Genome-wide transcriptome response of *Streptomyces tsukubaensis* to *N*-acetylglucosamine: effect on tacrolimus biosynthesis

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Abstract

Chitin is the second most abundant carbohydrate biopolymer present in soils and is utilized by antibiotic–producing *Streptomyces* species. Its monomer, *N*-acetylglucosamine (GlcNAc), regulates the developmental program of the model organism *Streptomyces coelicolor*. GlcNAc blocks differentiation when growing on rich medium whilst it promotes development on poor culture media. However, it is unclear if the same GlcNAc regulatory profile observed in *S. coelicolor* applies also to other industrially important *Streptomyces* species. We report here the negative effect of GlcNAc on differentiation and tacrolimus (FK506) production by *Streptomyces tsukubaensis* NRRL 18488. Using microarrays technology, we found that GlcNAc represses the transcription of *fkbN*, encoding the main transcriptional activator of the tacrolimus biosynthetic cluster, and of *ppt1*, encoding a phosphopantheteinyltransferase involved in tacrolimus biosynthesis. On the contrary, GlcNAc stimulated transcription of genes related to amino acid and nucleotide biosynthesis, DNA replication, RNA translation, glycolysis and pyruvate metabolism. The results obtained support those previously reported for *S. coelicolor*, but some important differences were observed; for example genes involved in GlcNAc transport and metabolism and genes encoding transcriptional regulators such as *crr*, *ptsI*, *nagE1*, *nagE2*, *nagB*, *chiA*, *chiJ*, *ngcE*, *dasR* or *atrA* are not significantly induced in *S. tsukubaensis* by GlcNAc addition. Differences in the GlcNAc transport systems, in the physiology of *S. tsukubaensis* and *S. coelicolor* and/or the different composition of the culture media used are likely to be responsible for the discrepancies observed between these species.

Keywords: S*treptomyces tsukubaensis*~~s~~; Tacrolimus; FK506; *N*-acetylglucosamine; Transcriptomics

1 Introduction

Gram positive bacteria of the genus *Streptomyces* are characterized by their mycelial growth and ability to produce secondary metabolites with a wide range of biological activities (Hopwood, 2007). As soil-dwelling bacteria, streptomycetes are adapted to changing environmental conditions and have a broad arsenal of ECF (extracytoplasmic function) sigma factors and two-component systems that allow them to detect and respond to external stimuli (Hutchings et al., 2004; Martín et al., 2012; Martín and Liras, 2010). In soils, the second most abundant carbohydrate biopolymer is chitin, a major component of fungal cell walls and of the exoskeleton of arthropods. Soil inhabiting *Streptomyces* degrade chitin using chit~~h~~inases (Nazari et al., 2011). The monomer of chitin, *N*-acetylglucosamine (GlcNAc), serves as nitrogen and carbon source for *Streptomyces* and exerts a dual regulatory role on its differentiation (Rigali et al., 2006, 2008). GlcNAc blocks morphological and biochemical differentiation in *Streptomyces coelicolor* growing on rich solid media such as R2YE (Rigali et al., 2006). On the contrary, on minimal medium (MM) agar plates GlcNAc triggers sporulation and antibiotic production (Rigali et al., 2008). The stimulatory effect of GlcNAc under poor nutritional conditions is a common phenomenon but not universal in *Streptomyces*. For example, it exerts an inhibitory effect on the growth of *Streptomyces roseosporus* cultured on MM with mannitol as carbon source (Rigali et al., 2008). In the same way, the blocking effect of GlcNAc on growth of *Streptomyces* species in R2YE is widespread but not general (Colson et al., 2008).

The regulatory effect of GlcNAc under limited nutritional conditions has been extensively studied in the model species *S. coelicolor*. A complete signaling cascade has been unraveled in which the pleiotropic regulator DasR, a member of the GntR family, plays a key role (Rigali et al., 2002, 2004). DasR binds to canonical sequences (named *dre* sites) in the promoter regions of several genes involved in chitin utilization and nitrogen transport and metabolism. In addition, ChIP-on-chip analysis revealed that DasR binds hundreds of other sites in the *S. coelicolor*'s genome that do not contain the canonical binding sequences (Świątek-Połatyńska et al., 2015). GlcNAc is transported through the

sugar phosphotransferase system (PTS; Nothaft et al., 2003) and internalized as *N*-acetylglucosamine-6-phosphate (GlcNAc-6-P). The product of GlcNAc-6-P metabolism, glucosamine-6-phosphate (GlcN-6-P), acts as an allosteric regulator of DasR, reducing its DNA-binding activity (Rigali et al., 2006). The release of DasR from its targets allows transcription of the GlcNAc regulon, which includes genes involved in chitin/GlcNAc metabolism (Rigali et al., 2006; Colson et al., 2007), antibiotic biosynthesis (Rigali et al., 2008), stress response (Nazari et al., 2013) and siderophore formation (Craig et al., 2012).

However, it is unclear if the same profile of GlcNAc regulation occurs in some important industrial producers of antibiotics or immunosuppressants, such as *Streptomyces tsukubaensis*. To our knowledge no studies about the regulatory effect of GlcNAc have been performed in this species, the major tacrolimus producer (Kino et al., 1987a, 1987b). Tacrolimus is a nitrogen containing macrolide immunosuppressant used in organ transplantation to avoid graft rejection and for the treatment of skin diseases like atopic dermatitis (Meier-Kriesche et al., 2006; McCormack and Keating, 2006; Ingram et al., 2009; Remitz and Reitamo, 2009). Since its discovery, the studies with *S. tsukubaensis* have been mainly focused on tacrolimus production enhancement through culture media optimization and genetic engineering of the producer strains (Singh and Behera, 2009; Barreiro and Martínez-Castro, 2014; Ban et al., 2016). Nevertheless, a detailed insight into the mechanisms that link nutritional signals and secondary metabolite formation is necessary to improve production yields. In this sense, our group has recently contributed with several works connecting phosphate and carbon regulation with tacrolimus biosynthesis (Ordóñez-Robles et al., 2017a, 2017b). Microarray-based genome-wide transcriptomic analysis is a powerful tool to elucidate differential expression of genes. However, no studies related to nitrogen metabolism in differentiation and, particularly, on the effect of GlcNAc have been performed so far. Therefore, we decided to study the effect of GlcNAc addition on tacrolimus production in *S. tsukubaensis* and its genome-wide transcriptional response using microarray technology.

