Signalling pathways that control development and antibiotic production in streptomyces
Urem, M.; Urem M.

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Author: Urem, M.
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CHAPTER V

SUPPRESSOR MUTANTS AS A TOOL TO IDENTIFY GLCN METABOLIC GENES IN *S. COELICOLOR*

Mia Urem, Magdalena A. Świątek-Połatyńska, Roger C. A. W. van Nasssau, Christian Rückert, Jörn Kalinowski and Gilles P. van Wezel

ABSTRACT

Streptomycetes are prolific producers of antibiotics, but many compounds are poorly expressed under laboratory conditions. Uncovering the control mechanisms governing the transcription of antibiotic biosynthetic genes is important for the activation and screening of cryptic antibiotics. The phosphorylated aminosugars glucosamine-6P (GlcN-6P) and *N*-acetylglucosamine (GlcNAc-6P) modulate the activity of the global antibiotic repressor DasR. While GlcNAc metabolism has been studied extensively, little is known of how GlcN is metabolised in *Streptomyces*. In an effort to define the GlcN metabolic pathway we analysed five mutants that are disturbed in GlcN metabolism, and had previously been obtained in a suppressor screen based on the lethality of GlcN to *nagB* null mutants. This identified mutations in *nagK* for the GlcNAc kinase and in *rokL6* (SCO1447) for a ROK-family protein. Deletion of either *nagK* or the GlcNAc deacetylase *nagA* in *nagB* mutants restores growth on GlcN, strongly suggesting that NagK and NagA are essential for GlcN metabolism; this suggests that after internalization, GlcN is first acetylated and phosphorylated and then follows the same metabolic route as GlcNAc-6P. Deletion of SCO1447 in *nagB* mutants relieved growth on GlcN but not on GlcNAc, identifying it as a GlcN-specific transcriptional regulatory gene.

Introduction

The emergence and rapid spread of infectious diseases involving multi-drug resistant (MDR) bacterial pathogens represents a major problem for the treatment of bacterial infections (WHO, 2014; O'Neill, 2014). One of the challenges in finding adequate novel antibiotics is replication, the rediscovery of previously identified antibiotics, while new molecules are rarely found; resulting in the extremely high cost and consequent drying up of industrial drug-discovery pipelines (Cooper & Shlaes, 2011; Kolter & van Wezel, 2016; Payne *et al.*, 2007). Multicellular, Gram-positive actinomycetes, like the streptomycetes, are producers of the majority of clinically employed antibiotics, and also a rich source of other secondary metabolites with medical and biotechnological importance, such as anticancer, antifungal and immunosuppressant drugs (Barka *et al.*, 2016; Bérdy, 2005; Hopwood, 2007). Finding the triggers and cues that activate the production of secondary metabolites is now a major challenge for novel drug discoveries, and the answers lie, among others, in the ecological context in which streptomycetes grow (Seipke *et al.*, 2012; Zhu *et al.*, 2014a).
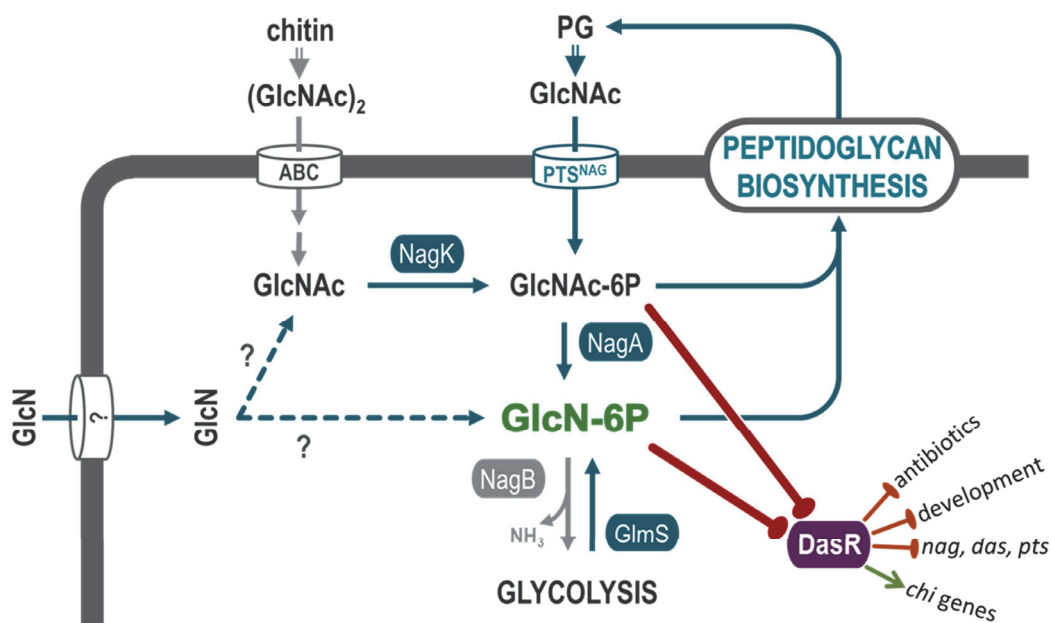

