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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**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-positive, 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. 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. 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.

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 mechanisms of predation leading to elimination of competing organisms. It is suggested that in natural habitat of bacteria,
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. 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 prodigiosine 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, which encode the synthase for the β-butyrolactone Scb1 and the Scb1-responsive transcriptional activator for Cpk biosynthesis, respectively. The molecular mechanism of GlcNAc signaling under rich nutritional conditions, which results in the blockage of sporulation and antibiotic production, is still unknown.

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

In S. coelicolor, the model organism of species, GlcNAc is transported via the phosphoenolpyruvate-dependent phosphotransferase system (PTS).

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 pglH), and then further onto enzyme IIA (EIIA; encoded by crr) and enzyme IIB (e.g., NagF).

NagF phosphorylates incoming via the permease ICE (NagE2) GlcNAc to form N-acetylmuramyl-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.

Recently we characterized the metabolic enzymes in S. coelicolor, namely GlcNAc-6P deacetylase (NagB; SCO4284), GlcN-6P deaminase (NagB; SCO5236) and GlcNAc kinase (NagK; SCO4285). 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.

Interestingly, some Streptomyces species, including S. bingchengensis, S. sattleyae, and S. violaceusniger, do not have a nagT ortholog. Genome mining revealed the presence of an open reading frame, which appears to be a hybrid of nagB/glmS and shows similarity to the nagB-II homologs containing the C-terminal domain of GlmS, but display only the carboxylic activity of NagB.

The fact that these three step-tomycetes also contain the PTS genes for internalization and phosphorylation of GlcNAc, including nagE2 (SBI_06066, SCATT_19300, Strvi_8260) and nagF (SBI_06887, SCATT_19290, Strvi_8239), as well as nagB (SBI_04996, SCATT_2457, Strvi_8766a) for the subsequent deacetylation of GlcNAc-6P to GlcN-6P, strongly suggests that indeed these organisms can carbodize 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). nagB 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.

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). This sheds new light on GlcN metabolism as an approach to enhance antibiotic production.
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 GlcN or GlcNAc, suppressor mutants were readily obtained, with a frequency of around 1:10^5, 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 GlcN when retested on MM + GlcN, while suppressor mutants with restored growth in the presence of GlcN had similar phenotypes on MM + GlcNAc. This suggests that suppressor mutations may have arisen in different genes.

Fifteen suppressor mutants obtained on GlcN, designated SMA1-SMA15 (Suppressor Mutants isolated on GlcN) and five independent suppressor mutants obtained on GlcNAc-containing media, SMG1-SMG5 (Suppressor Mutant isolated on GlcNAc), 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 (Figs. 3 and 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) (Figs. 3 and 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 GlcNAc 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 to the wild-type strain (Figs. 3 and 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 GlcN or GlcNAc (Fig. 3). The nagB mutant and most of the suppressor mutants obtained from either GlcN or GlcNAc 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 (Gki), respectively39 (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 nagA, or the metabolic genes nagF or nagC. These genes and nagE2, 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 controlled experiment, this suppressor mutant was complemented with plasmid pHJL401 harboring the nagA 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.38 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 nagE2 under the control of the nagE2 promoter (not shown).

In line with the idea that the accumulation of GlcN-6P was the cause of the toxicity, nagA 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.

The high frequency of second-site nag 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 database39). 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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