2 Materials and methods

2.1 Bacterial strains and culture conditions

S. tsukubaensis NRRL 18488 (Kino et al., 1987a) was cultured on ISP4 (Difco™, BD, NJ, USA) solid medium at 28 °C for spore obtention and for the assessment of morphological differentiation in the presence of GlcNAc. For the study of GlcNAc regulation the defined-rich liquid medium MGm-2.5 (optimized for tacrolimus production; Martínez-Castro et al., 2013) was used. MGm-2.5 contains starch as carbon source, glutamic acid as both carbon and nitrogen source and 2.5 mM phosphate. This medium allows the study of nutritional regulatory mechanisms, which would not be possible in complex media such as R2YE. For this purpose, 500 ml flasks containing 100 ml of MGm-2.5 medium were inoculated with 10^9 spores of *S. tsukubaensis* and incubated at 28 °C and 220 rpm. GlcNAc (SigmaAldrich, St. Louis, MI, USA) was added at the mid-exponential growth phase (i. e. 70 h) at a final concentration of 22.6 mM (0.5% w/v) since its blocking effect is especially strong over 20 mM (Rigali et al., 2006). In the practice, 3 ml of a stock solution of GlcNAc 16.7% w/v (or 3 ml of Milli-Q water in the control condition) were added to the cultures at 70 h. Flasks cultures in both experimental conditions were performed in duplicate.

2.2 Growth determination and tacrolimus concentration in the cultures

Samples for growth determination (measured as dry weight per ml) were taken at 70 h, 78.5 h, 89.5 h, 92 h, 100 h, 124 h, 148 h, 162.5 h and 235 h, and processed as indicated by Ordóñez-Robles et al. (2016). Samples for tacrolimus extraction with methanol and HPLC quantification were taken at 148 h, 162.5 h and 235 h. The procedures followed were those indicated by Ordóñez-Robles et al. (2017a).

2.3 RNA extraction, purification, labelling and hybridization

Considering the time points analysed in other GlcNAc-addition works (Świątek-Połatyńska et al., 2015), samples were taken immediately before the addition (i. e. 70 h), and 0.5 h, 1 h and 2 h after the addition. All the procedures related to RNA extraction and purification, labeled cDNA synthesis and the conditions of microarray hybridization were performed as previously described (Ordóñez-Robles et al., 2016).

2.4 Microarray design and data analysis

The design of the custom microarrays used in this work was previously described by Ordóñez-Robles et al. (2017a). Normalization of the fluorescence intensities and statistical analyses were conducted with the limma package v3.20 (Smyth, 2004), as indicated previously (Ordóñez-Robles et al., 2016). The processed fluorescence intensities are referred here as M_g values, which are normalized and $\log_2$-transformed values that represent an approximate measure of the abundance of the transcript of a particular gene with respect to its genomic copies (Mehra et al., 2006; Sidders et al., 2007). To find out genes affected by the GlcNAc addition, three interaction contrasts were conducted using limma. These contrasts can be expressed as '$(M_g^{tx} - M_g^{t70h})_{GlcNAc} - (M_g^{tx} - M_g^{t70h})_{Control}$', where *x* represent either one of the three culture times after the initial reference time (i.e., 70.5 h, 71 h or 72 h). The results of the contrasts comprise the differential transcription values, referred here as M_c values, and the associated *p*-values. The *p*-values were adjusted for multiple testing (named p_{FDR}) by the false discovery rate method of Benjamini and Hochberg (1995).

The microarray data discussed here has been deposited in NCBI's Gene Expression Omnibus database (Edgar et al., 2002) and are accessible under the accession number GSE110393.

2.5 Quantitative reverse transcription PCR (RT-qPCR)

To validate the microarray results by RT-qPCR we measured the transcript levels of *pfkA1*, *pfkA2*, *pfkA3* and *fkbN* at t70 h, t70.5 h, t71 h and t72 h. For normalizing assays, the gene *gyrB* was chosen since its M_g levels were almost constant throughout the time series. The sequences of the corresponding primer pairs were listed previously by Ordóñez-Robles et al. (2016, 2017b). The conditions and procedures of RT-qPCR were those indicated by Ordóñez-Robles et al. (2016). A high correlation (r^2 = 0.9947) between microarray and RT-qPCR transcriptional ratios validated the results (Fig S1).

3 Results

3.1 GlcNAc arrests differentiation and reduces significantly tacrolimus production in *S. tsukubaensis*

First of all, we determined the effect of GlcNAc on tacrolimus production and growth of *S. tsukubaensis*. To asses the effect on morphological differentiation, *S. tsukubaensis* was spread on ISP4 agar plates in the presence of GlcNAc 0.5% (w/v). As shown in Fig S2, GlcNAc exerted a negative effect on development, arresting sporulation. GlcNAc addition did not affect *S. tsukubaensis* growth on MGm-2.5 significantly, although 19% and 26% increases were observed at late times in the cultures (148 h and 162.5 h, respectively; see Fig. 1). In contrast, GlcNAc reduced significantly tacrolimus production yields in all samples to less than a half of the control yield. This result is in accordance to that reported previously in *S. coelicolor* (Rigali et al., 2008; Świątek-Połatyńska et al., 2015; Tenconi et al., 2015) and thus, we can include *S. tsukubaensis* in the group of streptomycetes whose secondary metabolism is negatively regulated by GlcNAc when growing under rich nutritional conditions.

