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

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Chapter 1

General Introduction

Streptomycetes are high-GC Gram-positive, multicellular mycelial bacterial that belong to the phylum Actinobacteria. Although primarily considered to be soil dwellers, they have been found in most environmental niches, including aquatic habitats and endophytic interactions (van der Meij *et al.*, 2017). Streptomycetes play a key role in soil ecology due to their exceptional ability to scavenge nutrients, 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). Additionally, they are prolific producers of enzymes and secondary metabolites, including more than 50% of all clinical antibiotics, which makes them highly attractive for medical, biotechnological, and industrial purposes (Berdy, 2005, Hopwood, 2007). With a complex life cycle, streptomycetes are morphologically similar to that of filamentous fungi. The life cycle of *Streptomyces* begins with a spore that germinates to form a branched network of hyphae, consisting of long, multinucleate filaments. 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 mycelium, which is part of a programmed cell death (PCD) mechanism that facilitates colony growth and development by releasing nutrients for the formation of aerial mycelium (Méndez *et al.*, 1985, Miguélez *et al.*, 1999, Wildermuth, 1970). Eventually, the aerial hyphae produce chains of unigenomic spores. The onset of development is closely linked to and coincides with the production of antibiotics, such as prodiginines, and other bioactive molecules (Bibb, 2005, van Wezel & McDowall, 2011).

Amino sugars, such as *N*-acetylglucosamine (GlcNAc) and its deacetylated derivative glucosamine (GlcN), are important carbon and nitrogen sources for bacteria (reviewed in **Chapter 2**). GlcNAc is the monomer of chitin, the second most abundant polysaccharide on earth after cellulose, and is ubiquitously distributed in both soil and aqueous environments (Plumbridge, 2015, Davies *et al.*, 2019, Elieh-Ali-Komi & Hamblin, 2016). Additionally, GlcNAc is a component of bacterial peptidoglycan (PG), which consists of chains of alternating GlcNAc and *N*-acetylmuramic acid (MurNAc) residues cross-linked via peptide bridges. In *Streptomyces coelicolor* A3(2), the best characterized representative of the *Streptomyces* genus, GlcNAc is a preferred carbon source and internalized by phosphotransferase system (PTS) (Nothaft *et al.*, 2010, Nothaft *et al.*, 2003a).

GlcNAc functions as an important signalling molecule in streptomycetes, modulating growth, development, and antibiotic production by differentiating between chitin-derived GlcNAc, indicative of nutrient abundance, and GlcNAc originating from cell wall lysis, which signals nutrient starvation (Rigali *et al.*, 2006, Rigali *et al.*, 2008). Under nutrient-rich conditions (*feast*), GlcNAc promotes vegetative growth, thereby blocking developmental processes. Conversely, in nutrient-poor conditions (famine), the accumulation of GlcNAc activates development and secondary metabolites production (van Wezel *et al.*, 2009, Rigali *et al.*, 2008). This metabolic control of development and antibiotic production is mediated via the

GntR-family regulator DasR (Rigali *et al.*, 2006, Rigali *et al.*, 2008). DasR acts as a global regulator of transcription, controlling the regulon for uptake and metabolism of GlcNAc, while DasR is also involved in the regulation of all pathway-specific regulatory genes for antibiotic and also siderophore biosynthesis (Craig *et al.*, 2012, Lambert *et al.*, 2014, Świątek-Połatyńska *et al.*, 2015). The DNA-binding activity of DasR is modulated by intracellular amino sugar metabolic intermediates, whereby *N*-acetylglucosamine-6-phosphate (GlcNAc-6P) and glucosamine-6-phosphate (GlcN-6P) allosterically induce the release of DasR from its recognition sites.

Thus, there is an intimate interplay between amino sugar metabolism and development and antibiotic production. However, our understanding of how amino sugars metabolically control *Streptomyces* development and secondary metabolite production is still far from complete. Another important aspect is the phenomenon of amino sugar toxicity that has been observed in various bacterial phyla. In *E. coli* and *B. subtilis*, both amino sugar phosphates *N*-acetylglucosamine-6-phosphate (GlcNAc-6P) and glucosamine-6-phosphate (GlcN-6P) are lethal when they are accumulated (Plumbridge, 2015). This is known as amino sugar sensitivity (Bernheim & Dobrogosz, 1970, Kadner *et al.*, 1992). Similarly, GlcNAc and GlcN are also toxic to *S. coelicolor nagB* mutants, likely due to the accumulation of the central metabolite GlcN-6P or its derivatives (Świątek *et al.*, 2012b). Notably, deletion of *nagA* relieves the toxicity of both GlcNAc and GlcN to *nagB* mutant, suggesting that NagA is either directly involved in or connected to the metabolic pathways of these amino sugars. More importantly, spontaneous suppressor mutants of *S. coelicolor nagB* were isolated, which by-passed the toxicity when *nagB* mutant spores were plated at high density on MM agar plates containing GlcNAc or GlcN. The identification of the responsible mutations revealed novel genes for enzymes and transcriptional regulators likely involved in amino sugar metabolism in *S. coelicolor* (Swiatek, 2012). Based on this, this thesis explored the metabolism and toxicity of GlcN, GlcNAc, and 2-deoxyglucose in *S. coelicolor* by studying these novel identified genes.