Figure 1. Peptidoglycan recycling and GlcN(Ac) metabolism leading to activation of the secondary metabolism in *S. coelicolor*.
Schematic representation of GlcNAc and GlcN metabolism and DasR regulation. Peptidoglycan recycling releases GlcNAc monomers which are transported into the cell and phosphorylated by the GlcNAc specific PTS. NagA deacetylates GlcNAc-6P to GlcN-6P, which is converted to Fru-6P and channelled into glycolysis. Also shown is GlcN-6P synthesis via GlmS and routes leading to cell wall synthesis. In contrast, hydrolysed chitin, in the form of GlcNAc dimers, is transported into the cell by ABC transporter DasABC, cleaved into monomers intracellularly and phosphorylated by GlcNAc kinase, NagK. The proposed pathway for GlcN metabolism is via GlcNAc metabolism by acetylation of GlcN to GlcNAc and then phosphorylation by NagK. Alternatively, and/or additionally, GlcN could be phosphorylated by an identified kinase directly to GlcN-6P. Allosteric effectors GlcNAc-6P and GlcN-6P inhibit DasR (in purple), the global, pleiotropic repressor of antibiotic production, development and GlcNAc metabolism (*nag*) and transport (*das* and *pts*) and activator of chitinolytic hydrolases (*chi*).
    Inhibition is represented by red lines (with ellipses), activation by green arrows. GlcN-6P, as an important central metabolic intermediate and signalling molecule, is highlighted in green. Metabolic pathways leading to PG synthesis and recycling are highlighted in blue.

A well-studied example of an antibiotic-activating signal is *N*-acetylglucosamine (GlcNAc); its regulatory role and metabolic pathway have been extensively studied in model organism, *Streptomyces coelicolor* (Figure 1; Świątek *et al.*, 2012a; Świątek *et al.*, 2012b; Rigali *et al.*, 2008; Urem *et al.*, 2016a). When *S. coelicolor* is grown under rich growth conditions (*feast*) supplemented with GlcNAc, both morphological and chemical differentiation (including antibiotic production) are repressed in favour of growth. Conversely, under poor growth conditions (*famine*), high concentrations of GlcNAc induce development and antibiotic production; presumably, the accumulation of GlcNAc, in an otherwise nutrient-depleted environment, is interpreted as a signal that PCD has been initiated (Rigali *et al.*, 2008). A key regulator in GlcNAc sensing is the global repressor DasR, which belongs to the family of GntR regulators. In *S. coelicolor*, all pathway-specific regulatory genes for antibiotic production are controlled by DasR (Świątek-Połatyńska *et al.*, 2015). The allosteric control of DasR by GlcNAc metabolic intermediates is shown schematically in Figure 1 and reviewed in Chapter II.

The utilisation of glucosamine (GlcN), which originates from chitosan (an *N*-deacetylated derivative of chitin) and de-acetylated GlcNAc portions of PG, likely also involves complex metabolic routes, in addition to the *csnR-K* operon that encodes the machinery required for the import and utilisation of GlcN dimers and chito-oligosaccharides (Viens *et al.*, 2015; Dubeau *et al.*, 2011). A suppressor screen, making use of the lethality of GlcN and GlcNAc to *S. coelicolor nagB* mutants, directly or indirectly due to the accumulation of GlcN-6P, showed ambiguity as to how aminosugars are metabolised in streptomycetes (Świątek *et al.*, 2012a; Świątek *et al.*, 2012b). Spontaneous suppressor mutants that survive on GlcNAc include those mutated in *nagA*, which is required for the formation of GlcN-6P from GlcNAc-6P during GlcNAc metabolism (Fig. 1, Chapter II). However, GlcN toxicity is also relieved by mutations in *nagA*, suggesting that GlcN may be metabolised via the GlcNAc metabolic route (Świątek *et al.*, 2012b), rather than taking a more direct pathway toward GlcN-6P by GlcN phosphorylation.

In this work, we aimed to identify genes involved in GlcN transport and metabolism, by screening *S. coelicolor nagB* suppressor mutants isolated in the presence of high concentrations of GlcN. Genome sequencing of selected mutants revealed mutations in SCO1447 (*rokL6*), a ROK-family transcriptional regulator, as well as GlcNAc metabolic genes.

