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Chapter IV

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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Invited addendum to *Bioengineered*
ABSTRACT

N-acetylglucosamine, 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 signalling 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 signalling 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 towards the improvement of antibiotic productivity or even the discovery of novel compounds.
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. Streptomyces 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 (Chater & Losick, 1997; Flärdh & Buttner, 2009). Streptomyces 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 (Hopwood, 2007). 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 (Sanchez et al., 2010; van Wezel & McDowall, 2011).

As saprophytic soil bacteria, streptomyces 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, this mechanisms is stimulated by production of antibiotics leading to thwart of growth of competing organisms (Hopwood, 2007).

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 (Challis & Hopwood, 2003). 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 (Baltz, 2008; Martin & Liras, 2010; van Wezel et al., 2009). Such approaches include engineering primary metabolism and regulatory cascades controlling antibiotic production (Butler et al., 2002; Chen et al., 2010; Martin & Liras,
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 favourable carbon and nitrogen sources for streptomycetes (Nothaft et al., 2010; van Wezel et al., 2006). Besides as nutrient, GlcNAc also serves as a signalling 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 (Rigali et al., 2008). We previously proposed a complete signalling 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 signalling cascade is GlcN-6P, which acts as an allosteric effector of DasR and therefore prevents its DNA binding ability (Rigali et al., 2006). 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 (van Wezel & McDowall, 2011), which encode the synthetase for the γ-butyrolactone Scb1 and the Scb1-responsive transcriptional activator for Cpk biosynthesis, respectively (D'Alia et al., 2011; Hsiao et al., 2007). The molecular mechanism of GlcNAc signalling 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) (Brückner & Titgemeyer, 2002; Nothaft et al., 2003a). 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<sup>Crr</sup>; encoded by <i>err</i>) and enzyme IIB (e.g. NagF) (Nothaft <i>et al.</i>, 2003b; Nothaft <i>et al.</i>, 2010). NagF phosphorylates incoming via the permease IIC (NagE2) GlcNAc to form <i>N</i>-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 (Rigali <i>et al.</i>, 2006).

Recently we characterized the metabolic enzymes in <i>S. coelicolor</i>, namely GlcNAc-6P deacetylase (NagA; SCO4284), GlcN-6P deaminase (NagB; SCO5236) and GlcNAc kinase (NagK; SCO4285) (Świątek <i>et al.</i>, 2012). 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 towards cell-wall synthesis and glycolysis. GlcN-6P is
converted by NagB to fructose-6-phosphate (Fru-6P), which enters the glycolytic pathway (Plumbridge & Vimr, 1999; Vogler & Lengeler, 1989), but is also incorporated into murein following its conversion to UDP-GlcNAc by the action of GlmM (phosphoglucomutase) 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 (Nothaft et al., 2010). 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. bingchenggensis, S. cattleya, and S. violaceusniger, do not have a nagB orthologue. 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 bacteria. These NagB-IIIs contain the C-terminal domain of GlmS, but display only the catabolic activity of NagB (Teplyakov et al., 1999; Yang et al., 2006). 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 signalling 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 (Świątek et al., 2012). 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) (Świątek et al., 2012 and Fig. 2). Toxicity of GlcN-6P was reported previously in Escherichia coli (Bernheim & Dobrogosz, 1970; Plumbridge, 2009; White, 1968). 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 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
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 aminosugar, while growth was restored to the nagAB double mutant and the nagB suppressor mutants, and also led to enhanced antibiotic overproduction.
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 grown on MM agar plates supplemented with GlcNAc overproduced antibiotics, and for SMG2 and SMG3 development was blocked (Fig. 3). Like for SMA suppressors, SMG mutants displayed three distinct phenotypes under this condition. 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 to the wild-type strain (Fig. 3 and Fig. 4).

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 (Glk), respectively (van Wezel et al., 2005) (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.

![Figure 4. Phenotypic diversity of nagB suppressor mutants.](image)

Identification second-site mutations relieving GlcN and GlcNAc toxicity to nagB mutants

To identify the second-site mutations the complete genome analysis of four independent suppressor mutants selected either on GlcN (called SMG1 and SMG4) or GlcNAc (SMA1 and SMA11) was performed. Whole-genome sequencing revealed many changes relative to the reference sequence. Most of these were identical mutations occurring in all mutants, and were therefore not considered further. The unique changes only occurring in one of the mutants are shown in Table 1. Since still quite a few different candidate mutations were identified in all of the mutants, we focused our initial attention to genes that were functionally related to primary metabolism, sugar transport or regulatory genes. The mutations discussed below were all verified by PCR followed
by DNA sequencing.

Table 1. Mutations identified by next-generation genome sequencing in the independent nagB suppressor mutants SMG1, SMG4 (obtained on GlcN), SMA1 and SMA11 (obtained on GlcNAc).