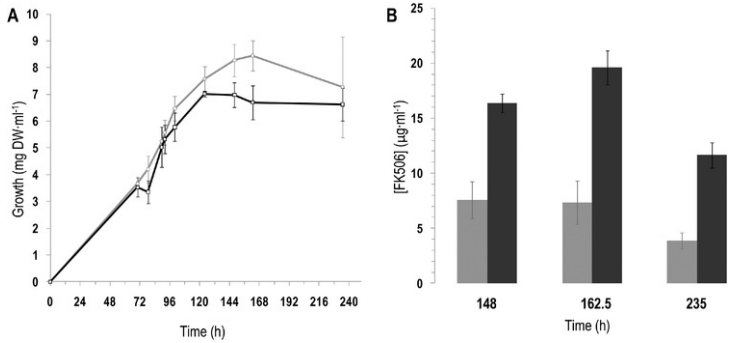

Fig. 1 Growth and tacrolimus production in the cultures. A) Growth is represented as dry weight. B) Tacrolimus production at 148 h, 162.5 h and 235 h in both experimental conditions. Data from control condition are represented as black line (panel A) and black bars (panel B) whils GlcNAc data are represented as grey line (panel A) and grey bars (panel B). In both panels the values represent the average and standard deviation of two biological replicates.

alt-text: Fig. 1

3.2 Transcriptional effect of GlcNAc addition

GlcNAc at a final concentration of 22.6 mM was added at 70 h, i. e. during the first growth phase and before the depletion of phosphate (Ordóñez-Robles et al., 2017b). This addition time was selected because it precedes the transition phase, in which tacrolimus biosynthesis is activated after phosphate is depleted. Adding GlcNAc before phosphate is exhausted avoids masking the effects of GlcNAc addition with those of the response to phosphate starvation. Samples for transcriptomic analysis were taken from duplicated cultures before the addition (i.e. 70 h), and 30 min, 1 h and 2 h later (time-points 70.5 h, 71 h and 72 h, respectively). The microarray data were analysed to find out which genes responded specifically to the addition of GlcNAc. For this, we compared the transcription profiles of the cultures with GlcNAc to those of the control cultures (without addition). This was done using the interaction contrasts of linear models that reveal the difference of differences; in other words, the different response through the time course between GlcNAc and control time series (see Materials and Methods for details). Those genes showing significant differential response ($p_{FDR} \leq 0.05$) in all the comparisons (i. e. at $t_{70.5\ h}$, $t_{71\ h}$ and $t_{72\ h}$ respect to t_{70-5h}) or at least in the two last time points (i.e. $t_{71\ h}$ and t_{72} h respect to t_{70-5h}) were selected. Thus, a total of 1320 genes were significantly affected by the addition (640 upregulated and 680 downregulated; data not shown). We focused our attention in those genes showing 2-fold or greater changes at the first (i. e. 70.5 h) and/or the second time points (i. e. 71 h); of these genes, 187 were upregulated and 392, downregulated (Table S1). These genes responding to the GlcNAc addition are grouped according to their metabolic roles and discussed in the next sections.

3.3 Effects of GlcNAc addition on transcription of genes involved in GlcNAc transport and metabolism

In *S. coelicolor* GlcNAc is transported through a dedicated PTS system (PTS^{GlcNAc}). The specific components IIB and IIC of the PTS^{GlcNAc} system are encoded by *nagF* and *nagE2*, respectively. In the model species, transcription of *nagF* and *nagE2* (belonging to the same operon) is induced by GlcNAc addition on MM plus glycerol (Nothaft et al., 2010). The incorporated GlcNAc-6-P is further metabolized by the subsequent actions of the deacetylase NagA and the deaminase/isomerase NagB. In a similar manner, transcription of *nagA* and specially *nagB* is upregulated after GlcNAc addition on MM plus mannitol in *S. coelicolor* (Świątek et al., 2012). Although MM determines poor growth, DasR is

released from its targets also in rich media (i. e. R5 medium), and this has been proven by ChIP-on-chip for *crr*, *ptsI* (encoding the enzymes EI and IIA^{ccr} from the PTS system, respectively), *nagE1*, *nagE2*, *nagB*, *chiA*, *chiJ*, *dasR*, *ngcE* and *atrA* (Świątek-Połatyńska et al., 2015). In *S. tsukubaensis*, transcription of *nagF* (STSU_23346) and *nagB* (STSU_31895) was not affected by GlcNAc addition under our conditions (the microarrays used did not contain probes for *nagA*), and transcription of STSU_23336, the likely orthologue to *nagE2*, was downregulated (Fig. 2a). In addition, most of the core DasR regulon genes mentioned above were not affected by the addition or were even repressed (Table S2).

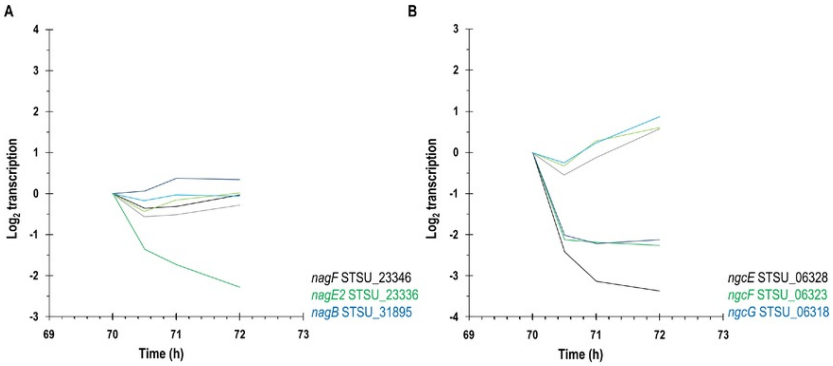

Fig. 2 Transcriptional profiles of genes involved in *N*-acetylglucosamine transport. Panel A: profiles of *nagF*, *nagE2* and *nagB*. Panel B: profiles of *ngcEFG*. The $\log_2$ transcription values (M_g) of selected genes are depicted for both the GlcNAc and control experimental conditions. In each panel, dark colors correspond to the GlcNAc condition whilst clear colors represent the control condition. Error bars have been omitted to facilitate the visualization of the results.

