Phosphate and carbon source regulation of two PhoP-dependent glycerophosphodiester phosphodiesterase genes of *Streptomyces coelicolor*

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Glycerophosphodiesterases are formed by deacylation of phospholipids. *Streptomyces coelicolor* and other soil-dwelling actinomycetes utilize glycerophosphodiesterases as phosphate and carbon sources by the action of glycerophosphodiester phosphodiesterases (GDPDs). Seven genes encoding putative GDPDs occur in the *S. coelicolor* genome. Two of these genes, *glpQ1* and *glpQ2*, encoding extracellular GDPDs, showed a PhoP-dependent upregulated profile in response to phosphate shiftdown. Expression studies using the *luxAB* genes as reporter confirmed the PhoP dependence of both *glpQ1* and *glpQ2*. Footprinting analyses with pure GST-PhoP of the *glpQ1* promoter revealed four protected direct repeat units (DRu). PhoP binding affinity to the *glpQ2* promoter was lower and revealed a protected region containing five DRu. As expected for *pho* regulon genes, inorganic phosphate, and also glycerol 3-phosphate, inhibited the expression from both *glpQ1* and *glpQ2*. The expression of *glpQ1* was also repressed by serine and inositol but expression of *glpQ2* was not. In contrast, glucose, fructose and glycerol increased expression of *glpQ2* but not that of *glpQ1*. In summary, our results suggest an interaction of phosphate control mediated by PhoP and carbon source regulation of the *glpQ1* and *glpQ2* genes involving complex operator structures.

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INTRODUCTION

Glycerophosphodiester metabolism has been described in organisms spanning the biological spectrum (from bacteria to humans), supporting the fundamental nature of this metabolism (Patton-Vogt, 2007). Glycerophosphodiesterases are formed by deacylation of phospholipids and differ according to their alcohol moiety; examples are glycerophosphoethanolamine, glycerophosphocholine, glycerophosphoinositol, glycerophosphoserine and glycerophosphoglycerol. These compounds are hydrolysed by glycerophosphodiester phosphodiesterases (GDPDs; EC 3.1.4.46), releasing *sn*-glycerol 3-phosphate (G3P) and the corresponding alcohol (Larson *et al.*, 1983).

The GDPD enzyme was first identified in *Escherichia coli* (Larson *et al.*, 1983). Two genes coding for GDPDs (*glpQ* and *ugpQ*) have been characterized in this organism. The

glpQ gene encodes a periplasmic enzyme of 333 amino acids while *ugpQ* encodes a cytosolic enzyme of 247 amino acids (Tomassen *et al.*, 1991). The *glpQ* gene forms part of the *glpTQ* operon, which belongs to the glycerol regulon and is induced by G3P (Larson *et al.*, 1983, 1987). The *ugpQ* gene forms part of the *ugpBAECQ* operon (Brzoska & Boos, 1988; Overduin *et al.*, 1988). This operon includes genes coding for an ABC transporter system and its expression is induced by the *pho* regulon (Brzoska & Boos, 1988).

In *Bacillus subtilis* there is only one gene coding for a GDPD (namely *glpQ*). This gene encodes a secreted GDPD and forms an operon with the *glpT* gene, which codes for a G3P permease. The *B. subtilis* *glpTQ* operon belongs to the glycerol regulon and is homologous to the *E. coli* *glpTQ* operon (Nilsson *et al.*, 1994). Under phosphate starvation *B. subtilis* *glpQ* is transcribed monocistronically from a PhoP-dependent promoter located upstream of the gene (Antelmann *et al.*, 2000).

In the *Streptomyces coelicolor* genome seven genes encoding putative GDPDs have been identified (Bentley *et al.*, 2002).

Abbreviations: CCR, carbon catabolite regulation; DRu, direct repeat unit(s); EMSA, electrophoretic mobility shift assay; G3P, *sn*-glycerol 3-phosphate; GDPD, glycerophosphodiester phosphodiesterase; GST, glutathione S-transferase; TSP, transcription start point.

