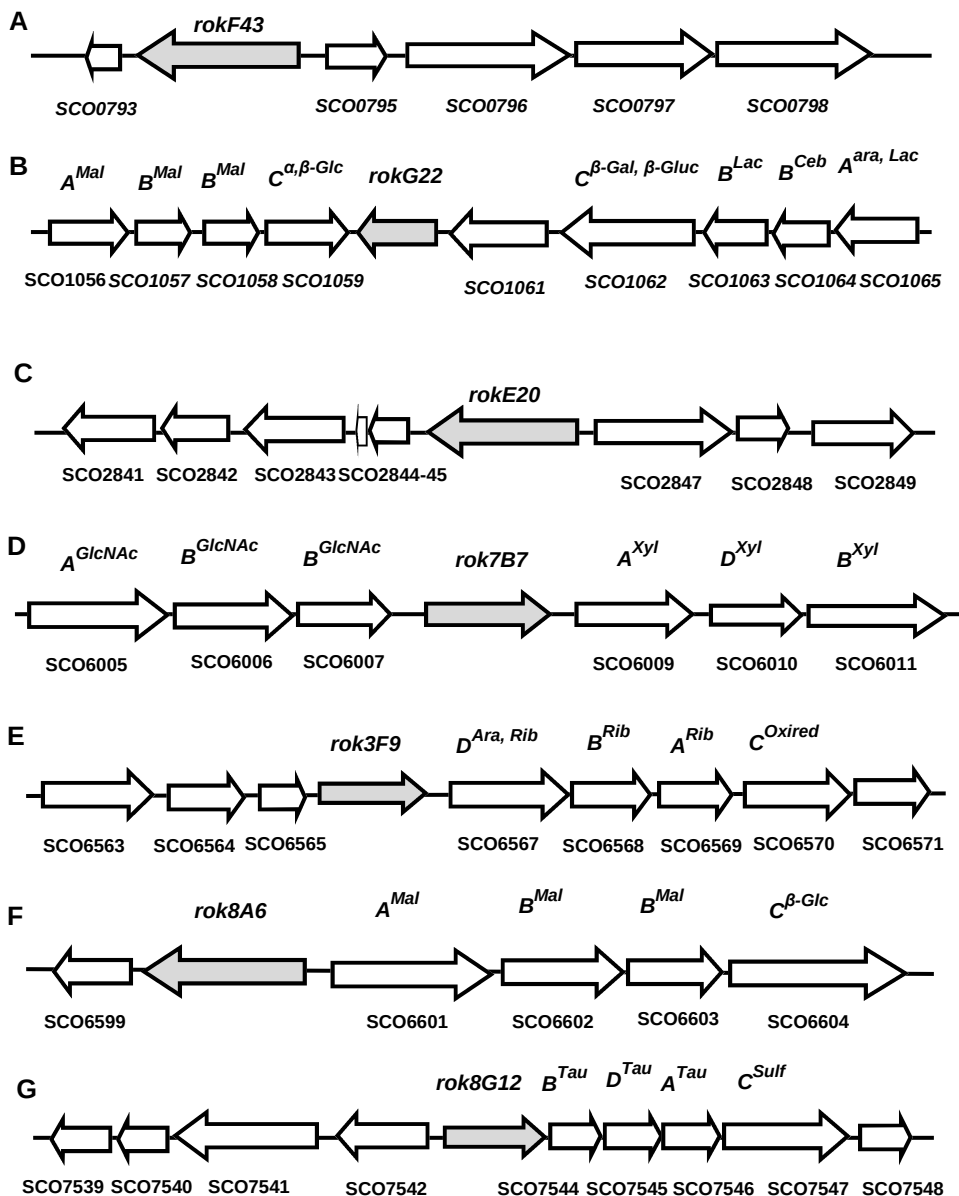


Figure 2. Genomic environment of the *rok* genes studied in this work. (A) *rokF43*, (B) *rokG22*, (C) *rokE20*, (D) *rok7B7*, (E) *rok3F9*, (F) *rok8A6*, (G) *rok8G12*. ORFs are indicated by open arrows. Genes encoding substrate-binding proteins, membrane proteins, ATPases and catabolic enzymes are marked with suffixes A, B, C and D, respectively. The known or predicted substrates of the ABC transporter are indicated. Abbreviations: Mal – maltose; α,β-Glu – α,β-glucosidase; β-Gal – β-galactosidase; β-Glc – β-glucosidase; Lac – lactose; Ceb – cellobiose; Ara – arabinose; GlcNAc- N-acetylglucosamine; Xyl – xylose; Rib – ribose; Oxired – oxidoreductase; Tau – taurine; Sulf – sulfonate.

upstream of a likely ribose ABC transporter operon, which shows strong phylogenetic linkage to a possible second likely ribose transporter operon (SCO4886-4888) and two nucleotide-related enzymes, namely a cytidine deaminase (SCO4889) and thymidine phosphorylase DeoA (SCO4890).

The gene synteny of *rokE20* (SCO2846) is also well conserved in streptomyces, and considering the small intergenic region (66 nt) its promoter most likely overlaps that of the divergently transcribed operon SCO2847-SCO2848, encoding an NAD(P) dependent oxidoreductase and a protein of unknown function, respectively, which also occur in firmicutes and proteobacteria. *rokE20* lies close to a gene for a hydrolase (SCO2843) with some similarity to *nagA* for GlcNAc-6P deacetylase; however, we previously demonstrated that SCO4284 encodes NagA (Świątek *et al.*, 2012), and the function of SCO2843 is unknown. *rok8A6* (SCO6600) is divergently transcribed from sugar transport operon SCO6601-SCO6604 involved in glucoside transport (Bertram *et al.*, 2004), with the gene products of SCO6601 (sugar binding protein), SCO6602 and SCO6603 (membrane permease) showing 28.9%, 32.6% and 36.7% amino acid identity to the maltose transporter MalEFG of *S. coelicolor* (van Wezel *et al.*, 1997a), while SCO6604 encodes a β -glucosidase.

rok8G12 (SCO7543) is also located next to transport operons, namely SCO7539-SCO7542 and SCO7544-SCO7548. SCO7547-SCO7544 encode a likely sulfonate ABC transporter with similarity (37.5%, 43.4% and 23.3% protein identity, respectively) to TauABC of *E. coli* (van der Ploeg *et al.*, 2001), while SCO7548 is similar to TauD, an alpha-ketoglutarate-dependent dioxygenase that preferentially liberates sulfite from taurine (2-aminoethanesulfonic acid). Interestingly, the system in *S. coelicolor* has an unusual reversed gene order, which corresponds to *tauDCBA*.

The gene synteny for *rokF43* (SCO0794) and *rokG22* (SCO1060) is not well conserved in other streptomyces. In the direct environment of *rokG22* are two sugar transport operons, for a likely galactose utilization operon (SCO1062-1065) and a second transport operon (SCO1056-1059) with similarity to the maltose operon *malEFG* (SCO2231-2234). *rokF43* lies upstream of a gene for a predicted fibronectin binding protein.

Phenotypic characterization of the *rok* mutants and effect on antibiotic production and aerial mycelium formation

As a first step towards the characterization of the *rok* genes, null mutants were constructed. For this, we first replaced the entire coding region of each gene of interest by the apramycin cassette (*aacC4*) flanked by *loxP* sequences. For each experiment five transformants exhibiting the desired double-crossover phenotype (Apra^R Thio^S) were selected and verified by PCR as described in the Materials and Methods section. Following removal of the *aacC4* cassette by the Cre recombinase, deletion (D) mutants were obtained and designed GAM30 (Δ SCO0794), GAM31 (Δ SCO1060), GAM32 (Δ SCO2846), GAM33 (Δ SCO6008), GAM34 (Δ SCO6566), GAM35 (Δ SCO6600) and GAM36 (Δ SCO7543). For all experiments both the apramycin resistant and the deletion mutants were analyzed. Considering that there were no phenotypic differences between them in terms of antibiotic production and development, from this point onwards we will only describe the results obtained for the deletion mutants unless stated otherwise.

The phenotype of all mutants was tested on MM and R2YE agar plates supplemented with glycerol, mannitol, arabinose, ribose, xylose, fructose, glucose, galactose, mannose, cellobiose, lactose, maltose, sucrose, trehalose, glucosamine (GlcN) or *N*-acetylglucosamine (GlcNAc) as the sole carbon source, while taurine was tested as a sulphur source for the *rok8G12* null mutant (Figure 3). The phenotypes of the tested mutants were similar to that of the parental strain in terms of colony morphology when grown on R2YE⁻ or MM agar plates supplemented with fructose, glucose, sucrose, trehalose and taurine. Additionally no difference between strains and the wild type was seen on MM agar plates supplemented with mannitol, cellobiose or maltose, and on R2YE supplemented with GlcNAc.

Interestingly, actinorhodin production and development of all strains (including the parent M145) were reduced when grown on MM or R2YE agar plates containing the C5 carbon source ribose as the sole carbon source. Similar effects were also observed for another pentose arabinose on MM agar plates. In fact, arabinose is a C'-2 carbon epimer of ribose. Ribose also repressed Red production. Carbon-source dependence of antibiotic production is widely reported in literature (reviewed in Sanchez *et al.*, 2010; van Wezel and McDowall, 2011), but the effect of C5 sugars is not widely acknowledged. Cellobiose also had a general repressing effect on development and antibiotic production.

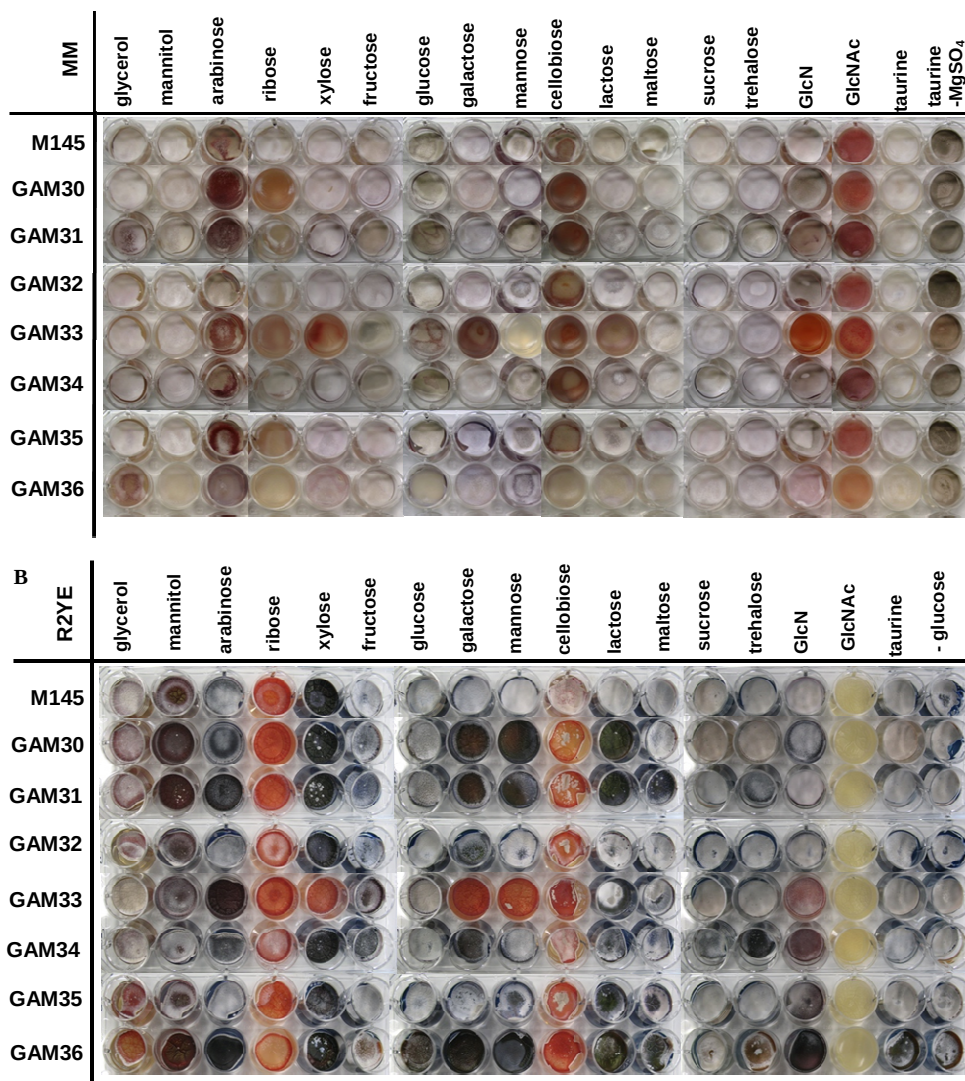


Figure 3. Phenotypic analysis of the *rok* mutants. Phenotypes of *S. coelicolor* M145 and its *rokF43*, *rokG22*, *rokE20*, *rok7B7*, *rok3F9*, *rok8A6*, *rok8G12* mutants (designated GAM30 to GAM36, respectively) were tested on (A) MM and (B) R2YE agar plates supplemented with 1% (w/v) of different carbon sources as indicated.

The most pleiotropic changes were observed in the *rok7B7* (GAM33) and *rok8G12* (GAM36) mutants (Figure 3). *In silico* predictions suggested that ROK7B7 might control GlcNAc and/or xylose utilization. Growth on MM and R2YE agar medium supplemented with GlcNAc did not show significant differences in morphology between M145 and the *rok7B7* deletion mutant. This observation supports the fact that GlcNAc uptake is mediated by the PTS (Nothaft *et al.*, 2010), which is under control of DasR

(Rigali *et al.*, 2006). The *rok7B7* mutant GAM33 showed delayed development and actinorhodin production on R2YE with xylose, galactose, mannose, cellobiose or GlcN (Figure 3B). However, after four days of growth the mutant showed enhanced sporulation and Act production in the presence of xylose (not shown). Furthermore, development of the *rok7B7* mutant was delayed on MM with arabinose, and blocked on MM with ribose, xylose, galactose, mannose, cellobiose, lactose or GlcN (Figure 3A).

The *rok8G12* null mutant GAM36 showed strong delay in development when grown on MM or R2YE, and on various carbon sources (Figure 3). Remaining strains also showed some differences in morphology and antibiotic production when compared to the wild type under other growth conditions and are presented in Figure 3. The effect of multiple sugars on development of M145 and the studied mutants again highlights the complexity of carbon regulation in *Streptomyces*.

Colorimetric determination of the Act and Red production

The ability of the multiple *rok* mutants to produce the pigmented antibiotics prodiginine (Red) and actinorhodin (Act) was further analysed by colorimetric assay (see Materials and Methods section). Samples were extracted from cultures grown on MM or R2YE agar plates supplemented with various sugar sources after five days of growth. Act functions as a pH indicator, and is red-pigmented at low pH, while blue at high pH. The lactone form of actinorhodin, γ -actinorhodin, is blue regardless of the pH (Bystrykh *et al.*, 1996). Prodiginine is red-pigmented at low pH and yellow at high pH (Rudd and Hopwood, 1980).

The isolated pigments were either mixtures of γ -Act and Act (blue), of Act and Red (green) or only Red (yellow/orange) (Supplemental Figure S1 and Table 3). Only small differences in antibiotic production were found between the strains when grown on MM agar plates (Figure S1A and Table 3). In line with its function in the control of ACT production (Park *et al.*, 2009 and this work), the *rok7B7* mutant GAM33 showed most changes (Figure S1A and Table 3). Act was also overproduced by GAM30 on arabinose and GAM 31 on trehalose, while Red by GAM35 and GAM36 on GlcNAc (Figure S1A and Table 3).

More significant differences were observed when the strains were grown on R2YE agar plates. In agreement with previous observations (Rigali *et al.*, 2008), GlcNAc

Table 3. Non-quantitative colorimetric assay for ACT and RED production in the *rok* mutants.

MM	M145	GAM30	GAM31	GAM32	GAM33	GAM34	GAM35	GAM36
		Δ 0794	Δ 1060	Δ 2846	Δ 6008	Δ 6566	Δ 6600	Δ 7543
glycerol	-							
mannitol	-							
arabinose	-	++			+			+
ribose	-							
xylose	-							
fructose	-							
glucose								
galactose								
mannose					+-			
cellobiose	-	+			++			
lactose	-							
maltose	-							
sucrose	-							
trehalose			++					
GlcN	-			+	++			+
GlcNAc							++	+

R5	M145	GAM30	GAM31	GAM32	GAM33	GAM34	GAM35	GAM36
		Δ 0794	Δ 1060	Δ 2846	Δ 6008	Δ 6566	Δ 6600	Δ 7543
glycerol			++					
mannitol	-	++						++
arabinose						++		
ribose						++		
xylose								
fructose								
glucose								
galactose		++						++
mannose								
cellobiose	-			++	++			++
lactose								++
maltose								
sucrose	-	++			++			
trehalose		++			++			
GlcN		++	++		++			
GlcNAc	-	-	-	-	-	-	-	-

Colour coding: light blue – Act; dark blue - γ -Act; violet – Act and γ -Act; red – Red; green – Act and Red; white, Act could not be distinguished from Red. ‘+(+)’ (strongly) enhanced antibiotic production; ‘+-’ decreased antibiotic production; ‘-’ low production of antibiotics or none.

blocked antibiotic production of all strains (Supplemental Figure S1B). Interestingly, in the presence of glucosamine, the wild-type strain produced both Act and Red, while three of the mutants (GAM30, GAM31, GAM33) showed enhanced Red production (Table 3, Supplemental Figure S1B). Increase in Act and Red production relative to the parental

strain M145 was observed for most mutants under one or more growth conditions (Figure S1B and Table 3). However, red production was mainly enhanced in GAM30, GAM33 and GAM36, while Act production was found in all mutants under various growth conditions (for details see Figure S1B and Table 3).

Global transcription profiling of the *rok7B7* null mutant by microarray analysis

Considering the effect of deletion of *rok7B7* on antibiotic production, and that it is the closest relative of the antibiotic-inducing *rep*, DNA microarray analysis was performed. For this, *S. coelicolor* M145 and the *rok7B7* mutant were grown on MM mannitol agar plates overlaid with cellophane discs and RNA was harvested after 14h (early vegetative growth), 24 h (corresponding to vegetative growth), 30 h (vegetative/aerial growth), 36 h (aerial growth), 42 h (aerial growth/sporulation) and 54 h (sporulation).

The transcription of over 700 genes were significantly (more than 1.5-fold) up- or down-regulated at one or more time points in the *rok7B7* mutant; of these, some 160 genes showed more than 2-fold and 68 genes > 2.5-fold change in transcription (Table 4, Supplemental Table S3). Classes of genes that were overrepresented were those related to (1) primary metabolism (sugar transport and metabolism, branched-chain amino acids biosynthesis and degradation); (2) protein biosynthesis; (3) secondary metabolism (carotenoid biosynthesis) (4) control of development.

Table 4. Number of genes more than 1.50 fold higher or lower expressed in the *rok7B7* mutant GAM33 as compared to the parent M145.

	1.50-2.00 fold		2.00-2.50 fold		2.50-3.00 fold		> 3.00 fold		total	
	up	down	up	down	up	down	up	down	up	down
14h	41	6	3	3	0	0	4	0	48	9
24h	41	8	1	2	0	0	3	0	45	10
30h	4	7	0	1	0	0	3	0	7	8
36h	49	82	5	6	2	1	3	0	59	89
42h	113	153	14	50	5	16	1	11	133	230
54h	151	91	29	12	17	1	18	0	215	104
total	368	333	51	72	24	17	22	7	426	424

Primary metabolism and protein biosynthesis

rok7B7 is flanked by two sugar transporter operons, which may be a direct target of its control. Indeed, deletion of *rok7B7* results in strong up-regulation of all three genes of the *xylFGH* operon in several time points (Figure 4A). In strong support of these transcriptional data, proteomics experiments showed that at the protein level components

of the XylFGH transporter are about 10-fold increased in the *rok7B7* mutant as compared to the parental strain M145 (J. Gubbens and G.P. van Wezel, manuscript in preparation).

glcP1 and *glcP2*, encoding identical glucose permease proteins, were also up-regulated (Figure 4A), although from previous work it is anticipated that the expression is mainly derived from *glcP1* (van Wezel *et al.*, 2005). However, the genes for glucose kinase and for the glycolytic enzymes were not changed significantly. At 36h up-regulation of *dagA* (SCO3471), which encodes the extracellular agarase precursor was observed (Figure 4A). Expression of *dagA* is subjected to carbon catabolite repression and is strongly repressed by glucose and other repressing carbon sources (Angell *et al.*, 1994; Kwakman and Postma, 1994; van Wezel *et al.*, 1997b). The role of Rok7B7 in the control of CCR is analysed further below.

Response of nitrogen-related genes suggested starvation for nitrogen and/or amino acids in the mutant. *glnK* (for a regulator of nitrogen metabolism), *glnA* and *glnII* (for glutamine synthetases) and *amtB* (for the ammonium transporter), were upregulated after 36 h, as were genes for the biosynthesis of branched amino acids (*ilvC2*, *ilvN*), while, conversely, genes of branched chain amino acid degradation (*acdH2*, *accD1*, *hmgL*, *acdH*, SCO6027) were down-regulated in the *rok7B7* mutant (Figure 4A). In *Streptomyces* branched-chain amino acids serve as precursors for branched-chain fatty acids and polyketide antibiotic synthesis (Denoya *et al.*, 1995; Potter and Baumberg, 1996; Stirrett *et al.*, 2009).

Surprising is the strong and global deregulation of protein biosynthetic genes, in particular almost all genes for ribosomal proteins, as well as genes for elongation factors EF-G, EF-P, EF-Ts and EF-Tu1, as well as the chaperonin GroES. Notably, transcription of the genes belonging to the larger ribosomal protein operons S10 (SCO4701-4711), L14 (*spc*; SCO4712-4722), S12 (*str*; SCO4659-4662) and S13 (SCO4727-4730) responded in the same way to the deletion of *rok7B7*, with down-regulation during late aerial growth (42 h), while the genes were up-regulated during sporulation (54 h). No major changes were found at earlier time-points, which correspond to vegetative and early aerial growth (Supplemental Table S3).

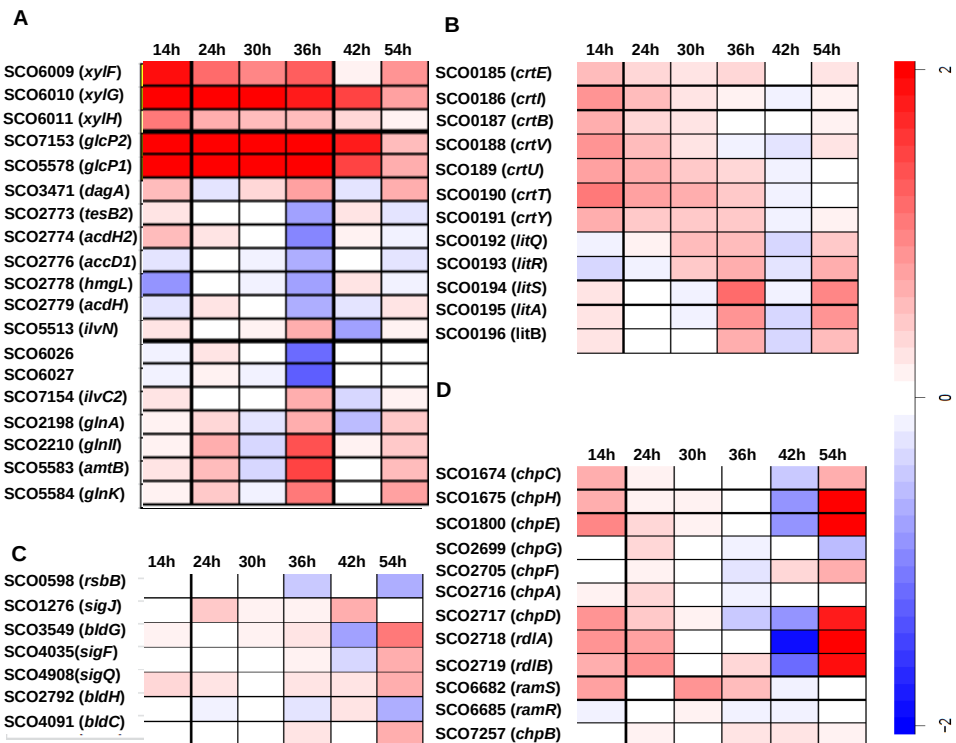


Figure 4. Selected classes of genes differentially expressed between the *rok7B7* null mutant GAM33 and its parent *S. coelicolor* M145. RNA was isolated from *S. coelicolor* M145 or its *rok7B7* mutant GAM33 after 14 h (early vegetative growth), 24 h (vegetative growth), 30 h (vegetative/aerial growth), 36 h (aerial growth), 42 h (aerial growth/sporulation) and 54 h (sporulation) of growth on MM agar plates supplemented with 1% mannitol. From the list of genes whose expression was increased or decreased more than 1.5 fold in GAM33 relative to M145, four classes of genes are shown, encoding (A) proteins related to sugar and branched-chain amino acid metabolism and transport; (B) proteins carotenoid biosynthesis, (C) proteins related to development, (D) SapB, chaplins, and rodlin. The heat map indicates the fold changes.

Control of carotenoid biosynthesis

The overall expression of antibiotics under the chosen growth conditions is typically very low, and indeed we could hardly detect the production of pigmented antibiotics. Still, some antibiotic-related genes were down-regulated, including *redZ* (54 h), which encodes the response regulator involved in the control of Red production and *actIORF1* (42 h) and *actIORF3* (42 h) encoding actinorhodin biosynthetic enzymes (Supplemental Table S3).

Interestingly, transcription of the carotenoid biosynthesis gene cluster consisting of two convergent operons, *crtEIBV* and *crtYTU*, was up-regulated at 14 h (early vegetative growth, Figure 4B). The *crt* gene cluster is flanked by a regulatory region that consist of two divergent operons, *litRQ* and *litSAB* (*lit*, light-induced transcription), where *litS* encodes σ^{LitS} , which acts as a light-induced sigma factor that directs transcription of the *crt* biosynthetic cluster (Takano *et al.*, 2005b). While the changes in the expression of a regulatory region were not detected at 14 h, strong up-regulation in the *rok7B7* mutant was observed at 36 h (aerial growth) and 54 h (sporulation) (Figure 4B).

Control of developmental genes

In terms of the control of developmental genes, there was striking overlap between the hits for the *dasR* mutant (Chapter V) and the *rok7B7* mutant (see General Discussion). Like seen also for the *dasR* null mutant, over 50 regulatory genes, including many genes for sigma factors and anti-sigma factor antagonists, were differentially expressed in the *rok7B7* mutant, with most changes detected during late aerial mycelium formation and sporulation (Supplemental Table S3). Genes for σ^{F} and σ^{J} , which are involved in the control of later stages of differentiation, were up-regulated during development (Figure 4C). The *bldG* gene, which encodes the anti-sigma factor antagonist BldG, which is required for the onset of development (Bignell *et al.*, 2000), was also differentially expressed during sporulation (Figure 4C). Additionally *rspB* and *adpA* (*bldH*) were down-regulated at 54 h, while *sigQ* and *bldC* were up-regulated at this time-point (Figure 4C). RsbB is the anti-anti-sigma factor for osmotic stress and differentiation sigma factor σ^{B} , AdpA is involved in the control of early stage of aerial mycelium formation (Wolański *et al.*, 2011), BldC is required for the onset of development (Hunt *et al.*, 2005), and σ^{Q} together with AfsQ form a pleiotropic but medium-dependent signal transduction system for the control of secondary metabolism and morphological development (Shu *et al.*, 2009).

The *chpABCDEFGH*, *rdlAB*, and *ramS* genes, encoding the surfactant proteins chaplins, rodmins and the spore-associated protein SapB, respectively, were also differentially expressed in the mutant, mostly down-regulated at 42 h and enhanced at 54 h (Figure 4D).

Finally, again similarly to the *dasR* null mutant, surprisingly many genes encoding putative transposases and DNA recombinases were differentially expressed in the *rok7B7* mutant, and most of these were up-regulated after 42 h.

Validation of microarray data by RT-PCR

To independently verify the expression patterns of the microarray experiment, semi-quantitative RT-PCR was performed on the same RNA as the microarray experiment, using oligonucleotide pairs for a selection of targets (*xylF*, *actII-ORF4*, *cpkC*, *redW*) with *rpsI* as the control (Figure 5). The relative transcript of *xylF* corresponded well to the results obtained from the microarray analysis, showing strong up-regulation in the *rok7B7* mutant. Surprisingly, while the changes in the expression of the *actII-ORF4*,

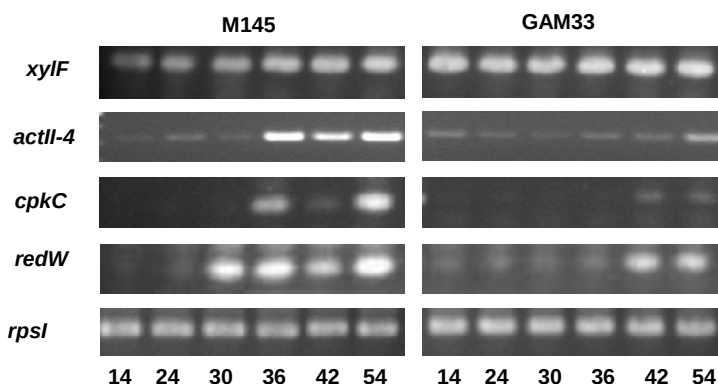


Figure 5. Validation of microarray data by semi-quantitative RT-PCR. RT-PCR was performed on RNA isolated from the parental strain M145 and the *rok7B7* mutant, using oligonucleotide pairs for a selection of targets (*xylF*, *actII-orf4*, *redW*, *cpkC*). *rpsI* (for ribosomal protein S9) was used as a control.

cpkC, *redW*, *nagE2* genes in the microarray experiment were not detected, the RT-PCR results showed that those genes were down-regulated in the *rok7B7* mutant. Down-regulation of antibiotic related genes (*actII-ORF4*, *cpkC*, *redW*) supports the idea that ROK7B7 acts as an activator of antibiotic production.

ROK7B7 and catabolite repression

Considering the up-regulation of the agarase gene *dagA* as apparent from the microarray analysis, we analysed the role of ROK7B7 in carbon catabolite control. Therefore, M145 and its *rok7B7* mutant GAM33 were compared on solid media containing either glucose

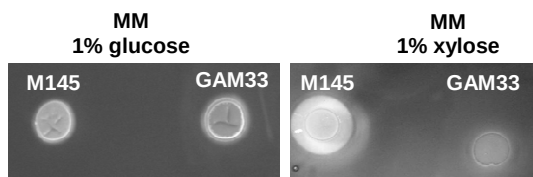


Figure 6. Effects of glucose and xylose on agarase production by M145 and GAM33 ($\Delta rok7B7$). 4 μ l of spores of either strain (10^9 cfu/ml) was spotted onto MM plates and agarase production was detected as a zone of clearing (for details see van Wezel *et al*, 1997b).

or xylose as the sole carbon source. On MM agar plates with glucose, neither strain produced significant amounts of agarase due to efficient glucose repression (Figure 6). Excitingly, on MM-xylose agar plates agarase production was also completely repressed in the *rok7B7* mutant, while no repression was observed for the parental strain M145 (Figure 6 and Table 5). The *rok7B7* mutant also showed enhanced CCR on MM agar plates with maltose or glucosamine as the sole carbon source, although not as strong as with xylose (Table 5). On R2YE, CCR was strongly enhanced on all carbon sources in the *rok7B7* mutant, with complete block of agarase production on R2YE with GlcNAc. These data suggest that ROK7B7 acts as a global regulator of CCR.

Table 5. Carbon catabolite repression measured as the zone of agarase clearing outside the colonies of *S. coelicolor* M145 and its *rok7B7* null mutant.