MATERIALS AND METHODS
Bacterial strains and growth media.
Bacterial strains used in this work are listed in Table S1. *E. coli* JM109 (Sambrook et al., 1989) was used as host for routine cloning, and *E. coli* ET12567 (Macneil et al. 1992) to produce non-methylated DNA for introduction into *Streptomyces* (Kieser et al., 2000). *Streptomyces coelicolor* A3(2) M145 was obtained from the John Innes Centre strain collection, and its *nagB* mutant was described previously (Świątek et al., 2012a). Cells of *E. coli* were grown in Luria-Bertani broth (LB) at 37°C. All *Streptomyces* media and routine *Streptomyces* techniques are described in the *Streptomyces* manual (Kieser et al., 2000). Yeast-extract malt extract (YEME; Kieser et al., 2000) was used to cultivate mycelia for preparing protoplasts and for genomic DNA isolation. R2YE (regeneration media with yeast extract) agar plates were used for protoplast regeneration, while SFM (soy flour mannitol) agar plates were used to prepare spore suspensions. Phenotypic characterization was done on R2YE and minimal media (MM) agar plates.

Gene replacement and knock-out mutants.
Deletion mutants of *S. coelicolor* were constructed according to a previously described method (Świątek *et al.*, 2012a). For the deletion of SCO1447, the -1251/LR+3 (left flank) and +1200/+2565 (right flank) regions, relative to the start of the gene, were amplified by PCR using primers described in Table S2. The left and right flanks were cloned into pWHM3 (Vara *et al.*, 1989), which is an unstable multi-copy vector that allows efficient gene disruption (van Wezel *et al.*, 2005). The apramycin resistance cassette *aac(3)IV* flanked by *loxP* sites (*apra-loxP*) was cloned into the engineered XbaI sites between the flanks to create knock-out constructs pGWS948. In the same fashion, the flank regions of SCO1448 (-1422/+9 and +1221/+2603) were cloned into pWHM3 with the *apra-loxP* to create pGWS955. The plasmids were introduced into *S. coelicolor* M145 or its *nagB* mutant for gene replacements with *apra-loxP*. The apramycin resistance cassette was then excised by introduction of Cre recombinase expression plasmid, pUWLcre, to create markerless deletion mutants. The correct recombination event in each of the mutants was confirmed by PCR. *apra-loxP* gene replacement mutants were previously made for mutants *nagAB* and *nagKAB* (Świątek *et al.*, 2012a) but the deletion mutants were created by transformation with pUWLcre here. For genetic complementation of *nagA* with its own promoter, which is located upstream of *nagK* (SCO4285), the *nagKA* operon promoter was cloned upstream of the *nagA* gene into pHJL401 (van Wezel & Kraal, 2000). The -136/-1 region (numbering relative to the translational start codon) encompassing the promoter region of *nagKA* and the +1/+1146 gene coding region of *nagA* were amplified from the *S. coelicolor* M145 chromosome using primers described (Table S2). pHJL401 is a low-copy number shuttle vector that is very well suited for genetic complementation experiments (van Wezel *et al.*, 2000a).

Genomic DNA isolation and sequencing.
Strains were grown for 24 h in 50 mL YEME for genomic DNA isolation. Genomic DNA was isolated by phenol-chloroform extraction as described (Kieser *et al.*, 2000) and sequenced.

Results

Characterization of GlcN-derived *S. coelicolor nagB* suppressors.

The metabolic pathway of glucosamine is largely undefined in *S. coelicolor* and to identify genes involved in its metabolism and transport, the occurrence of spontaneous suppressor mutants, that relieve the toxicity of GlcN to *S. coelicolor nagB* mutants, was exploited. As GlcN is lethal to the *nagB* mutant, only strains that have sustained a second-site mutation can survive (Świątek *et al.*, 2012a; Świątek *et al.,* 2012b). It is likely that these suppressor mutants (or, suppressors) are mutated in GlcN-related genes, preventing the toxic accumulation of intermediate metabolites. For this, spores of the *nagB* mutant were plated at a high density on minimal medium (MM) agar plates with 50 mM GlcN. Over 300 spontaneous suppressor mutants, that were able to grow in the presence of GlcN, were isolated.