<table>
<thead>
<tr>
<th>SMG1</th>
<th>SMG4</th>
<th>SMA1</th>
<th>SMA11</th>
<th>Function</th>
<th>aa changes</th>
<th>nt position*</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCO1859</td>
<td>putative aminotransferase</td>
<td>p.Val255Leu</td>
<td>763 (G→C)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SCO2672</td>
<td>p.Ala178Gly</td>
<td>2153 (C→G)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SCO2975</td>
<td>p.Gly1055Arg</td>
<td>3163 (G→C)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SCO3554</td>
<td>SCO4393</td>
<td>p.Gln117Lys</td>
<td>349 (G→A)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SCO4476</td>
<td>SCO4931</td>
<td>putative membrane protein</td>
<td>p.Ala346Pro</td>
<td>1036 (G→C)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SCO5236</td>
<td>p.Ala434Gly</td>
<td>1301 (C→G)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SCO6167</td>
<td>p.Ala346Pro</td>
<td>1036 (G→C)</td>
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Deletion-Insertion Polymorphism (DIP)

<table>
<thead>
<tr>
<th>SMG1</th>
<th>SMG4</th>
<th>SMA1</th>
<th>SMA11</th>
<th>Function</th>
<th>aa changes</th>
<th>nt position*</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCO1447</td>
<td>putative integral membrane protein</td>
<td>p.Arg9fs</td>
<td>26</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SCO4284</td>
<td>N-acetylglucosamine-6-phosphate deacetylase</td>
<td>p.Asp91fs</td>
<td>261-676</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

'fs' – frame shift; ‘*’ – nucleotide position relative to the start codon; ‘i’ – insertion; ‘d’ – deletion; ‘G, A, T, C’ – guanine, adenine, thymine, cytosine.

# Only those changes relative to the parent M145 are shown that were not found in all strains, and that gave rise to a change in the predicted protein sequence of the respective genes.

As mentioned above suppressor mutant SMG1 could grow on GlcN but not on GlcNAc (Fig. 3), which suggests that the mutation only relieves toxicity to the former non-acetylated aminosugar. A deletion-insertion polymorphism (DIP) analysis of the SMG1 genome revealed a frame-shift insertion of a single cytosine at nt position 26 in SCO1447, encoding a ROK-family protein. The ROK proteins are widespread among bacteria and many of them are involved in the control of aminosugar and/or sugar transport and utilization (Deutscher et al., 2006; Plumbridge, 2001; Chapter VI). Upstream of SCO1447 lies the divergently transcribed SCO1448, which encodes a probable transport protein containing a Major Facilitator Superfamily (MFS) domain. Examination of intergenic region between SCO1447 and SCO1448 identified the 29 bp inverted repeat GTAAA/ATCAGGGAGCTGCTGAGATAG, which is also found associated with these genes in all other streptomycete genomes analysed (Fig. 5). We anticipate that it is a possible binding site for the SCO1447 gene product. Considering that only a single element is found on genome, suggesting that if indeed SCO1447 binds there, SCO1448 may be its only direct target. Following this logic, since SMG1 only relieves toxicity of GlcN, we anticipate that SCO1448 may encode a GlcN transporter, and that its expression depends on SCO1447. This is currently being
SMG4 contained an out-of-frame deletion of 416 bp in nagA (nt positions 261-676). This result confirms our previous observation, that the toxicity of GlcN to nagB mutants can be relieved by additional deletion of nagA (Fig. 2). 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 (Świątek et al., 2012). Moreover, the mutants displayed enhanced antibiotic production (Fig. 2).

SMA1 has a G→T substitution at nt position 1468 in dasD (SCO5235), resulting in an Ala490Ser change in the protein. DasD is a predicted β-N-acetylglucosaminidase, which is a highly specific exoglucosidase that catalyzes the hydrolysis of terminal, non-reducing β-N-acetylglucosamine residues from oligosaccharides. Upstream of dasD lies the dasA-dasBC gene cluster for a oligo-GlcNAc-binding protein (DasA) and chitobiose permease (DasBC), respectively. Whereas dasA and dasBC are transcribed individually, dasD is probably co-transcribed with dasBC (Colson et al., 2008; Saito et al., 2007). It is anticipated that DasD might serve for hydrolysis of chitobiose [(GlcNAc)₂], which is taken up via the DasABC system (Colson et al., 2008; Saito et al., 2007). Downstream of dasD lies transcribed in the opposite direction nagB.