alt-text: Fig. 2

A possible explanation for these results might be that DasR is not released from its binding sequences under our experimental conditions. The DasR protein of *S. tsukubaensis* is highly similar to that of *S. coelicolor* (90% amino acid identity; 93% similarity) and putative *dre* sequences are found in *nagF*, *nagB* and *nagE2* (this promoter contains two putative sequences), differing only in 1 nt, 3 nt or 2 nt (see Fig. 3) from the canonical 16-nt A(G/C)TGGTCTAGACCA(G/C)T sequence, respectively. Nevertheless, experimental support through electromobility shift assays (EMSA) is necessary to confirm DasR binding to these putative *dre* elements. It is also important to note that the pattern of DasR binding varies according to the growth phase (see discussion) and that DasR binding is not only affected by GlcN-6-P but also by other metabolites such as phosphate, which increases the DasR binding ability (Tenconi et al., 2015). In fact, in the medium MGm-2.5, phosphate is depleted several hours after GlcNAc addition (80 h to 89 h; Ordóñez-Robles et al., 2017a, 2017b) and might reduce DasR release from its targets at the time points sampled. The confirmation of this hypothesis would require EMSA studies at different concentrations of phosphate.

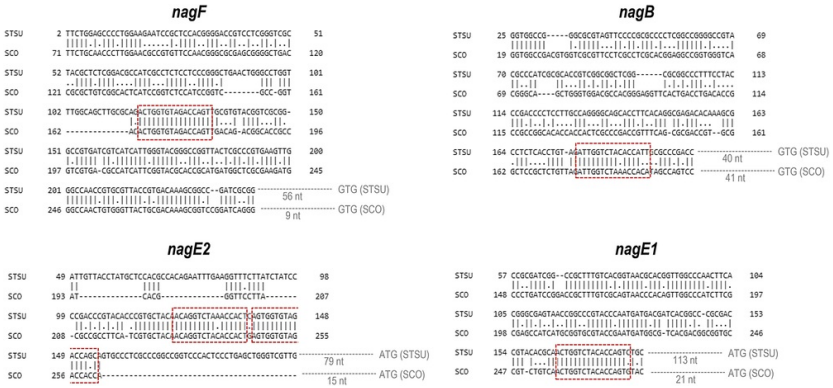

Fig. 3 Alignment of promoter regions of *nagF*, *nagB*, *nagE1* and *nagE2* between *S. tsukubaensis* and *S. coelicolor*. The alignments have been performed with the EMBOSS Matcher and EMBOSS Needle tools (EMBL-EBI). ~~Red~~ Black boxes indicate putative *dre* sequences. The distance in nucleotides to the corresponding start codon is indicated. ~~(For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).~~ (Delete this sentence since the legend has been updated)

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Another possibility is that *S. tsukubaensis* uses an alternative transporter for incorporation of GlcNAc, as similarly reported in *Streptomyces olivaceoviridis* (Xiao et al, 2002; Wang et al., 2013). *S. tsukubaensis* contains three genes

(STSU_06328, STSU_06323, STSU_06318) which are orthologues to *ngcEFG* from *S. olivaceoviridis* (Xiao et al., 2002), encoding an ABC transporter that mediates not only GlcNAc uptake but also chitobiose incorporation. Despite the high amino acid sequence identity between STSU_06328-STSU_06318 and *ngcEFG* (77%, 83% and 81%, respectively) it seems unlikely that GlcNAc is incorporated through this alternative transport system in *S. tsukubaensis*, since its mRNA levels were significantly reduced one hour after the addition rather than increased, which would be the expected response if GlcNAc is transported by this system (Fig. 2b). In *S. coelicolor* the orthologous genes (SCO6005-SCO6007) show low sequence similarity to *ngcEFG* (around 35% amino acid identity). For this reason, it has been suggested that, in the model species, the proteins encoded by these genes may transport a different molecule (Świątek et al., 2013).

3.4 Effects on the transport of alternative carbon and nitrogen sources

The mRNA levels of several genes that encode transporters for alternative carbon sources were reduced after the addition, which constitutes the basis of the concept of carbon catabolite repression (Magasanik, 1961; Sánchez et al., 2010). As shown in Figures S3.1 and S3.2a, this was the case for the genes encoding the glutamate ABC transporter (*gluABCD*; STSU_08033-STSU_08048), the maltose transporter (*malEFG*; STSU_26064-STSU_26054), the chitobiose transporter (*dasABC*; STSU_10781-STSU_10771) and the ATP-binding protein MsiK (STSU_19660). MsiK is involved in the transport of cellobiose and maltose in *Streptomyces* and is essential for chitobiose transport in *S. coelicolor* (Schlösser et al., 1997; Saito et al., 2008). Transcriptional repression of *dasA* by GlcNAc has been reported before in *S. coelicolor* (Saito et al., 2007; Colson et al., 2008).

Several genes coding for chitin binding proteins (i.e. STSU_32120) or chitinase enzymes (i.e. STSU_12805, STSU_11420 and STSU_11925 -orthologous to *chiA*-) were significantly downregulated after the addition (Fig S3.2b). This result is in good agreement with the GlcNAc-mediated transcriptional repression of *chiA* reported in *Streptomyces lividans* TK24 (Miyashita et al., 2000) and might reflect the availability of GlcNAc in the media, thus avoiding the need of chitin hydrolysis.

