Exploring the metabolism and toxicity of amino sugars and 2-deoxyglucose in Streptomyces

Li, C.

Chapter 6

General Discussion

Streptomycetes are high-GC, Gram-positive, multicellular mycelial bacteria belonging to the phylum Actinobacteria. They play a key role in soil ecology due to their exceptional ability to scavenge complex carbon sources, particularly through the hydrolysis of a broad spectrum of polysaccharides, such as cellulose, chitin, xylan, and agar, as well as other natural macromolecules (Chater *et al.*, 2010). Streptomycetes exhibit a complex life cycle that morphologically resembles that of filamentous fungi. This life cycle begins with the germination of a spore, leading to the formation of a branched network of long, multinucleate hyphae. Under adverse conditions, such as nutrient depletion, streptomycetes initiate a complex developmental program. A key event in this process is the lysis of the vegetative or substrate mycelia through programmed cell death (PCD), providing nutrients for the newly formed aerial mycelium (Méndez *et al.*, 1985, Miguélez *et al.*, 1999). Eventually, the aerial hyphae generate chains of unigenomic spores. The onset of development is closely linked to the production of antibiotics and other bioactive molecules (Bibb, 2005, van Wezel & McDowall, 2011).

N-acetylglucosamine (GlcNAc) is a preferred nutrient for streptomycetes and also acts as a signal indicative of the nutritional status of the environment, which is known as "*feast* and *famine*" ((Rigali *et al.*, 2008); reviewed in **Chapter 2**). Under nutrient-rich (*feast*) conditions, higher concentrations of GlcNAc (but not GlcN) repress development and antibiotic production, while conversely, under nutrient-limited conditions (*famine*) GlcNAc activates these processes. It is clear that the nutrient-sensory pleiotropic regulator DasR plays an important role; it has a large regulon consisting of genes for GlcNAc metabolism and transport, chitinases, siderophores and antibiotic production, whereby its DNA binding activity is controlled by amino sugar phosphates. However, while important, this is only one part of a complex jigsaw. Given that GlcNAc is also an essential component of bacterial cell walls (Vollmer *et al.*, 2008), its signaling role in streptomycetes may be linked to the perception of environmental nutrients, particularly in the context of mycelial lysis resulting from PCD due to nutrient depletion. This suggests a close connection between amino sugar metabolism, PCD and the development of *Streptomyces*.

State of the art at the start of this thesis

High concentrations of GlcNAc or GlcN are toxic in the absence of the enzyme glucosamine 6-phosphate deaminase (NagB) in *S. coelicolor* (Świątek et al., 2012a). On minimal media supplemented with 10 mM GlcNAc or GlcN, *nagB* mutants frequently sustain spontaneous second-site mutations that allows their survival (Świątek *et al.*, 2012a, Świątek *et al.*, 2012b). Suppressor mutations were predominantly found in the gene for *N*-acetylglucosamine-6-phosphate deacetylase (NagA), and on both GlcN and GlcNAc (Swiatek, 2012), suggesting a role for NagA in the metabolism of both amino sugars. This also indicated that the substrate of NagA, GlcNAc-6P, is non-toxic by itself. After all, this metabolite will accumulate in large

amounts in *nagA* mutants grown on GlcNAc. Besides in *nagA*, suppressor mutations were identified in *rokL6* (SCO1447) and SCO4393. Mutations in *rokL6* only arose on GlcN, while those on GlcNAc only arose on GlcNAc. RokL6 is a ROK-family regulator specifically involved in GlcN transport or metabolism, as frameshift mutations in this gene only alleviate GlcN toxicity (Urem, 2017). However, at the start of this thesis, the regulons of RokL6 and its roles in compensating for GlcN toxicity remained unknown. SCO4393 contains a predicted sugar isomerase (SIS) domain. Given its association with *N*-acetylglucosamine sensitivity, we recently renamed this gene *nagS*. Surprisingly, preliminary isothermal titration calorimetry (ITC) analysis showed that only GlcNAc-6P was the substrate of the enzyme (Urem, 2017), suggesting that NagS converts GlcNAc-6P into certain toxic products. However, this apparently conflicts the observation that *nagA-nagB* double mutants are completely insensitive to GlcNAc, despite accumulating large amounts of GlcNAc-6P. For a decade, this has been a major mystery within our laboratory. Although the structures of apo-NagS and GlcNAc-6P-bound NagS had been determined (Urem, 2017), its precise function and catalytic mechanism remained unresolved. Furthermore, apart from the role of DasR, the underlying mechanisms behind GlcNAc-mediated nutrient sensing, especially under *feast* conditions, were still not well understood.