Three of those genes (SCO1565, SCO1968 and SCO7550) have been predicted to encode secreted GDPDs and the other four (SCO1090, SCO1419, SCO3976 and SCO5661) cytoplasmic GDPDs. Moreover, the SCO1565 protein is exported out of the cell by the twin-arginine translocation (Tat) pathway, which is the major route of protein export in *S. coelicolor* (Widdick *et al.*, 2006). As occurs commonly with some other GDPD-encoding genes (Brzoska & Boos, 1988; Antelmann *et al.*, 2000; McLoughlin *et al.*, 2004; Schaaf & Bott, 2007), two of them (SCO1565 and SCO1968) are members of the *pho* regulon (Rodríguez-García *et al.*, 2007). The wealth of GDPD-encoding genes in *S. coelicolor* is intriguing and their roles in the metabolism of this bacterium need to be studied.

In this work we show that the regulation of two PhoP-dependent GDPD-encoding genes in *S. coelicolor* depends not only on the phosphate concentration in the medium but also on the concentration of carbon sources such as sugars, alcohols and amino acids. Additional information about the PhoP operator structure in *S. coelicolor* is provided, showing that the PhoP-dependent genes *glpQ1* and *glpQ2* contain class III complex operators that bind several PhoP molecules.

METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this work are listed in Table 1. *Streptomyces coelicolor* strains M145 (Kieser *et al.*, 2000) and INB201 (Δ *phoP*; Santos-Beneit *et al.*, 2009) were manipulated according to standard procedures (Kieser *et al.*, 2000). *E. coli* DH5 α was the general cloning host.

The *glpQ1* and *glpQ2* promoter regions were amplified by PCR using total DNA as template, as follows. The primers FSC19 (5'-TGTTGGATCCACACGGCCCGGATCGAAG-3') and FSC20 (5'-TGCGGTACCATATGGCCCTCCTGCCGTAGAC-3') amplified a 263 bp fragment encompassing the promoter region of *glpQ1* from the -247 to the -2 position (positions from the ATG translation start triplet). *Bam*HI (FSC19) and *Kpn*I/*Nde*I (FSC20) cloning sites (underlined) were introduced into the primer sequences. Primers FSC17 (5'-CCGGGATCCTCCCGGATTCTCCTGATG-3') and FSC18

(5'-CGCAGGTACCATATGTACTCCTCGCGTGAAC-3') were used to amplify a 274 bp fragment encompassing the promoter region of *glpQ2* from the -257 to the -1 position (positions from the translation start) flanked by the above-mentioned cloning sites. *glpQ1* and *glpQ2* *Bam*HI-*Kpn*I fragments were cloned into pBluescript II SK+, yielding pFS-*glpQ1* and pFS-*glpQ2*, respectively. The inserts of the plasmids were checked by sequencing. *Bam*HI-*Nde*I fragments from pFS-*glpQ1* and pFS-*glpQ2* were cloned into the promoter-probe vector pLUXAR-neo, yielding pLUX-*glpQ1* and pLUX-*glpQ2*, respectively.

Culture conditions. *S. coelicolor* cultures were performed in 100 ml defined MG-3.2 medium, containing 3.2 mM phosphate (Santos-Beneit *et al.*, 2008). The cultures were inoculated with 10^6 spores ml⁻¹ and grown in 500 ml baffled flasks (three replicates) at 30 °C and 300 r.p.m. For the time series cultures, samples were taken at 36 h, 40 h, 44 h, 48 h and 60 h of incubation.

In order to test the effect of adding different nutrients, the cultures were supplemented at 36 h with 15 mM of phosphate (KH₂PO₄+K₂HPO₄; Scharlau), phosphate plus glycerol (Merck), *rac*-glycerol 3-phosphate disodium salt hexahydrate (G2138, Sigma), L-serine (Sigma) or *myo*-inositol (Fluka); and with 2 % (w/v) of D-glucose (Merck), D-fructose (Sigma), maltose (Sigma) or glycerol (Merck). Samples were taken at 44 h and 48 h of incubation. In all cases three replicates were used.

