

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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Nacetylglucosamine (GlcNAc), 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 signaling 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 signaling 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 toward the improvement of antibiotic productivity or even the discovery of novel compounds.

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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.^{1,2} 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.³ 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.^{4,5}

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,

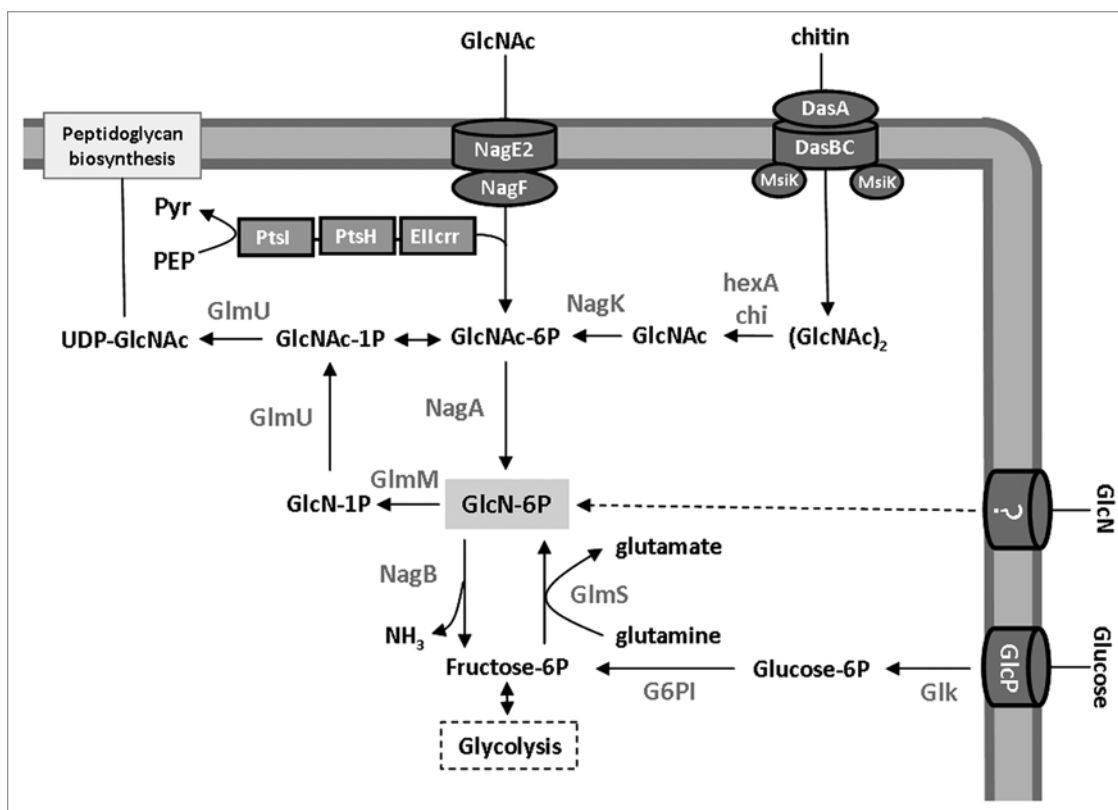


Figure 1. Glucose and aminosugar metabolism in *S. coelicolor*, with connecting pathways. Annotation is based on the KEGG database³¹ and experimental evidence. Arrows shows the direction of metabolism, and the responsible enzymes are presented in bold face. See text for further details.

this mechanisms is stimulated by production of antibiotics leading to thwart of growth of competing organisms.³

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.⁶ 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.⁷⁻⁹ Such approaches include engineering primary metabolism and regulatory cascades controlling antibiotic production.^{8,10,11}

Role of GlcNAc in Control of Development and Antibiotic Production

N-acetylglucosamine (GlcNAc), the monomer or chitin and constituent of peptidoglycan, and the related amino acid glutamate are preferred nutrients as they serve as highly favorable carbon and nitrogen sources for streptomycetes.^{12,13} Besides as nutrient, GlcNAc also serves as a signaling 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.¹⁴ We previously proposed a complete signaling 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 signaling cascade is GlcN-6P, which acts as an allosteric effector of DasR and therefore prevents its

DNA binding ability.¹⁵ 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*,⁵ which encode the synthetase for the γ -butyrolactone Scb1 and the Scb1-responsive transcriptional activator for Cpk biosynthesis, respectively.^{16,17} The molecular mechanism of GlcNAc signaling 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).^{18,19} 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).^{12,20} 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.¹⁵

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).²¹ 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 toward cell-wall synthesis and glycolysis. GlcN-6P is converted by NagB to fructose-6-phosphate (Fru-6P), which enters the glycolytic pathway,^{22,23} 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 uridylyltransferase) (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.¹² 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. bingchengensis*, *S. cattleya*, and *S. violaceusniger*, do not have a *nagB* ortholog. 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