MM	M145			$\Delta rok7B7$			fold [^]
	agarase	total*	ratio#	agarase	total	ratio#	
Agar	6	26	0.23	6	26	0.23	1
Mannitol	5	25	0.2	3.5	22	0.16	0.8
GlcN	5	23	0.22	3	19	0.16	0.73
Fructose	6	26	0.23	6	25	0.24	1.04
Maltose	6	26	0.23	5	26	0.19	0.83
GlcNAc	5	26	0.19	5	26	0.19	1
Glucose	2	17	0.12	2	14	0.14	1.21
Xylose	5	25	0.2	0	15	0	0
Chitin	6	26	0.23	6	27	0.22	0.96
R2YE	agarase	total*	ratio#	agarase	total	ratio#	fold [^]
	agarase	total*	ratio#	agarase	total	ratio#	fold [^]
Agar	5	22	0.23	1	15	0.07	0.29
Mannitol	5	22	0.23	1.5	16	0.09	0.41
GlcN	5	22	0.23	1.5	16	0.09	0.41
Fructose	5	23	0.22	2	17	0.12	0.54
Maltose	5	22	0.23	3	20	0.15	0.66
GlcNAc	2	17	0.12	0	14	0	0
Glucose	3	20	0.15	1.5	16	0.09	0.63
Xylose	5	25	0.2	2	17	0.12	0.59
Chitin	5	22	0.23	2	17	0.12	0.52

Agarase zones are indicated in mm and multiplied by two (zones were measured from magnified images to get higher accuracy). Abbreviations: * total: the radius of the agarase zone; # Ratio: length of agarase zone / combined length of agarase zone and colony; ^ Fold: ratio of $\Delta rok7B7$ / ratio of M145

DISCUSSION

The manipulation of pathway-specific regulatory genes in both native producers and heterologous hosts is an effective way to enhance antibiotic biosynthesis (Anzai *et al.*, 1987; Hung *et al.*, 2007; Malpartida *et al.*, 1990; Stratigopoulos *et al.*, 2004). One such study revealed the antibiotic enhancing activity of *rep*, a *rok*-family regulatory gene, which was obtained from a metagenomic library and strongly enhanced antibiotic production when introduced into *Streptomyces lividans* (Martinez *et al.*, 2005). In the present study, we performed an *in silico* and mutational analysis of seven *rok*-family regulatory genes, including those whose gene products show highest similarity to the Rep protein (Table 1).

Phenotypic analysis of the respective *rok* null mutants identified two possible pleiotropic regulatory genes, namely *rok7B7* and *rok8G12*. Based on *in silico* analysis we anticipated that ROK8G12 might be involved in the control of sulfonate metabolism. Surprisingly however, *rok8G12* null mutant GAM36 showed a strong delay in antibiotic production and aerial mycelium formation on almost all sugars tested, while it was not complemented by the addition of taurine as additional sulphur source. This suggests that the regulatory role of ROK8G12 is more complex than anticipated. Microarray experiments suggested that ROK7B7 may act as a repressor of the adjacent xylose operon *xylFGH* (SCO6009-6011), since expression of the operon was strongly enhanced in the mutant. However, recent proteomics data revealed the surprising enhanced expression of both ROK7B7 and XylFGH in *glk* null mutants (J. Gubbens and G.P. van Wezel, manuscript in preparation), which makes it less likely that ROK7B7 indeed represses *xylFGH*. In fact, the up-regulation of the xylose transporter operon may be an attempt to compensate for the absence of ROK7B7, perhaps because xylose-5P acts as an inducer molecule of ROK7B7 (see below). Furthermore, when the *rok7B7* mutant was grown on MM agar plates with xylose as the sole carbon source no extracellular agarase activity was detected, indicating that xylose is used so efficiently that it has become a favored carbon source, resulting in carbon catabolite repression (CCR). A similar observation was made for the control of maltose utilization by MalR in *S. coelicolor*, where *malR* deletion mutants showed complete catabolite control of agarase when maltose was used as the sole carbon source, while the wild-type strain hardly grows on

maltose (van Wezel *et al.*, 1997a). On R2YE agar media, CCR was more pronounced on all carbon sources, and a complete CCR was observed in presence of GlcNAc. These data also suggest that ROK7B7 may be a global regulator for CCR, which is at least in part due to enhanced expression of glucose kinase in this strain (J. Gubbens and G.P. van Wezel, manuscript in preparation). Excitingly, the glucose transporter gene *glcP* was strongly up-regulated in the *rok7B7* null mutant, suggesting direct control of glucose metabolism by ROK7B7. There are two identical *glcP* genes and these therefore can not be discriminated in microarray analysis (van Wezel *et al.*, 2005), so it is unclear if both paralogues are up-regulated. The membrane protein GlcP recruits Glk, and its release during sugar transport may be a key mechanism in the activation of CCR (van Wezel *et al.* 2007). The exact mechanism by which ROK7B7 controls CCR, as well as the role of xylose transport in this process, are currently under investigation.

Production of the blue-pigmented antibiotic actinorhodin was strongly delayed in the *rok7B7* mutant on R2YE agar plates. Using a DNA-affinity capture assay (DACA) ROK7B7 was shown previously to bind to the promoter regions of *actII*-ORF4 and *redD*, the pathway specific transcriptional regulators of Act and Red, respectively (Park *et al.*, 2009). Based on this and the phenotypic analysis presented here, we anticipate that ROK7B7 acts as an activator of the *act* cluster. However, microarray analysis did not reveal major changes in the expression of *actII*-ORF4 or other *act* genes, although RT-PCR did show reduced expression of *actII*-ORF4. This conflicting data may be explained by the very low overall expression of antibiotics under the tested growth conditions, which makes it difficult to analyse by microarray analysis. The general consensus from our data and those of others is therefore that ROK7B7 influences Act production by either (a) influencing metabolic pathways, which provide precursors for polyketide biosynthesis; (b) by directly controlling the promoter of *actII*-ORF4; or (c) interacting with a co-repressor. For the latter point, it is important to note that there is strong overlap between the targets identified by microarray analysis for the DasR (Chapter V) and ROK7B7 regulons. Around 400 differentially expressed genes were commonly found in both strains. Those were in particular genes belonging to the same classes, namely involved in primary and secondary metabolism, control of development and antibiotic production, protein biosynthesis, and DNA recombination (including many transposases).

This extensive functional overlap between DasR and ROK7B7, suggests that they act in concert to control a wide range of cellular processes (see General Discussion).

The microarray analysis also showed down-regulation of the degradation pathway for branched-chain amino acids (leucine, isoleucine and valine). This points at a role for ROK7B7 in precursor supply for antibiotic production, since the degradation products of these amino acids are precursors for biosynthesis of polyketides like actinorhodin (Stirrett *et al.*, 2009).

In this study we have also shown that the related C5 sugars arabinose and ribose act as strong suppressors of actinorhodin production in all of the tested strains. Whereas another C5 sugar, xylose, acts as the activator. Strong repression of antibiotic production was also detected in the presence of cellobiose. Carbon-source dependence of antibiotic production is widely reported in literature (reviewed in Sanchez *et al.*, 2010; van Wezel and McDowall, 2011). For instance, glucose depresses formation of aminoglycoside antibiotics (streptomycin, kanamycin, istamycin, neomycin, gentamicin), via repression of biosynthetic enzymes (Damain, 1989; Piepersberg W., 1997). One clue came from experiments in *Streptomyces lividans* that showed that glucose inhibits actinorhodin production by repressing the expression of *afsR2*, which encodes a global regulatory protein involved in the stimulation of secondary metabolite biosynthesis (Kim *et al.*, 2001). However, it is likely that the role of CCR in the control of antibiotic production is more global (Bibb, 2005; van Wezel and McDowall, 2011). Since cellobiose is a dimer of glucose (β -1,4), its strong repressing activity regarding antibiotic production may be explained by the accumulation of the glucose produced from its hydrolysis. The inhibitory effect of the C5 sugars ribose and arabinose on antibiotic production was already reported in *Streptomyces hygroscopicus* (Ilić *et al.*, 2010) and *Streptomyces olivocinereus* (Deianova *et al.*, 1988), but the mechanism of their repression remains unknown. To our knowledge there are no reports that exclusively study the stimulatory effect of xylose on antibiotic production. We have shown here that actinorhodin production was drastically delayed in the *rok7B7* mutant, when grown on media containing xylose. Therefore, we anticipate that xylose or its derivative (e.g. xylose-5P) acts as an effector molecule of ROK7B7, leading to its activation and linking nutrient-sensing machinery with antibiotic production and development.

To summarize, the phenotypic analysis of the *rok* mutants revealed pleiotropic effects on antibiotic production in *S. coelicolor*, which was strongly dependent on the carbon sources used. These proteins are potential targets for approaches towards the improvement of antibiotic production and again highlight the enormous complexity of the regulatory networks involved in the control of secondary metabolism.

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Chapter VII

Lack of A-factor production induces the expression of nutrient scavenging and stress-related proteins in *Streptomyces griseus*

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SUMMARY

The small γ -butyrolactone A-factor is an important autoregulatory signalling molecule for the soil-inhabiting streptomycetes. Starvation is a major trigger for development, and nutrients are provided by degradation of the vegetative mycelium via a process of programmed cell death, reusing proteins, nucleic acids and cell wall material. The A-factor regulon includes many extracellular hydrolases. Here we show via proteomic analysis that many nutrient scavenging and stress-related proteins are overexpressed in an A-factor nonproducing mutant (AFN) of *Streptomyces griseus* B2682. Transcript analysis shows that this is primarily due to differential transcription of the target genes during early development. The targets include proteins relating to nutrient stress, environmental stress and an orthologue of the *Bacillus* sporulation control protein Spo0M. The enhanced expression of these proteins underlines the stress that is generated by the absence of A-factor. Wild-type developmental gene expression is restored to AFN by the signalling protein Factor C, in line with our earlier observation that Factor C triggers A-factor production.

INTRODUCTION

Bacteria of the Gram-positive filamentous *Streptomyces* are a well-known model system for the study of prokaryotic multicellular differentiation. They have a complex mycelial life cycle starting with a vegetative mycelium that develops into aerial mycelium, which then produces chains of spores at the ends of the hyphae (Chater, 1993). The onset of development is triggered by nutritional signals (Rigali *et al.*, 2006) and temporally relates to the production of antibiotics and other secondary metabolites (Chater, 2006). Autoregulatory molecules play a key role in controlling both the onset of cellular differentiation and secondary metabolism. The best studied autoregulator is A-factor (2-isocapryloyl-3R-hydroxymethyl- γ -butyrolactone), a small microbial hormone-like molecule (243 Da) that induces both morphological and physiological differentiation in *Streptomyces griseus* (Horinouchi and Beppu, 1992; Horinouchi and Beppu, 1994). The γ -butyrolactone regulatory system is widespread in Streptomycetes. Virginiae butanolids control virginiamycin production in *Streptomyces virginiae* (Nakano *et al.* 1998) and SCB1 plays an important role in the control of actinorhodin and undecylprodigiosin biosynthesis and a cryptic, type I polyketide synthase (*cpk*) gene cluster in *Streptomyces coelicolor* (Takano *et al.*, 2001; Takano *et al.*, 2005). In *Streptomyces griseus* binding of A-factor to its cellular receptor ArpA derepresses expression of the transcriptional activator AdpA. While initially identified as the activator of streptomycin production through *strR*, this protein acts as a central switch, and the AdpA regulon includes several important positive regulators of development (*ssgA*, *amfR*, and *adsA* (*bldN*)) and secreted proteases, reviewed in (Ohnishi *et al.*, 2005; Horinouchi, 2007). A-factor deficient mutants are neither able to sporulate nor to produce antibiotics (streptomycin).

Another interesting autoregulator is the secreted signalling protein Factor C (Mw 34.555 KDa), originally isolated from the culture fluid of “*Streptomyces griseus* 45H” (Biró *et al.*, 1980) which was recently shown to be identical to a laboratory strain known as *Streptomyces flavofungini*, itself a member of the *Streptomyces albidoflavus* species group (Kiss *et al.*, 2008). The Factor C producer strain like *S. griseus* readily sporulates in submerged culture (Birkó *et al.*, 1999). Similarly to A-factor, Factor C also plays a key role in cellular communication and cytodifferentiation.

A-factor mutants fail to develop aerial hyphae and spores and are therefore

classified as bald mutants. Expression of *facC* from a low-copy plasmid in a spontaneous A-factor-deficient bald mutant of *S. griseus* NRRL B-2682 restored its A-factor production as well as aerial mycelium and spore formation on solid media (Birkó *et al.*, 2007). The wild-type strain itself does not produce Factor C, as shown by immunoblotting (Szeszák *et al.*, 1990) and by DNA hybridization studies (Biró *et al.*, 2000). Our previous results (Birkó *et al.*, 2007) indicate a connection between two highly divergent types of signalling molecules and possible interplay between their regulatory networks. In preliminary experiments we observed characteristic differences between the extracellular proteomes of the strains that prompted detailed further analysis, facilitated by the currently available DNA sequence of the genome of *S. griseus* IFO13350 (Ohnishi *et al.*, 2008). Here we showed that the bald AFN mutant overexpressed several ABC transporter solute-binding proteins and stress response proteins compared to the wild type *S. griseus* B2682 strain or to the *facC* transformant of the AFN mutant in an effort to supply the cells with nutrients.

EXPERIMENTAL PROCEDURE

Strains and preparation of extracellular protein fractions

Strains of *Streptomyces griseus* were grown on R2YE agar plates (Kieser *et al.*, 2000) covered with a PCTE membrane (Poretics 0.2 µm pore size). The strains were *S. griseus* NRRL B-2682 (parental strain; in short B2682), its A-factor non-producing bald mutant *S. griseus* NRRL B-2682 AFN (AFN) and a transformant of AFN (designated AFN/pSGF4) that harbors *facC* on the pHJL401-based low-copy-number plasmid pSGF4 (Biró *et al.*, 2000). Protein extracts were prepared from spent agar of surface-grown cultures by crumbling the solid medium and passing it through a syringe with frits at 4 °C by centrifugation. Samples of approximately 300 µg protein (measured using the Coomassie Protein Assay Reagent, Pierce) were purified using the ReadyPrep™ 2-D Cleanup Kit (Bio-Rad) according to the instructor's manual, and dissolved in Rehydration Buffer (8M urea, 2% CHAPS, 50 mM dithiothreitol (DTT), 0.2% 100x Bio-Lyte 3/10 (or 4/7) ampholyte, 0.002% Bromophenol Blue).

2D gel electrophoresis and image analysis

Separation of protein extracts (approximately 300 µg) in the first dimension was performed by isoelectric focusing using 17 cm long Immobiline DryStripsGels (IPG) in the pH range of 3-10 or 4-7 (Bio-Rad) on a Protean IEF cell (Bio-Rad). Samples were focused at 250 V for 15 minutes followed by an increase to 8000 V over 2.5 h and kept at this voltage for 45000 V-h. Focused strips were separated on the basis of relative molecular weight in the second dimension on 13% SDS-polyacrylamide gels in a Protean II XL vertical gel system (Bio-Rad). For quantitative comparison of extracellular protein profiles gels were stained with colloidal Coomassie G-250 (Candiano *et al.*, 2004). Gels were scanned using a GS-800 Imaging Densitometer (Bio-Rad) and images were analyzed with PDQuestTM software (Bio-Rad). Histograms comparing spot quantity were generated with this software. Two-fold differences between the mutant and the parental B2682 were considered as significant changes. The data below are from a single representative experiment but at least two additional, biological replicas were performed and they showed similar results.

In-gel digestion

Gel slices containing 2D PAGE-separated proteins were cut and diced and then washed with 25 mM NH₄HCO₃ in 50% (v/v) acetonitrile/water. After reduction with 20 ml 10 mM DTT (30 min at 56 °C) and alkylation with 25 ml 55mM iodoacetamide (30 min at room temperature, in the dark) the proteins were digested with side-chain-protected porcine trypsin (Promega, Madison, WI, U.S.A.) at 37 °C for 4 h. Tryptic digests were extracted into 1%FA/50%ACN/H₂O solution and desalted on C18 ZipTips (Millipore, Bedford, MA, U.S.A.). Mass spectrometric analysis of the unfractionated tryptic digests was performed in positive-ion, reflectron mode, on a Reflex III MALDI-TOF mass spectrometer (Bruker, Karlsruhe, Germany), using 2,5-dihydroxybenzoic acid as the matrix. Two-point external calibration was applied; this guarantees a mass accuracy within 200 ppm. The peak lists were generated with X-Tof (version 5.1.5) software. Mascot database search software (<http://www.matrixscience.com/>) was used to identify proteins in the full NCBI database (2007.11.16, 5633163 sequences). Searches also were performed using an in-house Mascot server (v2.2.04), and a *Streptomyces griseus* database (2008, 7138 sequences) downloaded from <http://streptomyces.nih.go.jp/gview/>

download.cgi?molecule=TG&item=sgr_cds_ami). Protein scores greater than 80 (full NCBI database search) or 51 (*S. griseus database*) were considered significant ($P < 0.05$). Search parameters were as follows: mass accuracy: 200 ppm; only tryptic cleavages were permitted; and 2 missed cleavages were considered. Carbamidomethylation of Cys-residues was a fixed modification; methionine oxidation, protein N-acetylation and pyroglutamic acid formation from N-terminal Gln residues (and Glu), and Me esterification of Asp and Glu residues (CBB-staining side reaction proven by PSD (20) were the considered variable modifications. Esterified peptides were only accepted when the peptide was also detected without the modification. Protein identification was confirmed by sequence information obtained from MS/MS (post source decay) spectra, acquired in 10-12 steps, lowering the reflector voltage by 25% in each step, then stitching the data together. Some MS/MS experiments were performed on an Agilent XCT Plus 3D ion trap equipped with an atmospheric pressure MALDI source using 4-OH-a-CN-cinnamic acid as the matrix. Search parameters for MS/MS data: Mass accuracy 200 ppm for the precursor ion and 1 Da for the fragment ions. Cleavage specificity and covalent modifications were considered as described above. MS/MS spectra are shown in supplementary Figures S1-S10. Instead of the accession number the SGR Locus number is listed in Table 1., except for protein ID#14 which is not present in the sequenced *S. griseus* IFO 13350 strain.

RNA isolation and RT-PCR

For transcript analysis, RNA was isolated from surface-grown cultures of *S. griseus* B2682 or its A-factor nonproducing derivative AFN. Mycelium was grown on R2YE agar plates and harvested after 36 h (onset of aerial growth) or 54 h (sporulation). RNA isolation and semi-quantitative RT-PCR was carried out twice, as described previously (21). 200 ng of RNA was used for each reaction (concentration was assessed using a Nanodrop® spectrophotometer). Oligonucleotides used for RT-PCR are presented in Supplementary material Table S1.

Table 1. Proteins with enhanced expression in *S. griseus* B2682 AFN.

ID #	SSP	Accession	Protein name	Match	Sequence confirmed by PSD or CID	Orthologue in SCO/SAV *
1	3002	SGR1460	OsmC-like ATP/GTP-binding protein	4/9	⁶ VAHTNWEGNIEGK ¹⁹ ²⁰ GVVTFDSSGIGDHPVSWP AR ³⁹	SCO6531 (83%), SAV1862 (84%)
2	0004	SGR2245	Superoxide dismutase (SodN)	11/15	⁸⁵ YPELHQLINDTLK ⁹⁷	SCO5254 (87%), SAV2988 (86%)
3	4603	SGR3109	Putative aminotransferase	31/43	² ADIQIPADIKPADGR ¹⁶ ²²⁶ HIPEFFSLPTAIDNSLK ²⁴²	SCO4366 (87%), SAV3883 (87%)
4	4507	SGR3109	Putative aminotransferase	13/29	² ADIQIPADIKPADGR ¹⁶ ²²⁶ HIPEFFSLPTAIDNSLK ²⁴²	SCO4366 (87%), SAV3883 (87%)
5	1502	SGR1498	Xylose ABC transporter solute-binding protein	32/34		SCO6009 (78%), SAV2247 (77%)
6	2402	SGR1498	Xylose ABC transporter solute-binding protein	22/27	⁴⁷ IGLLLPENQTAR ⁵⁸ ¹²⁵ AKDAGIPVVAFDR ¹³⁷ ²⁵⁴ GAGISPLPPVTGQDAELA GVQR ²⁷⁵	SCO6009 (78%), SAV2247 (77%)
7	2405	SGR1498	Xylose ABC transporter solute-binding protein	41/49	⁴⁷ IGLLLPENQTAR ⁵⁸ ¹²⁷ DAGIPVVAFDR ¹³⁷ ²⁵⁴ GAGISPLPPVTGQDAELA GVQR ²⁷⁵	SCO6009 (78%), SAV2247 (77%)
8	2404	SGR1498	Xylose ABC transporter solute-binding protein	31/41	⁴⁷ IGLLLPENQTAR ⁵⁸ ¹²⁷ DAGIPVVAFDR ¹³⁷ ²⁵⁴ GAGISPLPPVTGQDAELA GVQR ²⁷⁵	SCO6009 (78%), SAV2247 (77%)
9	8202	SGR5704	Putative SpoOM-type sporulation-control protein	16/24	³⁸ IQGGSVAAQIEGLNVGLQ AR ⁵⁷ ¹⁹² GLHQVELTFVADDR ²⁰⁵	SCO1793 (81%), SAV6485 (83%)
10	4305	SGR2237	Putative arginine/omithine binding protein	34/47	¹⁷⁸ SIEDLCGKPAAVQR ¹⁹¹ ²³¹ SGGSVAGVNDYPVAVDL AR ²⁴⁹ ²⁸⁶ EAVDAIIADGSYQK ²⁹⁹	SCO5260 (58%), SAV2982 (58%)
11	6701	SGR5275	Maltose binding protein MalE	21/45	⁷³ YVNVFPFGDAQNK ⁸⁴ ⁸⁷ NAAQAGDGAPDVIR ¹⁰⁰	SCO2231 (48%), SAV5977 (48%)
12	1307	SGR1737	Glutamate ABC transporter substrate-binding protein	33/35	⁷⁷ ADQPYLGFEDQATK ⁹⁰ ¹⁴⁹ QVGFAGPYK ¹⁵⁸ ¹⁹⁷ KPEYGASVVELAK ²⁰⁹	SCO5776 (40%), SAV2485 (55%)
13	1303	SGR1737	Glutamate ABC transporter substrate-binding protein	35/38	⁷⁷ ADQPYLGFEDQATK ⁹⁰ ¹³¹ GQVDYYVGTYTINDER ¹⁴⁶ ¹⁴⁹ QVGFAGPYK ¹⁵⁸ ¹⁹⁷ KPEYGASVVELAK ²⁰⁹	SCO5776 (40%), SAV2485 (55%)
14	4204		Manganese superoxide dismutase SodA	9/14	¹¹⁵ FKSDFAAAAAGR ¹²⁶	
15	2702	SGR5280	Putative alpha-amylase	21/25	²⁹⁵ SAVFVDNHDTER ³⁰⁶ ⁴⁰⁶ AYVAINHEGSALTR ⁴¹⁹ ¹⁰³ AAFAAMVNTCHAAGVK ¹¹⁸ ²²⁹ LTNPNVYWK ²³⁷	SCO7020 (78%) SAV5981 (73%)
16	1803	SGR5280	Putative alpha-amylase	25/46	²⁹⁵ SAVFVDNHDTER ³⁰⁶ ⁴⁰⁶ AYVAINHEGSALTR ⁴¹⁹	SCO7020 (78%) SAV5981 (73%)

Match: peptides identified/masses searched; * SCO, *S. coelicolor* database; SAV, *S. avermitilis* database; % age between brackets refers to aa identity between the predicted SGR and SCO proteins.

RESULTS AND DISCUSSION

Extracellular proteome analysis

The extracellular proteomes of *S. griseus* B2682, of its A-factor non-producing spontaneous mutant (AFN) and of AFN complemented with a plasmid expressing Factor C (called AFN/pSGF4; (Biró *et al.*, 2000)) were studied. AFN has a nonsporulating (bald) phenotype, and introduction of Factor C-expressing plasmid pSGF4 restores wild-type levels of sporulation to this strain (Biró *et al.*, 2000). The choice for the extracellular proteome is a logical consequence of our focus on extracellular signalling mechanisms, with many of the proteins that are part of the A-factor/AdpA response regulon extracellularly localized.

To obtain an initial assessment of the protein expression profiles in the different strains, SDS-PAGE was performed, which revealed that several protein bands varied strongly in intensity. Considering the low separative capacity of 1D-gels the samples were analyzed further by 2D- proteome analysis, and initial analysis with a pI range of 3-10 revealed that the vast majority of the proteins appeared in the pI range of 4-7, which was then used for all experiments. Over 200 detectable protein spots were identified on each gel by colloidal Coomassie staining (Figure 1). In total 42 spots differed significantly between the parent and the mutants and these were analyzed by MALDI-TOF mass spectrometry. In a previous study (Birkó *et al.*, 2007) we showed that Factor C acts by restoring A-factor production and normal sporulation to *S. griseus* AFN. Restoration of A-factor production also resulted in an increase in the expression of several A-factor responsive secreted proteases to wild-type levels. However, at the time detailed analysis of the proteomes of AFN and AFN/pSGF4 was severely hampered by the lack of adequate genome information of *S. griseus*. Though our data now indicate (see below) that most proteins studied are highly conserved in *S. coelicolor* and *S. avermitilis* a single amino acid substitution in the peptides will prevent protein identification. This significant hurdle was overcome once the full genome sequence of *S. griseus* had become publicly available (Ohnishi *et al.*, 2008).

The main question we sought to address this time is what proteins are expressed or overexpressed in the AFN mutant to compensate for the lack of extracellular hydrolases that normally supply the developing colonies with nutrients. 16 out of 42

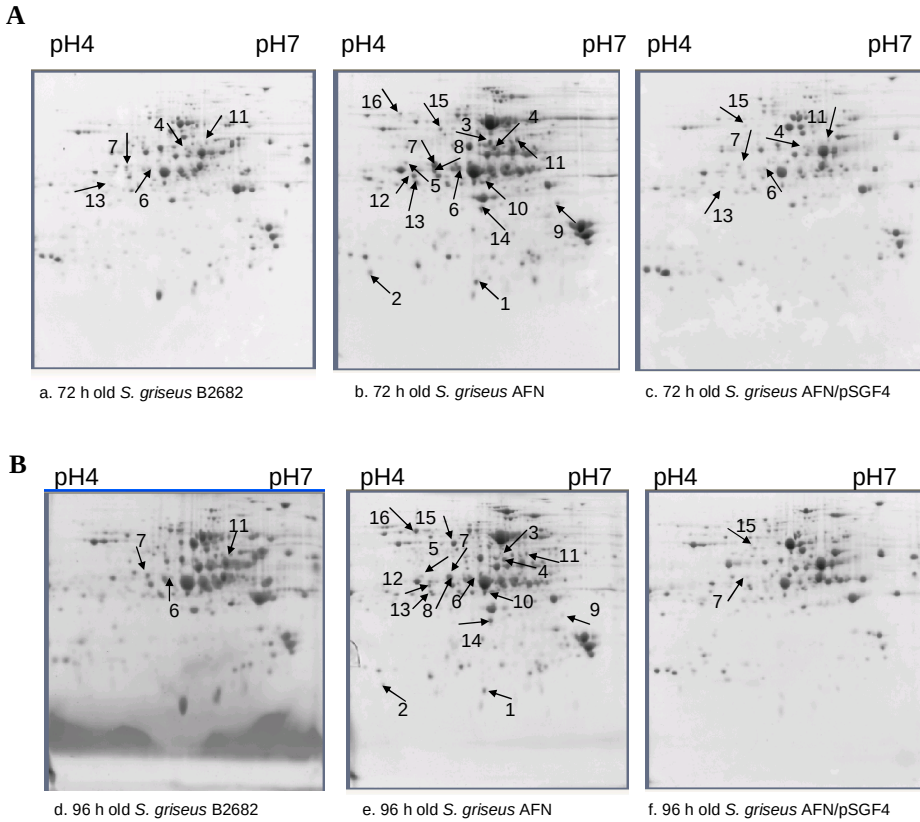


Figure 1. 2D-gel electrophoresis maps of secreted proteins extracted from 72 h old surface-grown cultures of (a) B2682, (b) AFN, (c) AFN/pSGF4, and from 96 h old surface-grown cultures of (d) B2682, (e) AFN, (f) AFN/pSGF4. Numbers correspond to the ID number of proteins in Table 1.

spots fulfilled the selection criteria (for details see the Materials and Methods section) and they were unambiguously identified (Table 1). The expression profiles of all 16 protein spots were very similar in the 72 and 96 h cultures, with the majority completely absent in wild-type cultures and in the *facC* complemented AFN strain. A few proteins were visible in B2682 and AFN/pSGF4 but with strongly reduced intensity (Fig. 1A-F. and Fig. 2A-B.). The 16 spots represented 10 different proteins, namely SGR1460 (#1), SGR2245 (#2), SGR3109 (#3-4), SGR1498 (#5-8), SGR5704 (#9), SGR2237 (#10), SGR5275 (#11) SGR1737 (#12-13), SGR5280 (#15-16) and SodA (#14). Interestingly, SodA or manganese superoxide dismutase is absent in the sequenced *S. griseus* strain IFO 13350. All proteins were overexpressed in both 72 h and 96 h surface-grown

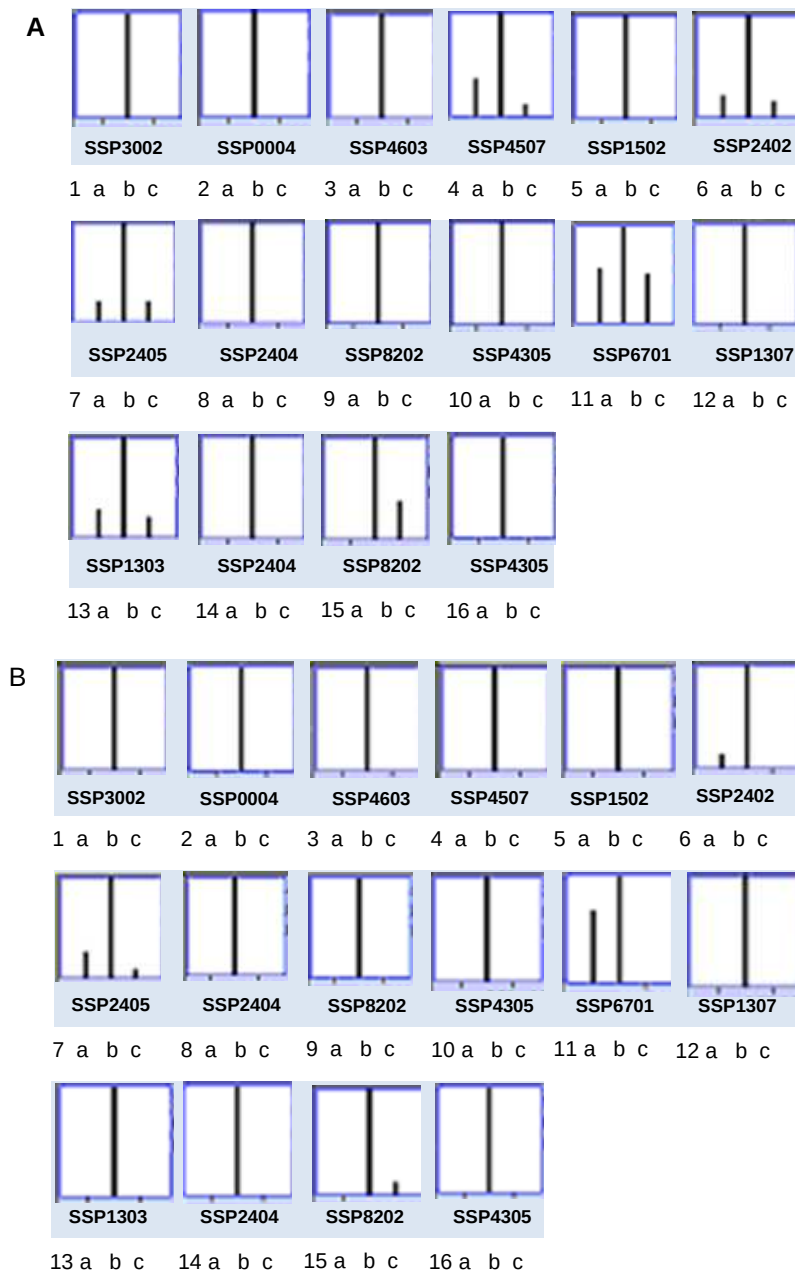


Figure 2. Quantitative image analysis of the proteins performed by PDQuest™ 2-D Analysis Software (Bio-Rad) in 72 h old (2A) and 96 h old (2B) cultures. Numbers underneath each plot correspond to the protein ID # in Figure 1. and Table 1. The bars represent the relative amount of the different proteins in the strains in the order of B2682 (a), AFN (b) and AFN/pSGF4 (c).

cultures (Figure 2 A and B). Detailed information regarding the identification is provided in Table 1.