Luciferase assay and growth determinations. The reporter luciferase activity was measured in a Luminoskan luminometer (Labsystems, Helsinki) as described by Rodríguez-García *et al.* (2007) and Santos-Beneit *et al.* (2008). For dry weight determination, culture samples of 2 ml were washed twice with MilliQ water and dried for 4 days at 80 °C.

DNase I footprinting assays. Non-radioactive DNase I footprinting assays of *glpQ1* and *glpQ2* promoters were performed using the pure GST-PhoP^{DBD} protein (Sola-Landa *et al.*, 2005) as described previously (Santos-Beneit *et al.*, 2009). Final protein concentrations were 1 μ M and 2 μ M for the *glpQ1* and *glpQ2* promoters, respectively. In the latter case, polyethylene glycol 1000 (PEG) was added at various concentrations to increase the effective protein concentration. DNA probes for both promoters were obtained by PCR using pFS-*glpQ1* and pFS-*glpQ2* as template. Electropherograms were analysed with PeakScanner v1.0 software (Applied Biosystems) to calculate the area under each fluorescence peak as well as the peak size. An Excel spreadsheet served to normalize peak areas and to calculate the degree of DNase I protection. Normalization factors for

Table 1. Bacterial strains and plasmids used in this work

Strain or plasmid	Characteristics	Origin
Strains		
<i>S. coelicolor</i> A3(2) M145	Wild-type	Kieser <i>et al.</i> (2000)
<i>S. coelicolor</i> A3(2) INB201	Δ <i>phoP</i> , Am ^r	Santos-Beneit <i>et al.</i> (2009)
<i>E. coli</i> DH5 α	F' ϕ 80 <i>dlacZ</i> Δ M15	Laboratory stock
<i>E. coli</i> ET12567(pUZ8002)	<i>dam dcm</i> mutant, Neo ^r Cm ^r	MacNeil <i>et al.</i> (1992)
Plasmids		
pBluescript II SK+	Cloning vector, Amp ^r	Promega
pFS- <i>glpQ1</i>	PCR product carrying <i>glpQ1</i> promoter cloned into pBSIISK+, Amp ^r	This work
pFS- <i>glpQ2</i>	PCR product carrying <i>glpQ2</i> promoter cloned into pBSIISK+, Amp ^r	This work
pLUXAR-neo	Cloning vector, Am ^r Neo ^r	Santos-Beneit <i>et al.</i> (2009)
pLUX- <i>glpQ1</i>	<i>Bam</i> HI- <i>Nde</i> I pFS- <i>glpQ1</i> fragment cloned into pLUXAR-neo, Am ^r Neo ^r	This work
pLUX- <i>glpQ2</i>	<i>Bam</i> HI- <i>Nde</i> I pFS- <i>glpQ2</i> fragment cloned into pLUXAR-neo, Am ^r Neo ^r	This work

each strand were obtained taking into account the regions shown in the respective figure, excluding the protected stretches, i.e. 50 nucleotides at both upstream and downstream sides.

Primer extension analysis. RNA samples were taken at 44 h from *S. coelicolor* M145 (containing pLUX-*glpQ1* or pLUX-*glpQ2*) cultures in MG-3.2 medium or in MG-3.2 supplemented with glucose or maltose. RNA was extracted with the RNeasy Midi kit (Qiagen). RNA concentration and quality were checked using a NanoDrop ND-1000 (Thermo Fisher Scientific) and a 2100 Bioanalyser (Agilent). Transcription start sites were determined as described by Santos-Beneit *et al.* (2008) using both LUX-FAM+47 and LUX-FAM+135 primers and Superscript III reverse transcriptase (Invitrogen). The primer extension reactions were incubated at 50 °C for 3 h.

