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Identification of the sequences involved in the glucose-repressed transcription of the *Streptomyces halstedii* JM8 *xysA* promoter

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Abstract

The expression of *xysA*, a gene encoding for an endoxylanase from *Streptomyces halstedii* JM8, is repressed by glucose. In order to define the regions involved in its regulation, several deletions were made in the 475 bp *xysA* promoter and were studied using the *melC* operon from *S. glaucescens* as a reporter. Four of the deleted versions obtained were seen to be derepressed when driving *melC* or its own *xysA* gene expression in *Streptomyces lividans*. Quantitative assays revealed that the activity of xylanase produced under the control of these four deleted promoters was higher than the original one in the presence of glucose. Three regions – RI, R16 and R21 – involved in glucose repression were defined in this analysis: RI is a palindromic sequence that is highly conserved among xylanase gene promoters from Actinomycetes (−213 GAAAxxTTTCxGAAA −197) and, R16 and R21 define two new seven-pair conserved motifs, respectively (−113 5′-CCTTCCC-3′ −106 in R16 and −76 5′-CGAACGG-3′ −69 in R21) located in the untranslated mRNA. Gel shift assays demonstrated the existence of proteins that bind specifically to these regions.

Keywords: CCR; Glucose repression; Xylanase; Gram-positive bacteria

1. Introduction

Owing to their saprophytic lifestyle, the Gram-positive high-GC bacteria of the genus *Streptomyces* synthesise a broad range of extracellular hydrolytic enzymes such as cellulases, chitinases, proteases, amylases and xylanases, which are used to obtain nutrient. Expression of the genes encoding these enzymes is generally activated by specific substrates and is repressed by the most energy-efficient carbon source, normally glucose (Nguyen et al., 1997). This regulatory phenomenon – called Carbon Catabolite Repression/Activation (CCR/CCA) (Stulke and Hillen, 1999) – has been well characterized in several groups of bacteria, such as *Escherichia coli* and *Bacillus subtilis*.

$EIIA^{glc}$, the *E. coli* glucose-specific transporter of PTS sugar transport, plays a central role in such regulation. This protein transmits carbon-regulatory signals via its phosphorylation state to several cellular targets, including CAP, the Catabolite Activator Protein (Titgemeyer and Hillen, 2002). $EIIA^{glc}$ also controls inducer exclusion, the main mechanism for CCR in this group of organisms. Inducer exclusion prevents the formation and/or activation of specific intracellular inducers of alternative carbon source operons under conditions of repression (Postma et al., 1993).

In *B. subtilis*, a low-GC Gram-positive bacterium, the bifunctional ATP-dependent enzyme HPr kinase/phosphatase (HPrK/P), is a key molecule in the main mechanism of CCR/CCA (Ramström et al., 2003). This enzyme can

Abbreviations: CCR, carbon catabolite repression; bp, base pair(s); GC, guanine plus citosine; CCA, carbon catabolite activation; Ser, serine; PTS, phospho-transferase system; His, histidine; MSA, manitol soya agar; w/v, weight/volume; v/v, volume/volume; Kb, kilobases; ORF, open reading frame; v/cm, volt/centimeter; *xysAp*, *xysA* gene promoter; PCR, polymerase chain reaction; wt, wild type; m.s., manuscript.

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phosphorylate and dephosphorylate the proteins HPr (a Histidine-containing protein from the PTS system) and Crh (catabolite repression HPr) at Ser-46, affording them regulatory functions. When these proteins are phosphorylated at Ser-46, they form a complex with CcpA (Catabolite Control Protein A) that recognizes some cis-acting operator sequences called *cres* (catabolite responsive elements), leading to the repression/activation of target genes or operon transcription. HPr is also part of the PTS system and is phosphorylated on His-15 by enzyme I (EI). The His-15 phosphoryl group can then be transferred to the sugar-specific enzymes II, which phosphorylate incoming PTS sugars.