The suppressor mutants were grouped by phenotypic characteristics (in particular by development and pigmentation) and representatives of these groups were selected and screened for their suppressor mutations. We previously identified suppressor mutations in *nagA,* and these mutants were filtered out by genetic complementation as described (Świątek *et al.*, 2012a; Świątek *et al.*, 2012b). In brief, a plasmid expressing *nagA* was introduced into the suppressor mutants, and transformants no longer able to grow on GlcN were discarded. Following this filtration of the group representatives, two suppressor mutants were considered for further analysis (designated SMG). In an attempt to minimise the likelihood of selecting *nagA* mutated suppressors, a second set of suppressors was selected by plating transformants of *nagB* mutants harbouring an extra copy of *nagA* (on plasmid pHJL401) on MM agar plates with GlcN. From this set of suppressors, two group representatives (designated SMG+) were selected as well. The phenotypic characteristics of this selected set of GlcN suppressors (SMG38, SMG42, SMG+3 and SMG+4) to which we added one suppressor mutant (SMG1) isolated in a previous screen (Świątek *et al.*, 2012a; Świątek *et al.*, 2012b), were analysed on solid minimal media (MM) with different sugars (Fig. 2).
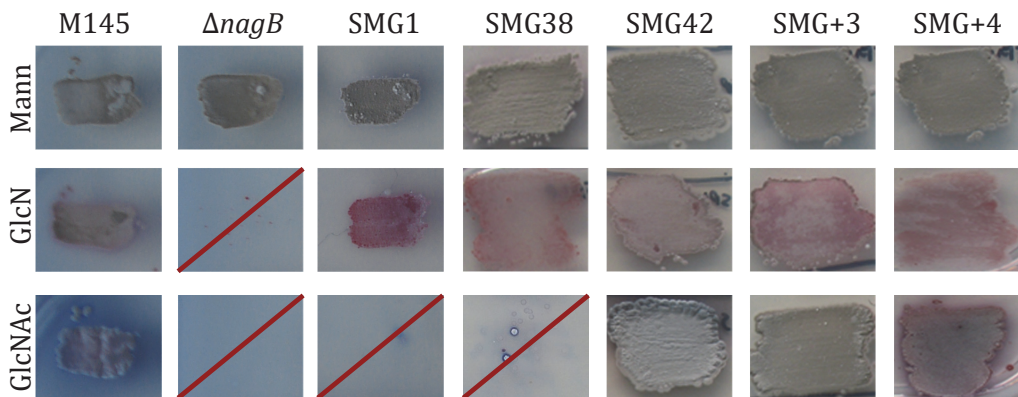

Figure 2. Phenotypic analysis of GlcN-derived *S. coelicolor nagB* suppressor mutants.
Spores of *S. coelicolor* wildtype M145, *nagB* deletion mutant and *nagB* suppressor mutants (SMG1, SMG38, SMG42, SMG+3 and SMG+4) were plated onto minimal medium and supplemented with 50mM of mannitol (Mann), glucosamine (GlcN) or *N*-acetylglucosamine (GlcNAc). SMG suppressor mutants are derived from *nagB* mutants on GlcN and SMG+ suppressors are derived from *nagB* harbouring a plasmid pGWS961 prior to isolation from GlcN. Mutants for which GlcN or GlcNAc was lethal are indicated with a red line. Suppressor SMG1 was first identified and described in Świątek, et al. (2012).

The five GlcN-derived *S. coelicolor nagB* suppressor mutants showed normal growth and development on MM agar with mannitol (Fig. 2). Interestingly, GlcN suppressor mutants SMG1 and SMG38 were unable to alleviate GlcNAc toxicity, which strongly suggests that mutations might have arisen in genes that are specific for the GlcN-metabolic pathway. However, growth of SMG1 and SMG38 was somewhat compromised on MM agar plates with GlcN, suggesting that the suppressor mutations could not fully alleviate the toxicity of the accumulation of GlcN-6P or (a) metabolic derivative(s) thereof. Suppressor mutants SMG42, SMG+2 and SMG+3 grew well on both sugars; however, development of all three is diminished on GlcN and altered on GlcNAc for SMG42, which is whiter in pigment, *i.e.* sporulation had been compromised. The three suppressor mutants produced antibiotics on GlcN but antibiotic production was blocked on GlcNAc for SMG42 and SMG+2 and altered for SMG+3 (loss of the production of the blue-pigmented actinorhodin). The loss of activation of antibiotic production suggests that these three suppressor mutants had, at least partially, lost their ability to sense GlcNAc under nutrient-limiting or *famine* conditions (MM agar) (Fig. 2). All five suppressor mutants had lost their ability to sense GlcNAc under rich growth conditions (*feast*; R2YE agar), given the loss of inhibition of antibiotic production in all strains and loss of inhibition of development in some (SMG42, SMG+2 and SMG+3, and somewhat in SMG38) (Fig. S1).

TABLE 1. Mutations identified in GlcN-derived *S. coelicolor nagB* suppressors.

SMG1 Type[a]	nt[b]	aa[c]	SMG38 Type[a]	nt[b]	aa[c]	Position[d]	SCO #[e]	Annotation
INS		fs	-	-	-	1543479i	SCO1447	transcriptional regulator (ROK family)
-	-	-	DEL	G̶	fs	1544534		
-	-	-	DEL	G̶	fs	3850016	SCO3485	LacI family transcriptional regulator
-	-	-	DEL	C̶	fs	3850017		
SNP	C>A	Q117K	-	-	-	3928899	SCO3554	putative integral membrane protein
SNP	A>C	H182P	-	-	-	6769952	SCO6167	proline rich protein (putative membrane protein)
-	-	-	SNP	C>G	H424Q	7265819	SCO6563	integral membrane transporter