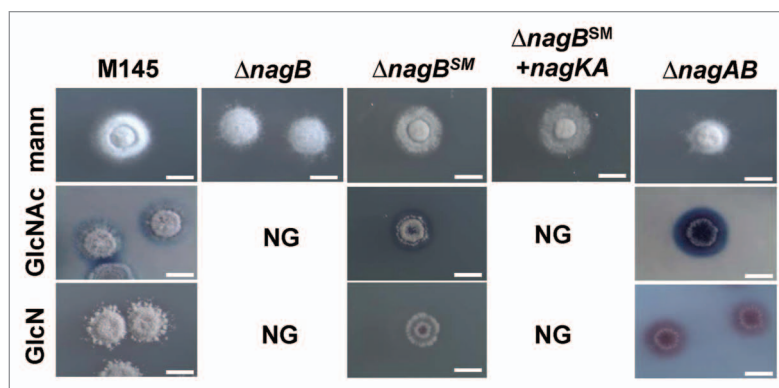


Figure 2. Phenotypic analysis of *nag* mutants. Stereomicrographs showing the phenotypes of *S. coelicolor* M145, its *nagB* and *nagAB* mutants, *nagB* suppressor mutant SMG4 (obtained on GlcN) that has a deletion in *nagA*, and the complemented suppressor mutant expressing *nagKA*. Strains were grown 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 amino-sugar, while growth was restored to the *nagAB* double mutant and the *nagB* suppressor mutants, and also led to enhanced antibiotic overproduction.

bacteria. These NagB-II homologs contain the C-terminal domain of GlmS, but display only the catabolic activity of NagB.^{24,25} 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 signaling 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.²¹ 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) (ref. 21 and Fig. 2). Toxicity of GlcN-6P was reported previously in *Escherichia coli*.²⁶⁻²⁸ 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 1:10⁵, which corresponds to expected single mutations or indels (insertions or deletions). This is a

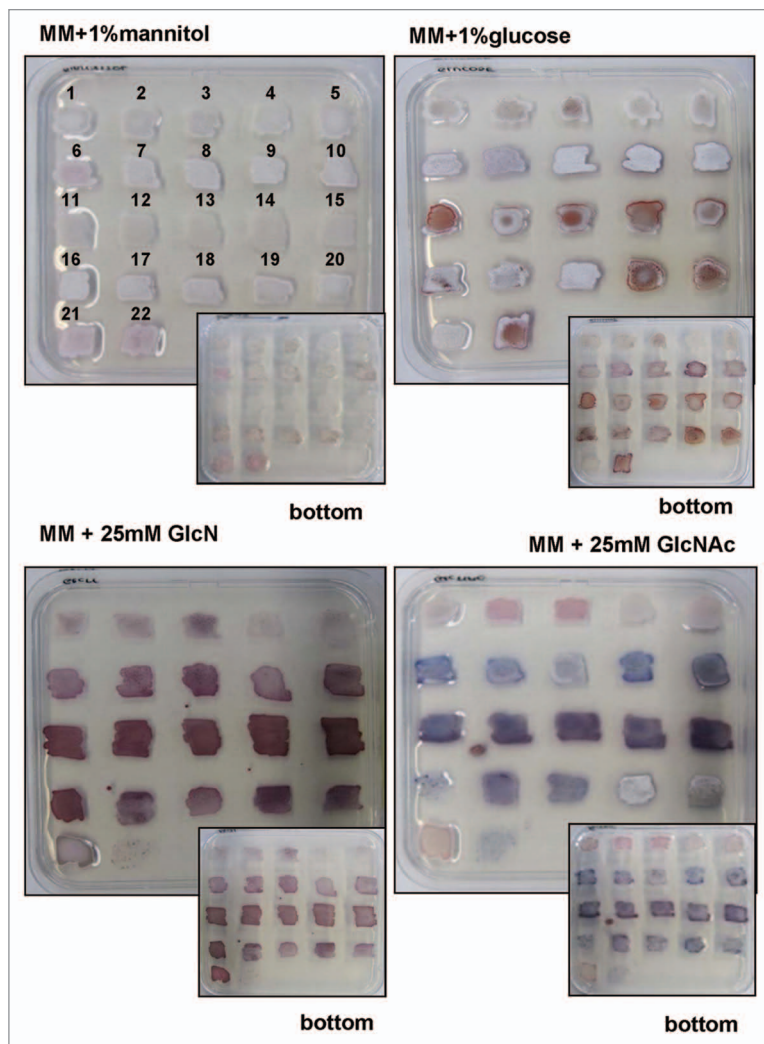


Figure 3. Analysis of antibiotic production by *nagB* suppressor mutants. Phenotypes of *nagB* suppressor mutants originally selected on GlcNAc (1–15; corresponding to SMA1–SMA15) or GlcN (16–20; corresponding to SMG1–SMG5). The parental strain M145 (21) and the *nagB* mutant (22) were used as controls. 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.

