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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.

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 chitinases (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

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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.