SMG+3 Type[a]	nt[b]	aa[c]	SMG+4 Type[a]	nt[b]	aa[c]	SMG42 Type[a]	nt[b]	aa[c]	Position	SCO #	Annotation
-	-	-	-	-	-	INS	+A	fs	521609i	SCO0492	peptide synthetase
-	-	-	SNP	G>C	P71R	-	-	-	1749870	SCO1635	hypothetical protein
SNP	C>A	A315S	-	-	-	-	-	-	1760847	SCO1647	Pup deamidase/depupylase
INS	+C	fs	-	-	-	INS	+C	fs	3849222i	SCO3485	LacI family transcriptional regulator
-	-	-	DEL	-	-				4692947 - 4699275	SCO4277-SCO4283	***
-	-	-	SNP	G>C	A354G				4699483	SCO4284	NagA, N-acetylglucosamine-6-phosphate deacetylase
SNP	T>C	K314R	SNP	T>C	K314R	DEL#			4699603		
SNP	C>T	G157E	SNP	C>T	G157E				4700074		
-	-	-	DEL	-	-				4700543 - 4701656	SCO4285	NagK, N-acetylglucosamine kinase
-	-	-		-	-						
-	-	-	INS	+6N	-	INS	+6N	-	5586261i	SCO5138	hypothetical protein
-	-	-	SNP	C>T	A139T	SNP	C>T	A139T	7050737	SCO6384	integral membrane lysyl-tRNA synthetase
-	-	-	SNP	A>C	K498Q	-	-	-	7190566	SCO6496	dehydrogenase
-	-	-	SNP	T>G	V388G	-	-	-	7778607	SCO7004	carbohydrate kinase

a – Type indicates whether the mutation is a SNP (single nucleotide polymorphism), deletion or insertion.

b – SNP substitutions are given as well as nucleotides that are inserted and, in case of single nucleotide deletions, the deleted nucleotide is given and strikethrough. +6N indicates the insertion of 6 nucleotides, please see Table S3 for details.

c – Amino acid substitutions are given if applicable and frameshifts are indicated by 'fs'.

d – Genomic position or range is given, 'i' indicates insertion.

e – *S. coelicolor* SCO numbers.

*** SCO4277 - putative tellurium resistance protein, SCO4278 - conserved hypothetical protein, SCO4279 - putative acetyltransferase, SCO4280 - putative reductase, SCO4281 – conserved hypothetical protein, SCO4282 - putative dimeric protein and SCO4283 - putative sugar kinase.

Region from position 4692947- 4701656 deleted in SMG42.

Identification of secondary mutations in GlcN-derived *S. coelicolor nagB* suppressors.
The genomic DNA of the five suppressor mutants was isolated from liquid-grown YEME cultures and sequenced by whole-genome sequencing. The genome of our laboratory-specific variant of *S. coelicolor* A3(2) M145 was also sequenced and used as a reference genome for detection of single nucleotide permutations (SNPs) and other mutations in the suppressor strains (Table S3). Only non-silent mutations, that were not present in the wild-type nor shared between all of the suppressor mutants, were considered (Table 1).

Analysis of the mutations identified in the suppressor mutants revealed that one of two genes, namely SCO1447 or *nagA*, were mutated or deleted in particular (Table 1). Surprisingly, despite the presence of a plasmid expressing *nagA* (SCO4284) in suppressor mutants SMG+3 and SMG+4, and the genetic complementation of SMG42 with that same plasmid, still all three were characterised by SNPs and/or deletions in *nagA* and additionally, large genomic disruptions in the *nagA* genomic context (region SCO4277-4285) were identified for SMG+4 and SMG42. This underlines the crucial role for *nagA* and potentially also its flanking genes (including *nagK*) in GlcN metabolism; the role of *nagK* (SCO4285) in GlcN metabolism is considered in more detail below. GlcN-specific suppressors SMG1 and SMG38 had single mutations in the ROK-family protein, SCO1447, in both cases resulting in a frame-shift mutation and hence an inactive gene product. Given that both strains had mutations that relieved GlcN toxicity but were unable to grow on GlcNAc, this strongly suggests that SCO1447 is a GlcN-specific transcriptional regulator and the first gene unique to GlcN metabolism and/or signalling that has been identified to date.

Other SNPs identified in the suppressor mutants were diverse and less likely to be involved in GlcN metabolism. These included genes for SCO0492, involved in the biosynthesis of the siderophore coelichelin, SCO1647, a proteasome accessory protein, SCO6384, a putative integral membrane lysyl-tRNA synthetase, SCO6496, a putative dehydrogenase, SCO6563, homolog of oxalate:format exchange protein from *Oxalobacter formigenes*, SCO7004, homolog of glycerol kinase from *Bacillus subtilis* and hypothetical genes SCO1635, SCO5138, SCO3554 and SCO6167. Considering the crucial role of *nagA* (Świątek *et al.*, 2012a; Świątek *et al.*, 2012b) and SCO1447 (see below) in relieving GlcN toxicity, none of these additional mutations were considered further.