Finally, a third and related toxic molecule exists, namely 2-deoxy-glucose (2-DG). GlcN is 2-amino-deoxyglucose. 2-DG is currently being developed as a chemotherapeutic drug against tumours (Zhang *et al.*, 2014, Bost *et al.*, 2016). However, its mode of action remains unclear, as glycolytic inhibition alone does not fully explain its toxicity to eukaryotic cells (Ralser *et al.*, 2008). In *S. coelicolor*, exposure to 2-DG resulted in spontaneous 2-DG resistant mutants, all of which mapped to *glkA*, encoding glucokinase, indicating that phosphorylation of 2-DG to 2-DG-6P is required for its toxicity (Van Wezel *et al.*, 2005).

Thus, this thesis started with the existence of three toxicity pathways in central metabolism, and aimed to answer some of the major questions mentioned above.

Solving the mystery around GlcN and 2-DG detoxification by RokL6-SCO1448

Making use of the GlcN toxicity, we aimed to identify the transporters of GlcN in *S. coelicolor* by evaluating its toxicity in several potentially related mutants. NagE1 and NagE2 are two closest homologs of GamP in *S. coelicolor*, one of the GlcN transporters in *B. subtilis*. Additionally, the transcription of genes encoding the ABC transporter CsnEFG (SCO2658-SCO2660) was significantly upregulated in the presence of GlcN (Li *et al.*, 2023). However, these mutants created on Δ*nagB*, Δ*nagB*Δ*nagE1*, Δ*nagB*Δ*nagE2*, and Δ*nagB*Δ*csnEFG* remain sensitive to GlcN (data not shown), indicating that GlcN transport and metabolism in *Streptomyces* is likely to be more complex than in *E. coli* and *B. subtilis*.

Mutations of *rokL6* in Δ*nagB* specifically relieve toxicity of GlcN, but not GlcNAc, indicating that this transcriptional regulator, RokL6, specifically controls GlcN-related

transport or metabolism (Urem, 2017). **Chapter 3** of this thesis aimed to identified the regulons of RokL6 and its role in GlcN toxicity. Our work showed that RokL6 represses transcription of the neighbouring and divergently transcribed SCO1448 and also acts as autoregulator, by binding to overlapping promoters in the *rokL6*-SCO1448 intergenic region. A conserved binding consensus for RokL6 (5' -C(T)TATCAGG - 7 nt - CCTGATAG(A)- 3') was identified. RokL6-independent expression of SCO1448 completely alleviated GlcN toxicity in *nagB* mutants. The high accumulation of GlcN-6P and/or its metabolic derivatives is lethal to *S. coelicolor*, consistent with observations in *E. coli* and *B. subtilis* (Plumbridge, 2015). SCO1448 encodes an MFS transporter, and considering that its overexpression relieves toxicity, it is logical to assume (but not proven) that it facilitates the export of one or more toxic intermediates. However, constitutive expression of SCO1448 specifically relieves the toxicity of GlcN but not GlcNAc, highlighting significant differences in the perception of GlcN and GlcNAc by *Streptomyces* and suggesting that GlcN and GlcNAc toxicity operate via independent pathways.