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* 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 overproduced antibiotics, and for SMG2 and SMG3 development was blocked (Fig. 3). 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 with the wild-type strain (Fig. 3 and Fig. 4). Like for SMA suppressors, SMG mutants grown on MM agar plates supplemented with GlcNAc displayed three distinct phenotypes.

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 (GlcK), respectively²⁹ (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.

Genetic Analysis of the *nagB* Suppressor Mutants Reveals Unsuspected Involvement of NagA in GlcN Utilization

Likely candidates for the suppressor mutations obtained on GlcNAc were the GlcNAc transporter genes *nagE2* and *nagF* or the metabolic genes *nagA* or *nagK*. These genes and *nagE1*, a direct homolog and neighboring gene of *nagE2*, were amplified by PCR and subjected to DNA sequencing. In total 10 individual suppressor mutants obtained on GlcN and 10 obtained on GlcNAc were analyzed. While GlcNAc suppressors did not reveal mutations in any of the sequenced genes or their promoter regions, one of *nagB* suppressor mutants selected on GlcN (SMG4) contained an out-of-frame deletion of 416 bp in *nagA* (nt positions 261- 676). In a control experiment, this suppressor mutant was complemented with plasmid pHJL401 harboring the *nagKA* operon under the control of its own promoter. pHJL401 is a low-copy number vector which is very suitable for complementation experiments due to its stability and low copy number.³⁰ Indeed, this transformant had regained sensitivity to both GlcN or GlcNAc, indicating that the deletion in *nagA* was the sole cause of the suppressor phenotype (Fig. 2). Full complementation of SMG4 was also achieved with pHJL401 harboring only *nagA* under the control of the *nagKA* promoter (not shown).

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) grew well regardless of whether they were challenged with GlcN or GlcNAc. Moreover, the mutants displayed enhanced antibiotic production (Fig. 2). To establish if other suppressor mutants had also sustained a mutation or indel in *nagA*, 19 additional suppressor mutants obtained on GlcNAc and 15 obtained on GlcN were selected and complemented with a wild-type copy of the *nagA* gene. Of the transformants, 10 GlcN-selected and three GlcNAc-selected *nagB* suppressor mutants lost their ability to grow in the presence of either aminosugar, suggesting they may also have been mutated in *nagA*.

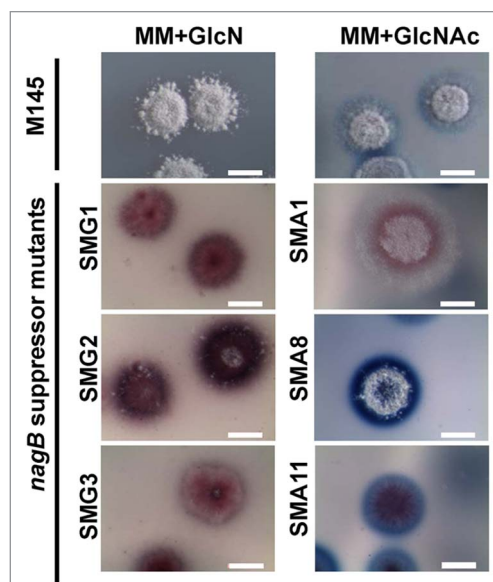


Figure 4. Phenotypic diversity of *nagB* suppressor mutants. Stereomicrographs illustrating the phenotypes of *S. coelicolor* M145 and representatives of three different classes of SMA mutants (SMA1, SMA8, SMA11) and of SMG mutants (SMG1-SMG3) grown on MM agar plates supplemented with GlcN (M145 and 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.

The high frequency of second-site *nagA* mutations relieving GlcN toxicity to *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 database³¹). This presents one example of the high relevance of the suppressor mutants, and closer analysis of GlcN and GlcNAc metabolism is currently in progress.

Since only three out of the 19 GlcNAc-selected *nagB* suppressor mutants were due to a second-site mutation in *nagA*, the 16 remaining mutants most likely sustained a mutation in one or more other genomic loci that could also relieve aminosugar toxicity of *nagB* mutants. We are currently analyzing the genomes of several of the *nagB* suppressors. Identification of these mutations and the corresponding genes will shed new light on aminosugar metabolism in streptomycetes. These

molecules play an important role in central metabolism and cell-wall biosynthesis and accumulation of GlcN(Ac)-derived metabolites serves as a trigger 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.

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