Deletion of nagK suppresses toxicity of GlcN and GlcNAc to nagB mutants.
Closer analysis of the *nagA*-mutated suppressors revealed that the majority of the genomic region from SCO4277 to SCO4285 is absent in suppressor mutants SMG+4 and SMG42 (Table 1 and S3). This region includes *nagK* (SCO4285) and *nagA* (SCO4284), regulated from the same promoter upstream of *nagK*, and a gene for a putative acetyltransferase (SCO4279). NagK is a GlcNAc kinase, phosphorylating (chitin-derived) GlcNAc intracellularly (Plumbridge, 2009; Świątek *et al.*, 2012a). To investigate whether NagK indeed plays a key role in GlcN metabolism, *nagK* deletion mutants were created by disrupting the gene in the parental strain *S. coelicolor* M145 and its *nagB, nagA* and *nagAB* mutant derivatives, followed by their characterization on MM and R2YE agar plates with or without GlcN or GlcNAc (Fig. 3).

Surprisingly, deletion of *nagK* restored the ability of *nagB* mutants to grow on both GlcN and GlcNAc. The alleviation of GlcNAc toxicity strongly suggests that either a significant portion of GlcNAc is imported as GlcNAc and not as GlcNAc-6P - *i.e.* by an ABC transporter instead of by the PTS - or that GlcNAc-6P is dephosphorylated at a high rate. Given that deletion of *nagK* also rescues the lethality of *nagB* mutants on GlcN, this implies that GlcN is metabolised via NagK substrate GlcNAc. Simultaneous deletion of both *nagK* and *nagA* in

nagB mutants also restored growth on GlcN and GlcNAc, despite the disruption of the GlcNAc metabolic genes. However, disruption of the *nag* genes (individually or simultaneously) had a profound impact on GlcNAc sensing (*feast* or *famine*) in addition to affecting development and antibiotics production in general. For example, GlcNAc sensing under feast conditions is compromised in all mutants; however, GlcNAc did not inhibit the production of antibiotics of any of the mutants on R2YE, and developmental inhibition by GlcNAc was lost in *nagA* and *nagKA* null mutants.
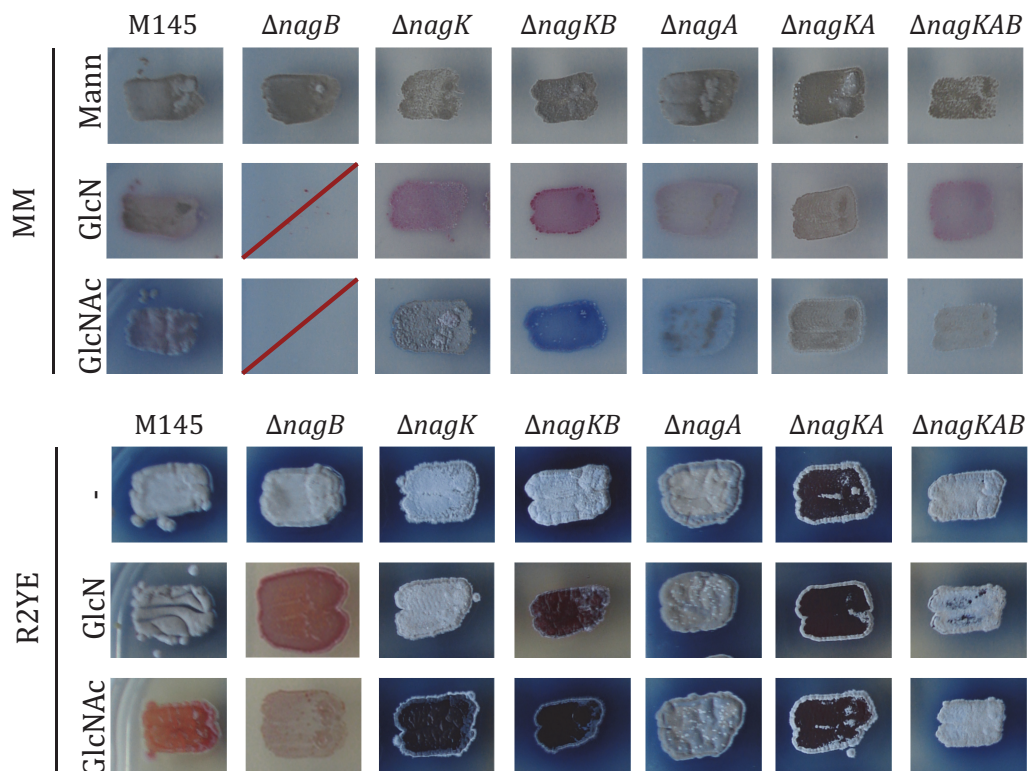

FIGURE 3. Phenotypic analysis of *S. coelicolor nagK* and other *nag* mutants on GlcN.
Spores of *S. coelicolor* wildtype (M145) and deletion mutants *nagB, nagK, nagKB, nagA, nagKA* and *nagKAB* were plated onto minimal medium (MM) or rich glucose-containing media (R2YE) and supplemented with 50mM of mannitol (Mann), glucosamine (GlcN) or *N*-acetylglucosamine (GlcNAc) as indicated.
Mutants for which GlcN or GlcNAc was lethal are indicated with a red line.