Interestingly, all proteins with the exception of SodA are well conserved in *S. avermitilis* and *S. coelicolor* (Table 1), which suggests that these proteins play a universal role in the *Streptomyces* life-cycle. In fact, the proteins are conserved (more than 50% aa identity for the gene products) in the sequenced genomes of *Streptomyces* species *S. clavuligerus* ATCC 27064, *Streptomyces* sp. Mg1, *S. sviveus* ATCC 29083, *S. pristinaspiralis* ATCC 25486, *Streptomyces* sp. SPB74, and *S. scabies* (data not shown). All identified proteins relate to stress, and may primarily be subdivided in environmental stress and nutrient stress (*i.e.* starvation) proteins. One exception is perhaps SGR5704, an orthologue of the *Bacillus* sporulation control protein Spo0M. However, sporulation results in the autolytic breakdown of the vegetative mycelium and in fact it is a major stress mechanism in bacteria (Rigali *et al.*, 2008, González-Pastor *et al.*, 2003).

The environmental stress control proteins are SGR1460, SGR2245 and SodA. SGR1460 is a putative ATP/GTP binding protein and a homologue of the *E. coli* osmotically inducible OsmC protein that is involved in osmotic stress response. Superoxide dismutase SodN (SGR2245) and SodA are involved in the control of oxidative stress. While these are predicted cytoplasmic proteins, SodF (SGR4906) is known to be autotransported as a leaderless protein (Birkó *et al.*, 2007; Henderson *et al.*, 2000) and our experiments suggest that the same is true for SGR1460, SodN and SodA.

Several of the proteins with enhanced expression in AFN are involved in supplying nutrients to the growing colonies, and the strongly enhanced expression in AFN suggests that this strain suffers from nutrient deprivation. SGR3109 is an orthologue of the phosphoserine aminotransferase SerC of *Mycobacterium tuberculosis* (Accession Z73101; 66% aa identity), an aminotransferase that plays an important role in the amino acid and vitamin biosynthesis, notably of glycine, serine, threonine and vitamin B6. SGR1498 is an ABC transporter solute binding protein that is known to be involved in xylose uptake (Bertram *et al.*, 2004). SGR2237 is a predicted arginine/ornithin binding protein and likely involved in the transport of polar amino acids. SGR5275 is MalE or maltose-binding protein which is the solute binding protein belonging to the maltose uptake system in streptomycetes (van Wezel *et al.*, 1997a, van Wezel *et al.*, 1997b). SGR1737 is a glutamate ABC transporter substrate-binding protein and likely involved in

sensing and processing of environmental information. SGR1498, SGR1737, SGR2237 and SGR5275 all have an amino-terminal secretion signal sequence and a conserved prokaryotic membrane lipoprotein lipid attachment site with a cysteine residue that is most likely a site for ADP-ribosylation.

Finally, SGR5704 is an orthologue of the well-studied sporulation control protein Spo0M of *Bacillus subtilis* and most likely in an operon with the phospholipid binding protein SGR5703. Mutation/deletion in spo0M or overexpression of spo0M in *B. subtilis* results in impaired sporulation (Han *et al.*, 1998).

The identified proteins are all overexpressed in the AFN mutant, which is deficient in A-factor production. A-factor as a pleiotropic autoregulator in *S. griseus* during normal development induces the biosynthesis of several secreted hydrolases whose function is to digest biopolymers and supply the differentiating colony with nutrients (Méndez *et al.*, 1985; Miguélez *et al.*, 1999; Hirano *et al.*, 2006). In the AFN mutant these hydrolases are not secreted (Birkó *et al.*, 2007), therefore the supply of small molecule nutrients is probably scarce.

Many of the proteins overexpressed in AFN are specific ligand-binding proteins associated to the transmembrane translocator component of the ABC transporters (Hirano *et al.*, 2006). It is likely that the overproduction of these nutrient scavenging proteins is a response to try and compensate for the reduced availability of nutrients. All of the ABC transporter substrate binding proteins have an amino-terminal secretion signal sequence and a properly positioned conserved prokaryotic membrane lipoprotein lipid attachment site with a cysteine residue. Secretion of these proteins is regulated by ADP-ribosylation. When the cysteine is ADP-ribosylated these proteins cannot be exported to the surface and are therefore nonfunctional in transport (Heide *et al.*, 2002). ADP-ribosylation-mediated inactivation of substrate binding proteins most likely takes place particularly during normal growth and development, when the supply of nutrients is plenty.

Transcriptional analysis of the overexpressed proteins

To our surprise, many of the overexpressed proteins detected extracellularly are predicted lipoproteins whose final destination is the cell surface, and their amount in the secretome therefore does not necessarily reflect their gene expression. To analyze if the enhanced accumulation of proteins in the AFN mutant was at least in part due to enhanced

transcription, RNA was isolated from surface-grown cultures of *S. griseus* B2682 and its AFN mutant derivative. For this, mycelium was grown on R2YE agar plates and harvested at the time when aerial mycelium was formed as well as during sporulation. We used *rpsI* (SGR2801, encoding ribosomal protein S9) as positive control, and reactions without reversed transcriptase step were performed to confirm the lack of DNA contamination in the samples. As shown in Figure 3, while *rpsI* transcript levels were the same in wild-type and mutant and did not vary in time, transcription of SGR1460, 1498, 1737, 2237, 2245, 3109, 5275, 5280 and 5704 was strongly up-regulated in the AFN mutant during early aerial growth. This corresponds to the critical phase in the *Streptomyces* life-cycle when programmed cell death is initiated to provide the cells with nutrients. The transcriptional analysis shows that the enhanced protein levels observed in the proteome of AFN (Figures 1-2) is at least in part due to enhanced gene expression in the absence of A-factor. Interestingly, differences in transcript levels were far less pronounced during sporulation, with only SGR1460 and SGR3109 up-regulated at this time point, while SGR5704 was lower in the mutant than in the parent. This strongly suggests that transcriptional up-regulation is specific for the time corresponding to programmed cell death.

Interestingly, we also observe elevated protein levels for the stress-related proteins in the AFN mutant during sporulation, which is not explained by differences at the transcriptional level alone. A likely explanation is provided by the fact that A-factor activates a multitude of extracellular proteases (Horinouchi, 2007; Birkó *et al.*, 2007), and hence secreted proteins are expected to be much more stable in the A-factor nonproducing mutant AFN, further contributing to the accumulation of proteins in the mutant

ABC transporters and the control of Streptomyces development

Streptomyces are soil-dwelling bacteria that are able to utilize almost all naturally occurring polymers, such as starch, cellulose, chitin and agar. These polymers are degraded by extracellular hydrolases and subsequently imported (mainly as monomers or dimers) via specific permeases, either via ABC transporters or via the phosphotransferase system PTS (Colson *et al.*, 2008; Saito and Schrempf, 2004; Nothaft *et al.*, 2003; Kamionka *et al.*, 2002). Permeases that are involved in sensing and transport therefore

substantially influence their metabolism and morphogenesis. An *in silico* survey (Bertram *et al.*, 2004) identified 81 ABC transporters in *S. coelicolor* that are involved in the uptake of sugars, oligopeptides and other nutrients. This large number of transporters and secreted hydrolases perfectly reflects the lifestyle of these microbes. In a recent analysis of the membrane-associated proteome (Kim *et al.*, 2005) in *S. coelicolor* it was noted that a large proportion of these characterized proteins related to ABC transporter systems, including SCO5776, SCO2231 and SCO6009 whose orthologues we found in this study, which was considered as a snapshot of the nutritional requirement of the organism.

Interestingly, recent studies revealed that in fact several sugar transporters play a crucial role in the control of development, and in particular those that relate to *N*-acetylglucosamine transport and metabolism. These include the universal PTS components EI, EIIA^{Crr} and HPr (Nothaft *et al.*, 2003; Rigali *et al.*, 2006) as well as components of the DasABC transporter. DasABC transports chitobiose, the dimer of *N*-acetylglucosamine, and DasA is a lipoprotein that functions as the chitobiose binding protein (Saito *et al.*, 2007). Besides in the transport of chitobiose DasA also plays a role in nutrient sensing, perhaps by interacting with the chiRS two-component system (Colson

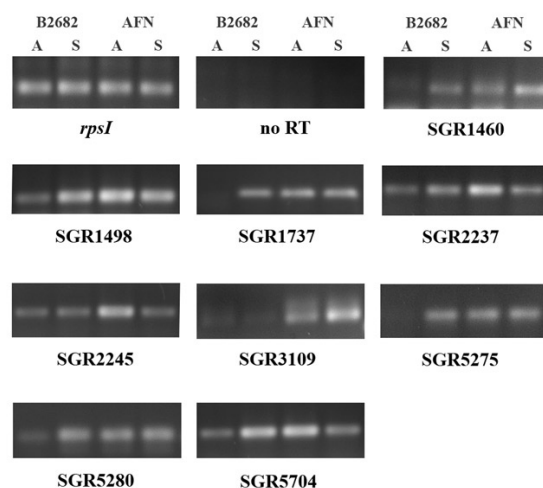


Figure 3. Transcriptional analysis by semi-quantitative RT-PCR. Samples were harvested from R2YE agar-grown mycelium of *S. griseus* B2682 and its AFN mutant. Time points: **A**, aerial growth (36 h); **S**, sporulation (54 h). No RT, the same as for RT-PCR with *rpsI* but without cDNA synthesis (control for absence of DNA contamination). Transcripts are indicated below the panels.

et al., 2008). The *dasABC* operon is developmentally regulated and plays a role in the control of sporulation in an A-factor independent manner in *S. griseus* (Colson *et al.*, 2008; Ohnishi *et al.*, 2002), and deletion of *dasA* results in a non-sporulating (bald) phenotype in *S. coelicolor* (Colson *et al.*, 2008). Other oligomeric transporters that relate to the onset of development are for example the BldK oligopeptide transporter of *S. coelicolor* that is essential for sporulation on rich and glucose-containing media (Nodwell *et al.*, 1996) and a second developmentally regulated ABC transporter - SCO7167 - involved in morphogenesis of *S. coelicolor* (Hillerich and Westpheling, 2006).

Superoxide dismutases in Streptomyces

Superoxide dismutases are a ubiquitous part of the cellular defense system against oxidative stress. They convert the toxic superoxide to hydrogen peroxide which is further degraded by the enzyme catalase. So far in *Streptomyces* two classes of superoxide dismutases have been described, namely the nickel containing SodN and the iron-zinc containing SodF. We detected an additional superoxide dismutase, which has high similarity to the *Bacillus pumilus* SAFR-032 manganese superoxide dismutase SodaA (YP_001487459). This protein so far has not been described in *Streptomyces* and may therefore represent a third class. In *S. pristinaespiralis* the expression of SodF is regulated by the SpbR protein, an analogue of the *S. griseus* ArpA and thus relating to the γ -butyrolactone regulon (Folcher *et al.*, 2001). It is also hypothesized that SodF is involved in adaptation to oxidative stress generated by metabolic shifts during colony development (Folcher *et al.*, 2001). In further support of this hypothesis, a catalase is also developmentally regulated and required for aerial mycelium formation in *S. coelicolor* (Cho *et al.*, 2000). Previously (Birkó *et al.*, 2007) we identified SodF in *S. griseus* as up-regulated in mutant strain AFN relative to the parental strain *S. griseus* B2682, suggesting that SodF is negatively controlled by AdpA. In this work we identified SodN as a protein up-regulated in the AFN mutant. Interestingly, the presence of nickel in a micromolar concentration in the growth media induced the expression of SodN in *S. coelicolor*, whereas the expression of SodF was repressed (Kim *et al.*, 1996). The differential expression of superoxide dismutases seem to suggest that a strict control of the level of SOD is of great importance to cellular defenses against oxidative stress.

The enhanced expression of the nutrient and environmental stress proteins in a strain that fails to produce (significant amounts of) A-factor underlines the stress that is generated by the absence of this signalling molecule.

The A-factor nonproducing strain of *S. griseus* detailed in this work is a valuable asset in the search for genes that control the production of A-factor and the response it elicits. The *afn* locus does not correspond to any of the known A-factor biosynthetic genes or regulatory genes, such as *afsA*, *adpA*, or *arpA*. Considering the fact that the *afn* mutant is able to produce A-factor under some conditions (such as after addition of the signalling protein Factor C) we anticipate that the mutation lies in a gene involved in the regulation of A-factor biosynthesis. The nature of the *afn* mutation and its role in the control of A-factor production is currently under investigation in our laboratories.

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Chapter VIII

General Discussion

GENERAL DISCUSSION

The soil is a thriving ecosystem, composed of both living and non-living matter with a multitude of interactions between them. It is considered as the most diverse microbial habitat on earth. Soil microorganisms perform important functions in recycling nutrients. They act as nutrient scavengers, obtaining nutrition from dead, decaying matter of plants and animals. Actinomycetes (including *Streptomyces*) are a large group of filamentous bacteria and may account even 30% of the total microorganisms in the soil rhizosphere, depending on nutrient availability (Barreto *et al.*, 2008; Kennedy, 1999). They are able to utilize broad range of naturally occurring polymers, including starch, cellulose, chitin and xylan. A sufficiency of utilisable organic material promotes the vegetative growth of these bacteria, thereby delaying morphological development and associated antibiotic production. My thesis focuses on understanding the molecular mechanisms by which *Streptomyces* link nutrient availability with antibiotic production and development.

Quorum sensing (QS) is one of the sophisticated mechanisms by which *Streptomyces* co-ordinate gene expression and control diverse cellular processes. The nature and mode of action of QS signals is highly diverse among bacteria. A-factor, a γ -butyrolactone isolated from *Streptomyces griseus*, was the first example of an extracellular signalling molecule that could control antibiotic production. As a pleiotropic autoregulator it also induces the biosynthesis of several secreted proteases (Birko *et al.*, 2007), which function to digest biopolymers and supply the differentiating colony with nutrients (Hirano *et al.*, 2006; Mendez *et al.*, 1985; Miguelez *et al.*, 1999). In the spontaneous A-factor non-producing mutant (AFN) these hydrolases are not secreted (Birko *et al.*, 2007). The work presented in this thesis shows that lack of A-factor induces expression of nutrient-scavenging proteins (Chapter VII), probably to compensate for the reduced availability of nutrients.

One of the important determinants of *Streptomyces* development (including antibiotic production) is the proper balance between cell proliferation and cell death (Miguelez *et al.*, 2000), as autolytic degradation of many substrate hyphae provides nutrients for emergence of aerial mycelium. *N*-acetylglucosamine (GlcNAc), a monomer unit of chitin and constituent of bacterial peptidoglycan is a preferred carbon and nitrogen source for streptomycetes. More importantly, it is also a crucial signalling molecule

whose availability, depending on growth conditions, diversely influences development. High concentrations of GlcNAc around *Streptomyces coelicolor* colonies lock them in the vegetative growth phase under rich growth conditions (*feast*), and activate development and antibiotic production under poor conditions (*famine*) (Rigali *et al.*, 2008). This reflects possible mechanisms occurring in nature where the accumulation of GlcNAc during cell-wall hydrolysis (*famine*) triggers development and antibiotic production, whereas utilization of chitin-derived GlcNAc (*feast*) should block development. The GlcNAc signal is transmitted through the GntR-family transcriptional regulator DasR, the core regulon of which revolves around GlcNAc-related genes (*pts*, *nag*, *chi*) (Colson *et al.*, 2007; Rigali *et al.*, 2008 and Chapters III and V). DasR was identified not only as a nutrient sensor, but also as a repressor of antibiotic production (Rigali *et al.*, 2008). Glucosamine-6P (GlcN-6P) acts as an effector molecule of DasR preventing its DNA-binding (Rigali *et al.*, 2008 and Chapter V). This results in DasR release from the promoter regions of specific activator genes for antibiotic production, including *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 and Chapter V).

In this study dissection of GlcNAc metabolism was performed in order to understand how a single molecule can deliver opposite information to the cell depending on the nutritional conditions (Chapter III). Mutational analysis of the *nag* metabolic genes, *nagA*, *nagB* and *nagK*, encoding respectively GlcNAc deacetylase (NagA), deaminase (NagB) and kinase (NagK), allow us to block metabolic roots and monitor the effect of accumulation of different GlcNAc-derivatives on antibiotic production and development. *nagA* null mutants lost GlcNAc sensing (Chapter III), which is explained by the model which predicts that they are blocked in the production of signalling molecule GlcN-6P. They showed strongly enhanced production of the blue-pigmented antibiotic actinorhodin (Act) on both rich (R2YE) and poor (MM) solid media containing GlcNAc (Chapter III). An additional effect observed in liquid and solid MM was growth reduction (Chapter III). *nagB* inactivation in *S. coelicolor* led to rapid growth inhibition and Act overproduction when *nagB* mutants were grown on solid R2YE medium supplemented with GlcNAc. On MM agar plates supplemented with either GlcNAc or its deacetylated derivative, glucosamine (GlcN) *nagB* mutants even failed to grow (Chapter

III and Chapter IV). Previous reports demonstrated the toxicity of GlcNAc to *nagA* and *nagB* deletion mutants in *E. coli* and other bacteria (Bernheim and Dobrogosz, 1970; Komatsuzawa *et al.*, 2004; White, 1968; Yadav *et al.*, 2011). This suggested that accumulation of GlcNAc-6P (in *nagA* mutants) and of GlcN-6P (in *nagB* mutants) is toxic for the cells. However, no direct evidence was presented, and toxicity of downstream metabolic products would be an alternative, such as UDP-activated glucosamine derivatives. In this light it is important to note that GlcN is just as toxic to *nagB* mutants of *S. coelicolor* as GlcNAc (Chapter IV). Our data show that prolonged germination of *nagB* mutant spores (which also resulted in the formation of many young vegetative hyphae) did not relieve GlcNAc sensitivity, though this sensitivity was overcome by exponentially growing cells. Most likely accumulation of GlcNAc-derivative is toxic to young vegetative hyphae. In the absence of NagB, the major route to deplete GlcN-6P is incorporation into murein following its conversion to UDP-GlcNAc by the action of GlmM (phosphoglucosamine mutase) and GlmU (*N*-acetylglucosamine-1-phosphate uridylyltransferase). A possible explanation may be that the young hyphae do not synthesize enough peptidoglycan to deplete the GlcN-6P pool sufficiently rapidly to cope with the toxicity. Alternatively, lower enzymatic activity of GlmM may also affect a sufficient incorporation of GlcN-6P into cell wall.

Interestingly, we identified few second-site mutations in *nagB* mutants obtained on GlcN or GlcNAc, which were able to relieve toxicity to aminosugar. Among mutated genes was *nagA*, but also three novel genes SCO1447, SCO4393 and *dasD*, encoding a ROK family transcriptional regulator, a highly conserved protein containing a sugar isomerase domain (SIS) and a predicted β -*N*-acetylglucosaminidase, respectively (Chapter IV). A mutation in *nagA* restored growth of *nagB* mutants not only on GlcNAc, but also on GlcN (Chapter IV). Up to date there are no reports discussing the involvement of NagA in GlcN metabolism. In *E. coli* GlcN is transported and phosphorylated to GlcN-6P by a mannose transporter (II^{Man} , encoded by the operon *manXYZ*) (Plumbridge, 2000). Immediately after internalization GlcN-6P is converted to fructose-6P by NagB or incorporated into peptidoglycan, with the first reactions carried out by GlmM and GlmU.

Whereas *nagA* mutations were commonly found in suppressor mutants obtained on GlcN or GlcNAc, SCO1447 was specific for GlcN-obtained mutants (Chapter IV). We suggest that product of SCO1447 likely controls transcription of the transporter gene

SCO1448, whose product may transport GlcN (Chapter IV). However, disruption of SCO1448 did not restore growth of *nagB* mutant on GlcN (data not shown), suggesting that even if the SCO1448 gene product is involved in GlcN transport, another transporters may import GlcN as well. Therefore, additional targets for transcriptional control by the SCO1447 gene product may exist. A strain of *E. coli* mutated in *manXYZ* is able to transport GlcN via glucose transporter II^{Glc} encoded by *ptsG*. It is possible due to spontaneous mutation in *ptsG*, which enhances *ptsG* expression (Jones-Mortimer and Kornberg, 1980; Plumbridge, 2000).

SCO4393 and *dasD* mutations were specific for GlcNAc-obtained suppressor mutants. Whereas *dasD* mutation was found only in one out of 20 screened suppressor mutants, SCO4393 occurred with much higher frequency. Gene synteny analysis revealed that SCO4393 is linked with *nag* and *pts* genes in many organisms (Chapter IV). Additionally, SCO4393 (251 aa) shows 27% aa identity in 185 aa overlap to YfeU [also known as MurQ, *N*-acetylmuramic acid-6-phosphate (MurNAc-6P) etherase, 298 aa] of *E. coli*. YfeU converts MurNAc-6P into GlcNAc-6P and lactate and plays an important role in peptidoglycan recycling (Uehara *et al.*, 2006). Taken together, this strongly suggests that SCO4393 is involved in GlcNAc utilization and its conversion into a toxic derivative. A number of questions regarding the substrate specificity of the SCO4393 product, but also the mode of action of SCO1447 encoded protein need to be addressed in the future in order to enhance our understanding of aminosugar metabolism.

Novel insight into GlcNAc transport and metabolism was also gained after studying the phenotype of *nagK* mutants. The sugar kinase NagK catalyzes the phosphorylation of GlcNAc to GlcNAc-6P. Based on current knowledge the PTS is the only GlcNAc transporter in *S. coelicolor* (Nothaft *et al.*, 2010). Hence GlcNAc is expected to be phosphorylated upon entering the cell (not requiring NagK). Interestingly, on R2YE agar plates with GlcNAc the *nagK* mutant showed partial loss of the GlcNAc repressing effect on antibiotic production. This suggests that at least some of the extracellular GlcNAc may be internalized in a PTS-independent manner under these growth conditions. Possible candidate is the ABC transporter encoded by SCO6005-6007, which various gene products share 30-40% aa identity with the orthologous components of NgcEFG, the GlcN/chitobiose transport system in *Streptomyces olivaceoviridis*. Another possibility would be hydrolysis of the accumulated GlcNAc-6P

to GlcNAc (which is not toxic) and subsequent phosphorylation via NagK in order to allow formation of the signalling molecule GlcN-6P.

Multiple reports provide information about the influence of different carbon sources on the production of antibiotics with the major focus on C6 sugar, glucose. Glucose is known to repress formation of the aminoglycoside antibiotics (streptomycin, kanamycin, istamycin, neomycin, gentamicin), via repression of biosynthetic enzymes (Damain, 1989; Piepersberg W., 1997). In *S. coelicolor* glucose inhibits actinorhodin production by repressing the expression of *afsR2* (also known as *afsS*), which encodes a global regulatory protein involved in the stimulation of secondary metabolite biosynthesis (Kim *et al.*, 2001; Lee *et al.*, 2009). In this thesis it is shown that cellobiose, a dimer of glucose (β -1,4) represses actinorhodin production. The most logical explanation for this is that this repressing activity of cellobiose regarding antibiotic production is caused by the accumulation of glucose following its hydrolysis.

One of the important observations in this thesis is that C5 sugars, namely arabinose, its C'-2 epimer ribose and xylose have a strong effect on antibiotic production (Chapter VI). The molecular basis of these mechanisms requires further investigation. Contrary to ribose and arabinose xylose stimulates actinorhodin production in *S. coelicolor* (Chapter VI). Xylose is the major unit of hemicellulose, one of the constituents of plant biomass (Sunna & Antranikian, 1997) and after internalization is converted to the common metabolic intermediate xylulose-5P (see KEGG database http://www.genome.jp/kegg-bin/show_pathway?map00040), contributing to e.g. pentose phosphate pathway. The pentose phosphate pathway is the major source of NADPH, which serves as the reducing agent for the synthesis of Act precursors (Avignone Rossa *et al.*, 2002). In this thesis the regulon is characterized for the pleiotropic transcriptional regulator ROK7B7, a member of the ROK family regulatory proteins (Repressors, QRFs and Kinases) (Chapter V). Among multiple processes ROK7B7 controls a) xylose transport, b) carbon catabolite repression, and c) Act production (Chapter V). Microarray analysis suggested that the expression of xylose transport operon *xylFGH* (SCO6009-6011) may be negatively controlled by ROK7B7 (Chapter V). However, recent proteomics data revealed the surprising enhanced expression of both ROK7B7 and XylFGH in *glkA* (encoding glucose kinase (Glk)) null mutants (J. Gubbens and G.P. van Wezel, manuscript in preparation), which makes it less likely that ROK7B7 indeed

represses *xylFGH*. In fact, the up-regulation of the xylose transporter operon may be an attempt to compensate for the absence of ROK7B7, perhaps because xylose-5P acts as an inducer molecule of ROK7B7. In this study we also showed that actinorhodin production was drastically delayed in the *rok7B7* mutant, when grown on R2YE agar plates containing xylose. DNA-affinity capture assay (DACA) showed that ROK7B7 bind to the promoter regions of *actII*-ORF4 and *redD*, the pathway specific transcriptional regulators of Act and Red, respectively (Park *et al.*, 2009). Therefore, the stimulating effect of xylose or its derivative (e.g. xylose-5P) may be explained through its activity as an effector molecule of ROK7B7, leading to its activation and subsequent induction of Act production. However, microarray analysis did not reveal significant changes in the expression of *actII*-ORF4 or other *act* genes. This may be explained by very low expression of antibiotics under the tested conditions (MM + mannitol). Instead, down-regulation of the degradation pathway for branched-chain amino acids (leucine, isoleucine and valine) was detected. This implies a role of ROK7B7 in precursor supply for antibiotic production, since the degradation products of these amino acids are precursors for biosynthesis of polyketides like actinorhodin (Stirrett *et al.*, 2009). Based on all information obtained so far, three different ROK7B7 modes of action may be proposed. ROK7B7 influences Act production by either (a) influencing metabolic pathways, which provide precursors for polyketide biosynthesis; (b) by directly controlling the promoter of *actII*-ORF4; or (c) interacting with a co-repressor. For the latter point, it is important to note that there is strong overlap between the targets identified by microarray analysis for the DasR (Chapter V) and ROK7B7 regulons (Chapter VI). Around 400 differentially expressed genes were commonly found in both *dasR* and *rok7B7* mutants. Those were in particular genes belonging to the same classes, namely involved in primary and secondary metabolism, control of development and antibiotic production, protein biosynthesis, and DNA recombination (including many transposases). In Chapter V it is shown that DasR has two classes of targets, namely Class I targets that are associated with a *dre* element and Class II targets that are not associated with a *dre*. The latter are probably not bound by DasR by itself and hence may require a co-repressor for DasR binding. Considering the large overlap in the number of differentially expressed genes in the mutants and that some of them belong to the Class II of the DasR direct targets, we propose that these two transcriptional regulators may bind

in concert to the DNA. It is apparently supported by the observations, which show that both regulators interact with *actII*-ORF4. DasR binds a *dre* element located between the -35 and -10 consensus boxes of the *actII*-ORF4 promoter, repressing its transcription, while ROK7B7 may activate *actII*-ORF4 expression, possibly upon binding to a different sequence in concert with DasR. However, it cannot be excluded that transcriptional changes detected in *dasR* and *rok7B7* mutants are at least in part due to secondary effects, caused by differential expression of other regulatory genes. In order to discriminate between primary and secondary effects, ChIP-on-chip experiments and protein interaction studies studying the ROK7B7-DNA interaction should be performed. This approach may allow for identification of novel ROK7B7 targets. Similarly during the study of the DasR regulon, chitinases belonging to the Class I of DasR targets were not found among differentially expressed genes due to the lack of chitin as an inducer (Chapter V). Here also additional experiments were needed to fully resolve the regulon. Therefore studying the ROK7B7 regulon under xylose inducing conditions should provide further insight into ROK7B7 regulatory role.

Another common feature between DasR and Rok7B7 is their involvement in the control of carbon catabolite repression (CCR). In *Streptomyces coelicolor*, CCR is controlled by Glk (Angell *et al.*, 1994; Angell *et al.*, 1992; van Wezel *et al.*, 2007). Disruption of *glkA* results among others in the loss of glucose repression of agarase, encoded by *dagA* (Angell *et al.*, 1994; Angell *et al.*, 1992; van Wezel *et al.*, 2007). We report here, that the *rok7B7* mutant completely lost the extracellular agarase activity when grown on MM agar plates supplemented with xylose. Combined with the observation obtained from microarray analysis, which showed enhanced expression of xylose transport operon *xylFGH* in the *rok7B7* mutant, we suggest that xylose is used so efficiently that it has become a favored carbon source, resulting in carbon catabolite repression (CCR). Another explanation may be enhanced expression of Glk in the *rok7B7* mutant (J. Gubbens and G.P. van Wezel, manuscript in preparation). Excitingly, microarray analysis revealed as well up-regulation of the glucose transporter gene *glcP* in this strain suggesting direct control of glucose metabolism (Chapter VI). Similarly, the *dasR* mutant showed hyper repression of agarase production when grown on MM agar plates supplemented with glucose (data not shown). Additionally, DasR overexpression leads to dramatic decrease in Glk protein levels and Glk-mediated CCR (S. Rigali,

personal communication). This suggests that DasR may relieve glucose repression under poor nutritional conditions. Disruption of either *rok7B7* or *dasR* led to hyper repression of agarase production and the exact mechanism by which both transcriptional regulators control CCR is currently under investigation.

All observations discussed above show cross-talk between nutrient sensors and at the same time global transcriptional regulators DasR and ROK7B7 in coordination of many complex cellular processes underlying CCR, and antibiotic production. Manipulating GlcNAc metabolism offer leads towards approaches directed at improving antibiotic production (Chapter IV). The antibiotic triggering effect of GlcNAc is common among streptomycetes (although not universal) (Rigali *et al.*, 2008), and combined with metabolic engineering strategies this may serve as an excellent tool to wake up cryptic antibiotic clusters, which are not expressed under standard laboratory conditions. Signalling molecules that trigger development of *Streptomyces* comes from the lytic dismantling of vegetative mycelium and they precisely modulate the timing of developmental programme. We hypothesize that DasR and A-factor may be involved in the control of degradation of cellular biopolymers, as their regulons include extracellular enzymes responsible for the utilization of cell-wall derivatives and proteins, respectively. Understanding how the onset of development and programmed cell death are correlated at the molecular level with such precision is a very interesting and challenging aspect of *Streptomyces* biology that is subject of current research in the group. The work presented in this thesis provides a strong foundation for further research of the interactions occurring between diverse components of transcriptional regulatory networks that control development and antibiotic production in *Streptomyces*.

Nederlandse samenvatting

De bodem is een zeer complex ecosysteem en de meest diverse microbiële habitat op aarde. Bodembacteriën spelen een cruciale rol in de recycling van voedingsstoffen, onder meer door het verwerken van nutriënten op basis van dood materiaal van planten en dieren. Actinomyceten (waaronder de *Streptomyces* bacterie die centraal staat in mijn proefschrift) vormen een grote groep schimmelachtige bacteriën en zo'n 30% van het totale aantal micro-organismen in de rhizosfeer, afhankelijk van de beschikbaarheid van voedingsstoffen (Barreto *et al.*, 2008; Kennedy, 1999). Zij zetten daarbij veel voorkomende natuurlijke polymeren als zetmeel, cellulose, chitine, xylaan en mannan om. Als er voldoende voedsel aanwezig is zal dit de vegetatieve groei van actinomyceten stimuleren, waardoor de morfologische differentiatie (productie van sporen) en de productie van antibiotica en andere secundaire metabolieten wordt uitgesteld. Dit proefschrift richt zich op het begrijpen van de moleculaire schakelmechanismen die *Streptomyces* in staat stellen de beschikbaarheid van voedingsstoffen te vertalen naar de correcte timing van het aanzetten van de antibioticumproductie en de ontwikkeling.