Chapter 2 provides a review of amino sugar transport and metabolism, and regulation of amino sugar utilization in the well-studied model bacteria *Bacillus subtilis*, *Escherichia coli* and *S. coelicolor*. This chapter also discusses the phenomenon of amino sugar sensitivity, with particular emphasis on the prolific antibiotic producer *S. coelicolor*.

Previous work had shown that challenge of *S. coelicolor nagB* mutants with GlcN or GlcNAc leads to suppressor mutations (Swiatek, 2012, Świątek *et al.*, 2012b). These form the basis for the work in this thesis. The analysis of suppressor mutants that arose after challenging *S. coelicolor nagB* mutants with GlcN, identified a novel ROK-family regulatory gene, *rokL6* (SCO1447). Mutations in this gene specifically alleviated GlcN toxicity, while cells remained sensitive to GlcNAc (Swiatek, 2012). So far, the function of RokL6 and its role in amino sugar toxicity remained unresolved, and this is elucidated in **Chapter 3**. We provide a comprehensive systems-wide analysis of RokL6 using RNA sequencing, ChIP-Seq, EMSAs, 5' RACE,

bioinformatics, and genetic approaches. We identified the function of the ROK-family regulator RokL6, including its regulons, binding sites, and its roles in the relief of GlcN toxicity in *S. coelicolor*. These findings shed new light on amino sugar sensitivity and on the control of amino sugar metabolism in *Streptomyces*.

Another gene that was identified in a lethal screen was SCO4393, whereby in this case mutants in SCO4393 relieved sensitivity of *nagB* mutants specifically to GlcNAc and not GlcN. We renamed SCO4393 to NagS, for *N*-acetylglucosamine sensitivity. The absence of NagS relieves the toxicity of GlcNAc in *nagB* mutants, suggesting that the enzyme is involved in the synthesis of toxic molecules that accumulate in *nagB* mutants when cultured on GlcNAc. The structures of the apoenzyme and GlcNAc-6P-bound NagS were previously resolved (Urem, 2017), but its precise catalytic function remained elusive. **Chapter 4** details the crucial role of NagS in both GlcNAc sensitivity and GlcNAc-mediated nutrient sensing. Additionally, we characterised the function of NagS in primary metabolism. Studies on the crystal structures of NagS in complex with its substrate GlcNAc-6P and inhibitor 6-phosphogluconate elucidated its binding mechanism and catalytic process. The work in this chapter also investigated the mechanisms underlying GlcNAc toxicity in *S. coelicolor nagB* mutants. This surprisingly identified a novel metabolic pathway for GlcNAc-6P, which involves the sequential dehydration and deacetylation catalysed by NagS and NagA. The work allowed us to propose a new model for the regulation of development and antibiotic production in streptomycetes, providing new insights into the phenomenon of *feast and famine*.

2-Deoxyglucose (2-DG) is a stable glucose analogue in which the hydroxyl group at the second carbon atom is replaced by a hydrogen. 2-DG is widely studied as glycolytic inhibitor and inhibits growth of both prokaryotic (Hodgson, 1982, Kornberg & Lambourne, 1994) and eukaryotic cells, (Ralser *et al.*, 2008, Laussel & Léon, 2020), making it a potential candidate for anticancer therapy. Despite decades of intensive study, the precise mechanism of 2-DG action remains unclear, and studies on its toxicity of 2-DG in bacteria are limited. In **Chapter 5**, we examined 2-DG toxicity and resistance in *S. coelicolor* by evaluating the 2-DG toxicity in various metabolic gene mutants and through a series of enzymatic assays. We showed that the pentose phosphate pathway (PPP) is the main route through which 2-DG exerts its toxicity in *S. coelicolor*. 2-DG-6P is dehydrogenated by glucose-6-phosphate dehydrogenases (Zwf) and then hydrolysed by 6-phosphogluconolactonase (Pgl) to obtain 6-phospho-2-deoxygluconate. The product of 2-DG-6P, 6-phospho-2-deoxyglucono-1,5-lactone, produced by Zwf is the key toxic molecule. SCO1448 was determined as a 2-DG resistance factor in *S. coelicolor*, most likely by pumping toxic intermediates out of the cells. This work provides new insights into the resistance mechanisms of 2-DG in bacteria and may contribute to the development of more effective 2-DG-related treatments for cancer therapy.

In **Chapter 6**, the results presented in this thesis are summarized and discussed.