Transcriptional regulator SCO1447 (RokL6) is involved in GlcN metabolism.
Suppressor mutants SMG1 and SMG38 are of great interest due to their ability to restore growth specifically on GlcN, but not on GlcNAc (Fig. 2). Both *nagB* suppressor mutants had second-site mutations in SCO1447, which encodes a ROK-family protein. We propose the name RokL6 for the protein, based on the nomenclature for ROK-family proteins that was proposed previously (Świątek *et al.*, 2013), with the numbering based on the location of the gene on the ordered cosmid library that contains parts of the *S. coelicolor* chromosome (Redenbach *et al.*, 1996). ROK-family proteins include sugar kinases and regulators, many of which are involved in the control and the utilisation of sugars in bacteria (Titgemeyer *et al.*, 1994), and several ROK-family proteins were implicated in the control of antibiotic production in streptomycetes (Bekiesch *et al.*, 2016; Świątek *et al.*, 2013). RokL6 is a

predicted transcriptional regulator, with similarity to other ROK-family regulators such the *nag* repressor NagC from *E. coli* (Plumbridge, 1995; Plumbridge, 1991). The *rokL6* gene shares its upstream region with the divergently transcribed SCO1448, which encodes a major facilitator superfamily (MFS) transporter. To verify if either RokL6 or SCO1448 plays a role in GlcN metabolism, deletion mutants were created for either gene.

The deletion mutations were phenotypically characterized on MM agar in the presence of GlcN and GlcNAc, as shown in Figure 4. *rokL6-nagB* double mutants grew on MM with GlcN, though much as the original suppressor mutants, not on GlcNAc. Deletion of *rokL6* relieved the toxicity of GlcN to *S. coelicolor nagB* mutants (Fig. 4A), confirming its involvement in the accumulation of toxic intermediates and hence a likely role in GlcN metabolism. The *rokL6* deletion mutant could grow on both sugars, though antibiotic production was blocked by GlcNAc on minimal medium and disruption of *rokL6* in either strain did not significantly affect the phenotype on R2YE agar (Fig. S1).

Considering its genomic location next to and divergently transcribed from *rokL6*, we hypothesized that perhaps SCO1448 may play a role in the internalization of GlcN. However, deletion of the gene for this MFS transporter did not relieve the toxicity of GlcN or GlcNAc to *nagB* null mutants on MM (Fig. 4) nor did its disruption affect antibiotic production or morphology on MM or R2YE agar (Fig. S1). This suggests that, at least under the conditions tested, SCO1448 is not (solely) responsible for the internalization of GlcN.
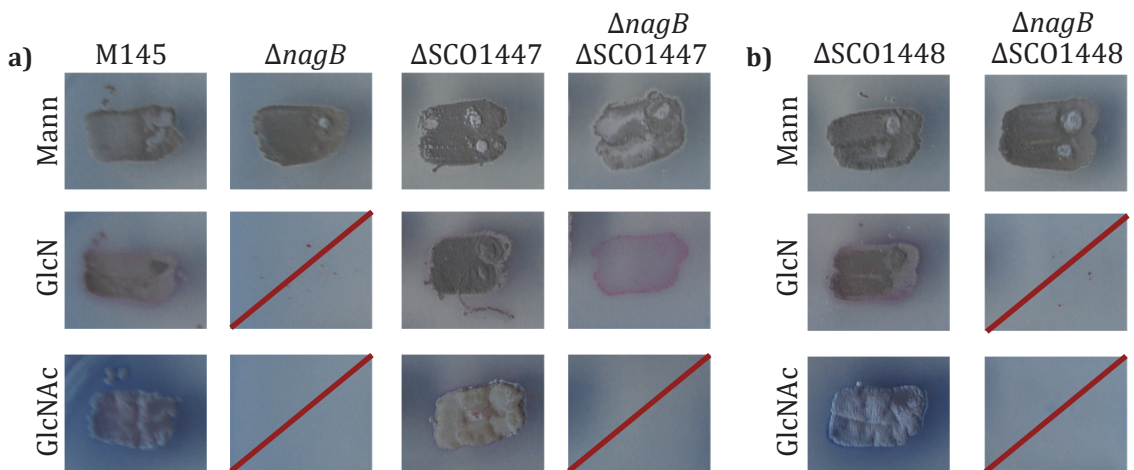

FIGURE 4. Phenotypic analysis of *S. coelicolor* SCO1447 and SCO1448 mutants.
Spores of *S. coelicolor* wildtype (M145) nagB and deletion mutants of SCO1447 (a) and 1448 (b) were plated onto minimal medium (MM) or rich media (R2YE) and supplemented with 50mM of mannitol (Mann), glucosamine (GlcN) or N-acetylglucosamine (GlcNAc).
Mutants for which GlcN or GlcNAc was lethal are indicated with a red line.