Quorum sensing (QS) is een van de geavanceerde mechanismen waarmee *Streptomyces* de controle van diverse cellulaire processen coördineert. De aard en werking van QS signalen is zeer divers onder bacteriën. A-factor, een γ -butyrolacton geïsoleerd uit *Streptomyces griseus*, was het eerste voorbeeld van een extracellulair hormoonachtig signaalmolecuul dat de antibioticumproductie reguleert. Het is namelijk verantwoordelijk voor het aanzetten van de productie van streptomycine, vroeger een zeer belangrijk antibioticum tegen tuberculose en het eerste antibioticum dat ooit bij streptomyceten is ontdekt (Waksman *et al.*, 1945). Daarnaast controleert dit zogenaamde autoregulator molecuul ook de biosynthese van verschillende afgescheiden proteases, noodzakelijk voor het hergebruik van eiwitten (Birko *et al.*, 2007), zodat de differentiërende kolonie voorzien kan blijven van nutriënten (Hirano *et al.*, 2006; Mendez *et al.*, 1985; Miguelez *et al.*, 1999). In een spontaan verkregen mutant die geen A-factor kan produceren (AFN), worden die proteases niet langer uitgescheiden (Birko *et al.*, 2007). Het werk beschreven in dit proefschrift laat zien dat een gebrek aan A-factor de expressie van eiwitten betrokken bij de opname van suikers en aminozuren induceert (Hoofdstuk VII), als teken dat er inderdaad een gebrek aan voedingsstoffen is.

Een van de belangrijkste determinanten van de ontwikkeling van streptomyceten (inclusief antibioticumproductie) is een juiste balans tussen celproliferatie en celdood (Miguel *et al.*, 2000). Tijdens de vorming van luchthyfen wordt het vegetatieve mycelium (ook wel substraatmycelium genoemd) afgebroken, om zo de nutriënten voor de volgende levensfase te garanderen. *N*-acetylglucosamine (GlcNAc) is de monomeer waaruit chitine is opgebouwd maar ook (samen met *N*-acetylmuraminezuur ofwel MurNAc) een van de hoofdbestanddelen van peptidoglycan, wat de bacteriële celwand vormt. GlcNAc is een belangrijke koolstof- en stikstofbron voor streptomyceten, maar functioneert ook als signaalmolecuul. Bij hoge concentraties GlcNAc blijven *Streptomyces coelicolor* kolonies doorgroeien als er tevens andere nutriënten aanwezig zijn (*feest*), terwijl in de afwezigheid van andere nutriënten het juist een signaal is voor afbraak en de start van de ontwikkeling en antibioticumproductie (*hongersnood*) (Rigali *et al.*, 2008). Dit weerspiegelt de situatie in de bodem, waar het vrijkomen van GlcNAc in de afwezigheid van andere nutriënten eigenlijk alleen van de celwand kan komen, oftewel tijdens de celdood. Dit signaal leidt automatisch tot differentiatie als zelfbescherming, teneinde sporen en antibiotica te maken. Het GlcNAc signaal wordt doorgegeven via de GntR-familie transcriptionele regulator DasR, waarvan het centrale regulon draait om genen betrokken bij GlcNAc metabolisme (Nag eiwitten) en transport (eiwitten van de PTS, hetgeen staat voor *PEP-dependent phosphotransferase system*) (Colson *et al.*, 2007; Rigali *et al.*, 2008 en de Hoofdstukken III en V). DasR werd niet alleen geïdentificeerd als een belangrijke sensor voor specifieke nutriënten, maar ook als een repressor van de antibioticumproductie (Rigali *et al.*, 2008). Glucosamine-6-fosfaat (GlcN-6P), een afgeleide van GlcNAc, bindt aan DasR en fungeert zo als effector, dat wil zeggen het verhindert de binding van DasR aan DNA (Rigali *et al.*, 2008 en Hoofdstuk V). Dit resulteert in het loslaten van DasR van zijn DNA targets, waaronder de genen die coderen voor de specifieke activators voor antibioticumproductie, met name de genen *actII-ORF4* (voor actinorhodine productie) en *redZ* (prodiginine productie) (Rigali *et al.*, 2008 en Hoofdstuk V).

In dit proefschrift is onder meer het GlcNAc metabolisme ontrafeld, om beter inzicht te krijgen in hoe een enkel molecuul zulke tegenovergestelde informatie aan de cel kan geven afhankelijk van de voeding rijkheid van de omgeving (Hoofdstuk III). Mutatieanalyse en manipulatie van de *Nag* metaboliet genen, *nagA*, *nagB* en *nagK*, die

coderen voor respectievelijk GlcNAc deacetylase (NagA), deaminase (NagB) en kinase (NagK), stellen ons in staat metaboliet routes te blokkeren en te analyseren wat het effect is van de accumulatie van verschillende GlcNAc-derivaten op antibioticumproductie en ontwikkeling. Inactiveren van *nagB* in *S. coelicolor* leidde tot een snelle groeiremming en overproductie van antibiotica, wanneer deze *nagB* mutanten werden gekweekt op rijke voedingsbodems met GlcNAc. Op minimale media met N-acetylglucosamine (GlcNAc) of glucosamine (GlcN), konden *nagB* mutanten überhaupt niet groeien, hetgeen suggereert dat de intracellulaire accumulatie van GlcN-6P (welke in *nagB* mutanten niet verder kan worden omgezet) toxisch is (Hoofdstuk III en Hoofdstuk IV).

Toch ontstonden er spontaan mutanten die wel in staat waren om te groeien op GlcNAc, oftewel ze hadden een tweede mutatie opgelopen die compenseerde voor de afwezigheid van *nagB*. Dit kan zijn door te zorgen dat GlcNAc niet wordt geïmporteerd of omgezet, of dat de (overigens nog onbekende) oorzaak voor de letaliteit van GlcN-6P wordt opgeheven. Genoomsequencing van een aantal van deze mutanten liet zien dat de mutaties gelegen waren in de volgende genen: *nagA* (zonder welke GlcN-6P niet kan worden gevormd), en de genen SCO1447, SCO4393 en *dasD*. De laatste drie coderen voor respectievelijk een transcriptionele regulator uit de ROK familie, een sterk geconserveerd eiwit met een suiker isomerase domein (SIS) en een β -N-acetylglucosaminidase (Hoofdstuk IV). Tot onze verbazing herstelde de mutatie van *nagA* niet alleen de groei van *nagB* mutanten op N-acetylglucosamine maar ook op glucosamine, hoewel er in de literatuur (onder meer de KEGG database) geen directe rol voor NagA bij het metabolisme van glucosamine bekend is. Dit is een bijzonder interessante ontdekking, die erop wijst dat de huidige kennis van deze centrale routes niet volledig is.

SCO4393 is met name interessant, omdat het wellicht een nieuw enzym is in centraal metabolisme rond aminosuikers (waaronder GlcNAc en GlcN). Mutatie van dit gen heft specifiek de toxiciteit op van GlcNAc voor *nagB* mutanten, maar niet die GlcN. Het gen is sterk geconserveerd in vele bacteriële phyla en vaak gelegen in directe nabijheid van genen betrokken bij GlcNAc metabolisme en transport (Hoofdstuk IV). Er is significante homologie met MurQ, een celwandenzym welke MurNAc-6P omzet in GlcNAc-6P en lactaat, een belangrijke stap in de peptidoglycan recycling (Uehara *et al.*, 2006). Echter, dat verklaart niet waarom deletie van SCO4393 de toxiciteit van GlcNAc

voor *nagB* mutanten opheft. Er zijn dus sterke aanwijzingen voor een rol van SCO4393 bij GlcNAc metabolisme, maar de precieze substratspecificiteit moet nog worden opgehelderd. Daarmee zal het inzicht in centraal metabolisme rond aminosuikers verder kunnen worden verbeterd.

Een andere ontdekking die beschreven staat in dit proefschrift is de belangrijke rol van C5-suikers, namelijk arabinose, zijn C'-2 epimeer ribose en xylose, op de productie van antibiotica (Hoofdstuk VI). De moleculaire basis hiervan wordt momenteel onderzocht. Een aanwijzing die in dit proefschrift staat beschreven is het regulon van de pleiotrope transcriptionele regulator ROK7B7, een regulator van onder meer xylose metabolisme en lid van de ROK (Repressors, ORFs en Kinases)-familie van regulatie-eiwitten (Hoofdstuk V). ROK7B7 speelt een rol bij de controle van regulons gerelateerd aan onder meer xylose transport, globale koolstofregulatie (kataboliet repressie), productie van het antibioticum actinorhodine en precursorsupply voor actinorhodine en andere polyketide antibiotica (Hoofdstuk V). Analyse middels DNA microarrays liet zien dat de expressie van het xylose transportoperon *xylFGH*, welke naast *rok7B7* op het chromosoom ligt, hoger is in de *rok7B7* mutant. Wellicht is deze verhoogde expressie een poging tot compensatie voor de afwezigheid ROK7B7, want recente proteomics data lieten verrassenderwijs verhoogde expressie van zowel ROK7B7 en XylFGH zien in sommige mutanten (J. Gubbens, ingediend voor publicatie), wat het minder waarschijnlijk is dat ROK7B7 de expressie van *xylEFG* onderdrukt. In dezelfde studie hebben we ook aangetoond dat actinorhodine productie drastisch werd vertraagd in de *rok7B7* mutant. Werkhypothese is momenteel dat xylose of een afgeleid metaboliet daarvan (bijvoorbeeld xylose-5-fosfaat) het effectormolecuul van Rok7B7 is, met als hypothese dat het ROK7B7 activeert, leidend tot verhoogde antibioticumproductie. Op basis van alle informatie die tot nu toe is verkregen, kunnen drie verschillende werkingsmechanismen worden voorgesteld voor de controle van actinorhodine productie door ROK7B7, namelijk (a) het beïnvloeden van de routes in het primaire metabolisme die de precursors verzorgen voor polyketide biosynthese, (b) directe controle van de promotor voor *actII-ORF4*, het activator gen voor actinorhodine productie, of (c) interactie met cq controle van een ander DNA bindend eiwit wat de actinorhodine gencluster controleert.

Mede in het licht van het laatste is het interessant op te merken dat er een sterke

overlap bestaat tussen de regulons voor ROK7B7 en DasR, want niet minder dan 400 genen laten sterk veranderde expressie zien in zowel de *dasR* als de *rok7B7* mutant. Deze 400 genen zijn met name betrokken bij primair en secundair metabolisme, de ontwikkeling en antibioticumproductie, eiwit biosynthese en DNA recombinitie. Feitelijk is DasR in *Streptomyces* een zeer pleiotrope regulator, welke direct en indirect zo'n 20% van het gehele genoom beïnvloedt. In bijvoorbeeld *Bacillus* is dat veel minder, want de DasR homolog (NagR) controleert daar vrijwel uitsluitend de genen van het core regulon, namelijk *N*-acetylglucosamine metabolisme en transport (Bertram *et al.*, 2011).

Globale analyse van alle bindingsplaatsen voor DasR liet zien dat dat er zo'n 300 zijn, welke in twee klassen uiteenvallen, zogenaamde klasse I targets die zijn gekoppeld aan een duidelijk herkenbaar bindingselement (*dre*, voor DasR-responsief element) en Klasse II targets waarvoor geen duidelijk herkenbaar element (*dre* of anderszins) is gevonden. De laatste worden wellicht niet direct door DasR herkend, omdat zuiver DasR eiwit niet in staat is deze targets *in vitro* te binden. Echter, we hebben zoveel onafhankelijk bewijs voor binding *in vivo* dat het waarschijnlijk is dat DasR inderdaad bindt, wellicht in samenwerking met een ander eiwit. Het idee dat dit ROK7B7 zou kunnen zijn is speculatief, maar het wordt kennelijk ondersteund door de grote overlap tussen de targets voor de twee eiwitten. Een ander gemeenschappelijk kenmerk ligt in de controle van de kataboliet repressie, oftewel het mechanisme wat ervoor zorgt dat bij keuze uit diverse suikers eerst de suiker met de hoogste energetische waarde wordt gebruikt. Een bekend voorbeeld hiervan is de repressie van het lactose operon door glucose in *E. coli*, waarbij glucose de genen voor gebruik van lactose onderdrukt (Yudkin and Moses, 1969). In tegenstelling tot de gangbare regulatiemechanismen voor kataboliet repressie in de meeste andere bacteriën, speelt in *Streptomyces coelicolor* het enzym glucose kinase een centrale rol (Angell *et al.*, 1994; Angell *et al.*, 1992; van Wezel *et al.*, 2007). Verstoring van de expressie van glucose kinase resulteert onder andere in het verlies van glucose repressie van het extracellulaire enzym agarase, gecodeerd door *dagA*, welke een makkelijk te screenen marker vormt.

Onderzoek beschreven in dit proefschrift laat tevens zien dat zeer sterke kataboliet repressie plaatsvindt in de *rok7B7* mutant die is gegroeid in de aanwezigheid van xylose. In combinatie met de verhoogde expressie van xylose transport in de *rok7B7*

mutant, lijkt het aannemelijk dat xylose zo efficiënt wordt gebruikt dat het een favoriete koolstofbron wordt, resulterend in kataboliet repressie. Het is belangrijk op te merken dat in de mutant ook sterk verhoogde expressie van het glucosetransporteiwit GlcP te zien is, hetgeen een direct link met glucose-utilisatie lijkt aan te tonen (Hoofdstuk VI). Ook in de *dasR* mutant vindt zeer sterke onderdrukking van agarase productie plaats bij groei op glucose, en verhoogde expressie van DasR onderdrukt de productie van glucose kinase (niet gepubliceerde data en S. Rigali, pers. comm.), wat een sterke aanwijzing is dat DasR de kataboliet repressie onderdrukt. Verder onderzoek moet uitwijzen wat de relatie tussen de DasR en Rok7B7 regulons is en hoe ze het primaire metabolisme en de controle ervan zo drastisch kunnen beïnvloeden.

Deze data laten een duidelijk communicatie zien tussen de globale transcriptieregulators DasR en ROK7B7 en daarmee tussen de vele complexe cellulaire processen zoals kataboliet repressie en antibioticumproductie. Het manipuleren van het GlcNAc metabolisme is een interessante aanpak gericht op het verbeteren van de productie van antibiotica (Hoofdstuk IV). Het antibioticum activerende effect van GlcNAc is ook waargenomen in andere streptomyceten (al is het niet universeel; Rigali *et al.*, 2008) en in combinatie met *metabolic engineering* strategieën kan dit dienen als een benadering om zogenaamde slapende antibiotica wakker te maken. Het blijkt namelijk dat actinomyceten vaak clusters van genen hebben die erop wijzen dat bepaalde antibiotica kunnen worden gemaakt, maar die dat onder de standaard laboratoriumcondities niet doen. Een van de signaalmoleculen die de ontwikkeling van *Streptomyces* kolonies aanzetten is dus GlcNAc, hetgeen vrijkomt bij de lytische ontmanteling van het vegetatieve mycelium. Het ligt in de lijn der verwachting dat ook andere metabolieten een dergelijke rol kunnen spelen, bijvoorbeeld bij afbraak van DNA, RNA, eiwitten of lipiden en dat die moleculen ook aangrijpen op grote regulons. Zoals hierboven beschreven leidt het hormoonachtige signaalmolecuul A-factor tot de secretie van enzymen die eiwitten afbreken en hebben xylose en ribose - onderdeel van nucleïnezuren (DNA/RNA) - mogelijk ook een signaalfunctie. Een van de uitdagingen waar we nu voor staan is om beter te begrijpen hoe het begin van de ontwikkeling en geprogrammeerde celdood op moleculair niveau wordt geregeld. Het onderzoek in dit proefschrift biedt een goede basis voor verder onderzoek naar de interacties tussen de verschillende regulatoire netwerken in streptomyceten, als basis voor verder inzicht in

hoe de ontwikkeling en de productie van antibiotica in deze complexe micro-organismen wordt gecontroleerd.

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Appendices

Supplemental Information - Chapter III

Table S1. Plasmids used and constructed in this study.

Plasmid	Description	Reference
pWHM3	Cloning vector, <i>colE1</i> replicon, pSG5 replicon, TsrR, AmpR	Vara <i>et al.</i> , 1989
pHJL401	Complementation vector, SCP2*, pUC19 replicon, TsrR, AmpR	Larson and Hersherberger, 1986
pUWLcre	pUWLoriT derivative with <i>cre(a)</i> gene under <i>ermE*</i> promoter	Fedoryshyn <i>et al.</i> , 2008
pGAM1	pWHM3 containing flanking regions of <i>S. coelicolor</i> SCO4284 with <i>apraloxP_XbaI</i> inserted between them in pWHM3 <i>EcoRI-HindIII</i>	This work
pGAM2	pWHM3 containing flanking regions of <i>S. coelicolor</i> SCO5236 with <i>apraloxP_XbaI</i> inserted between them in pWHM3 <i>EcoRI-HindIII</i>	This work
pGAM3	pWHM3 containing flanking regions of <i>S. coelicolor</i> SCO4285 with <i>apraloxP_XbaI</i> inserted between them in pWHM3 <i>EcoRI-HindIII</i>	This work
pGAM4	pWHM3 containing flanking regions of <i>S. coelicolor</i> SCO4284-SCO4285 operon with <i>apraloxP_XbaI</i> inserted between them in pWHM3 <i>EcoRI-HindIII</i>	This work
pGAM5	pHJL401 containing <i>nagKA</i> gene with its promoter	This work
pGAM6	pHJL401 containing <i>nagB</i> genes with its promoter	This work

Table S2. Oligos used in this study. Restriction sites used for cloning are in bold and underlined. GAATTC, *EcoRI*; TCTAGA, *XbaI*; AAGCTT, *HindIII*

Name	5'-3' Sequence	Purpose and restriction sites
nagA_LF-1365	GTCAG <u>GAATTC</u> ACGTCGTTCCAGGAGTAGACGGTG	Cloning, <i>EcoRI</i>
nagA_LR+6	GTCAT <u>TCTAGA</u> AGGCCATCAGGTGGTTACC	Cloning, <i>XbaI</i>
nagA_RF+1133	GTCAT <u>TCTAGA</u> CACCTGGGCTGATCCGGCTCC	Cloning, <i>XbaI</i>
nagA_RR+2484	GTCAA <u>AAGCTT</u> TGAACGTGCGCTACGGCATCC	Cloning, <i>HindIII</i>
nagB_LF-1185	GTCAG <u>GAATTC</u> AAGAGCGACCGCTTGTCGCCGAA	Cloning, <i>EcoRI</i>
nagB_LF+6	GTCAT <u>TCTAGA</u> ATTCACGCTGGCCTGCCGTTT	Cloning, <i>XbaI</i>
nagB_RF+770	GTCAT <u>TCTAGA</u> TGGCAGGCATCTGAGCTGTC	Cloning, <i>XbaI</i>
nagB_RR+1918	GTCAA <u>AAGCTT</u> TGTGATGAGCGCCCATCCTGG	Cloning, <i>HindIII</i>
nagK_LF-1450	GTCAG <u>GAATTC</u> ATGTACGGCACCGTAACGCCCA	Cloning, <i>EcoRI</i>
nagK_LR+6	GTCAT <u>TCTAGA</u> CTTCATCCCGGTGCCGCCACATC	Cloning, <i>XbaI</i>
nagK_RF+963	GTCAT <u>TCTAGA</u> GAGGTAACCACCTGATGGCCCCAAG	Cloning, <i>XbaI</i>
nagK_RR+2570	GTAC <u>AAGCTT</u> CTCGTTGAGCTGGGTGGTGTCG	Cloning, <i>HindIII</i>
APRA_loxL	CTAGG <u>TCTAGA</u> GGTGATGGATAACTTCGTATAGCATACATT ATACGAAGTTATACTTATGAGCTCAGCCAATCG	Cloning, <i>XbaI</i>
APRA_loxR	CTAGG <u>TCTAGA</u> GATGCGGATAACTTCGTATAATGTATGCT ATACGAAGTTATCCCCGAAGCAGGGTTATGCAG	Cloning, <i>XbaI</i>
5236compl-454	GTCAG <u>GAATTC</u> GCACGGCGGTGATGCCGGACAAC	Cloning, <i>EcoRI</i>
5236compl+773 (796)	GTCA <u>AAGCTT</u> TGCGGGACAGCTCAGATGCCCTGC	Cloning, <i>HindIII</i>

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nagA_FOR-198	TCACCTTCCAGAACTGCCGGAG	PCR confirmation, sequencing
nagA_REV+1417	AGCCGGTGACCGTGACCTCGTGG	PCR confirmation, sequencing
nagB_FOR-336	CGCCCGGCATCATCGACACGGAC	PCR confirmation
nagB_REV+1098	TCGCGGGCGTCTGACGATCACC	PCR confirmation
nagK_FOR-208	AGGACCGCCGTCATGCCAGTG	PCR confirmation
nagK_REV+1310	ACGATGTCGCCCTGCTGCGCCAG	PCR confirmation
SCO4285for_seq	GTCAG <u>GAATTC</u> AGTGCAGACACCACCGCGATCG	Sequencing, <i>EcoRI</i>
SCO4285rev_seq	GTCA <u>AAGCTT</u> AGAACCTTGCTTGGGGCCATCAG	Sequencing, <i>HindIII</i>
2907for_seq	GTCAG <u>GAATTC</u> GCGCCTGTGATCAGGGGACTTGG	Sequencing, <i>EcoRI</i>
2907rev_seq	GTCA <u>AAGCTT</u> TGTACGAGATCTGAGCCCGCGAC	Sequencing, <i>HindIII</i>
2905for_seq	GTCAG <u>GAATTC</u> TGACCGCGCTGTCGGCACTC	Sequencing, <i>EcoRI</i>
2905rev_seq	GTCAG <u>GAATTC</u> TGACCGCGCTGTCGGCACTC	Sequencing, <i>HindIII</i>
2906for_seq	GTCAG <u>GAATTC</u> AGTCGGGTGATGCAGCCTTCG	Sequencing, <i>EcoRI</i>
2906rev_seq	GTCA <u>AAGCTT</u> TCGCGAGACCACTCCCAAAGG	Sequencing, <i>HindIII</i>
4284RTfor	ACATCGTTGGCATCCAATT	RT-PCR
4284RTrev	CGCCAGCGTCATCATCTT	RT-PCR
4285RTfor	CGTCACCTTCCAGAACTGC	RT-PCR
4285RTrev	CTGTGGAAGACGGAGCAGA	RT-PCR
rpsI RTfor	GTAGCGGTTGTCCAGCTCGAGCA	RT-PCR
rpsI RTrev	GAGACCACTCCCGAGCAGCCGC	RT-PCR
5236_RT1	CGACTCGCGGTGCTCGGCGGG	RT-PCR
5236_RT2	GATGCCAAGGCGGGCGGCGAAC	RT-PCR
dredasA	CAAGCTCCCCGTACTGGTCTACACCATTGGTCCAGGTCCC	EMSA
drenagB	CCGCTCTGTTAGATTGGTCTAAACCACATAGCCAGTCCCG G	EMSA
drenagKA	CGTACACCCGGGAGAGGTCTAGTCCACTGCGGTGGTGT G	EMSA
blal cis-acting element OP1	GAAAGTATTACATATGTAAGATTTAAATGC	EMSA
SCO4285- 84compl-512	GTCAG <u>GAATTC</u> ACCGGGATGGAGAGCACGTCGTC	Cloning, <i>EcoRI</i>
SCO4285- 84compl+2240(62)	GTCA <u>AAGCTT</u> ACGGTGAGGATCACCGTGCCGA	Cloning, <i>HindIII</i>

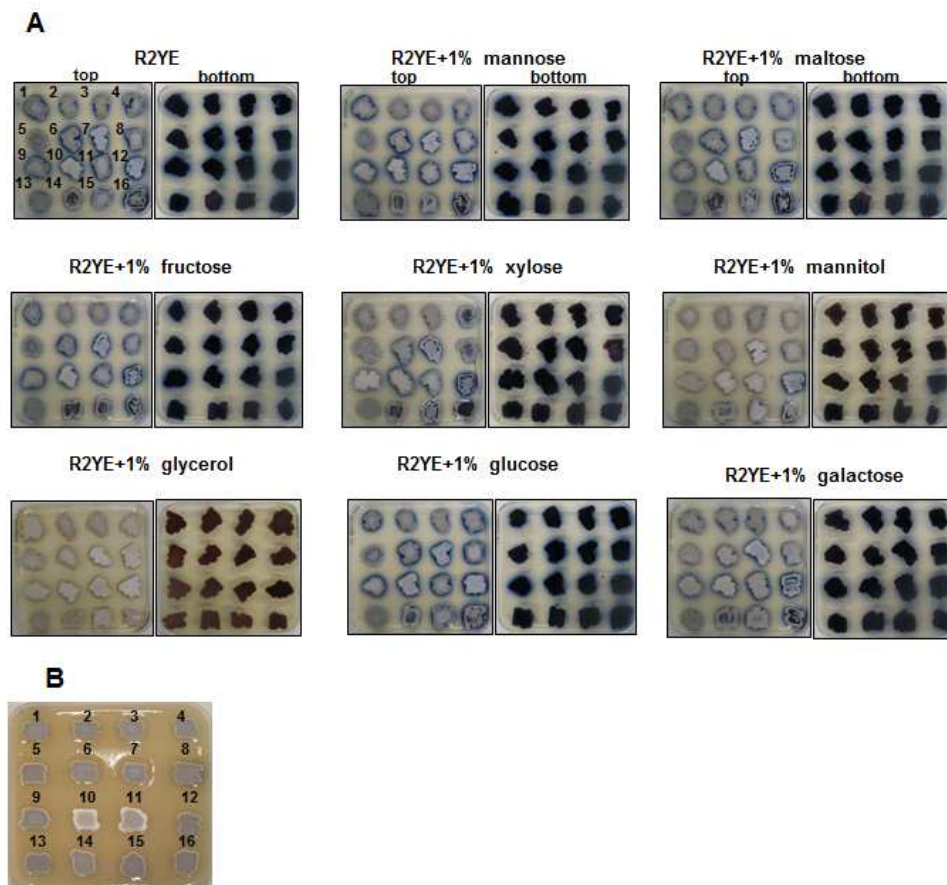


Figure S1. Phenotypes of the *nag* mutants. Strains were grown for four days on (A) R5- agar plates supplemented with 1% (w/v) of different carbon sources (glucose, fructose, galactose, glycerol, maltose, mannitol, mannose, or xylose) as indicated, or (B) on SFM agar plates. For each plate the top (left) and bottom (right) view is presented. Strains: **1.** *S. coelicolor* M145 (parental strain), **2.** GAM1 (M145 Δ *nagA*), **3.** GAM4 (M145 Δ *nagA*^{IFD}), **4.** GAM4 + pHJL401/*nagA*, **5.** M145 Δ *nagE2*, **6.** GAM2 (M145 Δ *nagB*), **7.** GAM5 (M145 Δ *nagB*^{IFD}), **8.** GAM5 + pHJL401/*nagB*, **9.** GAM3 (M145 Δ *nagK*), **10.** GAM6 (M145 Δ *nagK*^{IFD}), **11.** GAM6 + pHJL401/*nagKA*, **12.** M145 Δ *nagA*^{IFD} Δ *nagB*, **13.** GAM9 (M145 Δ *nagB*^{IFD} Δ *nagA*), **14.** GAM8 (M145 Δ *nagKA*^{IFD}), **15.** M145 + pHJL401, **16.** GAM10 (M145 Δ *nagKA*^{IFD} Δ *nagB*). For strains see further Table 1 and the text. **IFD**, in-frame deletion mutant.

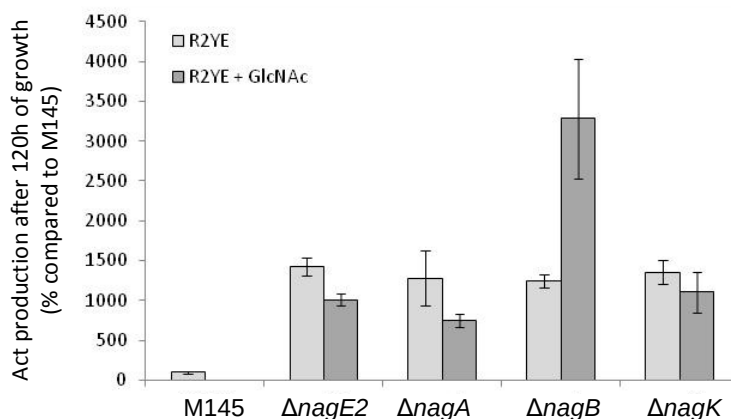


Figure S2. Quantification of antibiotic production after 120 h of growth. Production of actinorhodin was quantified relative to the production by the parental strain *S. coelicolor* M145 (which was set to 100%). Cultures were grown for 120 hr on R2YE agar plates with (dark bars) or without (light bars) GlcNAc. For 42 h and 48 h see main text and Fig. 6.

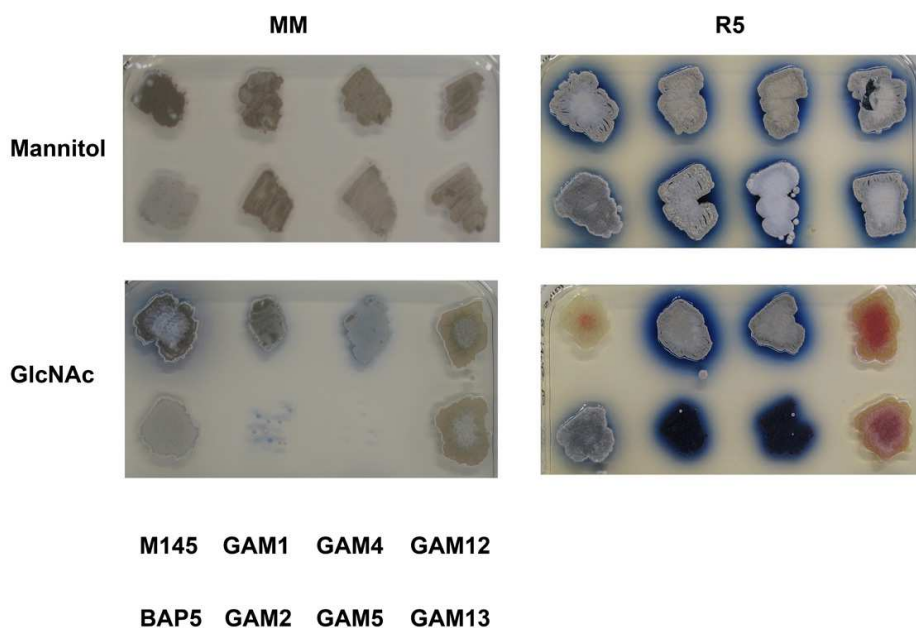


Fig. S3. Complementation of the *nagA* and *nagB* mutants. Left, patches of *S. coelicolor* M145 and derivatives on MM agar plates with either mannitol (top) or GlcNAc (bottom) as the sole carbon source. Right, identical but then on R5 agar plates. Note that complementation of *nagA* IFD mutant GAM4 with a plasmid harbouring the *nagKA* operon (strain GAM12) restores sensitivity to GlcNAc on R5, while complementation of *nagB* IFD mutant GAM5 with a plasmid harbouring the *nagB* gene (strain GAM13) restores normal growth and viability on MM and R5 with GlcNAc and normal GlcNAc sensing on R5. The *nagE2* mutant BAP5, which is insensitive to GlcNAc, was used as a control. Patches were grown for 4 days at 30°C.