3.5 Effect on central carbon pathways

The addition of GlcNAc exerted common effects to that previously reported for glycerol and, especially, glucose addition (Ordóñez-Robles et al., 2017b), stimulating transcription of glycolytic and fatty acid biosynthetic genes and reducing transcription of gluconeogenic genes. Such results make sense considering that both glucose and GlcNAc share a common catabolic pathway from fructose-6-phosphate (Fig. 4).

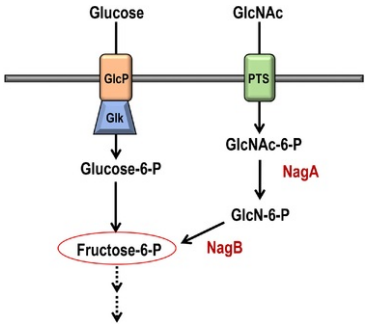

Fig. 4 Scheme of GlcNAc and glucose transport and metabolism. Glucose is incorporated through the combined activity of the glucose permease GlcP and the glucose kinase GlkA and GlcNAc is transported through the PTS system. Note that both compounds are internalized as phosphorylated intermediates and further metabolized to fructose-6-phosphate.

alt-text: Fig. 4

GlcNAc upregulated transcription of the glycolytic genes *glk* (encoding the glucose kinase), *ppgK* (encoding the polyphosphate glucokinase) and *pfkA3* (encoding the 6-phosphofructokinase 3) and downregulated transcription of gluconeogenic genes such as STSU_12200 and *glpX* (encoding a phosphoenolpyruvate carboxykinase and the fructose-1,6-biphosphate aldolase, respectively; Fig S3.3). In agreement with these results, transcription of *gap2* (encoding the glyceradehyde-3-phosphate dehydrogenase 2), which product has been proposed to be involved in glucose regeneration rather than in its consumption, was downregulated after GlcNAc addition. Interestingly, transcription of the glycolytic *pfkA1* gene (encoding the 6-phosphofructokinase 1) was downregulated after GlcNAc addition, indicating a different regulation than that of the paralog *pfkA3,* which was upregulated (Fig S3.3).

Conversion of phosphoenolpyruvate to oxaloacetate might be stimulated after GlcNAc addition since transcription of the *ppc* gene (encoding a phophoenolpyruvate carboxylase) increased. In agreement with the upregulation of carbon catabolic genes, we observed a downregulation in some genes involved in anabolic pathways such as STSU_24836 (encoding a pyruvate phosphate dikinase that regenerates phosphoenolpyruvate from pyruvate) or STSU_10621 (encoding a malate dehydrogenase that transforms malate to oxalacetate (Fig S3.4a). GlcNAc addition decreased transcription of some genes from the tricarboxylic acid cycle such as *sdhB, sdhA* (encoding succinate dehydrogenase subunits), STSU_11380 (paralog of STSU_02380) and *fumC* (encoding a fumarase) that are also downregulated by glucose addition (Fig S3.4b; Ordóñez-Robles et al., 2017b).

GlcNAc stimulated transcription of genes involved in the formation of acetyl-CoA from acetate (i. e. *pta* and *ackA*, encoding the phosphate acetyltransferase and the acetate kinase, respectively) and in the biosynthesis of fatty acids. For example, *fabH* (encoding a β-oxoacyl-CoA synthase III responsible for the initiation of fatty acid biosynthesis; Revill et al., 2001; Han et al., 1998) and *fasR*, encoding a transcriptional activator of the *fabD-fabH-acpP-fabF* operon (Arabolaza et al., 2010), increased their mRNA levels after the addition (Fig S3.5a). In addition, *fabG* and *fabG3* (encoding a 3-ketoacyl-ACP reductase involved in fatty acid biosynthesis) showed opposite transcriptional profiles (being *fabG* upregulated and *fabG3* downregulated). This observation has been reported previously after glucose and glycerol additions, suggesting different transcriptional regulation for these paralog genes (Ordóñez-Robles et al., 2017b). In agreement with the positive effect of GlcNAc on fatty acid biosynthesis, we observed a transcriptional downregulation of some genes involved in fatty acid catabolism such as STSU_06268 (encoding an acetyl-CoA acetyl transferase) and *acdH2* (encoding an acyl-CoA dehydrogenase; Fig S3.5b).

3.6 Upregulation effect of GlcNAc on key genes of the PHO regulon

GlcNAc addition led to an increase in the mRNA levels of several genes of the PHO regulon involved in the response to phosphate scarcity (Fig S3.6a). This was the case for *phoRP*, encoding the two component system that senses phosphate starvation (Sola-Landa et al., 2003); the divergently transcribed STSU_19400 (*phoU*), encoding a modulator of the phosphate response (Martín-Martín et al., 2017), and (This sentence continues in a different paragraph, please attach the sentence correctly.)

STSU_18950-STSU_18935 (*pstSCAB* operon), encoding a high affinity phosphate transport system (Sola-Landa et al., 2005; Martín, 2004). These results indicate that GlcNAc utilization interacts with phosphate regulation through the *phoRP* regulatory genes and supports the concept of the cross-regulation between major transcriptional regulators involved in nutrient assimilation (Sola-Landa et al., 2013; Martín et al., 2016). A similar response has been reported previously after glucose and glycerol additions in *S. tsukubaensis* (Ordóñez-Robles et al., 2017a,b), which might reflect an increased need of phosphate for the catabolism of carbon sources, including GlcNAc.

