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

Swiatek, M.A.

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 *act*II-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 uridyltransferase). 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 carrie 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 phopshate 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, ORFs 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.*