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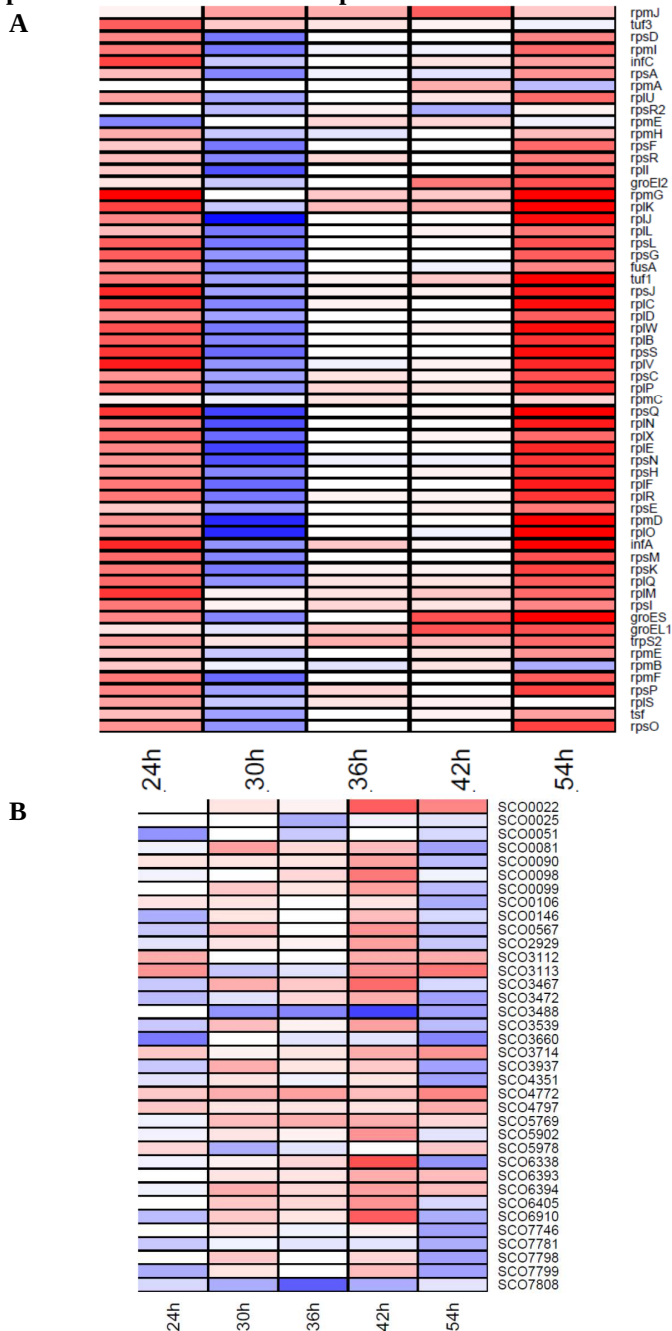


Figure S1. Selected classes of genes differentially expressed between the *dasR* mutant and its parent *S. coelicolor* M145. RNA was isolated from mycelium grown on MM with 1% mannitol during vegetative growth (24 h), vegetative/aerial growth (30 h), aerial growth (36 h), aerial growth/sporulation (42 h) and sporulation (54 h). From the list of genes with a fold change (AdasR expression/M145 expression) of more than 1.5, four classes of genes are shown: (A) protein biosynthesis, (B) transposases and DNA recombination. The levels of the fold

changes are indicated with colours as represented by the scale bar.

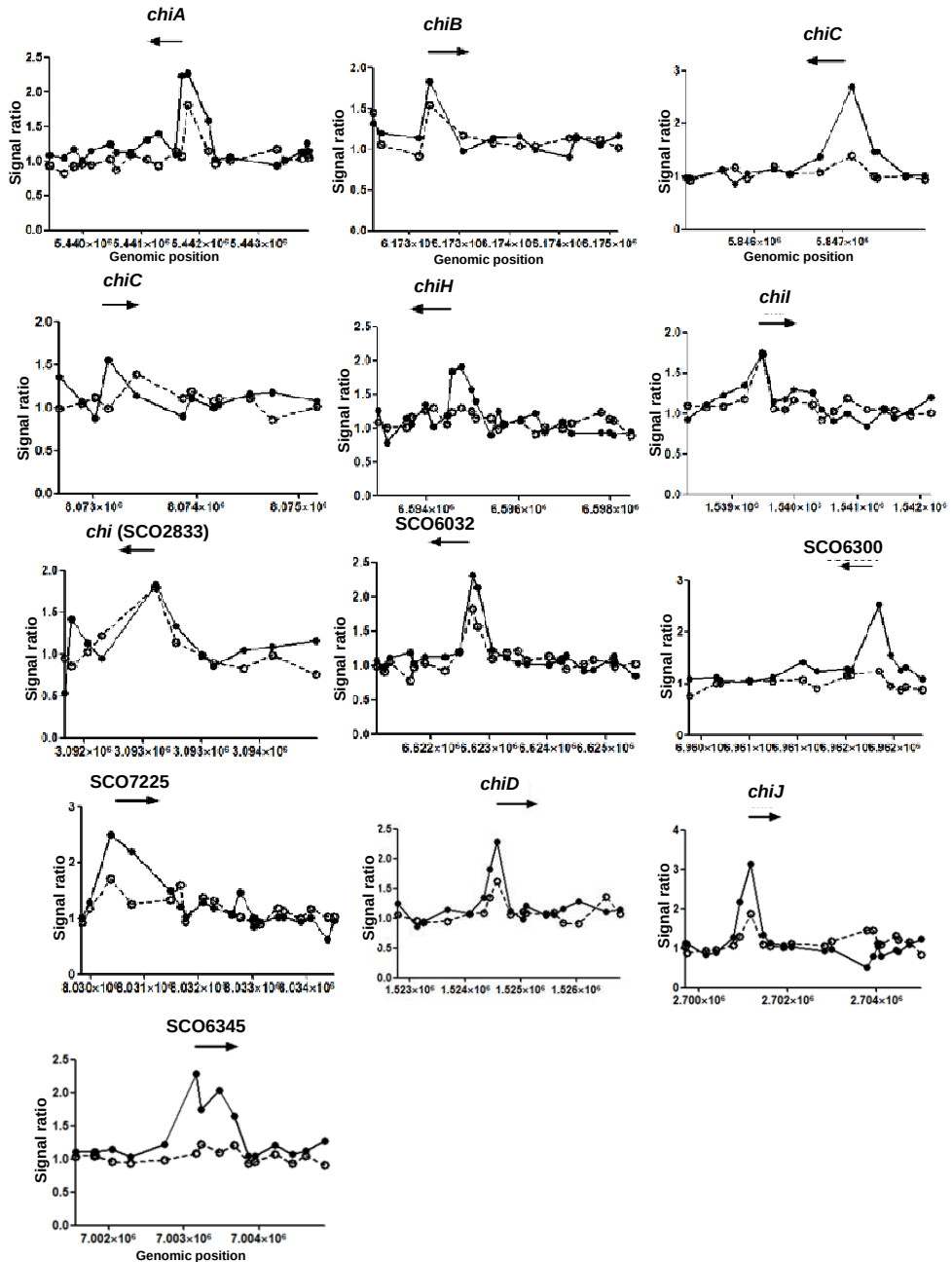
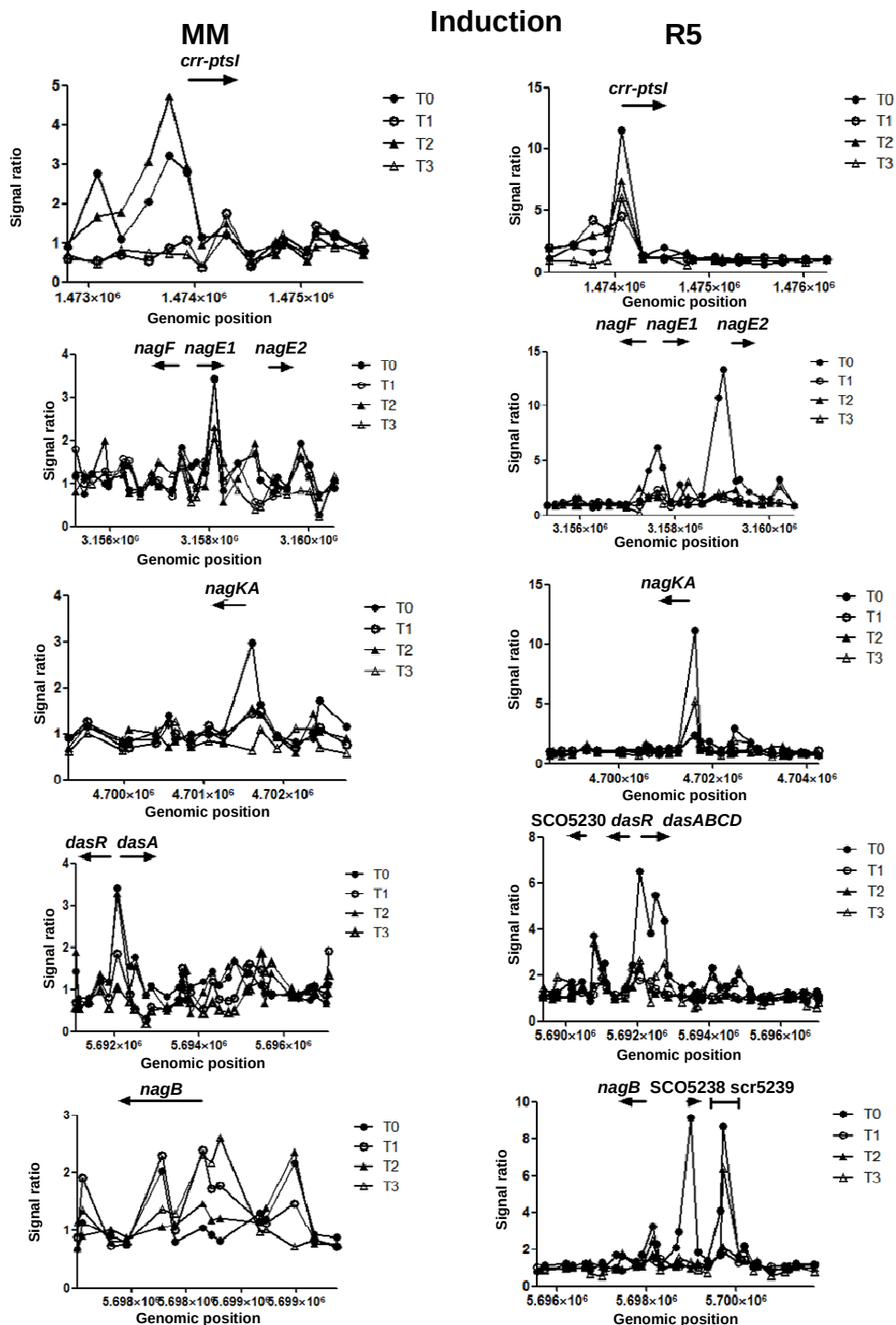


Figure S2. Chip-on-chip data for selected *DasR* targets detected in time-course experiment. Peaks indicate the presence of *DasR* binding sites in the promoter regions of *chi* genes. Plots presents *DasR* binding at 24 h (solid line, close circles) and 54 h (dashed line, open circles). The arrows indicate the orientation of the target gene.



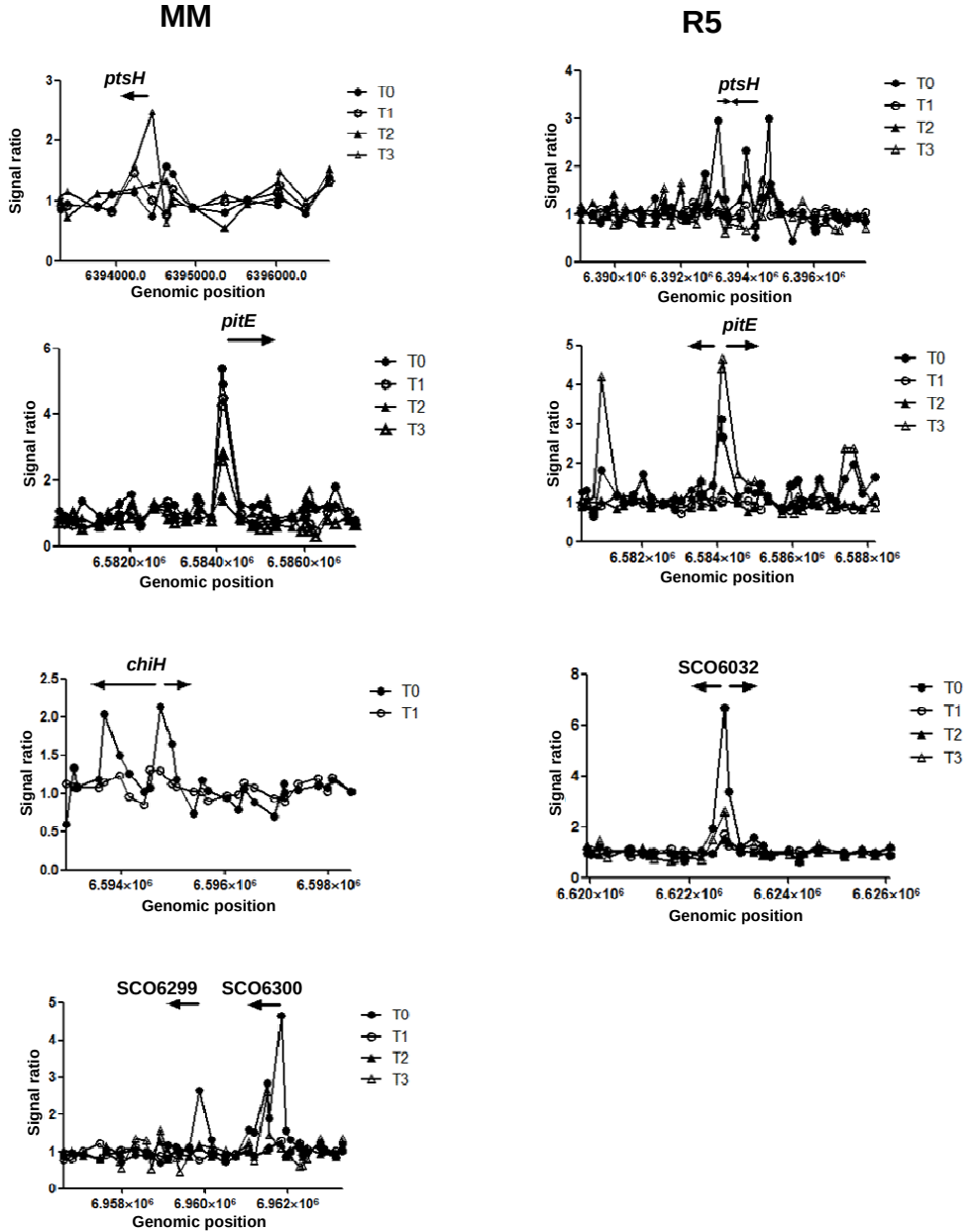


Figure S3. Chip-on-chip data for selected DasR targets detected in the GlcNAc induction experiment. Samples were collected prior to (T₀, closed circles) and 30 (T₁, open circles), 60 (T₂, closed triangle) or 120 min (T₃, open triangle) after GlcNAc addition. Plots indicate changing of the DasR binding to the promoter regions of 10 known DasR targets (*crr-ptsI*, *nagE1-nagE2*, *nagKA*, *dasR-dasABC*, *nagB*, *ptsH*, *pitE*, *chiH*, SCO6032, SCO6300) upon amino sugar addition to MM (left panel) and R5 (right panel). The arrows indicate the orientation of targeted gene.

Table S1. List of differentially expressed genes between the *dasR* mutant and wild-type

	D/M_24h	D/M_30h	D/M_36h	D/M_42h	D/M_54h						
SCO0019	-1.43	0.45	0.40	1.20	-1.10	SCO1914	0.77	-1.48	-0.21	-0.50	1.38
SCO0022	0.07	0.21	0.17	1.27	0.95	SCO1978	0.52	-1.14	-0.07	-1.50	0.41
SCO0098	-0.10	0.04	0.40	1.04	-0.13	SCO1982	1.08	1.20	0.43	-0.22	-0.66
SCO0107	1.00	0.85	-0.25	-0.40	-1.20	SCO2008	0.85	1.39	1.63	2.01	1.61
SCO0141	-0.24	-0.22	-0.38	-0.20	-1.25	SCO2012	0.44	0.90	1.28	1.64	1.04
SCO0193	0.01	0.50	0.13	0.43	1.11	SCO2113	-1.39	-1.51	-1.16	-0.84	0.20
SCO0194	-0.76	0.29	-0.21	0.48	1.24	SCO2151	0.23	-1.02	-0.42	0.03	1.50
SCO0195	-0.59	0.17	-0.26	0.27	1.21	SCO2155	-0.02	-0.57	-0.25	0.15	1.00
SCO0196	-1.20	0.16	-0.11	0.44	0.61	SCO2156	0.90	-1.16	-0.21	-0.11	1.50
SCO0381	0.02	1.06	0.73	0.92	0.28	SCO2195	-0.21	0.15	0.63	0.40	1.04
SCO0383	-0.04	0.94	1.00	1.30	0.17	SCO2205	0.08	1.26	1.67	1.65	0.24
SCO0396	-0.31	0.91	0.92	1.32	0.45	SCO2207	1.45	1.03	1.57	1.80	0.93
SCO0399	0.08	0.86	0.74	1.23	0.48	SCO2210	0.98	0.49	0.72	0.91	1.47
SCO0400	-0.18	0.88	0.67	1.09	0.36	SCO2217	1.15	0.15	1.62	2.48	2.45
SCO0494	0.94	0.26	0.19	1.03	-0.09	SCO2218	0.48	0.40	0.84	1.12	0.11
SCO0500	1.02	0.45	-0.54	0.50	-0.61	SCO2236	1.20	0.47	-0.60	0.30	-0.21
SCO0502	1.18	-1.47	0.02	-0.21	0.22	SCO2261	-0.48	-0.70	-0.50	-1.71	-0.79
SCO0527	1.43	-0.66	-0.01	-0.30	0.71	SCO2288	-0.07	-0.08	-1.23	-0.48	0.20
SCO0569	0.17	0.71	0.70	1.21	0.45	SCO2379	1.44	0.90	0.27	0.49	-0.16
SCO0591	-0.03	-0.40	-1.36	-0.64	0.98	SCO2389	0.07	-0.84	-0.68	-0.07	1.01
SCO0644	0.92	0.09	0.97	1.37	1.18	SCO2492	0.94	1.15	0.89	1.21	0.86
SCO0736	1.03	-0.13	1.30	0.57	0.89	SCO2496	0.73	0.77	0.90	1.28	0.29
SCO0850	-0.05	-0.33	-1.28	-0.28	0.52	SCO2512	-0.32	0.12	-0.29	0.23	-1.22
SCO0923	0.10	-1.11	-0.05	0.00	-0.13	SCO2530	-1.06	-0.26	-0.31	-0.30	0.10
SCO0930	1.01	0.34	1.44	1.78	1.41	SCO2573	0.48	0.47	1.06	1.22	1.01
SCO0932	-1.42	-0.44	-0.35	0.19	-0.02	SCO2574	0.60	1.02	0.69	1.15	0.12
SCO0933	0.13	-1.11	-0.28	-0.87	0.22	SCO2591	0.13	0.89	1.07	1.01	0.19
SCO0934	-0.15	-1.53	-2.11	-1.07	0.76	SCO2597	0.74	-0.75	0.02	0.22	1.11
SCO0955	-0.19	-0.27	-0.63	0.10	1.06	SCO2604	-0.43	1.13	0.45	0.99	-0.61
SCO0996	0.15	1.13	0.89	1.21	-0.42	SCO2636	-0.43	0.01	-0.04	-0.74	-1.30
SCO1050	0.13	-0.34	-1.03	-0.72	0.15	SCO2658	-0.29	0.15	0.05	1.05	0.77
SCO1121	1.15	0.19	1.33	1.79	1.23	SCO2699	-0.83	-1.16	-1.57	-1.03	-0.30
SCO1143	-0.19	-0.34	-1.40	-0.94	-0.65	SCO2705	-0.83	-2.30	-1.70	-0.93	0.59
SCO1174	-1.99	0.14	0.06	-0.03	-0.12	SCO2716	-1.06	-1.48	-1.49	-0.87	-0.19
SCO1189	0.00	0.42	0.71	1.09	0.83	SCO2717	-1.30	-2.40	-0.86	-0.37	0.80
SCO1192	-2.42	0.62	0.14	0.04	-0.41	SCO2718	-1.57	-3.31	-1.30	-1.02	1.65
SCO1195	-0.10	-0.37	-1.04	-0.51	-0.43	SCO2719	-1.79	-2.64	-1.17	-0.79	1.30
SCO1236	-0.01	-0.03	0.03	0.37	1.00	SCO2739	-0.30	-0.02	-0.25	-0.04	-1.03
SCO1240	-0.06	-0.07	-1.11	-0.34	0.73	SCO2778	0.20	-1.16	-0.47	-0.46	-0.15
SCO1255	0.61	-1.16	0.13	-0.47	-0.93	SCO2789	1.12	0.93	0.96	0.91	0.71
SCO1276	-0.55	0.73	0.01	0.12	-1.63	SCO2819	0.26	1.18	0.64	0.93	0.48
SCO1288	-0.16	0.48	0.35	1.24	-0.66	SCO2823	-0.07	-0.03	-0.13	0.48	1.31
SCO1321	1.21	0.48	0.26	0.17	-0.13	SCO2905	3.46	1.96	2.47	2.80	3.64
SCO1322	1.07	0.12	-0.05	0.06	-0.03	SCO2907	1.62	1.06	1.71	2.11	1.65
SCO1345	-1.09	-0.31	-0.33	-0.03	-0.19	SCO2924	-0.47	-1.10	-1.20	-1.93	-1.06
SCO1356	0.86	0.39	1.24	1.56	0.98	SCO2953	1.16	-0.03	1.00	2.00	1.21
SCO1357	0.39	0.54	1.06	1.23	0.50	SCO2954	0.77	0.53	1.11	1.71	1.44
SCO1415	0.25	-0.98	-1.36	-1.03	-0.15	SCO2976	0.26	0.91	0.70	1.21	1.35
SCO1426	-0.76	-0.40	-0.52	-0.55	-1.09	SCO3086	1.74	1.55	1.08	-0.93	0.45
SCO1427	-1.83	-0.37	-0.59	-0.17	0.29	SCO3092	0.55	-0.04	0.61	0.72	1.21
SCO1474	-1.19	-0.44	-0.44	-0.34	-0.28	SCO3105	1.36	1.16	0.65	0.17	-0.04
SCO1480	0.93	-0.06	0.18	0.60	1.53	SCO3106	1.28	0.92	0.57	0.10	-0.01
SCO1505	0.95	-1.04	0.01	0.00	0.96	SCO3113	0.86	-0.43	-0.28	0.81	1.04
SCO1557	0.18	-0.08	1.19	0.60	0.28	SCO3121	0.13	1.14	0.08	0.57	-1.00
SCO1573	0.46	0.43	0.87	1.12	0.87	SCO3218	-1.56	0.11	-0.04	0.09	-0.54
SCO1575	0.22	0.28	0.89	1.21	0.65	SCO3220	-0.89	-0.37	-0.20	-0.54	-1.11
SCO1599	1.06	-1.09	-0.15	-0.17	1.18	SCO3222	-2.17	-0.27	0.01	-0.06	-0.36
SCO1600	1.42	-0.40	0.06	0.24	0.80	SCO3224	-1.11	0.17	-0.05	0.28	-1.20
SCO1604	-0.74	1.21	0.46	1.16	-0.66	SCO3244	-1.15	-0.25	-0.26	-0.13	-0.76
SCO1659	-1.07	-0.51	-0.34	-0.30	0.02	SCO3262	-0.62	0.83	-0.27	0.30	-1.05
SCO1660	-1.53	-0.27	-0.16	-0.01	0.16	SCO3263	2.96	2.59	4.76	5.53	3.99
SCO1674	-1.07	-1.09	-0.39	-0.27	0.44	SCO3265	2.12	2.17	3.25	3.45	2.81
SCO1675	-0.53	-2.23	-0.44	-0.39	1.39	SCO3266	0.51	0.93	1.33	1.77	0.61
SCO1700	0.86	0.54	0.99	0.32	1.38	SCO3268	2.31	1.83	3.45	4.08	4.16
SCO1721	0.78	-0.85	-0.04	0.06	1.08	SCO3269	-0.61	-0.03	-0.48	-0.21	-1.06
SCO1756	-1.03	-0.28	-0.54	-0.14	-0.31	SCO3271	-0.30	-0.37	-1.34	-0.34	-0.50
SCO1795	2.16	1.05	0.36	0.47	-0.35	SCO3278	0.00	-0.48	0.09	-1.12	-0.36
SCO1800	-1.73	-2.21	-1.25	-0.25	1.03	SCO3299	2.48	0.42	0.31	0.17	-0.60
SCO1828	-0.46	-1.27	-1.85	-0.88	0.18	SCO3309	-0.55	-0.53	-0.14	-1.17	-0.52
SCO1839	0.22	0.06	-0.70	0.17	1.43	SCO3323	0.29	0.01	0.01	0.73	1.35
SCO1905	1.90	0.85	0.28	0.24	-0.18	SCO3327	0.56	-0.91	-0.07	-0.06	1.56
						SCO3343	0.60	0.31	1.02	0.76	-0.03
						SCO3356	1.35	-0.12	0.83	0.06	1.00

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SCO3467	-0.41	0.61	0.48	1.11	-0.39	SCO4649	1.33	-0.88	0.17	0.38	1.96
SCO3475	-0.01	-0.65	-0.98	-1.26	-0.88	SCO4651	1.21	1.17	0.77	0.44	0.30
SCO3476	-0.28	-0.29	-0.59	-0.93	-1.02	SCO4652	1.00	-1.84	-0.02	-0.09	1.89
SCO3479	-0.28	-0.51	-0.66	-1.00	-0.77	SCO4653	0.51	-1.05	0.00	0.20	1.06
SCO3480	-0.15	-0.27	-0.64	-1.02	-0.84	SCO4659	1.30	-1.08	-0.05	-0.09	1.40
SCO3481	0.11	-0.85	-0.98	-1.20	-0.90	SCO4660	1.27	-0.83	-0.07	0.02	1.27
SCO3482	-0.40	-0.68	-0.94	-1.16	-0.53	SCO4662	1.08	-0.79	0.14	0.42	1.99
SCO3483	0.04	-1.12	-1.00	-1.30	-0.61	SCO4663	0.94	0.13	0.54	0.84	1.08
SCO3484	-0.28	-0.83	-1.18	-1.47	-1.24	SCO4677	0.68	1.18	0.78	0.99	1.54
SCO3487	-0.18	-1.57	-1.54	-1.76	-1.42	SCO4687	0.72	0.53	0.39	1.06	1.13
SCO3488	0.08	-0.81	-0.94	-1.42	-0.72	SCO4701	1.65	-0.79	0.13	0.18	1.75
SCO3492	-0.12	-0.02	0.20	-0.70	-1.17	SCO4702	1.41	-0.98	0.18	0.02	1.82
SCO3549	0.54	-0.60	-0.04	0.39	1.01	SCO4703	0.81	-0.76	0.02	0.02	1.29
SCO3561	-0.13	-1.07	-2.11	-1.48	-1.48	SCO4704	1.39	-1.04	-0.03	0.20	1.84
SCO3579	0.58	-0.16	0.01	0.08	1.13	SCO4705	1.23	-0.96	-0.09	0.05	1.54
SCO3607	-0.60	0.15	0.20	-0.01	-1.14	SCO4706	1.53	-1.11	0.07	0.03	1.87
SCO3660	-1.01	0.08	-0.26	-0.26	-0.90	SCO4707	1.79	-0.89	-0.11	0.15	1.66
SCO3662	0.01	0.46	0.32	1.14	1.13	SCO4708	0.88	-0.78	0.21	0.15	1.35
SCO3663	-0.10	-1.15	-0.64	-0.73	0.20	SCO4709	1.15	-0.83	0.33	0.30	1.58
SCO3671	0.78	-0.25	0.19	0.24	1.21	SCO4711	1.53	-1.49	0.01	0.15	2.08
SCO3720	-0.91	-0.24	1.08	0.48	-0.55	SCO4712	1.00	-1.30	0.10	0.06	1.71
SCO3731	1.28	-0.58	-0.26	-0.30	0.59	SCO4713	1.15	-1.15	-0.05	0.12	1.17
SCO3748	1.28	-0.44	-0.01	-0.24	1.04	SCO4714	0.98	-1.48	-0.03	-0.08	1.67
SCO3762	-0.70	-0.72	-0.46	-0.45	-1.03	SCO4715	0.81	-1.37	-0.14	-0.10	1.57
SCO3793	0.82	1.14	1.20	0.12	0.18	SCO4716	0.88	-0.97	0.05	0.13	1.60
SCO3863	0.37	-2.14	-1.65	-1.20	-0.50	SCO4717	1.09	-1.15	0.07	0.04	1.77
SCO3871	-0.32	-0.41	-1.41	-0.76	-0.63	SCO4718	1.03	-1.03	0.18	0.16	1.52
SCO3899	0.08	-0.94	0.07	0.56	1.23	SCO4719	0.47	-0.75	0.04	0.17	1.03
SCO3900	-0.03	-1.14	0.02	0.15	1.13	SCO4720	0.83	-1.68	-0.09	-0.01	2.21
SCO3906	0.50	-1.07	0.08	-0.09	1.18	SCO4721	0.81	-1.66	0.00	-0.11	1.92
SCO3908	0.55	-0.94	0.31	0.09	1.06	SCO4723	0.73	0.53	0.77	0.78	1.07
SCO3909	0.42	-1.32	0.00	-0.01	1.06	SCO4725	1.63	-0.88	0.44	0.19	2.12
SCO3918	-0.52	0.01	0.26	0.29	-1.40	SCO4727	1.19	-0.91	0.00	0.02	1.37
SCO3982	4.08	3.87	5.16	6.00	5.55	SCO4728	1.09	-1.01	0.20	0.15	1.44
SCO3983	3.46	2.79	4.08	4.53	4.60	SCO4729	0.95	-0.86	0.38	0.02	1.14
SCO3984	1.79	2.02	2.68	2.92	2.44	SCO4730	1.19	-0.86	0.27	0.27	1.26
SCO3985	3.05	3.09	4.26	4.84	4.19	SCO4734	1.59	0.13	0.28	0.45	1.18
SCO3986	2.80	1.95	3.61	3.94	3.67	SCO4735	1.09	0.15	0.34	0.26	0.91
SCO3987	3.28	2.99	3.93	4.51	4.16	SCO4740	0.42	-0.02	0.04	0.37	1.36
SCO3988	2.06	1.61	2.25	2.67	2.49	SCO4756	0.18	0.50	0.51	1.07	0.57
SCO3989	-0.52	0.65	0.55	1.10	-0.32	SCO4759	0.00	-0.06	-1.02	-0.28	-0.28
SCO3990	-0.47	0.69	0.45	1.00	-1.22	SCO4761	0.99	-0.91	0.04	1.32	2.32
SCO4002	-0.93	-0.74	-1.03	-0.80	-1.44	SCO4762	0.22	-0.25	0.41	1.36	1.37
SCO4032	-0.17	-1.18	-0.55	-0.60	-0.35	SCO4770	0.79	0.50	0.80	1.01	0.97
SCO4057	-0.12	-1.06	-1.52	-1.30	-0.63	SCO4779	1.33	0.85	1.04	-0.17	0.44
SCO4063	-0.23	0.39	0.66	1.29	-0.59	SCO4789	0.93	0.70	1.15	1.49	1.26
SCO4091	0.37	0.22	0.19	0.47	1.27	SCO4794	0.61	0.42	0.64	1.02	0.55
SCO4177	1.47	0.15	0.08	0.47	0.68	SCO4800	0.36	0.67	1.00	0.64	1.15
SCO4178	1.81	0.36	0.25	0.58	0.95	SCO4808	0.71	-0.45	0.67	0.76	2.00
SCO4187	-0.57	0.98	0.50	0.16	1.54	SCO4809	0.39	-0.39	0.40	0.58	1.54
SCO4198	-0.39	-1.24	-0.75	-0.43	0.75	SCO4823	0.30	1.05	-0.22	0.90	0.19
SCO4212	0.25	-1.86	-0.08	-1.20	0.73	SCO4838	0.52	-1.09	-0.20	-0.56	0.58
SCO4213	-0.58	0.11	0.08	0.22	-1.14	SCO4839	0.74	0.29	0.65	0.58	1.13
SCO4216	0.30	0.81	0.33	0.74	-1.14	SCO4840	1.05	0.56	0.37	0.66	0.95
SCO4239	-0.98	-1.07	-0.78	-0.07	-0.18	SCO4854	0.68	0.30	0.85	1.12	1.70
SCO4251	-1.34	-0.29	-0.28	-0.01	-0.11	SCO4855	1.05	0.52	0.60	0.57	0.98
SCO4252	-1.79	0.11	0.18	0.72	-0.13	SCO4856	1.25	0.45	0.55	0.78	1.04
SCO4266	-0.26	-0.55	-1.28	0.26	0.19	SCO4858	0.99	0.55	0.49	0.77	1.36
SCO4285	1.24	0.79	1.46	1.59	1.50	SCO4861	0.32	1.31	1.21	1.70	0.68
SCO4289	1.11	0.72	0.92	-1.04	-0.16	SCO4883	1.15	0.79	0.80	0.95	1.61
SCO4296	0.27	-0.41	0.02	1.04	1.38	SCO4884	0.30	0.98	1.15	0.62	0.46
SCO4442	0.32	-0.49	0.12	0.19	1.91	SCO4894	0.81	1.05	1.24	1.98	0.89
SCO4516	-0.30	1.12	0.26	0.61	-0.87	SCO4895	0.53	0.85	0.92	1.04	0.73
SCO4540	-0.50	1.20	-0.45	0.15	-1.33	SCO4902	-1.48	-1.15	-0.50	-0.12	0.84
SCO4543	-1.01	-0.13	-0.21	-0.05	-0.35	SCO4903	0.70	0.55	0.61	1.39	0.74
SCO4562	-0.73	-0.39	-1.08	-0.40	0.00	SCO4904	1.32	1.13	0.97	-0.13	0.43
SCO4611	-0.04	0.38	0.65	0.72	1.06	SCO4908	1.16	1.07	0.73	-0.42	1.35
SCO4630	1.63	1.59	1.11	1.44	0.39	SCO4920	0.31	0.71	0.64	0.74	1.38
SCO4635	2.37	-0.03	0.48	0.42	2.03	SCO4968	0.59	-0.45	0.10	0.35	1.41
SCO4636	0.73	0.87	0.61	0.70	1.38	SCO5029	0.75	-0.69	0.36	0.42	1.86
SCO4642	0.62	-0.23	0.25	0.30	1.19	SCO5071	-0.45	-0.27	-0.47	1.23	0.30
SCO4648	1.49	-0.48	0.53	0.64	2.09	SCO5074	-1.04	-0.57	-0.36	1.52	0.84