Surprisingly, deletion of *rokL6* in *S. coelicolor* Δ*nagB* not only relieve the toxicity of GlcN but also that of 2-deoxyglucose (2-DG) (Urem, 2017), linking the metabolism of these two glucose analogues in *S. coelicolor*. 2-DG is a stable glucose analogue that inhibits the growth of both normal and malignant cells. Similar to its effects on *E. coli*, yeast and mammalian cells, 2-DG inhibits the growth of filamentous *Streptomyces*. Constitutive expression of SCO1448 can also alleviate 2-DG toxicity in M145 (**Chapter 5**), suggesting that the metabolic pathways leading to the accumulation of toxic compounds derived from 2-DG and GlcN may overlap in *Streptomyces*, with SCO1448 acting as an exporter to pump out the toxic molecules derived from them. In particular, why would streptomycetes have a cryptic exporter to protect themselves from toxic intermediates related to GlcN and 2-DG? The system is likely important, as its gene synteny and also (the location of) the RokL6 binding site are highly conserved in *Streptomyces* (**Chapter 3**). This high conservation includes the RokL6 binding site, which predicts that the expression of SCO1448 is repressed by RokL6 in many if not all streptomycetes. While the biological significance of the RokL6-SCO1448 regulatory system remains unclear, this protein may play a crucial role in preventing the accumulation of excess toxic intermediates under specific growth conditions in the natural environment. However, several questions remain unanswered; (i) how and when is the inhibitory effect of RokL6 on SCO1448 relieved within the cell; (ii) what is the ligand that alters the DNA binding capacity of RokL6 and when is the system relieved; and (iii) the nature of the substrates transported by SCO1448. These questions warrant further investigation.

2-DG toxicity is mediated via the pentose phosphate pathway

We next explored 2-DG toxicity by targeting the downstream enzymes of glucose-6-phosphate as 2-DG-6P is likely further metabolised via these routes. We found that 2-DG-6P was not a

substrate of glucose-6-phosphate (Pgi) but it is dehydrogenated by glucose-6-phosphate dehydrogenase (Zwf) from *S. coelicolor*, *E. coli* and *Saccharomyces cerevisiae*. We also confirmed the hydrolysis of 6-phospho-deoxyglucono-1,5-lactone (6-PDL) by the 6-phosphogluconolactonases (Pgl) from *S. coelicolor* and *S. cerevisiae*. Thus, 2-DG-6P is metabolized via the PPP in both prokaryotes and eukaryotes, and this is the main cause of its toxicity; 2-DG-6P is dehydrogenated to 6-PDL by Zwf, followed by hydrolysis to form 6-phospho-deoxygluconate by Pgl (**Chapter 5**). Deletion of genes encoding Zwf significantly reduces 2-DG toxicity, while deletion of *pgl* enhances it, providing strong evidence that the Zwf product, 6-PDL, is the key toxic molecule responsible for growth inhibition by 2-DG. Indeed, chemically synthesized 2-deoxyglucono-1,5-lactone exhibits high toxicity to *S. coelicolor*, and around one order of magnitude higher than 2-DG. The alleviation of 2-DG toxicity in Δ*nagB*Δ*rokL6* is not solely due to enhanced SCO1448 expression, as *rokL6* single mutants with comparable SCO1448 expression remain sensitive to 2-DG. Since GlcN-6P, the substrate of NagB, inhibits Zwf (Kanji *et al.*, 1976), its accumulation in Δ*nagB*Δ*rokL6* reduces the production of 6-PDL, explaining the observed 2-DG resistance in Δ*nagB*Δ*rokL6* but not in Δ*rokL6*.

In *S. cerevisiae*, 2-DG-6P phosphatases (Dog1 and Dog2) also play a role in 2-DG detoxification (Defenouillère *et al.*, 2019). However, there are no homologues of these enzymes in *Streptomyces*. This suggests differences in the mechanisms of 2-DG resistance between prokaryotes and eukaryotes. Daran and Pronk from TU Delft conducted preliminary studies on 2-DG toxicity in *zwf1* mutants and *sol3-sol4* double mutants of the eukaryotic yeast *S. cerevisiae*. The *sol* genes encode 6-phosphogluconolactonase. However, neither Δ*zwf1* nor Δ*sol3*Δ*sol4* showed altered sensitivity to 2-DG. Thus, the results found in yeast could not yet be aligned with those in *S. coelicolor*, and further research is required to test the toxicity of the accumulation of 6-PDL in *S. cerevisiae* and to assess the potential eukaryotic cytotoxicity of 2-deoxyglucono-1,5-lactone.