Bioinformatic analysis. SCO1565 and SCO1968 orthologous genes were sought using the *Streptomyces* annotation server (<http://strepdb.streptomyces.org.uk>). For the analysis of the PhoP-binding sites, we calculated the individual information content (the R_i value; Schneider, 1997a) of each 11 nt direct repeat unit (DRu) using model 1 of the PhoP-binding site (an R_i matrix that gathers the information of 37 DRu that are part of operator cores; Sola-Landa *et al.*, 2008). This model also served to make the sequence walkers (Schneider, 1997b).

RESULTS

Analysis of the PhoP control of seven GDPD genes

There are seven genes encoding putative GDPDs in the *S. coelicolor* genome (Rodríguez-García *et al.*, 2007). All of these genes contain the Pfam motif characteristic of GDPDs. According to the presence or absence of signal peptide and transmembrane domains we propose that the genes SCO1565, SCO1968, and SCO7550 encode secreted proteins, and that the four other genes (SCO1090, SCO1419, SCO3976, and SCO5661) encode cytoplasmic proteins. Following the annotation of the *E. coli* GDPD, in this work, the three extracytoplasmic proteins were named GlpQ1, GlpQ2 and GlpQ3, respectively; and the four cytosolic ones UgpQ1, UgpQ2, UgpQ3 and UgpQ4, respectively.

Of these genes, only *glpQ1* (SCO1565) and *glpQ2* (SCO1968) showed a PhoP-dependent upregulated profile A0 (ascending transcription in the wild-type response to phosphate shift-down and null change in the Δ *phoP* mutant) in our previous transcriptomic analysis (Rodríguez-García *et al.*, 2007; Fig. 1). The binding of PhoP to these two promoter regions was demonstrated by electrophoretic mobility shift assay (EMSA) (Rodríguez-García *et al.*, 2007). Since the other five genes (SCO7550, SCO1090, SCO1419, SCO3976 and SCO5661) are not phosphate regulated and we did not find any PHO box in their promoter regions, we concluded that of the seven genes coding for GDPD in the *S. coelicolor* genome, only *glpQ1* and *glpQ2* belong to the *pho* regulon.

In order to confirm the PhoP-dependent expression of *glpQ1* and *glpQ2*, reporter studies were performed with the *S. coelicolor* wild-type and Δ *phoP* mutant strains grown in

Fig. 1. Transcriptomic profiles of the seven *S. coelicolor* GDPD-encoding genes in the wild-type and Δ *phoP* mutant backgrounds from the microarray studies of Rodríguez-García *et al.* (2007). The samples were taken from phosphate-replete MG cultures immediately before the shift-down (t_0), and 7.5 h after phosphate shift-down ($t_{7.5}$). (a) Fold changes were calculated from transcription values at t_0 (M_g values, see below). Grey lines, wild-type strain; black lines, Δ *phoP* mutant strain. Only *glpQ1* (SCO1565) and *glpQ2* (SCO1968) showed significant changes (A0 profiles). (b) M_g values (normalized $\log_2$ of Cy3/Cy5 intensities) and standard deviations of four biological replicates. The Cy3 signal corresponded to the labelled cDNA and the Cy5 signal to the labelled gDNA as the common reference.

MG-3.2 medium. This medium is limited in phosphate and thus is optimal for the expression of *pho* genes (Santos-Beneit *et al.*, 2008). As shown in Fig. 2, wild-type cultures yielded high levels of luciferase activity, while the Δ *phoP* mutant activities were almost null (less than 3% of the wild-type), indicating that the transcription of both genes is PhoP-dependent.

Luciferase activities of *glpQ1* and *glpQ2* promoters were very low during the rapid growth phase (Fig. 2; 36–40 h) and reached maximum values during the transition growth

Fig. 2. Promoter activity and growth of *S. coelicolor* exconjugants containing pLUX-*glpQ1* and pLUX-*glpQ2* in the wild-type and Δ *phoP* mutant backgrounds. Cultures were grown in MG-3.2 medium at 300 r.p.m. and 30 °C. Three replicates were used. Samples were taken at 36, 40, 44, 48 and 60 h of culture. In all cases, the black symbols represent the wild-type strain and the open symbols the Δ *phoP* mutant strain. The discontinuous lines represent the growth of the wild-type (●) and Δ *phoP* mutant (○). The solid lines represent the promoter activities of the exconjugants containing pLUX-*glpQ1* (●, ○) or pLUX-*glpQ2* (▼, ▽). Error bars correspond to the SD.