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SCO5075	-0.26	-0.26	-0.24	1.07	0.45
SCO5078	-0.37	-0.34	-0.09	1.18	0.35
SCO5082	-0.24	-0.58	-0.18	1.14	1.34
SCO5083	-0.07	-0.86	-0.49	1.14	0.89
SCO5084	-0.02	-1.23	-0.49	1.48	1.22
SCO5085	-0.89	-0.48	-0.06	1.31	0.77
SCO5089	-0.40	-0.33	-0.45	1.04	0.36
SCO5112	0.23	0.64	0.46	0.85	1.06
SCO5113	0.04	0.61	0.66	1.00	1.16
SCO5137	-1.06	-0.49	-0.39	-0.44	-0.23
SCO5163	0.25	0.08	0.10	0.12	1.11
SCO5189	2.22	0.75	0.13	0.10	0.12
SCO5190	3.31	0.82	-0.10	0.35	-0.39
SCO5191	3.15	0.22	0.31	0.35	-0.56
SCO5232	1.38	1.43	1.47	1.58	0.64
SCO5233	0.49	0.39	0.78	1.11	0.29
SCO5234	0.44	0.41	1.10	1.11	0.29
SCO5236	1.89	1.48	2.04	2.06	2.01
SCO5249	-1.46	-0.77	-0.91	-0.68	-0.25
SCO5272	-0.55	-0.79	-0.53	-1.55	-0.60
SCO5276	-0.52	-0.06	-0.53	-0.36	-1.14
SCO5314	0.36	-0.15	-0.93	-0.53	1.04
SCO5316	0.01	-0.18	-0.99	-0.66	1.22
SCO5319	0.00	-1.27	-1.18	-0.46	0.69
SCO5326	-0.13	0.02	0.48	1.46	-0.97
SCO5329	-0.61	1.44	0.51	1.05	-0.66
SCO5332	-0.47	1.24	0.41	0.38	-1.64
SCO5389	-2.36	-0.27	-0.54	0.02	-0.37
SCO5476	0.50	0.94	1.13	1.15	0.65
SCO5477	0.91	1.01	0.85	1.40	1.10
SCO5478	0.69	0.80	0.99	1.12	0.68
SCO5498	0.57	0.10	0.21	0.46	1.06
SCO5521	1.04	1.01	0.70	1.05	0.86
SCO5542	0.18	0.48	0.84	1.35	0.66
SCO5556	-0.13	-1.60	-1.66	-0.77	0.30
SCO5571	1.07	-1.14	-0.07	-0.07	1.29
SCO5576	-0.11	-0.03	-0.09	-0.15	-1.03
SCO5582	0.04	0.07	-0.01	0.19	1.13
SCO5583	0.31	0.13	0.43	0.68	1.23
SCO5584	0.55	0.05	0.21	0.50	1.16
SCO5590	-0.42	-0.16	0.03	0.16	1.01
SCO5591	0.93	-0.74	0.34	0.03	1.44
SCO5592	1.30	-0.40	-0.09	0.23	1.33
SCO5609	-1.34	-0.61	-0.82	-0.98	-1.21
SCO5610	1.17	1.74	0.65	0.58	-0.38
SCO5611	0.10	0.72	0.42	1.48	-0.77
SCO5614	-0.86	0.55	0.10	1.20	-0.98
SCO5632	0.16	1.39	1.26	1.59	2.61
SCO5638	-0.91	0.82	-0.37	0.18	-1.45
SCO5639	-1.29	0.82	0.10	0.94	-1.34
SCO5640	-0.87	0.66	-0.49	0.11	-1.25
SCO5650	0.54	0.23	0.41	0.57	1.03
SCO5672	-0.20	-1.00	-0.69	-1.35	-0.80
SCO5705	1.08	-0.24	-0.08	0.03	0.83
SCO5736	0.85	-0.83	0.00	0.05	1.44
SCO5789	-1.80	-0.49	-0.59	0.15	0.04
SCO5796	1.05	0.63	0.28	-0.01	-0.05
SCO5826	-1.17	0.05	-0.11	0.70	0.92
SCO5841	1.51	1.30	1.18	1.23	1.59
SCO5885	-0.80	0.18	0.91	1.02	0.04
SCO5920	0.98	1.29	0.16	-1.14	-0.48
SCO5990	-0.18	-0.55	-1.13	-0.82	-0.58
SCO6005	1.07	1.23	1.45	2.10	1.98
SCO6006	0.60	0.94	1.11	1.39	1.47
SCO6007	0.78	0.85	1.35	1.84	1.27
SCO6027	-1.24	-0.51	-0.23	-0.72	-0.29
SCO6029	0.08	-1.00	-0.83	-0.50	0.26
SCO6042	0.09	-0.24	0.14	0.44	1.07
SCO6149	1.86	1.15	0.43	0.43	-0.21
SCO6197	0.35	1.56	1.57	2.41	0.67
SCO6240	0.90	1.35	0.08	0.79	-0.61
SCO6266	1.10	0.28	0.32	0.54	0.00

SCO6276	-1.07	0.91	0.10	0.25	0.05
SCO6279	-1.02	1.03	0.13	0.27	0.10
SCO6282	-1.59	0.53	0.29	0.25	0.86
SCO6283	-1.05	0.10	0.05	0.18	0.07
SCO6338	-0.19	0.11	0.39	1.35	-0.89
SCO6373	-0.29	-0.09	-1.10	-0.39	-0.35
SCO6374	-0.03	-0.16	-1.07	-0.45	-0.60
SCO6401	-0.31	1.09	0.33	1.00	-0.66
SCO6433	-0.45	0.62	0.62	1.50	0.10
SCO6440	-0.04	-0.02	-0.29	-0.33	-1.04
SCO6441	-0.10	0.00	-0.50	-0.35	-1.12
SCO6543	-0.25	-0.02	-0.28	0.16	-1.02
SCO6592	0.46	0.98	1.04	0.86	-0.62
SCO6624	-0.68	-1.64	-0.58	0.41	1.53
SCO6631	0.15	1.02	0.69	0.97	0.64
SCO6650	1.41	0.13	1.79	2.46	2.75
SCO6652	0.56	0.44	1.02	1.08	0.85
SCO6655	0.66	0.46	1.34	2.02	1.38
SCO6682	-4.44	-3.13	-2.72	-1.73	-0.77
SCO6715	0.40	1.42	-0.38	-1.92	-0.80
SCO6736	0.09	-0.02	1.04	0.09	-0.45
SCO6810	-0.13	-1.14	-0.38	-0.76	0.29
SCO6812	0.37	1.42	0.20	0.10	-0.43
SCO6836	-1.27	0.01	0.40	-0.17	0.29
SCO6906	-0.14	1.21	0.37	1.05	-0.14
SCO6909	-0.46	0.56	-0.06	-0.24	-1.29
SCO6910	-0.52	0.49	0.24	1.29	-0.60
SCO6952	1.05	0.62	0.05	0.06	-0.52
SCO7106	0.02	-1.05	-0.72	-0.89	-0.52
SCO7190	-0.23	0.85	0.36	1.02	-0.21
SCO7195	-0.13	0.73	0.11	0.56	-1.05
SCO7252	-0.44	0.24	0.28	0.65	1.31
SCO7257	-1.18	-0.48	-0.45	0.22	0.20
SCO7336	-0.19	-0.21	-2.00	-0.99	0.23
SCO7387	0.24	-1.63	-0.16	-0.30	0.14
SCO7434	0.11	-0.91	-1.92	-1.48	-0.65
SCO7449	0.21	-1.12	-1.91	-1.28	0.83
SCO7452	-0.12	-1.20	-1.40	-1.17	-0.03
SCO7453	-0.10	-2.05	-1.91	-1.33	0.07
SCO7536	0.04	0.51	1.11	1.15	0.19
SCO7643	0.21	-0.04	0.12	1.09	-0.43
SCO7657	-0.25	-0.38	0.38	1.26	0.96
SCO7658	-0.01	-0.05	0.92	1.55	0.96
SCO7676	-0.46	-1.05	-0.58	-0.70	-0.48
SCO7699	-1.09	-0.43	-0.78	-0.69	-0.55
SCO7701	-1.02	-0.20	-0.82	-0.65	-0.90
SCO7717	-0.93	-0.33	-0.24	-0.29	-1.36
SCO7800	0.06	0.56	0.16	1.10	-0.97
SCO7801	-0.41	0.81	0.27	0.80	-1.07
SCO7805	-0.48	-0.46	-0.64	-1.11	-0.74
SCO7808	-0.31	-0.67	-1.28	-0.67	-0.26

Table S2. Binding by DasR as identified by genome-wide ChIP-on-chip experiments. Expression, trend in microarray analysis.

Nr	Gene ID	Annotation	Name	24h	54h
1	SCO0003 intragenic	putative DNA-binding protein		NB	B (1)
2	SCO0005 intragenic	putative transposase		NB	B (1)
3	SCO0018 upstream	hypothetical protein		NB	B (1)
4	SCO0020 intragenic	putative transposase		NB	B (2)
5	SCO0046 intragenic	putative hydrolase		NB	B(1)
6	SCO0050 intragenic	hypothetical protein		NB	B(1)
7	SCO0053 upstream	putative transposase		NB	B(1)
8	SCO0055 intragenic	putative membrane-associated oxidoreductase		NB	B(1)
9	SCO0072 intragenic	putative secreted protein		NB	B(1)
10	SCO0073 upstream	hypothetical protein		NB	B(1)
11	SCO0079 intragenic	putative integral membrane transport protein		NB	B(1)
12	SCO0086 upstream	putative ribosylglycohydrolase (putative secreted protein)		NB	B(1)
13	SCO0090 intragenic	putative transposase		NB	B(1)
14	SCO0096 upstream	putative noncomposite transposon transposase		NB	B(1)
15	SCO0097 upstream	putative integral membrane protein		NB	B(3)
16	SCO0103 intragenic	probable flavohemoprotein		NB	B(1)
17	SCO0130 intragenic	possible beta-lactamase		NB	B(1)
18	SCO0166 intragenic	possible regulator		NB	B(1)
19	SCO0239 intragenic	hypothetical protein		NB	B(1)
20	SCO0261 upstream	probable acetyltransferase (SCO0261)		NB	B(2)
21	SCO0271 intragenic	possible binding-protein-dependent transport protein		NB	B(1)
22	SCO0287 intragenic	possible regulatory protein		NB	B(1)
23	SCO0311 intragenic	probable ligase		NB	B(1)
24	SCO0319 intragenic	hypothetical protein		NB	B(1)
25	SCO0368 intragenic	transposase		NB	B(1)
26	SCO0382 upstream	probable UDP-glucose/GDP-mannose family dehydrogenase (putative secreted protein)		B(1)	NB
27	SCO0432 upstream	probable secreted peptidase		B(1)	NB
28	SCO0511 intragenic	possible dehydrogenase (putative secreted protein)		B(1)	NB
29	SCO0588 intragenic	possible sensor kinase	<i>cvnA11</i>	B(1)	NB
30	SCO0615 intragenic	putative secreted protein		B(1)	NB
31	SCO0661 intragenic	possible binding protein dependent transport protein		NB	B(1)
32	SCO0915 upstream	hypothetical protein		NB	B(1)
33	SCO0923 intragenic	probable reductase flavoprotein subunit		NB	B(1)
34	SCO0947 intragenic	possible integral membrane protein		NB	B(1)
35	SCO0961 intragenic	glucose-1-phosphate adenylyltransferase	<i>glgC</i>	NB	B(1)
36	SCO0968 upstream	hypothetical protein		NB	B(1)
37	SCO0976 intragenic	hypothetical protein		B(1)	NB
38	SCO1061 intragenic	probable bifunctional protein (secreted sugar binding protein/sugar hydrolase)		NB	B(1)
39	SCO1109 intragenic	probable oxidoreductase		NB	B(1)
40	SCO1116 upstream	hypothetical protein (SCO1116)		NB	B(1)
41	SCO1166-67 intragenic	putative integral membrane protein (SCO1166)		NB	B(1)
		probable helicase (fragment) (SCO1167)			
42	SCO1276 upstream	RNA polymerase ECF sigma factor	<i>sigJ</i>	NB	B(2)
43	SCO1321 upstream	elongation factor TU-3	<i>tuf3</i>	B(1)	NB
44	SCO1327 intragenic	possible integral membrane protein		NB	B(1)
45	SCO1390-SCO1391 ups	PTS sugar phosphotransferase component IIA	<i>crr</i>	B(1)	B(1)
		phosphoenolpyruvate-protein phosphotransferase PtsI	<i>ptsI</i>	B(1)	NB
46	SCO1429 upstream	chitinase	<i>chiD</i>	B(2)	B(1)
47	SCO1444 upstream	chitinase	<i>chiI</i>	B(1)	B(1)
48	SCO1471 intragenic	putative transposase		NB	B(1)
49	SCO1488-89 intergenic	pyrimidine operon regulatory protein DNA-binding protein BldD	<i>pyrR</i> <i>bldD</i>	NB	B(1)
50	SCO1499 intragenic	possible integral membrane protein		NB	B(1)

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51	SCO1538 intragenic	probable transport system membrane protein		NB	B(1)
52	SCO1645 intragenic	conserved hypothetical protein		NB	B(2)
53	SCO1793 intragenic	putative secreted protein		NB	B(1)
54	SCO2013 upstream	probable two-component system response regulator		NB	B(1)
55	SCO2062-63 intragenic	hypothetical protein (SCO2062)		NB	B(1)
		putative small hydrophilic protein (SCO2063)			
56	SCO2064-65 intragenic	DNA polymerase III alpha chain (SCO2064)	<i>dnaE</i>	NB	B(1)
		conserved hypothetical protein			
57	SCO2078 upstream+intr	putative membrane protein		NB	B(2)
58	SCO2107 intragenic	possible oxidoreductase		NB	B(1)
59	SCO2125 upstream	hypothetical protein		NB	B(1)
60	SCO2188 intragenic	possible peptidase (putative secreted protein)		B(1)	NB
61	SCO2198 intragenic	glutamine synthetase I	<i>glnA</i>	NB	B(1)
62	SCO2371 intragenic	pyruvate dehydrogenase E1 component	<i>aceE2</i>	NB	B(1)
63	SCO2381 upstream+intr	conserved hypothetical protein		NB	B
64	SCO2474 intragenic	putative metalloproteinase (putative secreted protein)		B(1)	NB
65	SCO2490 intragenic	putative dehydrogenase (putative secreted protein)		B(1)	NB
66	SCO2494-95 intragenic	putative pyruvate phosphate dikinase (SCO2494)		NB	B(1)
		putative membrane protein (SCO2495)			
67	SCO2503 upstream	chitinase	<i>chiJ</i>	B(2)	B(1)
68	SCO2504-05 intergenic	glycyl-tRNA synthetase	<i>glyS</i>	NB	B(2)
		putative ABC-transporter metal-binding lipoprotein (SCO2505)			
69	SCO2528-29 intragenic	2-isopropylmalate synthase (SCO2528)	<i>leuA</i>	NB	B(1)
		putative metalloprotease (SCO2529)			
70	SCO2532 upstream+intr	PhoH-like protein		NB	B(2)
71	SCO2561 upstream	possible long-chain fatty-acid CoA ligase		NB	B(1)
72	SCO2562-63 intergenic	GTP-binding protein	<i>lepA</i>	NB	B(1)
		30s ribosomal protein S20 (SCO2563)	<i>lpsT</i>		
		putative integrase; Contains TTA leucine codon, possible target for bldA regulation		NB	B(1)
73	SCO2603 intragenic			NB	B(1)
74	SCO2629 intragenic	putative membrane protein		B(1)	NB
75	SCO2632 intragenic	putative transposase		NB	B(1)
76	SCO2650 upstream	putative secreted protein		NB	B(1)
77	SCO2669 intragenic	hypothetical protein		B(1)	NB
78	SCO2681 intragenic	possible ATP/GTP-binding protein		NB	B(1)
79	SCO2724-25 intergenic	putative amino acid transporter (SCO2724)		NB	B(1)
		possible lipoprotein			
80	SCO2729 intragenic	possible acetyltransferase		B(1)	NB
81	SCO2738 intragenic	hypothetical protein		NB	B(1)
82	SCO2783 intragenic	probable monooxygenase	<i>desB,des</i>	NB	B(1)
83	SCO2833 upstream	secreted chitin binding protein	<i>chb</i>	B(1)	B(1)
84	SCO2836 intragenic	possible glycosyl transferase		NB	B(1)
85	SCO2868 intragenic	conserved hypothetical protein		NB	B(1)
86	SCO2898 upstream	molecular chaperone	<i>sugE</i>	B(1)	B(1)
87	SCO2902 intragenic	conserved hypothetical protein		B(1)	NB
88	SCO2904 upstream	putative ribonuclease PH	<i>rph</i>	B(1)	NB
89	SCO2906 upstream	PTS transmembrane component NagF	<i>nagF</i>	B(2)	B(2)
90	SCO2907 upstream	PTS transmembrane component NagE2	<i>nagE2</i>	B(2)	B(2)
91	SCO2945 intragenic	putative sugar transporter membrane protein		NB	B(1)
92	SCO2946 upstream	putative sugar transporter sugar-binding protein		NB	B(1)
93	SCO2977 intragenic	hypothetical protein		NB	B(2)
94	SCO2986-87 upstream	conserved hypothetical protein	<i>ohrA</i>	NB	B(1)
		putative regulatory protein (SCO2987)	<i>ohrR</i>		
95	SCO2990-91 intragenic	hypothetical protein (SCO2990)		B(1)	NB
		hypothetical protein (SCO2991)			

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96	SCO3005 intragenic	preprotein translocase	<i>secA</i>	NB	B(1)
97	SCO3043 upstream	conserved hypothetical protein		NB	B(1)
98	SCO3067-68 intergenic	putative anti anti sigma factor	<i>arsI</i>	NB	B(1)
		putative RNA polymerase sigma factor	<i>sig15, sigI</i>		
99	SCO3079 upstream	putative thiolase		B(1)	NB
100	SCO3092 upstream	ncRNA		B(3)	B(4)
101	SCO3112 intragenic	unknown		NB	B(1)
102	SCO3138-39 intergenic	galactose-1-phosphate uridylyltransferase (SCO3138)	<i>galT</i>	NB	B(1)
		putative sodium:solute symporter (SCO3139)			
103	SCO3218 downstream	putative small conserved hypothetical protein		B(1)	NB
104	SCO3230 intragenic	CDA peptide synthetase I	<i>cdaPSI</i>	NB	B(3)
105	SCO3231 intragenic	CDA peptide synthetase II	<i>cdaPSII</i>	NB	B(1)
106	SCO3261 intragenic	putative ATP-binding protein		NB	B(1)
107	SCO3262 intragenic	hypothetical protein		NB	B(3)
108	SCO3266 intragenic	hypothetical protein		NB	B(1)
109	SCO3305 intragenic	putative membrane protein		NB	B(1)
110	SCO3323 upstream	putative RNA polymerase sigma factor	<i>bidN</i>	NB	B(1)
111	SCO3365 upstream	unknown		NB	B(1)
112	SCO3398 upstream	probable dihydropteroate synthase	<i>folP</i>	B(1)	B(1)
113	SCO3468 intragenic	transposase		NB	B(1)
114	SCO3469 intragenic	transposase		NB	B(3)
115	SCO3471 intragenic	extracellular agarase precursor	<i>dagA</i>	NB	B(1)
116	SCO3473 intragenic	probable aldolase		NB	B(1)
117	SCO3476 intragenic	probable dehydrogenase		NB	B(1)
118	SCO3479 upstream	possible beta-galactosidase		B(1)	NB
119	SCO3482 intragenic	possible sugar-permease (SCO3482)		NB	B(2)
120	SCO3484 intragenic	possible secreted sugar-binding protein,		NB	B(1)
121	SCO3487 upstream	putative hydrolase		NB	B(1)
122	SCO3490 intragenic	transposase		NB	B(2)
123	SCO3498 intragenic	putative secreted protein		NB	B(1)
124	SCO3503 intragenic	putative binding protein dependent transport protein		NB	B(1)
125	SCO3504 intragenic	putative binding protein dependent transport protein		NB	B(1)
126	SCO3510-11 intergenic	putative DNA methylase (SCO3510)		NB	B(1)
		possible lipoprotein (SCO3511)			
127	SCO3545 upstream	putative transferase		B(1)	NB
128	SCO3567 intragenic	probable serine protease		NB	B(1)
129	SCO3649 upstream	putative fructose 1,6-bisphosphate aldolase	<i>fba</i>	B(1)	NB
130	SCO3664 upstream	possible regulatory protein		NB	B(2)
131	SCO3679 upstream	regulatory protein		B(2)	B(1)
132	SCO3713 upstream	hypothetical protein		NB	B(1)
133	SCO3718 intragenic	probable cation transport system component	<i>kdpA</i>	NB	B(1)
134	SCO3746 intragenic	putative membrane protein		NB	B(1)
135	SCO3787 upstream	putative integral membrane protein		B(1)	NB
136	SCO3888 intragenic	conserved hypothetical protein		NB	B(3)
137	SCO3922 intragenic	putative membrane protein		NB	B(2)
138	SCO3938 intragenic	probable thiamin biosynthesis protein ThiC	<i>thisC</i>	B(1)	NB
139	SCO3986 intragenic	putative GntR-family transcriptional regulator		NB	B(2)
140	SCO3989 intragenic	possible ribonuclease inhibitor		NB	B(1)
141	SCO3993 intragenic	hypothetical protein		NB	B(1)
142	SCO4089-90 intergenic	valine dehydrogenase (SCO4089)	<i>vdh</i>	NB	B(1)
		conserved hypothetical protein (SCO4090)			
143	SCO4138 intragenic	phosphate transport protein	<i>pitH</i>	NB	B(1)
144	SCO4152 intragenic	possible secreted 5'-nucleotidase		NB	B(1)
145	SCO4183 intragenic	putative transposase		B(1)	B(1)
146	SCO4219 upstream	putative hydrolase		NB	B(2)
147	SCO4231-32 intergenic	putative lipoprotein (SCO4231)		NB	B(1)
		putative transcriptional factor regulator (SCO4232)			
148	SCO4281 intragenic	conserved hypothetical protein		NB	B(1)
149	SCO4284-85	N-acetylglucosamine-6-phosphate deacetylase (SCO4284)	<i>nagA</i>	B(3)	B(2)
		N-acetylglucosamine kinase	<i>nagK</i>		
150	SCO4333-34 intergenic	putative integral membrane protein (SCO4333)		NB	B(1)
		possible integral membrane protein (SCO4334)			

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151	SCO4342 intragenic	conserved hypothetical protein		NB	B(1)
152	SCO4344 upstream + int	transposase		NB	B(2)
153	SCO4354 intragenic	hypothetical protein		NB	B(1)
154	SCO4391-92 intragenic	conserved hypothetical protein (SCO4391)		NB	B(1)
155	SCO4432 intragenic	hypothetical protein		NB	B(1)
156	SCO4466 intragenic	hypothetical protein		NB	B(1)
157	SCO4521 intragenic	putative secreted protein		NB	B(2)
158	SCO4526 intragenic	possible protein kinase		NB	B(1)
159	SCO4529 intragenic	putative integral membrane protein		NB	B(1)
160	SCO4540 upstream	hypothetical protein		NB	B(3)
161	SCO4589 upstream	probable aminopeptidase (putative secreted protein)		B(1)	NB
162	SCO4630 intragenic	hypothetical protein		NB	B(1)
163	SCO4632 intragenic	putative ATP/GTP binding protein		NB	B(1)
164	SCO4644 downstream	SCO4644-add, adenosine deaminase;	<i>add</i>	NB	B(1)
165	SCO4698 intragenic	putative insertion element IS1652 transposase		NB	B(2)
166	SCO4699 intragenic	putative Rhs protein		NB	B(3)
167	SCO4800 intragenic	isobutyryl CoA mutase	<i>icm</i>	NB	B(1)
168	SCO4816 intragenic	hypothetical protein (fragment)		NB	B(1)
169	SCO4859 intragenic	hypothetical protein		NB	B(1)
170	SCO4874 intragenic	putative integral membrane protein		NB	B(1)
171	SCO4914 intragenic	putative deoxyribose-phosphate aldolase		NB	B(1)
172	SCO4915 upstream	putative integral membrane protein		NB	B(1)
173	SCO4934 upstream	possible lipoprotein		NB	B(1)
174	SCO4979 upstream	probable phosphoenolpyruvate carboxykinase		NB	B(1)
175	SCO4987-88 intergenic	putative D-amino acid deaminase (SCO4987)		NB	B(1)
		putative carbohydrate kinase		NB	B(1)
176	SCO4997 intragenic	hypothetical protein			
177	SCO5003-04 intergenic	chitinase (SCO5003)	<i>chiA</i>	B(3)	B(1)
		hypothetical protein (SCO5004)			
178	SCO5013 intragenic	putative secreted protein		NB	B(1)
179	SCO5022 intragenic	putative lipoprotein		NB	B(1)
180	SCO5032 intragenic	alkyl hydroperoxide reductase	<i>ahpC</i>	NB	B(1)
181	SCO5117 intragenic	possible peptide transport system secreted peptide-binding protein		NB	B(1)
182	SCO5161 upstream	conserved hypothetical protein		B(1)	B(1)
183	SCO5189 intragenic	hypothetical protein		NB	B(1)
184	SCO5231-32 intergenic	DasR	<i>dasR</i>	B(5)	B(4)
		sugar binding protein DasA	<i>dasA</i>		
185	SCO5236 upstream	glucosamine phosphate isomerase	<i>nagB</i>	B(2)	NB
186	SCO5237-38 intergenic	putative oxidoreductase (SCO5237)		B(2)	NB
		putative TetR-family protein (SCO5238)			
187	SCO5239 upstream	scr5239		B(3)	B(2)
188	SCO5300 intragenic	conserved hypothetical protein		B(1)	B(1)
189	SCO5322 intragenic	conserved hypothetical protein;		B(1)	NB
190	SCO5331 intragenic	possible DNA methylase		NB	B(1)
191	SCO5332 intragenic	hypothetical protein		NB	B(1)
192	SCO5342 intragenic	spdB2 protein		NB	B(1)
193	SCO5365 intragenic	putative transferase		NB	B(1)
194	SCO5368 upstream	ATP synthase C chain	<i>atpE</i>	NB	B(1)
195	SCO5376 upstream	chitinase	<i>chiC</i>	B(3)	NB
196	SCO5411 intragenic	putative integrase/recombinase;		NB	B(1)
197	SCO5428 intragenic	putative integral membrane transport protein		NB	B(1)
198	SCO5431 intragenic	putative secreted nucleosidase		B(1)	NB
199	SCO5443 intragenic	possible alpha-amylase	<i>pep1A</i>	NB	B(2)
200	SCO5458 intragenic	putative lipoprotein		NB	B(1)
201	SCO5535 upstream	probable carboxyl transferase	<i>accB</i>	B(1)	NB
202	SCO5550 downstream	tRNA-Gln-Glu		B(8)	B(9)
203	SCO5564 intragenic	rpmB, 50S ribosomal protein L28		NB	B(1)

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204	SCO5613 intragenic	hypothetical protein		NB	B(1)
205	SCO5616 intragenic	hypothetical protein		NB	B(1)
206	SCO5641 intragenic	transposase		NB	B(1)
207	SCO5644 intragenic	hypothetical protein		NB	B(3)
208	SCO5672 upstream	hypothetical protein		NB	B(1)
209	SCO5673 upstream	chitinase	<i>chiB</i>	B(1)	B(1)
210	SCO5702-03 intragenic	putative lipoprotein (SCO5702)		NB	B(1)
		hypothetical protein (SCO5703)			
211	SCO5727 intragenic	hypothetical protein		NB	B(1)
212	SCO5728 intragenic	putative secreted protein		NB	B(1)
213	SCO5751 upstream	putative membrane protein		NB	B(2)
214	SCO5827 intragenic	probable transmembrane transport protein		NB	B(1)
215	SCO5840 upstream	probable transcriptional regulator		B(1)	NB
216	SCO5841-42 intragenic	phosphocarrier protein hpr (SCO5841)	<i>ptsH</i>	B(4)	B(3)
		conserved hypothetical protein (SCO5842)			
217	SCO5874 intragenic	putative membrane protein		NB	B(1)
218	SCO5916 intragenic	hypothetical protein		NB	B(1)
219	SCO5922 upstream	possible oxidoreductase		NB	B(1)
220	SCO5952 intragenic	putative membrane protein		NB	B(1)
221	SCO6004-05 intergenic	secreted ATP/GTP binding protein (SCO6004)		NB	B(1)
		lipoprotein (SCO6005)	<i>ngcE, pitE</i>		
222	SCO6012-13 intergenic	chitinase	<i>chiH</i>	B(3)	NB
		probable 1-deoxyxylulose-5-phosphate synthase			
223	SCO6032-33 intergenic	putative hydrolase (SCO6032)		B(2)	B(2)
		hypothetical protein with phytanoyl-CoA dioxygenase domain (SCO6033)			
224	SCO6127 intragenic	putative carboxylesterase		B(1)	B(1)
225	SCO6167 intragenic	proline rich protein (putative membrane protein)		B(1)	NB
226	SCO6169-70 intergenic	putative regulatory protein (SCO6169)		NB	B(1)
		probable oxidoreductase subunit (SCO6170)			
227	SCO6236 intragenic	possible DNA-binding protein		NB	B(1)
228	SCO6240 intragenic	conserved hypothetical protein		NB	B(2)
229	SCO6273 intragenic	type I polyketide synthase	<i>cpkC</i>	NB	B(2)
230	SCO6274 coding	type I polyketide synthase	<i>cpkB</i>	NB	B(7)
231	SCO6275 intragenic	type I polyketide synthase (fragment)	<i>cpkA</i>	NB	B(1)
232	SCO6300 upstream	secreted β -N-acetylglucosaminidase		B(3)	NB
233	SCO6304 intragenic	putative oxidoreductase		NB	B(1)
234	SCO6321 intragenic	possible integral membrane protein		NB	B(1)
235	SCO6325 intragenic	putative membrane protein		NB	B(1)
236	SCO6336 intragenic	hypothetical protein		NB	B(1)
237	SCO6344-45 intergenic	putative secreted amidase (SCO6344)		B(4)	NB
		putative secreted chitinase (SCO6345)	<i>chi</i>		
238	SCO6373 intragenic	putative integral membrane protein		NB	B(2)
239	SCO6377 upstream	putative lipoprotein		NB	B(1)
240	SCO6381 intragenic	putative lipoprotein		NB	B(1)
241	SCO6383 intragenic	putative integral membrane protein		NB	B(1)
242	SCO6384 intragenic	putative integral membrane lysyl-tRNA synthetase		NB	B(1)
243	SCO6385 intragenic	probable integral membrane protein,		NB	B(1)
244	SCO6387 intragenic	hypothetical protein		NB	B(1)
245	SCO6390 intragenic	putative integral membrane protein		NB	B(1)
246	SCO6393 intragenic	putative transposase		NB	B(1)
247	SCO6400 intragenic	putative IS117 transposase		NB	B(2)
248	SCO6401 intragenic	conserved hypothetical protein		NB	B(1)
249	SCO6470 intragenic	hypothetical protein		NB	B(1)
250	SCO6474 upstream	possible transcriptional regulator;		B(1)	NB
251	SCO6486 intragenic	putative transport associated protein		B(1)	NB
252	SCO6512 intragenic	ABC transporter ATP-binding protein		B(1)	NB
253	SCO6513 intragenic	hypothetical protein		B(1)	NB