The phosphosugar dehydratase NagS with totally novel function

Challenging *nagB* mutants with GlcNAc leads to suppressor mutations in *nagS* (SCO4393). The *nagS* gene is divergently expressed from *dmdR1* (SCO4394) on the *S. coelicolor* genome, and gene synteny surrounding *nagS* is highly conserved among all *Streptomyces* species, particularly in relation to *dmdR1*. DmdR1 is the global iron-homeostasis regulator in *Streptomyces* that also controls the production of the siderophores coelichelin and desferrioxamine (Flores & Martín, 2004). The addition of Fe^{2+} to R2YE media supplemented with GlcNAc restores development and antibiotic production in *S. coelicolor*, also in the presence of GlcNAc (Lambert *et al.*, 2014). In addition, *nagS* null mutants had lost the ability to suppress siderophore production on R5 with added GlcNAc, suggesting a close link between NagS and iron utilization in *Streptomyces*. Annotated as a phospho-sugar isomerase, NagS

exhibits a distinct function from other sugar isomerases, dehydrating both GlcNAc-6P and ManNAc-6P to produce the corresponding 2,3-dehydro derivative (**Chapter 4**), a reaction that has not yet been described in the textbooks. By structural and mutagenesis analysis, we identified the active sites of NagS, which include His53, Arg64, Glu94, Asp179, and proposed a mechanism for ring-opening of GlcNAc-6P during catalysis. Besides, we determined the co-crystal structure of NagS and 6-phosphogluconate (6-PG). As an important pentose phosphate pathway intermediate, 6-PG was identified as a competitive inhibitor of NagS, suggesting an inhibitory role of 6-PG in the formation of GlcNAc derived toxic molecules. Study of NagS has revealed the characteristics of a totally new enzyme involved in amino sugar metabolism, providing new clues to amino sugar utilization and GlcNAc sensitivity in *Streptomyces*.

GlcNAc toxicity and the role of NagS and NagA

The basis for GlcNAc sensitivity in *S. coelicolor nagB* mutants was also investigated in **Chapter 4**. We studied the essential roles of two enzymes, NagA and NagS, in GlcNAc toxicity, and also discovered a new catalytic substrate of NagA as well as a novel metabolic route for GlcNAc. Based on this, we clarified the origin of GlcNAc toxicity in *S. coelicolor*. In addition to being deacetylated by NagA, GlcNAc-6P is dehydrated by NagS to yield compound **1**, which is further deacetylated by NagA to yield the potential toxic molecules, **2** and **3** (**Chapter 4**). The discovery of this new metabolic pathway solves the previously mentioned mystery of why *nagA* mutants, which accumulate large quantities of GlcNAc-6P, grow well on GlcNAc: NagA has a second, promiscuous activity, allowing deacetylation of the product of NagS, which then yields a toxic compound that resembles ribose. Without NagA, this pathway is closed. The addition of ribose attenuates GlcNAc toxicity, suggesting that GlcNAc sensitivity is linked to impaired nucleotide synthesis, which is essential for cell growth and signal transduction. We hypothesize that the accumulated compound **3** is the likely toxic agent, as it has a similar chemical structure to ribose-5P. This hypothesis needs to be confirmed through metabolomic studies of wild-type and Δ*nagS* mutants, as well as by assessing their structurally correct nucleic acid content *in vivo* when exposed to GlcNAc.