Little is known about the regulation of carbon source utilization and carbohydrate transport in Gram-positive bacteria with high G+C such as the Streptomycetes. It is accepted that in *Streptomyces*, glucose kinase (GlkA) plays a regulatory role in CCR. Its deletion causes a loss of repression by glucose in almost all systems studied, although glucose phosphorylation is restored by, for example, activation of a normally silent glucose kinase (GlkII). Lack of GlkA not only affects glucose repression, but also the repression exerted by other carbon sources, such as fructose, that are not metabolised via glucose kinase (Kwakman and Postma, 1994). However, in several *Streptomyces* species, some genes are repressed by glucose through GlkA-independent pathways (Ingram and Westpheling, 1995). Recently, Ramos et al. (2004) have suggested that in *Streptomyces peucetius* var. *caesius*, GlkA alone is not sufficient to elicit CCR, and that provided the first evidence supporting the hypothesis that some of the products of glucose catabolism are involved in CCR in *Streptomyces*. In *Streptomyces coelicolor*, two PTS energy-coupling enzymes – enzyme I (EI) and HPr, as well as the fructose-specific permease (enzyme II) – have been identified and have been suggested to be involved in CCR. Nevertheless, deletion of the *ptsH* gene encoding HPr has no effect on glucose repression and, additionally, no HPr kinase activity has been found, suggesting that it does not play a direct role in CCR (Nothaft et al., 2003).

Specific regulators implied in CCR have been described in *Streptomyces*, such as: GylR in the *gylCABX* glycerol utilization operon of *S. coelicolor* (Hindle and Smith, 1994), MalR, implied in maltose utilization in *S. coelicolor* (Van Wezel et al., 1997) and its homologue in *Streptomyces lividans*, $MalR_{sl}$ (Reg1) that has some effect in the repression of α-amylase and induction of the four chitinases existing in *S. lividans* (Nguyen et al., 1997). More recently, it has been described the implication of BxlR in the regulation of the uptake system of xylan degradation products operon, *bxlEFGA*, this protein might also act as a repressor of genes involved in xylan degradation of *S. thermoviolaceus* OPC-520 (*stxI* and *stxII* xilanases, *stxIII* acetylxylan esterase and *stxIV* α-L-arabinofuranosidase) (Tsujibo et al., 2004).

Cis-acting elements in the promoter regions of genes encoding hydrolytic enzymes have also been described as target sequences of the specific regulators. This is the case of (i) the 12 bp direct repeat sequences described for chitinases (Saito et al., 2000), (ii) the six hexamers overlapping with two pairs of direct repeat sequences in the *S. coelicolor* and *S. lividans galP1* galactose utilization operon, (iii) the palindromic sequence of 14 bp in the promoter of all cellulases described to date in *Streptomyces* and related actinomycetes, such as *Thermomonospora fusca* (Spiridonov and Wilson, 1999), (iv) the direct and inverted repeats in the promoter regions of all known *Streptomyces* α-amylase genes (Yin et al., 1997) and (v) the four inverted repeat that specifically bound to BlxR (Tsujibo et al., 2004).

In the case of xylanase promoters, it seems that the cis element involved in CCR is an inverted duplicate sequence of 4 bp: 5′-CTTT-Nx-AAAG-3′ (Giannotta et al., 2003).

In a previous work, by Western blot analysis of culture supernatants of *Streptomyces halstedii* JM8, it was shown that the production of xylanase Xys1L and its truncated form Xys1S was repressed by glucose and induced by xylan and xylose (Ruiz-Arribas et al., 1997). The promoter region of the *xysA* gene encoding Xys1 has been successfully used in heterologous expression of different bacterial and fungal proteins in *S. lividans* (Díaz et al., 2004) and hence an understanding of its regulation in this strain should be a useful tool for improving protein production systems.

Here we report a detailed deletion-based analysis of the *xysA* promoter region. This allowed us to define important regions in CCR. The involvement of glucose kinase GlkA in glucose repression of this gene was also determined, as well as the importance of DNA-binding proteins in its regulation.

2. Materials and methods

2.1. Bacterial strains and culture conditions

The strains used in this work are listed in Table 1. *E. coli* was grown in Luria Broth (LB) (Sambrook et al., 1989) at 37 °C, supplemented with neomycin (50 μg/ml) when needed. For *Streptomyces* growth, R2YE agar plates (Kieser et al., 2000) were used at 28 °C. MSA plates were used to obtain spores of transformants carrying the different constructions. All the *Streptomyces* strains were stored as spore suspensions in 20% (v/v) glycerol at −20 °C. Carbon sources: glucose or oat spelt xylan (Sigma) was added at a final concentration of 1% (w/v). *Streptomyces* media were supplemented with neomycin (15 μg/ml) when required.

The production of melanin due to *melC* expression was accomplished in NMMP solid minimal medium (Kieser et al., 2000) supplemented with 0.03% tyrosine (w/v).

To determine xylanase activity, liquid cultures were performed in baffled flasks with 1/10 volume of Neutral YES medium: 1% yeast extract, 10.3% filtered sucrose and 5 mM $MgCl_2$, buffered to pH 7, with 50 mM NaH_2PO_4, 50 mM Na_2HPO_4 and 50 mM NaCl. 2.5×10^6 spores were