Discussion

Aminosugar metabolism plays an important role in growth and nutrient sensing of streptomycetes. GlcNAc serves as both a carbon and nitrogen source and is also involved in the signalling of various major cellular processes, including development and antibiotic production (Rigali *et al.*, 2008; Urem *et al.*, 2016a). The *S. coelicolor* genome encodes around 700 regulatory proteins in addition to a variety of transport systems and sensory proteins that coordinate the highly complex regulatory networks in this organism (Bentley *et al.*, 2002). One of these regulators is the pleiotropic antibiotic repressor DasR, which controls a large regulon including many metabolic and transport genes relating to aminosugar and polysaccharide utilisation, development and secondary metabolism (Craig *et al.*, 2012; Nazari *et al.*, 2012; Rigali *et al.*, 2006; Świątek-Połatyńska *et al.*, 2015). DasR responds to the metabolic status of the cell, in particular to metabolic intermediates of GlcNAc and GlcN metabolism (Rigali *et al.*, 2008; Tenconi *et al.*, 2015a). With this in mind, we previously investigated the potential of engineering aminosugar metabolism as a means to activate the production of antibiotics in *Streptomyces* (Świątek *et al.*, 2012b).

The GlcNAc metabolic pathway results in the formation of GlcN-6P which is then metabolised, depending on the cellular requirements, via NagB to fructose-6P for entry into glycolysis or, alternatively, via the Mur enzymes towards cell-wall synthesis. For GlcN metabolism, the most straightforward route to GlcN-6P would be the direct phosphorylation of the internalized sugar. However, the genomic analysis of GlcN-derived *nagB* suppressor mutants of *S. coelicolor* showed that NagA plays an important role in GlcN metabolism (Świątek *et al.*, 2012b). This suggested that GlcN is converted to GlcNAc-6P in order to be metabolised, which would most likely occur via acetylation to GlcNAc and subsequent phosphorylation by NagK to produce GlcNAc-6P. Indeed, our data show that NagK is involved in GlcN metabolism on minimal media. *nagKB* or *nagKAB* deletion mutants were both able to grow on in the presence of either GlcN or GlcNAc (Fig. 3). We propose that after internalization, GlcN is acetylated by a yet unidentified protein, phosphorylated by NagK and then joins the core *nag* pathway as GlcNAc-6P. This hypothesis will need to be investigated by metabolic flux analysis. Glucosamine acetylases have previously been identified in *Clostridium acetobutylicum* (Reith & Mayer, 2011) and we are currently investigating potential candidates in *S. coelicolor*, including the acetyltransferase SCO4279 that is located in the close vicinity of *nagKA.* However, we cannot rule out the possibility that multiple pathways are available for GlcN metabolism.

The suppressor screen identified a novel gene involved in GlcN-specific metabolism, namely SCO1447 (*rokL6*), which encodes the ROK-family regulator RokL6 (discussed in greater detail in Chapter VI). Disruption of this regulatory gene in the *nagB* mutant of *S. coelicolor* restored growth on GlcN, while GlcNAc remained toxic to the double mutant. This combined with the presence of a DNA binding motif implies that RokL6 is a regulatory protein that is involved in the control of GlcN metabolism and possibly controls the transporter gene SCO1448 which lies adjacent to (and is transcribed divergently from) *rokL6*. However, SCO1448-*nagB* double mutants failed to grow on GlcN, and it is therefore unlikely that SCO1448 is (solely) responsible for GlcN transport. There may be redundancy in terms of GlcN transport, with multiple transporters involved in the import of the aminosugar. Studies are currently ongoing in our laboratory that focus on the GlcN regulon and on identifying the ligand of RokL6, aimed at better understanding of GlcN metabolism and at the identification of the toxic molecule that accumulates inside the cell when *nagB* null mutants are grown on aminosugars.

In conclusion, in addition to enhancing antibiotic production by controlling metabolic

flux via *nag* gene deletions, the emergence of suppressor mutants has proven to be an indispensable tool in the discovery of new metabolic genes and the better understanding of the aminosugar metabolic pathways in *S. coelicolor*. Through the analysis of GlcN-derived *nagB* suppressors, we have been able to identify a new transcriptional regulatory gene involved in GlcN metabolism, and have obtained new insights into the GlcN metabolic route under poor growth conditions. This includes the surprising observation that NagK plays a key role in the metabolism of GlcN, while a direct route via GlcN-6P was expected. We are currently dissecting the GlcN and GlcNAc pathways to extend our knowledge of this complex system that plays such a key role in streptomycetes as nutrient and as signalling molecule for the onset of development and antibiotic production.