3.7 Effect on amino acid and nucleotide metabolisms, transcription and translation

GlcNAc addition exerted a positive effect on the transcription of several genes involved in the biosynthesis of L-glutamate, L-phenylalanine, L-serine, L-threonine, L-tyrosine, L-histidine, L-leucine, L-cysteine, L-methionine or L-tryptophan (Table S1). Many of these genes were also upregulated by glucose addition in *S. tsukubaensis* (Ordóñez-Robles et al., 2017b), such as the L-glutamate synthase coding genes *gltBD*. The upregulation in the transcription of the glutamate dehydrogenase coding gene *gdhA* after GlcNAc addition, suggests an increase in ammonium assimilation (Fig S3.6b). In agreement with these results, genes involved in glutamate consumption like *gdhD* (NAD glutamate dehydrogenase) and STSU_29012 (glutamine synthetase; Rexer et al., 2006) were transcriptionally downregulated (Fig S3.6b). ~~It was also observed a~~An increase in the mRNA levels of genes involved in nucleotide biosynthesis (Table S1), DNA replication and RNA translation (i. e. DNA polymerase subunits, ribosomal proteins and RNAt-aa synthetases) was observed. Such results suggest that GlcNAc addition was sensed as a positive nutritional stimulus.

3.8 Effect on respiration-related genes

GlcNAc addition caused a decrease in the mRNA levels of the NADH dehydrogenase I genes *nuoABCDE* and *nuoN* (Fig S3.7a). This enzymatic complex regenerates NAD^+ in the electron transport chain. A similar effect was observed previously after glucose and glycerol addition in *S. tsukubaensis* and it is likely due to an increased flux through the respiratory chain (Ordóñez-Robles et al., 2017b). Interestingly, the paralogous genes *nuoL2*, *nuoB2*, *nuoI2*, *nuoD2* and *nuoJ2* were upregulated, indicating an opposite regulation (Fig S3.7b). Transcription of *atpH*, encoding the delta subunit of the ATPase involved in the oxidative phosphorylation, decreased in all the time points tested after GlcNAc addition (Fig S3.7b).

The transcriptional repressor Rex responds to the $NADH/NAD^+$ balance (Brekasis and Paget, 2003): under high $NADH/NAD^+$ ratios Rex is released from its binding sequences allowing transcription of target genes such as *rex* and the *cydABCD* operon (encoding a cytochrome oxydase and an ABC transporter). Transcriptions of both *rex* and *cydBCD* were upregulated after GlcNAc addition (Fig S3.8a), indicating a situation of low availability of NAD^+ in the cultures.

GlcNAc addition upregulated transcription of *oxyR* (encoding the oxidative stress response regulator OxyR) and the OxyR target genes *ahpC* and *ahpD*, which encode alkyl hydroperoxyde reductases (Fig S3.8b). The same effect has been observed in this species after glucose and glycerol additions and has been attributed to an increase in the activity of the respiratory chain (Ordóñez-Robles et al., 2017b). In addition, we observed an increase in the mRNA levels of *senS* (Fig S3.8b), which encodes the sensor kinase of a two component system that is possibly involved in the response to redox balances in *S. coelicolor* (Ortiz de Orué Lucana and Groves, 2009). GlcNAc, as well as glucose and glycerol, increased transcription of oxidative stress response genes and reduced that of the NADH dehydrogenase I operon, indicating a status of low availability of NAD^+, which in turn, reflects alterated fluxes through the central metabolic pathways.

3.9 GlcNAc effect on genes involved in morphological and biochemical differentiation

A high number of genes involved in morphological differentiation showed a reduced transcription after GlcNAc addition, which is coherent with the developmental blockage observed on ISP4 agar plates. These include several

bld and *whi* genes like the *bldK* operon (STSU_11350-STSU_11330; Fig S3.9a), encoding the oligopeptide transporter responsible for the initiation of the signaling cascade that leads to morphological differentiation in this genus (Nodwell et al., 1996). Transcription of *bldC* (STSU_18523), *bldM* (STSU_13050), *bldG* (STSU_15482), *bldN* (STSU_14448) and *bldH* (STSU_23624) were significantly reduced one hour after the addition (Fig S3.9b). Transcription of *bldK*, *bldM* and *bldN* has been reported to be under the regulation of DasR in *S. coelicolor* (Świątek-Połatyńska et al., 2015). It is interesting to note that BldC is necessary to maximize transcription of *actII-orf4* and *redD* in *S. coelicolor* (Hunt et al., 2005). We also detected reduced mRNA levels of *wblA* (STSU_15654), *wblI* (STSU_11505) and *whiH* (STSU_07863; Fig S3.10a). WblA is a key sigma factor for the sporulation of some streptomycetes (Rabyk et al., 2011; Fowler-Goldsworthy et al., 2011) and has been proven to be directly targeted by DasR (Świątek-Połatyńska et al., 2015). WblA downregulates antibiotic production and reduces the response to oxidative stress (Kang et al., 2007; Kim et al., 2012). As *wblA*, *bldN* (encoding the RNA polymerase sigma factor) and the *bldK* operon have been reported to decrease their mRNA levels after glucose and glycerol addition in *S. tsukubaensis* (Ordóñez-Robles et al., 2017b).

Other genes encoding RNA polymerase sigma factors downregulated after GlcNAc addition include *hrdA* (STSU_24896), *hrdB* (STSU_07858), *sigU* (STSU_22934) and *sigN* (STSU_18170; Fig S3.10b). In *Streptomyces aureofaciens*, the transcription of *hrdA* correlates with the formation of the aerial mycelium (Kormanec and Farkasovský, 1993) and in *S. tsukubaensis* is downregulated after glucose and glycerol additions (Ordóñez-Robles et al., 2017b).