Our data combined with previous data allowed us to propose a new model for the control of development and antibiotic production in *Streptomyces* in **Chapter 4**. This model integrates NagS, GlcNAc metabolism, the regulation by DasR and intracellular iron accumulation, to explain the onset of termination of PCD in the mycelia, as the gateway to the onset of morphological and chemical differentiation. Undoubtedly, the concentration of GlcN(Ac)-6P is crucial. As vegetative growth progresses, the mycelia are degraded to support the new biomass. This leads to the accumulation of building blocks like amino acids, nucleotides, and GlcNAc. Elevated levels of GlcNAc triggers a PCD-like process by activating NagS/NagA toxicity pathways, iron import and prodiginine production, all of which are toxic to cells. High GlcNAc levels in regions undergoing extensive mycelial lysis inhibits DasR through

the allosteric effect of GlcNAc-6P and GlcN-6P, thereby activating DmdR1. This reduces iron import and limits cell death from Fe^{2+}-mediated reactive oxygen species (ROS) production (Cornelis *et al.*, 2011). NagS inactivation likely occurs post-translationally via a salvage pathway that inhibits NagS and stops toxic compound production (**Chapter 4**). The key discovery is that the pentose phosphate pathway (PPP) intermediate 6-phosphogluconate inhibits NagS. Deletion of *nagB* will prevent the conversion of GlcN-6P to Fru-6P and then via the PPP to the NagS inhibitor 6-PG. The salvage pathway is not accessible, and NagS remains active. This explains at least in part why *nagB* mutant cells are so sensitive to amino sugars. Finally, once PCD is switched off due to reduce iron, prodiginines and NagS activity, nutrient depletion serves as a major signal for the onset of morphological and chemical differentiation.

Concluding remarks and look ahead

In conclusion, the work described in this thesis answers several long-standing mysteries around amino sugar and/or 2-deoxyglucose metabolism in *Streptomyces*, but inevitably has also created new questions to be resolved in the future. The biggest mystery perhaps was the one around *nagS* and *nagA*. When *nagA* mutants are challenged with high concentrations of GlcNAc, this will result in high intracellular concentrations of GlcNAc-6P, which apparently are non-toxic (because *nagA*-*nagB* double mutants are totally insensitive to either amino sugar). How then can a suppressor of the *nagB* mutant be in the gene for an enzyme that uses GlcNAc-6P as a substrate? This was resolved by showing that in fact NagS and NagA form a novel pathway, whereby deacetylation by NagA of the product of GlcNAc dehydratase NagS is the key step towards toxicity. Thus we discovered both a totally new enzyme in primary metabolism (NagS) as well as a novel promiscuous function for NagA. The work also shows that NagS plays a central role as gatekeeper of nutrient control of development and antibiotic production, thus enhancing our understanding of the underlying mechanisms of amino sugar sensitivity and “feast and famine”. We also resolved the role of the ROK-family regulator RokL6 in GlcN toxicity, showing that RokL6 acts as a transcriptional repressor of SCO1448, for an MFS transporter. This transporter provides resistance to both GlcN and 2-DG toxicity. Furthermore, we also showed that that 2-DG toxicity is mediated via the PPP, with 6-phospho-2-deoxyglucono-1,5-lactone (6-PDL) as the cytotoxic agent.

Looking ahead, one of the important questions that still lies on the table is, why does deleting *nagA* prevent toxicity of GlcN? And what is the precise cross-talk between the toxicity pathways around GlcN, 2-DG an GlcNAc? Generally speaking, we still understand very little of GlcN metabolism in *Streptomyces*, which is one key step towards answering the major questions that still have to be addressed. Furthermore, to better understand the mechanisms controlling the onset of development, elucidation of the spatiotemporal coordination of NagS-NagA, iron utilization and prodiginine production is necessary. Given that SCO1448 participates in the detoxification of both GlcN and 2-DG, identifying its substrate(s) is crucial

for uncovering the basis of GlcN toxicity in bacteria. The work on 2-DG has significant implications in the field of cancer research, considering its high toxicity for human cells. Better understanding of the precise mechanism by which 2-DG acts on eukaryotic cells is important for its application in the clinic, among others to prevent drug resistance. For this, we need further analysis of 6-PDL in eukaryotic model organisms, which will hopefully provide deeper insights for developing more effective anti-tumour therapies based on 2-DG. Thus, we have come a long way working out the molecular basis for amino sugar and deoxysugar toxicity in *Streptomyces*. At the same time – as always – new avenues have been opened for further exploration. Further research on this exciting topic should provide further insights into the role of the toxicity pathways in the control of growth, development and antibiotic production by streptomycetes.