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254	SCO6622 intragenic	putative ATP/GTP binding protein		NB	B(1)
255	SCO6625 intragenic	hypothetical protein		NB	B(1)
256	SCO6627 intragenic	putative adenine-specific DNA methyltransferase gene	<i>pglX</i>	NB	B(4)
257	SCO6632 upstream	hypothetical protein		NB	B(1)
258	SCO6640 intragenic	putative ATP-dependent helicase		NB	B(1)
259	SCO6807 upstream	hypothetical protein		NB	B(1)
260	SCO6834 intragenic	possible thioredoxin reductase	<i>trxB2</i>	NB	B(1)
261	SCO6859 intragenic	hypothetical protein		NB	B(1)
262	SCO6860 intragenic	hypothetical protein		NB	B(1)
263	SCO6861 intragenic	protein kinase-like protein	<i>pk3</i>	NB	B(2)
264	SCO6862 intragenic	hypothetical protein		NB	B(1)
265	SCO6871 intragenic	putative lipoprotein		NB	B(1)
266	SCO6876 intragenic	hypothetical protein		NB	B(1)
267	SCO6908 intragenic	hypothetical protein		NB	B(1)
268	SCO6948 intragenic	hypothetical protein		NB	B(1)
269	SCO6952 intragenic	hypothetical protein		NB	B(3)
270	SCO6953 intragenic	conserved hypothetical protein		NB	B(2)
271	SCO7018 intragenic	hypothetical protein		NB	B(2)
272	SCO7019 intragenic	secreted alpha-amylase (EC 3.2.1.1)	<i>amil</i>	NB	B(1)
273	SCO7037 intragenic	putative secreted protein;		NB	B(1)
274	SCO7056 upstream	possible gntR-family transcriptional regulator		B(2)	B(2)
275	SCO7059 upstream	putative oxidoreductase		B(1)	NB
276	SCO7077 intragenic	putative integral membrane protein		NB	B(1)
277	SCO7080 intragenic	putative insertion element transposase		NB	B(1)
278	SCO7120 intragenic	ubiquinol-cytochrome C reductase cytochrome B subunit	<i>qcrB2</i>	NB	B(1)
279	SCO7165 intragenic	probable sugar-binding integral membrane transport protein		NB	B(1)
280	SCO7197 intragenic	probable amino acid transport integral membrane		NB	B(1)
281	SCO7225 upstream	chitinase	<i>chi</i>	B(2)	B(1)
282	SCO7226 upstream	putative integral membrane protein		B(1)	B(1)
283	SCO7227 intragenic	possible secreted protein		B(1)	NB
284	SCO7263 upstream	chitinase	<i>chiF</i>	B(1)	NB
285	SCO7274 upstream	possible membrane protein		B(1)	NB
286	SCO7279 intragenic	possible DNA-binding protein	<i>popR</i>	B(1)	NB
287	SCO7330 intragenic	putative membrane protein		B(1)	NB
288	SCO7335 intragenic	possible alpha-amylase	<i>pep1B</i>	NB	B(2)
289	SCO7350 intragenic	putative membrane efflux protein		NB	B(1)
290	SCO7392 intragenic	hypothetical protein		NB	B(1)
291	SCO7410 intragenic	putative binding-protein dependent transport protein.		B(1)	NB
292	SCO7416 upstream	hypothetical protein		B(1)	NB
293	SCO7493 intragenic	conserved hypothetical protein		B(1)	NB
294	SCO7494-95 itergenic	putative membrane protein (SCO7494) pseudogene, hypothetical protein		NB	B(1)
295	SCO7520 upstream	putative integral membrane protein		B(1)	NB
296	SCO7742 intragenic	putative MarR-family transcriptional regulator		NB	B(1)
297	SCO7743 intragenic	hypothetical protein		NB	B(1)
298	SCO7752 intragenic	putative integral membrane protein		NB	B(2)
299	SCO7775 intragenic	putative secreted protein		NB	B(2)
300	SCO7785 intragenic	putative transcriptional regulator		NB	B(1)
301	SCO7792 intragenic	hypothetical protein		B(1)	NB
302	SCO7798 intragenic	transposase		NB	B(2)
303	SCO7803 intragenic	putative insertion element transposase		NB	B(1)
304	SCO7804 upstream	putative membrane protein		NB	B(1)
305	SCO7826 upstream	hypothetical protein		NB	B(1)
306	SCO7827 intragenic	putative transposase		NB	B(1)
307	SCO7837-38	putative membrane protein (SCO7837) conserved hypothetical protein (SCO7838)		NB	B(1)
308	SCO7842 intragenic	putative transposase		NB	B(1)
309	SCO7844 intragenic	putative DNA-binding protein		NB	B(1)
310	SCO4123 ^d	<i>rrnA</i>		-	B (>2)
311	SCO1792 ^d	<i>rrnB</i>		-	B (>2)

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312	SCO1390	rrnC		-	B (>2)
313	SCO5746	rrnE		-	B (>2)
314	SCO3334	rrnF		-	B (>2)

NB, no binding; B, binding; numbers between brackets indicate the number of probes that were significant enriched.

Table S3. DasR binding before and after addition of GlcNAc to liquid-grown MM and R5 cultures.

Nr	Gene ID	Name	Function	MM				R5			
				T0	T1	T2	T3	T0	T1	T2	T3
			PTS, GlcNAc metabolism, chitinases and sugar related								
1	SCO1390- SCO1391	<i>crr-pts</i>	PTS sugar phosphotransferase component IIA and phosphoenolpyruvate-protein phosphotransferase	B	N	B	N	B	B	B	B
2	SCO1429	<i>chiD</i>	chitinase	N	N	N	N	B	N	N	B
3	SCO1444	<i>chiI</i>	chitinase	N	N	N	N	N	N	N	N
4	SCO2503	<i>chiJ</i>	chitinase	N	N	N	N	B	N	N	B
5	SCO2833	<i>chb</i>	secreted chitin binding protein	B	N	B	N	B	N	B	N
6	SCO2906	<i>nagF</i>	GlcNAc PTS transmembrane component	N	N	N	N	B	B	B	B
7	SCO2907	<i>nagE</i>	GlcNAc PTS transmembrane component	N	N	N	N	B	N	B	N
8	SCO4284- sco4285	<i>nagK</i>	putative GlcNAc-6P deacetylase (SCO4284); sugar kinase	B	N	N	N	B	B	B	B
9	SCO5003- sco5004	<i>chiA</i>	chitinase (SCO5003); hypothetical protein (SCO5004)	B	N	N	N	B	N	N	N
10	SCO5231- SCO5232	<i>dasR</i>	GntR-family transcriptional regulator DasR	B	N	B	N	B	N	N	N
		<i>dasA</i>	sugar transporter sugar binding protein								
11	SCO5236	<i>nagB</i>	glucosamine phosphate isomerase	N	N	N	N	B	N	N	B
12	SCO5376	<i>chiC</i>	chitinase	N	N	N	N	N	N	N	N
13	SCO5673	<i>chiB</i>	chitinase	B	B	B	B	B	N	N	N
14	SCO5841	<i>ptsH</i>	phosphocarrier protein Hpr (SCO5841)	N	N	N	N	N	N	N	N
15	SCO6004- SCO6005	<i>ngcE</i>	secreted ATP/GTP binding protein (SCO6004); lipoprotein (SCO6005)	B	B	B	B	B	N	N	B
16	SCO6012- SCO6013 intergenic	<i>chiH</i>	chitinase (SCO6012); probable 1-deoxyxylulose-5-phosphate synthase (SCO6013)	N	N	N	N	B	N	N	N
17	SCO6032- SCO6033 intergenic		putative hydrolase (SCO6032) hypothetical protein with phytanoyl-CoA dioxygenase domain (SCO6033)	N	N	N	N	B	N	N	B
18	SCO6300		secreted β -N-acetylglucosaminidase	N	N	N	N	B	N	N	N

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19	SCO6344- SCO6345 intergenic	<i>chi</i>	putative secreted amidase (SCO6344) putative secreted chitinase (SCO6345)	N	N	N	N	B	N	N	N
20	SCO7225	<i>chi</i>	chitinase	N	N	N	N	B	N	N	N
			Development and antibiotic production								
21	SCO3230	<i>cdaPS</i>	CDA peptide synthetase I	B	N	B	N	B	N	N	B
22	SCO3231c oding	<i>cdaPS</i>	CDA peptide synthetase II	B	B	N	N	B	N	N	B
23	SCO6273	<i>cpkC</i>	type I polyketide synthase	B	B	N	B	N	N	N	N
24	SCO6274	<i>cpkB</i>	type I polyketide synthase	B	B	N	B	N	N	N	N
25	SCO5085	<i>actII-4</i>	actinorhodin cluster activator protein	B	N	B	N	B	N	N	B
26	SCO5877	<i>redD</i>	transcriptional regulator RedD	B	B_r	B_r	N	B	N	N	N
27	SCO5881c oding	<i>redZ</i>	response regulator	B	N	B	N	B	N	N	B
28	SCO5114	<i>bidKC</i>	oligopeptide transport system integral membrane prote	B	B_r	B_r	N	N	N	N	N
29	SCO4118- SCO4119 intergenic	<i>atrA</i>	possible tetR-family transcriptional regulator (SCO4118) possible NADH dehydrogenase (SCO4119)	B	B	B_r	B_r	B	N	N	B
30	SCO2686- SCO2687 intergenic	<i>ribA1</i>	possible luxR-family transcriptional regulator (SCO2686) GTP cyclohydrolase II	B	B_r	B_r	N	B	N	N	B
31	SCO3258	<i>pra</i>	regulatory protein	B	B	B	B	N	N	N	N
			Transposases and DNA recombination								
32	SCO0098- SCO0099 intergenic		transposases	B	N	B	N	N	N	N	N
33	SCO0567		insertion element transposase	B	B	B	N	N	N	N	N
34	SCO1471		transposase	B	N	N	N	N	N	N	N
35	SCO1603		transposase	B	B_r	B_r	N	N	N	N	N
36	SCO3466		transposase	B	B	B	N	B	N	N	B
37	SCO3467		transposase	B	B	B	N	N	N	N	N
38	SCO3468		transposase	B	B	B	N	B	N	N	B
39	SCO6400		probable transposase	B	B_r	B_r	N	B	N	N	B
40	SCO6910- SCO6911 intergenic		transposases	B	B	B	N	B	N	N	B
			Other targets								
41	SCO1327		possible integral membrane protein	B	B	N	B	B	N	N	B
42	SCO1606		Penicillin Binding Protein (putative)	B	B	B_r	B_r	N	N	N	B
43	SCO1967- SCO1968 intergenic	<i>glpQ2</i>	possible integral membrane protein (SCO1967) probable secreted hydrolase (SCO1968)	B	B	B_r	B_r	B	N	N	B
44	SCO2078		putative membrane protein	B	B	B_r	B_r	N	N	N	B
45	SCO2427- SCO2428 intergenic		probable secreted protein (SCO2427) possible phosphate binding protein (SCO2428)	B	B	N	N	N	N	N	N
46	SCO2687		ribA1, GTP cyclohydrolase II	B	B_r	B_r	B_r	B	N	N	B
47	SCO2724- SCO2725 intergenic		possible amino acid transporter (SCO2724) putative lipoprotein (SCO2725)	B	B	B_r	B_r	N	N	N	N
48	SCO2741		possible secreted protein	B	B	N	N	N	N	N	N
49	SCO3249		probable acyl carrier protein	B	B	N	N	N	N	N	N

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50	SCO3262		unknown	B	B	N	N	N	N	N	N	B
51	SCO3362		putative membrane protein	B	B	B	N	N	N	N	N	N
52	SCO3503		possible binding protein transport dependent protein	B	B	B_r	B_r	N	N	N	N	N
53	SCO3567		probable serine protease	B	B	B	N	N	N	N	N	B
54	SCO3594- SCO3595	<i>dldH</i> <i>ddlA2</i>	probable D-lactate dehydrogenase probable D-alanine:D-lactate ligase	B	B_r	B_r	N	B	N	N	N	B
55	SCO3748	<i>f40</i>	cold shock protein	B	B	B	N	B	N	N	N	B
56	SCO3871		possible decarboxylase	B	B	B	N	N	N	N	N	N
57	SCO4219		possible hydrolase (dienelactone hydrolase family)	B	B	B	B_r	N	N	N	N	N
58	SCO4540		unknown	B	B_r	B_r	B_r	B	N	N	N	B
59	SCO5014- SCO5015		possible secreted protein (SCO5014) putative secreted protein (SCO5015)	B	B	B	B_r	N	N	N	N	N
60	SCO5190	<i>wbIC</i>	DNA-binding protein	B	B	B_r	B_r	N	N	N	N	N
61	SCO5423	<i>pyk2</i>	pyruvate kinase	B	B	B_r	B_r	N	N	N	N	N
62	SCO6108	<i>fusH</i>	esterase	B	B	B_r	B_r	B	N	N	N	B
63	SCO6401		unknown	B	B	B_r	B_r	N	N	N	N	N
64	SCO6806		putative phage integrase	B	B	B_r	B_r	N	N	N	N	N
65	SCO6816		possible ABC transporter binding lipoprotein	B	B	N	B	N	N	N	N	N
66	SCO7496- SCO7497 intergenic		conserved hypothetical integral membrane protein (SCO7496) possible transcriptional regulator (SCO7497)	B	B	N	N	N	N	N	N	N
67	SCO1020- SCO1021.in tergenic		hypothetical protein	N	N	N	N	B	N	N	N	B
68	SCO3503		putative binding protein dependent transport protein (SCO3503)	B	B	B	N	B	N	B	B	B
69	SCO3660		hypothetical protein	B	B	B	B_r	B	N	N	N	B
70	SCO3844		putative secreted protein	B	B	B	N	B	N	N	N	B
71	SCO3978- SCO3979 intergenic		possible oxidoreductase (SCO3978) possible TetR-family transcriptional regulator (SCO3979)	N	N	N	N	B	N	N	N	B
72	SCO3998- SCO3999 intergenic		unknown (SCO3998) possible lipoprotein (SCO3999)	B	B	N	B	B	N	N	N	B
73	SCO5144- SCO5145 intergenic		probable acyl CoA isomerase (SCO5144) conserved hypothetical protein (SCO5145)	B	B_r	B_r	N	B	N	N	N	B
74	SCO5331		possible DNA methylase	N	N	N	N	B	N	N	N	B
75	SCO5639		hypothetical protein	B	B	B	N	B	N	N	N	B
76	SCO6499	<i>gvpO</i>	probable gas vesicle synthesis protein	N	N	N	N	B	N	N	N	B
77	SCO6553- SCO6554 intergenic		probable integral membrane efflux protein probable transcriptional regulatory protein	N	N	N	N	B	N	N	N	B
78	SCO7056		possible gntR-family transcriptional regulator	N	N	N	N	B	N	N	N	N
79	SCO7073- SCO7074 intergenic		hypothetical protein possible membrane protein	B	N	N	N	B	N	N	N	B
80	SCO7763		possible membrane protein	N	N	N	N	B	N	N	N	B
81	SCO1488- SCO1489 intergenic intergenic	<i>pyrR</i> <i>blbD</i>	pyrimidine operon regulatory protein (SCO1488) DNA-binding protein BldD (SCO1489)	N	N	N	N	B	B	B	B	B
82	SCO1677		conserved hypothetical protein	N	N	N	N	B	B	B	B	B
83	SCO3663- SCO3664 intergenic		possible membrane protein (SCO3663) possible regulatory protein (SCO3664)	N	N	N	N	B	B	B	B	B
84	SCO4010- SCO4011 intergenic		possible secreted protein (SCO4010) possible integral membrane protein (SCO4011)	N	N	N	N	B	B	B	B	B
85	SCO4075	<i>ragA</i>	ABC transport protein, ATP-binding subunit	N	N	N	N	B	B	B	B	B

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86	SCO4092	<i>hrpA</i>	ATP-dependent helicase tRNA-Phe-Asp-Glu	N	N	N	N	B	B_i	B_i	B_r
87	SCO4107		possible integral membrane protein tRNA-Met	N	N	N	N	B	B	B	B
88	SCO4635	<i>rmpG</i>	50S ribosomal protein L33	B	B	B	B	B	B	B	B
89	SCO4646	<i>secE</i>	preprotein translocase SecE subunit	N	N	N	N	B	B	B	B
90	SCO4987- SCO4988 intergenic		possible D-amino acid deaminase (SCO4987) probable carbohydrate kinase (SCO4988)	N	N	N	N	B	B	B	B
91	SCO5205		hypothetical protein	N	N	N	N	B	B	B	B
92	SCO5549- SCO5550		tRNA-Gln-Glu	B	N	B	N	B	B	B	B
93	SCO1547		anthranilate synthase	N	N	N	N	N	B	B	N
94	SCO2461		possible secreted protein	N	N	N	N	B	B	B	B
95	SCO3087		hypothetical	N	N	N	N	N	B	B	B
96	SCO3092		ncRNA	N	N	N	N	N	B	B	B
97	SCO3843		unknown	B	B	N	N	N	B	B	B
98	SCO3945	<i>cydA</i>	cytochrome oxidase subunit I	N	N	N	N	N	B	B	N
99	SCO4067	<i>dnaZ</i>		N	N	N	N	N	B	B	B
100	SCO5579		possible transmembrane protein	B	B	B	B	N	N	N	N
101	SCO5610- SCO5611 intergenic		unknown prophage gene (SCO5610) possible prophage transcriptional regulator (SCO5611)	N	N	N	N	N	N	N	B
102	SCO5705		hypothetical protein	N	N	N	N	N	B	B	N
103	SCO6588- SCO6589 intergenic	<i>fusB</i>	unknown (SCO6588) possible alternative elongation fact or G	N	N	N	N	N	B	B	N
104	SCO3138- SCO3139 intergenic	<i>galT</i>	galactose-1-phosphate uridylyltransferase possible sodium:solute symporter (SCO3139)	N	N	N	N	B	N	N	B
105	SCO3496		possible lyase precursor	N	N	N	N	N	N	N	B
106	SCO5332		unknown	N	N	N	N	N	N	N	B
107	SCO4123 ^d	<i>rrnA</i>	ribosomal RNA	B	N	B	N	N	B	B	B
108	SCO1792 ^d	<i>rrnB</i>	ribosomal RNA	B	N	B	N	N	B	B	B
109	SCO1390	<i>rrnC</i>	ribosomal RNA	B	N	B	N	N	B	B	B
110	SCO5746	<i>rrnE</i>	ribosomal RNA	B	N	B	N	N	B	B	B
111	SCO3334	<i>rrnF</i>	ribosomal RNA	B	N	B	N	N	B	B	B

T0, before induction; T1, T2, T3; time 30, 60 and 120 min after the addition of GlcNAc. B, binding; N, no binding; B_re, B_r, binding reduced; B_i, binding enhanced.

Table S4. Colour coding for figure 4B

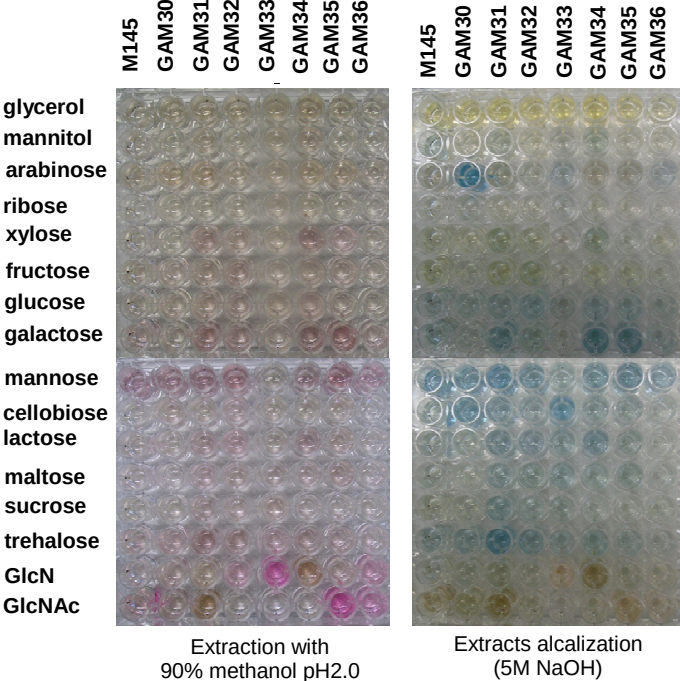
Colour	Protein Classification		
lightpink	3.3.1.5 Pool, multipurpose conversions of interned. met'm (11)	lime	2.2.3 DNA - replication, repair, restr./modific'n (85)
pink	3.5.1 Aerobic respiration (9)	forestgreen	3.8.0 Secondary metabolism (165)
crimson	3.1.9 Glycine (3)	green	1.7.1 Cell division (20)
lavenderblush	2.2.1 Amino acyl tRNA syn; tRNA modific'n (34)	darkgreen	3.2.0 Biosynthesis of cofactors, carriers (5)
palevioletred	2.2.0 Macromolecule synthesis, modification (4)	lawngreen	6.3.1.2 MerR (22)
hotpink	3.4.2 Amino acids (28)	chartreuse	3.1.5 Chorismate (9)
deeppink	3.3.1.3 Phosphorus compounds (8)	greenyellow	1.6.1 Adaptations, atypical conditions (25)
mediumvioletred	3.2.1.6 biotin carboxyl carrier protein (BCCP) (2)	darkolivegreen	2.2.1.0 Proteins - translation and modification (36)
orchid	3.5.9 ATP-proton motive force (10)	yellow green	2.1.3 Degradation of polysaccharides (92)
thistle	3.4.4 Fatty acids (59)	olivedrab	3.3.1.0 Nucleotide hydrolysis (3)
plum	3.1.1.8 Threonine (4)	ivory	2.2.7 Phospholipids (14)
violet	7.0.0 Not classified (included putative assignments) (565)	beige	3.1.2.0 Tyrosine (1)
fuchsia	3.1.1.5 Phenylalanine (3)	lightyellow	3.3.1.9 Sulfur metabolism (13)
fuchsia	6.3.2 AraC (33)	lightgoldenrod	3.8.1 PKS (102)
darkmagenta	1.4.2 Detoxification (90)	yellow	3.4.5 Other (10)
purple	3.2.1.3 Riboflavin (7)	olive	6.3.5 LacI (34)
mediumorchid	3.1.1.9 Tryptophan (9)	darkkhaki	4.1.7 Gram +ve exported/lipoprotein (439)
darkviolet	4.2.2 Ribosomal proteins - synthesis, modification (62)	palegoldenrod	6.1.2 Response regulator (79)
darkorchid	3.5.2 Anaerobic respiration (22)	lemonchiffon	3.1.6 Cysteine (4)

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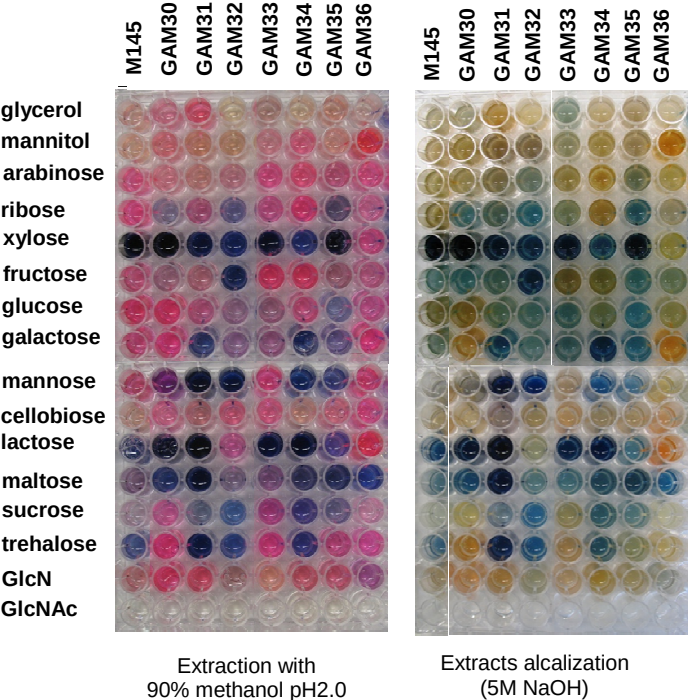
indigo	1.3.1	Chaperones (15)	khaki	6.2.3	anti sigma factor antagonist (15)
blueviolet	3.1.1	Alanine (2)	gold	3.3.4	Gluconeogenesis (4)
mediumpurple	3.3.8	Misc. glycerol metabolism (1)	cornsilk	4.1.6	Gram +ve membrane (836)
mediumslateblue	3.1.7	Glutamate (6)	goldenrod	3.2.1 0	Pantothenate (4)
slateblue	3.5.7	Pyruvate dehydrogenase (10)	darkgoldenrod	6.3.1 3	ArsR (15)
darkslateblue	5.1.4	Transposon/insertion element-related functions (85)	floralwhite	3.1.2	Arginine (10)
ghostwhite	5.1.2	Phage-related functions and prophages (25)	oldlace	1.5.3	Carbohydrates, organic acids and alcohols (9)
lavender	3.1.8	Glutamine (6)	wheat	3.8.2	NRPS (10)
blue	3.2.3	Cobalamin (19)	orange	3.3.1 1	Nucleotide interconversions (25)
mediumblue	3.2.1 4	Thiamin (7)	moccasin	6.2.2	anti sigma factor (7)
darkblue	6.3.9	ROK (19)	papayawhip	3.3.1 4	Polyamine biosynthesis (6)
navy	6.3.4	IcIR (12)	blanchedalmon	3.1.4	Aspartate (2)
midnightblue	3.4.1	Amines (12)	navajowhite	6.3.1	AsnC (14)
royalblue	1.2.1	Chromosome replication (8)	antiquewhite	2.2.2	Basic proteins - synthesis, modification (1)
cornflowerblue	3.2.9	Molybdopterin (8)	tan	0.0.2	Conserved in organism other than Escherichia coli
lightsteelblue	2.2.6	Lipoprotein (2)	burlywood	3.1.1 7	Serine (6)
lightslategray	6.3.8	TetR (122)	darkorange	2.2.4	Glycoprotein (11)
slategray	3.1.2 1	Valine (10)	bisque	3.2.1 1	Pyridine nucleotide (7)
dodgerblue	2.2.1 1	RNA synthesis, modification, DNA transcription (33)	linen	3.5.4	Fermentation (6)
aliceblue	1.5.2	Cations (40)	peru	3.3.1 7	Salvage of nucleosides and nucleotides (2)
steelblue	6.5.0	Others (237)	peachpuff	3.3.1 6	S-adenosyl methionine (3)
lightskyblue	3.1.1 3	Lysine (13)	sandybrown	3.4.3	Carbon compounds (91)
skyblue	3.7.2	Pyrimidine ribonucleotide biosynthesis (9)	chocolate	2.2.8	Polysaccharides - (cytoplasmic) (25)
deepskyblue	3.3.2 1	other (4)	saddlebrown	1.5.1	Amino acids and amines (34)
lightblue	3.2.6	Heme, porphyrin (16)	seashell	3.5.3	Electron transport (71)
powderblue	4.2.3	Ribosomes - maturation and modification (5)	sienna	3.2.5	Folic acid (9)
cadetblue	3.6.0	Fatty acid biosynthesis (3)	lightsalmon	3.1.1 0	Histidine (11)
darkturquoise	6.4.0	Protein kinases (2)	coral	1.6.3	Fe storage (11)
azure	2.1.2	Degradation of RNA (8)	orangered	3.3.1 8	Sugar-nucleotide biosynthesis, conversions (11)
lightcyan	3.5.8	TCA cycle (26)	darksalmon	5.1.0	Laterally acquired elements (2)
paleturquoise	1.4.0	Protection responses (1)	tomato	3.1.0	Amino acid biosynthesis (8)
aqua	6.4.1	Serine/threonine (37)	salmon	4.1.8	Gram +ve surface anchored (1)
aqua	3.2.1	Acyl carrier protein (ACP) (2)	mistyrose	4.1.9	Gram +ve peptidoglycan, teichoic acid (40)
darkcyan	3.3.1 2	Oligosaccharides (1)	lightcoral	3.7.1	Purine ribonucleotide biosynthesis (21)
teal	3.3.2	Amino sugars (3)	snow	1.5.5	Other (11)
darkslategray	6.3.3	GntR (51)	rosybrown	1.6.2	Osmotic adaptation (7)
mediumturquoise	0.0.0	Unknown function, no known homologs (1049)	indianred	3.1.1 6	Proline (3)
lightseagreen	3.6.1	Fatty acid and phosphatidic acid biosynthesis (53)	red	1.5.0	Transport/binding proteins (520)
turquoise	2.2.9	Protein modification (12)	brown	3.5.5	Glycolysis (25)
aquamarine	6.3.1 1	LuxR (GerR) (23)	firebrick	3.2.7	Lipoate (3)
mediumaquamarine	6.3.6	LysR (38)	darkred	2.1.1	Degradation of DNA (13)
mediumspringgreen	3.2.2	Biotin (7)	maroon	5.1.3	Plasmid-related functions (27)
mintcream	1.8.1	Differentiation/sporulation (11)	white	3.2.1 2	Pyridoxine (2)
springgreen	3.3.5	Glyoxylate bypass (5)	whitesmoke	3.3.1	2'-Deoxyribonucleotide metabolism (2)
mediumseagreen	3.2.8	Menaquinone, ubiquinone (12)	gainsboro	3.1.1 4	Methionine (8)
seagreen	3.3.6	Incorporation metal ions (1)	lightgrey	3.2.1 5	Thioredoxin, glutaredoxin, glutathione (8)
honeydew	3.1.1 2	Leucine (5)	silver	6.1.1	Sensor kinase (85)
darkseagreen	2.1.4	Degradation of proteins, peptides, glycoproteins (126)	darkgray	6.3.1 0	DeoR (13)
palegreen	6.2.1	sigma factor (66)	gray	3.3.9	Non-oxidative branch, pentose pathway (8)
lightgreen	3.5.6	Oxidative branch, pentose pathway (9)	dimgrey	3.5.0	Energy metabolism, carbon (1)
limegreen	6.3.7	MarR (40)	black	6.1.3	Sensor kinase/response regulator fusion (1)

Supplemental Information—Chapter VI

A



B



Supplemental Figure S1. Non-quantitative colorimetric assay for Act and Red production in the *rok* mutants, when grown on (A) MM and (B) R2YE agar plates. Strains were grown for five days on MM and R2YE- agar plates supplemented with 1% (w/v) of different carbon sources (glycerol, mannitol, arabinose, ribose, xylose, fructose, glucose, galactose, mannose, cellobiose, lactose, maltose, sucrose, trehalose, GlcN, GlcNAc, taurine). Actinorhodin and undecylprodigiosin were assessed as described in Material&Methods.