In agreement with the transcriptional repression of developmental genes we detected reduced mRNA levels of genes related to sporulation and chromosome segregation such as *ssgR* (STSU_17758), *ssgA* (STSU_17763), *ssgB* (STSU_29352), *parB* (STSU_17583) or STSU_26804, encoding the othologue of SepF, which is involved in the stabilization of the Z-ring for cell division in *Bacillus* (Hamoen et al., 2006; Fig S3.11a). Transcription of *ragB* (STSU_18448) increased after GlcNAc addition (Fig S3.11a). This gene encodes the permease of an ABC transporter and is part of the *ragABKR* operon, which modulates the formation of aerial hyphae and sporulation (San Paolo et al., 2006). GlcNAc addition upregulated transcription of the GTPase-coding gene *obg,* which is also upregulated after glucose and glycerol additions in *S. tsukubaensis* (Ordóñez-Robles et al., 2017b). This GTPase avoids aerial mycelium formation in *S. coelicolor* (Okamoto and Ochi, 1998). Moreover, *otsA* (STSU_19947) and *treSI* (STSU_09859), which seem to be involved in trehalose biosynthesis, decreased their transcription after GlcNAc addition (Fig S3.11b). Trehalose is the main storage carbohydrate in *Streptomyces* spores and can account for ~~u~~as much as the 25% of their dry weight (McBride and Ensign, 1987).

3.10 Downregulation effect of GlcNAc on tacrolimus biosynthetic genes

Regarding tacrolimus biosynthesis, we observed a decrease in the mRNA levels of *fkbN* (encoding the main transcriptional activator of the tacrolimus biosynthetic cluster) and of *ppt1* (encoding a 4′-phosphopantetheinyl transferase) after GlcNAc addition (Fig. 5). Transcription of *ppt1* is FkbN-dependent in *S. tsukubaensis* NRRL 18488 (Ordóñez-Robles et al., 2016) and its product is involved in tacrolimus biosynthesis in *S. tsukubaensis* L19 (Wang et al., 2016). Transcription of *scoT* (STSU_07618), encoding a type II thioesterase II which is also affected by *fkbN* inactivation (Ordóñez-Robles et al., 2016), was downregulated after GlcNAc addition (Fig. 5).

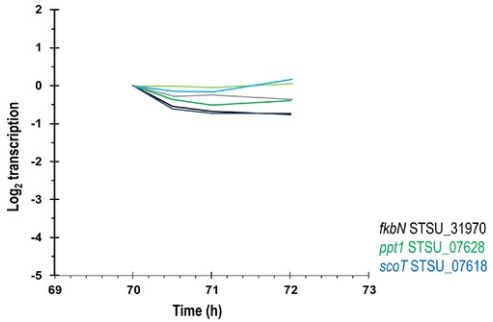

Fig. 5 Transcriptional profiles of genes involved in tacrolimus biosynthesis. The $\log_2$ transcription values (M_g) of *fkbN*, *ppt1* and *scoT* are depicted for both the GlcNAc and control experimental conditions. In each panel, dark colors correspond to the GlcNAc condition whilst clear colors represent the control condition. Error bars have been omitted to facilitate the visualization of the results.

alt-text: Fig. 5

3.11. Effect of GlcNAc on genes encoding transcriptional factors that might influence tacrolimus biosynthesis **(This is the heading of a subsection of the text.)**

The transcriptional downregulation of several genes that are involved in biochemical differentiation in *Streptomyces* might contribute to the GlcNAc negative effect observed on tacrolimus production. Among them we include *atrA* (STSU_18801), encoding a transcriptional activator of *actII-orf4* and *strR* promoters in *S. coelicolor* and *S. griseus*, respectively (Uguru et al., 2005; Vujaklija et al., 1993) and *eshA* (STSU_03599) which product regulates in a positive manner actinorhodin and streptomycin production in *S. coelicolor* and *S. griseus*, respectively (Saito et al., 2006; Kawamoto et al., 2001; Fig S3.12). It is interesting to note that *eshA* transcription is also downregulated by glucose and glycerol in *S. tsukubaensis* and that *atrA* shows a transcriptional profile similar to that of *fkbN*, encoding the main transcriptional positive regulator of the tacrolimus biosynthesis cluster (Ordóñez-Robles et al., 2017b; Fig S3.12). In the model species *S. coelicolor*, DasR binding to *atrA* target sites is relieved after GlcNAc addition on the rich medium R5 (Świątek-Połatyńska et al., 2015), allowing transcription. The different transcriptional behavior of *atrA* after GlcNAc

addition in *S. coelicolor* and *S. tsukubaensis* might be due to different regulation between *Streptomyces* species and/or to different composition of the media. In *S. tsukubaensis* we found several two-component systems related to antibiotic production in streptomycetes that were affected by GlcNAc addition. For example, transcription of *absA2* (STSU_09809), encoding the response regulator of a two component system that represses transcription of antibiotic biosynthetic clusters in *S. coelicolor* (McKenzie and Nodwell, 2007; Santos-Beneit et al., 2013), was significantly upregulated after the addition (Fig S3.12). In a similar manner, transcription of *afsQ1* (STSU_12480) was upregulated (Fig S3.12). AfsQ1 is the response regulator of a two component system that is also involved in biochemical and morphological differentiation in *S. coelicolor* and regulates genes for nitrogen metabolism and phosphate transport under high glutamate concentrations (Wang et al., 2013).

On the contrary, the transcription of the two component system coding genes *cutRS* (STSU_06603-STSU_06608) decreased after GlcNAc addition (Fig S3.12). CutR-CutS, which might regulate copper metabolism (Tseng and Chen, 1991), show a negative impact on actinorhodin production in *S. lividans* (Chang et al., 1996).