Suppressor mutant SMA11 has a G→A substitution at nt position 535 in SCO4393, resulting in a Asp179Asn change in the SCO4393 gene product. SCO4393 encodes a highly conserved protein across Streptomyces species. It contains a SIS (Sugar ISomerases) domain characteristic of phosphosugar isomerases and phosphosugar binding proteins. The best known examples of SIS domain containing proteins are bacterial helix-turn-helix (HTH) transcriptional regulators belonging to the RpiR family. The RpiR regulators control the expression of genes encoding sugar metabolic enzymes. This control is modulated through direct interaction of the domain with substrates of regulated metabolic enzymes. It is unlikely that the SCO4393 product is a transcriptional regulator, since it lacks a clear DNA binding motif, and we expect it may have an
enzymatic function. Glucosamine-6P synthetase (GlmS) is an example of a metabolic enzyme containing a SIS domain. Excitingly, gene syntenic analysis revealed that SCO4393 is linked to nagA in many organisms. In Gram-negative alpha-proteobacteria the orthologs of SCO4393 are clustered with nagA and nagB, while in firmicutes they are adjacent to pts genes (Fig. 6). These results highlight the likely involvement of the SCO4393 gene product in the aminosugar utilisation, and strongly suggest that indeed the Asp179Asn change in SCO4393 may be the cause of the suppressor phenotype.

In a control experiment, each of these four nagB suppressor mutants was complemented with the low-copy-number vector pHJL401 (Larson & Hershberger, 1986) harboring a wild-type copy of the putative target genes sustaining the suppressor mutation transcribed from either the native promoter or - in case a promoter was not identified in the upstream region - from the ermE* promoter. For the latter we used the plasmid pHJL401-SuperP, which contains the ermE* promoter and the ribosome binding site from the tuf1 gene, which allows very efficient translational initiation (Vijgenboom et al., 1994). SMG1 was complemented with pGAM30 (pHJL401/SCO1447), SMG4 with pGAM5 (pHJL401/nagKA), SMA1 with pGAM31 (pHJL401-SuperP/dasD) and SMA11 with pGAM32 (pHJL401/SCO4393). The sensitivity of all strains to aminosugars was tested on MM agar plates. Importantly, in all cases indeed the complemented suppressor mutants failed to grow on amino sugars, strongly suggesting that these specific mutations
were the cause of the restored growth (Fig. 7).

![Figure 7. Complementation of nagB suppressor mutants. Phenotypes of S. coelicolor M145, the nagB mutant, nagB suppressor mutants SMG1, SMG4, SMA1, SMA11 and the complemented suppressor mutants expressing respectively SCO1447, nagA, dasD and SCO4393 are shown. 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 aminosugar.](image)

![Figure 8. Phenotypic analysis of the double nagB-SCO1447 mutant. Patches of S. coelicolor M145 and its derivatives on MM agar plates with either GlcN (top) or GlcNAc (bottom) as the sole carbon source are shown. Note that nagB mutants, SMG1 and double nagB-SCO1447 mutants do not grow on GlcNAc, whereas growth of the last two is restored on GlcN. Patches were grown for 5 days at 30°C.](image)

To know if more suppressor mutants contained the same second-site mutations, fifteen additional suppressor mutants obtained on GlcN and nineteen suppressor mutants obtained on GlcNAc were transformed with all complementation constructs. Two of the GlcN-selected suppressor mutants regained sensitivity to this aminosugar upon introduction of a construct expressing SCO1447, while on MM agar plates supplemented with GlcNAc, 13 of the GlcNAc-selected suppressor mutants regained sensitivity to GlcNAc following introduction of pGAM32 harbouring SCO4393, while none of the tested strain was sensitive to GlcNAc upon transformation with plasmid pGAM31 harbouring dasD. 10 GlcN-selected and three GlcNAc-selected nagB suppressor mutants...
lost their ability to grow in the presence of either aminosugar, when complemented with a wild-type copy of the nagA gene.

The high frequency of second-site nagA mutations relieving GlcN toxicity of 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 database; http://www.genome.jp/kegg-bin/show_pathway?org_name=sco&mapno=00520). This presents one example of the high relevance of the suppressor mutants, and closer analysis of GlcN and GlcNAc metabolism is currently in progress.

In contrast to SMG4, SMG1 harboring the second site mutation in SCO1447 could grow only in the presence of GlcN (Fig. 3 and Fig. 7). In a control experiment, SCO1447 was deleted from the nagB mutant and subsequently growth of the obtained strain was tested on both aminosugar. Again, the double mutant was able to grow on GlcN, but not GlcNAc (Fig. 8). Therefore SCO1447 is specifically involved in the control of GlcN utilization, perhaps by governing control the transporter gene SCO1448, which may transport GlcN. However, disruption of SCO1448 did not restore growth of nagB mutant on GlcN (data not shown).

Thus, the identification of second-site mutations in nagB mutants restoring growth on GlcNAc and/or GlcN has allowed us to identify novel candidate genes that are somehow involved in aminosugar metabolism in streptomycetes. These molecules play an important role in central metabolism and cell-wall biosynthesis and accumulation of GlcN(Ac)-derived metabolites, which among others serve as a signal for the onset 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.

Acknowledgements
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