Table S1. Plasmids used and constructed in this study

Plasmid	Description	Reference
pWHM3	Cloning vector, <i>colE1</i> replicon, pSG5 replicon, TsrR, AmpR	Vara et al, 1989
pHJL401	Complementation vector, SCP2*, pUC19 replicon, TsrR, AmpR	Larson and Hershberger, 1986
pUWLcre	pUWLori T derivative with <i>cre(a)</i> gene under <i>ermE*</i> promoter	Fedoryshyn et al, 2008
SuperP401		
pGAM7	pWHM3 containing flanking regions of <i>S. coelicolor</i> SCO0794 with apraloxP	This work
pGAM8	pWHM3 containing flanking regions of <i>S. coelicolor</i> SCO1060 with apraloxP	This work
pGAM9	pWHM3 containing flanking regions of <i>S. coelicolor</i> SCO2846 with apraloxP	This work
pGAM10	pWHM3 containing flanking regions of <i>S. coelicolor</i> SCO6008 with apraloxP	This work
pGAM11	pWHM3 containing flanking regions of <i>S. coelicolor</i> SCO6566 with apraloxP	This work
pGAM12	pWHM3 containing flanking regions of <i>S. coelicolor</i> SCO6600 with apraloxP	This work
pGAM13	pWHM3 containing flanking regions of <i>S. coelicolor</i> SCO7543 with apraloxP	This work
pGAM14	pHJL401 containing SCO6008 gene with its promoter	This work

Table S2. Plasmids used and constructed in this study

Name	5'-3' sequence	Purpose and restriction sites
0794LF-1497	gtcagaatttcaccggttgattgaaccgcaggct	Cloning, <i>EcoRI</i>
0794LR+6	gaagttatccatcacctctagacgccatgtggctcctccgta	Cloning, <i>XbaI</i>
0794RF+1285	gaagttatcgcgcattcttagacactgaggtttcgccggtcgc	Cloning, <i>XbaI</i>
0794RR+2699	gtcaaaagcttcgggcagcaaaatcggtggtcacg	Cloning, <i>HindIII</i>
1060LF-1387	gtcagaatttcggctgggacatcgaggcaccgct	Cloning, <i>EcoRI</i>
1060LR+9	gaagttatccatcacctctagagttcatcatggtgctgtcgag	Cloning, <i>XbaI</i>
1060RF+1228(48)	gaagttatcgcgcattcttagacgggacgcggctgacacgga	Cloning, <i>XbaI</i>
1060RR+2552(74)	gtcaaaagcttcgggacgcgtcatctgggacacc	Cloning, <i>HindIII</i>
2846LF-1369	gtcagaatttccttgcgggcgattccaggcgctcgt	Cloning, <i>EcoRI</i>
2846LR+9	gaagttatccatcacctcttagacctgttcacaccccatgtgc	Cloning, <i>XbaI</i>
2846RF+1224	gaagttatcgcgcattcttagacggggggacggcggtgagac	Cloning, <i>XbaI</i>
2846RR+2637	gtcaaaagctttcagaccgcttcacagaggtcc	Cloning, <i>HindIII</i>
6008LF-848new	gtcagaatttcgatcgtcgtggtctcggcactg	Cloning, <i>EcoRI</i>
6008LR+147new	gaagttatccatcacctcttagaccggacgatgttcgagaccgtcg	Cloning, <i>XbaI</i>
6008RF+1201loxPnew	gaagttatcgcgcattcttagagccttacttagagcacggatgg	Cloning, <i>XbaI</i>
6008RR+2675(97)	gtcaaaagcttagctctacgtcggtaggcgcct	Cloning, <i>HindIII</i>
6566LF-1406	gtcagaatttcgcgccgacaagatgtccgaggtc	Cloning, <i>EcoRI</i>
6566LR+6	gaagttatccatcacctcttagaggacacgacgaggccgcccgg	Cloning, <i>XbaI</i>
6566RF+1398	gaagttatcgcgcattcttagagcgtagccaccaccttcaatt	Cloning, <i>XbaI</i>
6566RR+2677	gtcaaaagcttagaccgacacgttgccgggtgac	Cloning, <i>HindIII</i>
6600LF-1371	gtcagaatttcctgaactgcgactgtcgggca	Cloning, <i>EcoRI</i>
6600LR+6	gaagttatccatcacctcttagaggccatccggccctcccttt	Cloning, <i>XbaI</i>
6600RF+1321(41)	gaagttatcgcgcattcttagaggatgagggcgtgcgcggcg	Cloning, <i>XbaI</i>
6600RR+2671(93)	gtcaaaagctttgccgctgagctacgtcgacctg	Cloning, <i>HindIII</i>
7543LF-1253	gtcagaatttcacgggtgcgctcgccgttgac	Cloning, <i>EcoRI</i>
7543LR+6	gaagttatccatcacctcttagagggtcaaggtagggaggctcga	Cloning, <i>XbaI</i>
7543RF+1270(90)	gaagttatcgcgcattcttagaccttgacaagcggagtgccga	Cloning, <i>XbaI</i>
7543RR+2635(57)	gtcaaaagctttccatcagcaggatcgaggggtc	Cloning, <i>HindIII</i>
0794check-451	gtcagaatttcgttcaggcacagcgcgcacatg	PCR confirmation, <i>EcoRI</i>
0794check+1728(51)	gtcaaaagcttagttctctgcggggactgcggcag	PCR confirmation, <i>HindIII</i>
1060check-501	gtcagaatttcctggcaccggcaaccagtgccag	PCR confirmation, <i>EcoRI</i>
1060check+1695(1718)	gtcaaaagcttgccacggacaaccgctacgagg	PCR confirmation, <i>HindIII</i>
2846check-687	gtcagaatttcactcggcgtaacctgcgtgacc	PCR confirmation, <i>EcoRI</i>
2846check+1886(1907)	gtcaaaagcttaccctgctccatgtcgggtgagc	PCR confirmation, <i>HindIII</i>
6008check-689	gtcagaatttcacagttgcagaacttcgggctg	PCR confirmation, <i>EcoRI</i>

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6008check+1923(46)	gtca <u>aaagctt</u> tacaccttgcgctcgaggacggag	PCR confirmation, <i>HindIII</i>
6566check-541	gtcaga <u>aattc</u> acaccgagggctgggtcgatctg	PCR confirmation, <i>EcoRI</i>
6566check+1898(1921)	gtca <u>aaagctt</u> tccggctcgtgaccgaggtggacg	PCR confirmation, <i>HindIII</i>
6600check-549	gtcaga <u>aattc</u> tcgaagtcttctggcgtcccagg	PCR confirmation, <i>EcoRI</i>
6600check+1880(1901)	gtca <u>aaagctt</u> tggtagcgcgccgggatctcg	PCR confirmation, <i>HindIII</i>
7543check-626	gtcaga <u>aattc</u> agccacacgaagatgccgtcg	PCR confirmation, <i>EcoRI</i>
7543check+1894(1917)	gtca <u>aaagctt</u> acgcggcggaagatcgtggcgtc	PCR confirmation, <i>HindIII</i>
SCO6008F-272	gtcaga <u>aattc</u> ggatcgccggttcgggtgcgcag	Cloning, <i>EcoRI</i>
SCO6008R+1229	gtca <u>aaagctt</u> aacgggtgccatccgtgctcta	Cloning, <i>HindIII</i>
xylF_for	agcctgcagaaccagcaggtcg	RT-PCR
xylF_rev	tcgccttcgctcttgcgcagcgt	RT-PCR
actII-o4_for	gctgcagacgtacgtgtaccacac	RT-PCR
actII-o4_rev	ggtcgcatacggagctgcattcc	RT-PCR
cpkC_for	ggctcggcgggtcaaccaggacg	RT-PCR
cpkC_rev	acttcagcgaccccaggtacagc	RT-PCR
redW_for	cttcttcggactgaccacctt	RT-PCR
redW_rev	agaagatgctcgccccggagc	RT-PCR
rpsI_for	gagaccactcccagcagccgc	RT-PCR
rpsI_rev	gtagcggttgtccagctcgagca	RT-PCR

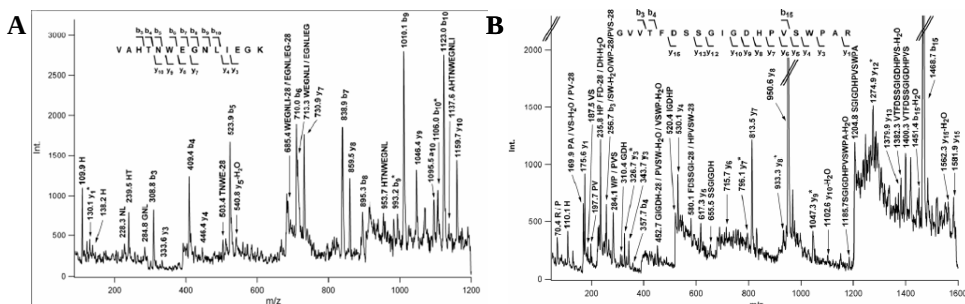
Table S3. List of differentially expressed genes between the *Rok7B7* mutant and wild-type

Gene	R/M_14	R/M_24	R/M_30	R/M_36	R/M_42	R/M_54		SCO2354	0.959	1.084	1.122	0.992	0.590	2.517
SCO0007	1.050	0.832	0.943	0.938	0.420	1.856		SCO2494	0.473	0.406	0.582	0.839	0.753	0.897
SCO0013	1.137	0.800	1.001	0.838	0.407	0.965		SCO2636	0.881	0.925	1.005	1.017	1.814	0.503
SCO0022	1.389	1.203	1.199	1.252	1.222	2.618		SCO2698	1.272	1.423	1.133	0.431	1.175	1.048
SCO0045	0.809	1.387	0.889	0.768	2.038	1.013		SCO2717	1.856	1.389	1.077	0.754	0.537	3.267
SCO0056	1.185	0.993	0.917	1.012	0.727	2.008		SCO2718	1.789	1.717	1.056	0.996	0.279	8.120
SCO0182	0.842	0.906	1.034	1.029	0.483	1.464		SCO2719	1.607	1.795	1.041	1.240	0.448	3.617
SCO0190	2.091	1.703	1.538	1.360	0.931	0.958		SCO2727	0.915	1.074	1.080	1.182	0.484	1.314
SCO0194	1.199	1.019	0.886	2.152	0.872	1.974		SCO3113	1.302	1.328	1.303	1.134	0.965	4.222
SCO0251	1.895	0.692	0.751	0.825	0.204	2.823		SCO3152	0.941	1.043	1.058	0.895	2.054	0.668
SCO0436	0.864	0.737	1.335	0.871	1.207	2.109		SCO3255	1.103	0.874	0.998	0.814	0.459	1.390
SCO0527	1.304	1.080	0.939	1.062	0.486	2.048		SCO3262	1.454	1.432	1.013	1.208	1.372	0.408
SCO0779	0.950	0.716	0.954	0.882	0.337	1.498		SCO3263	0.995	1.245	1.157	0.525	1.697	0.481
SCO0861	1.212	1.238	1.353	2.037	1.707	0.961		SCO3289	2.078	1.586	1.168	1.182	1.115	0.922
SCO0949	1.057	0.871	1.010	0.974	0.491	1.194		SCO3327	1.411	1.191	1.060	0.965	0.433	2.943
SCO0989	0.933	0.665	0.983	0.768	0.394	1.077		SCO3342	0.779	1.406	0.610	0.839	1.926	0.465
SCO1027	1.053	1.099	0.993	0.878	0.393	2.787		SCO3461	1.120	0.919	1.057	0.908	0.470	1.699
SCO1082	1.045	1.152	1.025	0.431	1.119	0.610		SCO3475	1.429	0.848	1.128	1.904	0.692	2.169
SCO1255	1.049	0.938	1.032	0.809	0.424	1.946		SCO3476	1.255	0.865	1.049	1.493	0.774	2.015
SCO1389	1.104	1.182	0.967	1.110	1.225	2.570		SCO3478	1.235	0.832	1.019	1.663	0.846	2.022
SCO1426	0.973	0.982	1.107	1.461	2.096	0.697		SCO3481	1.268	0.767	1.181	1.705	0.622	2.628
SCO1431	1.024	0.845	1.158	0.757	0.437	2.868		SCO3482	1.311	0.856	1.087	1.674	0.732	2.304
SCO1480	0.945	0.978	1.124	1.117	0.625	2.264		SCO3483	1.123	0.788	1.196	1.474	0.708	2.849
SCO1599	1.421	1.100	1.008	1.130	0.337	4.874		SCO3484	1.385	0.744	1.178	1.667	0.688	2.982
SCO1675	1.622	1.107	1.072	0.988	0.542	4.044		SCO3485	1.095	0.962	1.142	1.260	1.062	2.099
SCO1721	0.989	0.870	1.055	1.083	0.427	1.726		SCO3486	1.231	0.855	1.081	1.234	0.833	2.445
SCO1800	1.869	1.281	1.084	0.988	0.555	4.642		SCO3487	1.217	0.793	1.080	1.884	0.444	3.733
SCO1828	0.995	1.050	1.127	1.407	0.513	2.893		SCO3488	1.052	0.784	1.143	1.933	0.695	2.817
SCO1839	1.536	1.347	1.125	0.930	0.474	2.984		SCO3492	1.011	1.137	1.065	1.311	0.904	6.844
SCO1914	1.394	1.012	0.999	0.898	0.154	8.244		SCO3549	1.133	1.054	1.119	1.213	0.576	2.049
SCO1978	1.224	0.886	0.969	0.779	0.387	2.856		SCO3731	1.458	1.117	0.960	0.893	0.516	2.024
SCO2113	0.400	0.605	0.996	1.386	0.779	0.907		SCO3748	1.594	1.267	0.815	0.911	0.423	3.122
SCO2156	1.216	1.033	1.030	1.122	0.465	1.338		SCO3762	1.103	1.053	0.959	0.980	0.344	2.599
SCO2210	1.076	1.535	0.787	2.612	1.108	1.368		SCO3868	0.972	0.825	1.013	0.820	0.422	1.092
SCO2261	1.055	0.810	0.901	0.741	0.454	0.919		SCO3887	0.964	0.965	1.074	1.090	0.465	2.004
SCO2338	0.951	0.818	0.916	1.179	0.531	2.164		SCO3906	1.411	1.132	0.933	1.047	0.473	1.122

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SCO3908	1.489	1.164	0.983	1.088	0.437	1.305	SCO6010	5.801	4.696	4.511	3.305	2.717	1.656
SCO3938	1.144	0.914	1.036	1.118	0.197	4.461	SCO6011	2.056	1.566	1.464	1.459	1.295	1.110
SCO3990	1.049	1.146	0.857	0.606	2.213	0.785	SCO6026	0.903	1.209	0.939	0.465	0.973	1.031
SCO3992	1.381	1.624	1.180	0.843	2.708	0.921	SCO6027	0.906	1.076	0.895	0.435	0.954	0.973
SCO3993	1.065	1.430	1.245	0.948	2.192	0.529	SCO6045	0.996	0.992	0.990	0.984	1.363	0.476
SCO4005	0.941	1.218	0.940	1.027	1.640	0.483	SCO6290	1.327	1.050	1.055	1.100	0.333	1.675
SCO4011	0.950	0.903	1.024	0.738	1.491	0.439	SCO6338	0.984	1.337	1.123	0.356	1.516	0.551
SCO4063	1.174	1.070	0.965	0.565	2.092	0.669	SCO6403	1.136	1.129	0.681	0.726	0.498	1.125
SCO4212	1.102	0.547	0.968	0.512	0.048	2.180	SCO6480	1.250	1.042	0.698	0.700	0.438	1.762
SCO4225	1.039	0.940	1.014	0.686	1.630	0.450	SCO6499	0.471	0.594	1.016	1.140	1.474	0.952
SCO4261	1.176	0.832	0.954	0.885	0.306	1.570	SCO6547	1.441	1.399	1.079	1.781	0.690	2.041
SCO4289	0.752	1.241	0.879	0.928	2.713	0.369	SCO6600	1.056	0.490	1.092	1.440	0.720	2.381
SCO4289	0.752	1.241	0.879	0.928	2.713	0.369	SCO6624	0.746	0.820	1.018	0.815	0.815	4.175
SCO4424	1.009	0.887	0.937	1.237	1.735	0.480	SCO6642	0.973	1.283	1.321	0.600	2.150	1.089
SCO4442	1.452	1.129	1.310	0.909	0.338	3.005	SCO6715	1.731	0.995	1.153	2.376	1.584	0.829
SCO4635	1.315	0.991	1.095	1.028	0.404	2.221	SCO6761	0.934	0.951	0.795	0.584	1.483	0.462
SCO4652	1.175	0.984	0.971	1.075	0.428	1.825	SCO6779	1.065	1.212	0.795	0.495	1.361	0.925
SCO4659	1.388	1.055	0.942	1.004	0.398	1.470	SCO6810	1.220	0.717	0.920	1.133	0.368	1.878
SCO4660	1.488	1.086	0.943	1.021	0.423	1.405	SCO6910	0.987	1.307	1.158	0.733	2.189	0.775
SCO4701	1.459	1.131	0.911	0.887	0.419	1.558	SCO6925	0.910	1.195	1.105	0.521	2.121	0.623
SCO4702	1.599	1.106	0.917	0.968	0.402	1.799	SCO6933	0.924	1.005	1.103	0.901	0.479	1.998
SCO4704	1.280	1.035	1.006	0.923	0.430	2.667	SCO7086	0.963	0.910	1.028	0.934	0.488	1.286
SCO4706	1.651	1.075	0.942	1.001	0.327	2.397	SCO7087	1.075	1.066	0.991	0.817	0.514	2.358
SCO4711	1.364	1.086	1.033	0.894	0.272	4.002	SCO7106	1.111	0.893	1.157	0.980	0.368	1.548
SCO4712	1.386	1.115	0.983	0.900	0.418	1.893	SCO7153	8.047	6.510	6.128	4.418	3.350	1.514
SCO4713	1.325	1.076	0.998	0.878	0.440	0.995	SCO7217	1.123	0.996	0.975	1.172	1.063	2.339
SCO4714	1.461	1.070	0.927	0.929	0.344	1.793	SCO7528	0.953	0.792	0.966	0.858	0.456	2.030
SCO4715	1.251	0.993	0.973	1.003	0.413	2.338	SCO7552	1.196	1.083	0.959	1.174	0.383	2.315
SCO4716	1.322	1.035	0.913	0.951	0.480	1.548	SCO7636	1.155	1.099	1.034	0.631	0.345	1.305
SCO4717	1.341	0.996	0.977	1.000	0.493	1.485	SCO7645	0.939	0.866	0.944	0.867	0.462	1.254
SCO4720	1.161	0.993	0.946	1.012	0.366	3.901	SCO7742	1.283	1.149	1.019	1.291	0.475	2.133
SCO4721	1.261	1.052	0.936	1.018	0.359	2.015	SCO7752	1.009	0.933	1.045	1.029	0.466	1.625
SCO4730	1.390	1.047	0.918	0.963	0.475	2.452	SCO7774	1.119	0.955	0.924	0.928	0.436	1.223
SCO4761	0.960	0.872	0.949	1.391	0.435	1.524	SCO7800	1.009	1.781	1.172	0.590	2.237	0.833
SCO4894	0.956	0.910	1.080	0.635	2.013	0.849	SCO7833	1.254	0.934	0.918	0.823	0.488	1.442
SCO4903	0.921	1.088	0.982	0.822	1.762	0.460	SCO7836	1.107	0.976	0.987	1.203	0.413	3.370
SCO5029	1.290	1.032	1.088	1.126	0.497	1.622							
SCO5191	1.276	1.454	0.995	1.298	0.319	1.708							
SCO5227	0.812	0.782	1.033	0.985	0.529	2.819							
SCO5240	1.519	1.201	0.936	1.328	0.484	2.311							
SCO5326	0.966	1.569	1.012	0.490	2.565	0.477							
SCO5490	1.127	0.921	0.909	0.768	0.386	1.260							
SCO5578	8.691	7.028	6.642	5.109	2.671	1.519							
SCO5583	1.172	1.508	0.797	2.727	1.021	1.485							
SCO5584	1.136	1.329	0.878	2.116	0.976	1.710							
SCO5609	1.171	0.743	0.974	1.055	0.088	3.108							
SCO5639	1.584	1.564	1.169	0.708	2.045	0.678							
SCO5742	1.042	1.944	0.923	0.538	2.107	0.808							
SCO5824	0.980	0.834	0.956	0.777	0.495	1.338							
SCO5907	1.042	0.748	0.758	0.761	0.405	1.952							
SCO5918	1.359	1.019	0.504	0.587	1.201	1.051							
SCO5920	1.282	1.003	0.406	0.807	1.233	0.936							
SCO5978	0.947	0.888	0.964	0.990	0.641	2.240							
SCO5987	1.118	0.938	1.154	1.228	2.318	0.419							
SCO6009	3.530	2.202	1.982	2.337	1.105	1.803							

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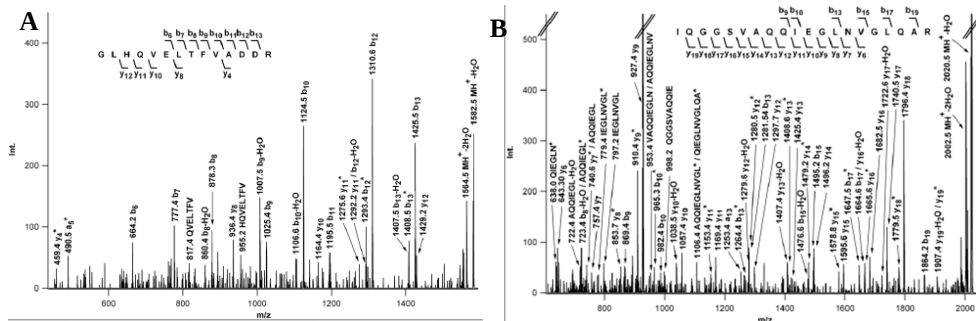


Figure S5. PSD-spectra of MH+ = 1599.81 (S5a) and MH+ = 2038.10 (S5b) peptides corresponding to GLHQVELTFVADDR [192-205] and IQGGSVAQIQIEGLNVGLOAR [38-57] of hypot. Pro. Sporulation-control protein

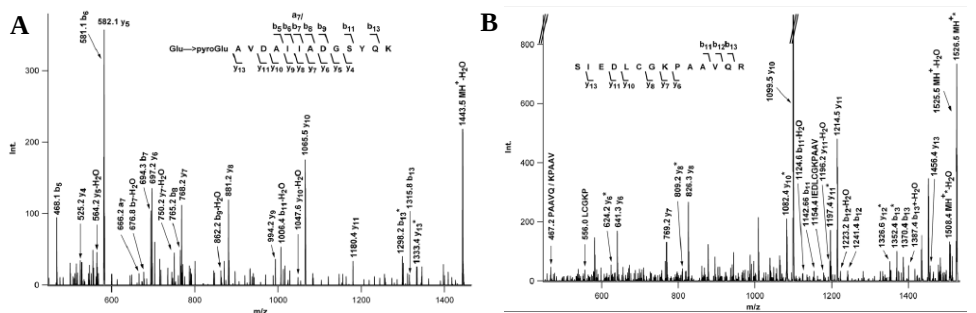


Figure S6. PSD-spectra of MH+ = 1461.72 (S6a) and MH+ = 1543.79 (S6b) peptides corresponding to the Glu->pyroGlu AVDAIIADGSYQK [286-299], SIEDLCGKPAAVQR [178-191].

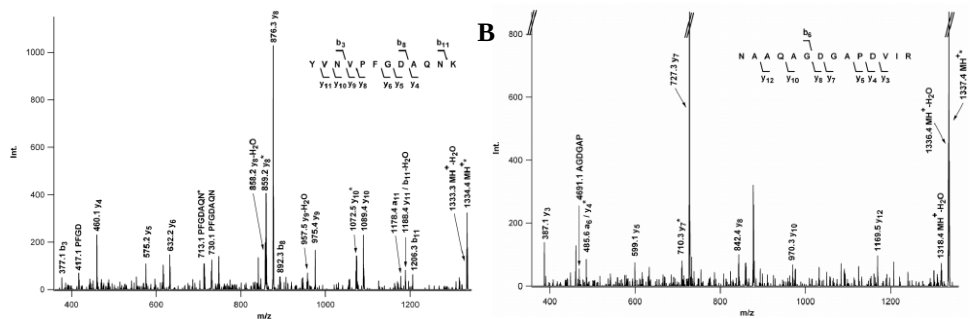


Figure S7. CID-spectra of MH+ = 1351.66 (S7a) and MH+ = 1354.67 (S7b) peptides corresponding to YVNPFGDAQNK [73-84] and NAAQAGDGAPDVIR [87-100] of putative ABC transporter maltose-binding protein.

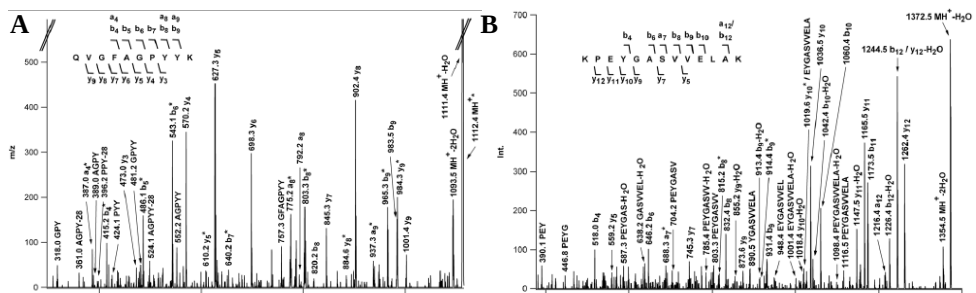


Figure S8. PSD-spectra of MH+ = 1129.57 (S8a) and MH+ = 1390.76 (S8b) peptides corresponding to QVGFAGPYK [149-158] and KPEYGASVVELAK [197-209] of putative glutamate ABC transporter substrate-binding protein.

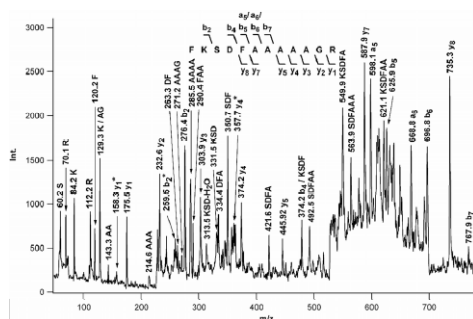


Figure S9. PSD spectrum of $MH^+ = 1211.61$ peptide corresponding to the FKSDFAAAAAGR [115-126] sequence of manganese (Mn) superoxide dismutase Soda.

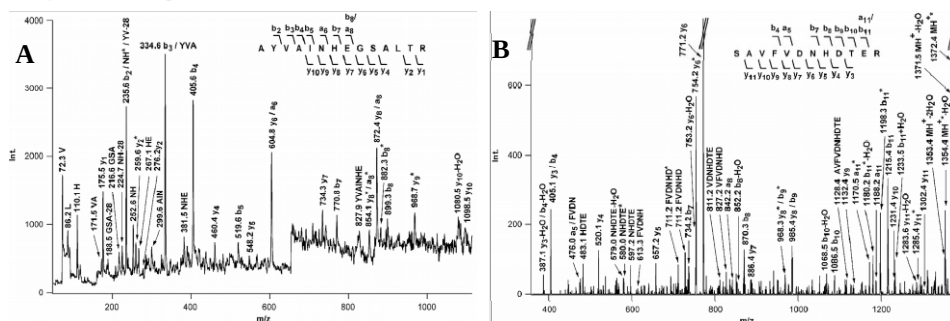


Figure S10. PSD spectrum of $MH^+ = 1501.78$ (S10a) and CID spectra of $MH^+ = 1389.64$ (S10b) peptides, corresponding to the AYVAINEHGSALTR [406-419] and SAVFDNHDTER [295-306] sequences of putative alpha-amylase.

Table S1. Oligonucleotides used for semi-quantitative RT PCR analysis.

Oligonucleotide	Target	Position 5' end #	Sequence (5' → 3')
RT_1460+169f	SGR1460	+169	CACTCCAGCTGCTCTCGAT
RT_1460+419c	SGR1460	+419c	AGCGAGGCGCTCAGGGTGATC
RT_1498+105f	SGR1498	+105	AGGAGGTTCCGAGAAGAAGG
RT_1498+313c	SGR1498	+313c	CCCTGTTGGTGATCATGGTG
RT_1737+286f	SGR1737	+286	GACATCGAGATCGCCAGAT
RT_1737+558c	SGR1737	+558c	GATCGAGCGACCTTCTGTGC
RT_2237+514f	SGR2237	+514	AACCGCGAGAACATCAAGTC
RT_2237+729c	SGR2237	+729c	GACCGGGTAGTCGTTGACAC
RT_2245+118f	SGR2245	+118	GAGAAGTACCAAGGCCAACGA
RT_2245+396c	SGR2245	+396c	TCAGGCCCTTCTGTTCTCC
RT_3109+429f	SGR3109	+429	CTTCACCCACACGAGACCT
RT_3109+695c	SGR3109	+695c	GAGAAGAACTCCGGGATGTG
RT_5275+215f	SGR5275	+215	AGTACGTCACGCTGCCCTTC
RT_5275+496c	SGR5275	+496c	CCCTCTTGAGCAGCTCTCTTG
RT_5280+478f	SGR5280	+478	TCCAGATCGGGAACAACCTA
RT_5280+719c	SGR5280	+719c	GCCTCGTCTCTCCAGTAGAC
RT_5704+530f	SGR5704	+530	CGTTCTACAGGAGATCGAGTT
RT_5704+749c	SGR5704	+749c	AGCCACTGGTTGAGGTATGC

Oligonucleotides ending with 'f' were used as forward primers in the amplification step, those ending with 'r' for cDNA synthesis with reversed transcriptase and as reverse primers during amplification. #, position is indicated relative to the start of the target gene; c, complementary to coding strand.

CURRICULUM VITAE

Magdalena A. Świątek was born on the 22nd of August 1983 in Nowa Ruda (Poland). In 2002, she obtained the diploma for secondary education at the Stanisław Staszic School in Nowa Ruda. Between 2002 and 2007 she studied at the Wrocław University of Technology (Poland), where she obtained a Master's degree in Biotechnology. During 2005-2007, she completed an internship at the Institute of Immunology and Experimental Therapy in the Laboratory of Molecular Biology of Microorganisms (Polish Academy of Science, Wrocław, Poland) under the supervision of Prof. Jolanta Zakrzewska-Czerwińska and Dr Agnieszka Kois-Ostrowska. In October 2007 she obtained a personal fellowship from the EU (Leonardo da Vinci) and completed a research project at the John Innes Centre in the laboratory of Prof. Mervyn Bibb, Department of Molecular Microbiology (Norwich, UK). In the summer of 2008, she started as a Ph.D. student at Leiden University (the Netherlands). She worked under the supervision of Prof. Gilles P. van Wezel in Molecular Biotechnology, LIC. The work done as a Ph.D. student is presented in this thesis. During this time she participated in two international meetings and presented her work there. After her Ph.D. she will join the laboratory of Prof. Lotte Søggaard-Andersen at Max Planck Institute in Marburg (Germany).

LIST OF PUBLICATIONS

- Świątek MA**, Urem M, Tenconi E, Rigali S, van Wezel GP (2012) Engineering of *N*-acetylglucosamine metabolism for improved antibiotic production in *Streptomyces coelicolor* A3(2) and an unsuspected role of NagA in glucosamine metabolism. *Bioengineered* 3(5): 280-285
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