4 Discussion

GlcNAc, the monomer of chitin, exerts a dual regulatory role in *Streptomyces* depending on growth media composition: in poor nutritional conditions GlcNAc triggers differentiation whilst on rich conditions blocks it. The regulatory mechanisms of GlcNAc under poor nutritional conditions have been studied in depth in the model organism *S. coelicolor* and involve the transcriptional regulator DasR. DasR binds to the so-called DasR-responsive elements (*dre* sites) ~~that~~, which are formed by 16 nucleotides. This canonical *dre* element is present in genes involved in GlcNAc transport and metabolism but, in addition, DasR binds to many sites in the genome at non canonical sequences (Świątek-Połatyńska et al., 2015). This indicates that DasR is a pleiotropic regulator that affects many genes involved both in chitin utilization and in secondary metabolism. Nevertheless, little is known about the mechanisms operating under defined rich growing conditions, wich are optimal for tacrolimus production in *S. tsukubaensis*. This work provides a new example of GlcNAc-mediated negative regulation of morphogenesis and antibiotic production in the industrially important species *S. tsukubaensis*. We performed a genome-wide study of the transcriptional response to GlcNAc addition on a rich culture media (MGm-2.5) that, contrary to R5, is defined, enabling the performance of nutritional studies (Ordóñez-Robles et al., 2017b). GlcNAc addition reduced the transcription of *fkbN* (encoding the main positive transcriptional regulator of the tacrolimus biosynthetic cluster) and other genes involved in polyketide biosynthesis; thus, we conclude that the negative effect of GlcNAc on tacrolimus biosynthesis is played, at least in part, at the transcriptional level. This study also revealed a transcriptional downregulation of developmental genes, related to biochemical and morphological differentiation, which is in agreement with the developmental arrest observed on ISP4 agar plates and is likely to contribute to the repression of secondary metabolite biosynthesis. As it has been suggested in previous works (Romero-Rodríguez et al., 2016; Ordóñez-Robles et al., 2017b), the developmental blockage exerted by carbon sources might take place at very early stages such as the detection of peptidic signals that trigger morphological differentiation (i. e. oligopeptide transport through the BldK transporter).

In the model species *S. coelicolor* growing on R5 medium, DasR is dissociated from its targets after GlcNAc addition, allowing transcription of genes such as *crr*, *ptsI*, *nagE1*, *nagE2*, *nagB*, *chiA*, *chiJ*, *dasR*, *ngcE* or *atrA*. Unexpectedly, in *S. tsukubaensis* most of these genes showed no variations in their transcription after GlcNAc addition and *nagE2*, *chiA*, *ngcE* and *atrA* were even repressed. Such results might be due to physiological differences in GlcNAc transport and metabolism between *S. coelicolor* and *S. tsukubaensis* and/or to the different composition of the culture media used (i. e. R5 and MGm-2.5).

The DasR protein of *S. tsukubaensis* is highly similar to DasR of *S. coelicolor* (90% identity, 93% similarity) and we found possible DasR responsive elements (*dre* sequences) in several genes involved in GlcNAc transport and metabolism. Nevertheless, direct binding of DasR to these sequences cannot be confirmed at this point and further studies (such as electrophoretic mobility shift assays) must be addressed in future works taking in consideration the nutritional environment present in our culture conditions (i. e. DasR binding ability varies depending on phosphate availability and, in our culture media, it was not depleted at the time of GlcNAc addition) and the stage of growth (in *S. coelicolor* growing on MM plus mannitol, metabolic and transport genes such as *chi*, *nag* or *ptsH* show higher DasR binding at the vegetative state whilst developmental and secondary metabolite genes are DasR-bounded during the sporulation phase; Świątek-Połatyńska et al., 2015). In addition, we cannot exclude the lack of effect of other transcriptional regulator(s) under our experimental conditions and/or phase of growth tested to explain the differences observed between species. In any case, these observations highlight the importance of interpreting results within the context of the composition of the growing media and the use of chemically defined ones when studying nutritional networks.

GlcNAc stimulated transcription of genes related to amino acid and nucleotide biosynthesis, DNA replication, RNA translation, glycolysis, pyruvate metabolism and phosphate scavenging. On the contrary, it reduced transcription of genes involved in the uptake of alternative carbon sources, which is coherent with the concept of carbon catabolite repression (Sánchez et al., 2010). These responses are similar to those observed after glucose addition in *S. tsukubaensis* growing in the same culture media (Ordóñez-Robles et al., 2017b), suggesting that GlcNAc exerts a mechanism similar to that of glucose carbon catabolite regulation on many intermediary metabolism genes. For example, under our experimental conditions, both GlcNAc and glucose downregulate transcription of genes involved in GlcNAc transport such as *nagE2* (Ordoñez-Robles et al, 2017b). The high similarity between glucose and GlcNAc responses reinforces the tight interconnection between their regulatory networks that has been reported before; for example, glucose carbon catabolite repression is enhanced after *dasR* inactivation in *S. coelicolor* (Colson et al., 2008).

5 Conclusions

- GlcNAc blocks morphological differentiation in *S. tsukubaensis* on ISP4 agar plates.
- GlcNAc arrests tacrolimus production in *S. tsukubaensis* growing on the defined MGm-2.5 medium, which is in accordance with the transcriptional downregulation of *fkbN*.
- GlcNAc has an important pleiotropic role on the expression of many genes involved in primary and secondary metabolism in *S. tsukubaensis*.
- There are some important differences beween *S. coelicolor* and *S. tsukubaensis* regarding the GlcNAc effect on the transcription of genes involved in chitin utilization and GlcNAc transport and metabolism. Such differences might be attributable to regulatory differences and/or to the different composition of the culture media used.
- The GlcNAc effects are similar to those exerted by glucose in this strain and, thus, the regulatory mechanisms of this aminosugar resemble those of carbon catabolite repression.

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Conflict of interest

María Ordóñez-Robles declares that she has no conflict of interest.

Juan F. Martín declares that he has no conflict of interest.

Antonio Rodríguez-García declares that he has no conflict of interest.

This article does not contain any studies with human participants or animals performed by any of the authors.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.micres.2018.08.014.

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Appendix A. Supplementary data

The following is Supplementary data to this article:

Multimedia Component 1

Queries and Answers

Query: Fig. 3 will appear in black and white in print and in color on the web. Based on this, the respective figure caption has been updated. Please check, and correct if necessary.
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