phase (44–48 h). When maximum promoter activities are compared, the *glpQ1p* activity is more than three times higher than that of *glpQ2p* (Fig. 2). The induction rates of both genes were highest at 44 h and very similar for both genes (fold ratios, referred to 40 h activities, of 8 and 6 for *glpQ1* and *glpQ2*, respectively). This pattern of expression correlates with the drop of inorganic phosphate (P_i) below 0.1 mM, which triggers the *pho* response, as is seen for the *pstS* gene (Rodríguez-García *et al.*, 2007; Santos-Beneit *et al.*, 2008).

Complex PhoP operator structures regulate *glpQ1* and *glpQ2* expression

In order to determine the nucleotide sequences where PhoP binds in the *glpQ1* and *glpQ2* promoters, footprinting studies were done using GST-PhoP^{DBD} as described in Methods. The *glpQ1* promoter was protected in a region located 50 nt upstream of the translation start codon (Fig. 3a, b). GST-PhoP^{DBD} protein at 1 μ M produced $63\% \pm 16\%$ protection (mean peak ratios and SD) against DNase I digestion in the coding strand, while in the complementary strand a stretch of 46 nt was protected to a higher extent ($64\% \pm 13\%$) than the following sequence of 15 nt ($34\% \pm 11\%$). This region was analysed by means of information theory tools (see Methods) to determine the structure of the direct repeat units (DRu) that are the 11 nt sequences bound by each PhoP monomer (Sola-Landa *et al.*, 2008). Four DRu were located from –93 to –49 with respect to the translation start codon and all showed positive R_i values, which indicate PhoP binding (Fig. 3c).

Although upstream of DRu-A there are 18 protected nucleotides in both strands, this sequence lacked any detected DRu. There is one nucleotide separating DRu-1 and DRu-A. This gap hampers the cooperative binding of PhoP monomers to DNA. Thus, DRu-A may function as an extension support DRu (E_S ; Sola-Landa *et al.*, 2008). Since we observed three retarded DNA–protein complexes in the EMSA analysis (Rodríguez-García *et al.*, 2007) and taking into account the R_i values of the four DRu, it is proposed that DRu-1 and DRu-2 form the core of the operator. The fastest-migrating complex probably involves the binding of two PhoP monomers to the core (DRu-1 and DRu-2). An additional PhoP monomer would bind DRu-3, named E for extension (Sola-Landa *et al.*, 2008), and would explain the second retarded complex. Finally, binding of DRu-A would result in the third complex observed in the EMSA analysis.

The GST-PhoP^{DBD} protein protected the *glpQ2* promoter from DNase I to a lesser extent than the *glpQ1* promoter because we needed to increase the protein concentration to 2 μ M to obtain similar protection. PEG was included in the reaction mixture to favour the protein–DNA binding. In the coding strand two protected stretches of 39 nt and 14 nt were intercalated with hypersensitive sites (Fig. 3d). This bipartite pattern was also found in the complementary strand, although a partially protected sequence, instead of hypersensitive sites, separated the two main protected stretches of 27 nt each (Fig. 3e). Protection was $55\% \pm 8\%$ in the coding strand, and $42\% \pm 11\%$ in the complementary strand. The bound region encompasses five DRu, which are located from positions –105 to –48 with respect to the translation start codon. Four of the five DRu have positive R_i values: DRu-A (1.3 bits), DRu-1 (2.5 bits), DRu-2 (3.7 bits), and DRu-4 (3.9 bits). DRu-3, with a negative R_i (–8.0 bits), coincided with the region of hypersensitive sites in the upper strand and low protection in the bottom strand (Fig. 3f). As occurs in the *glpQ1* promoter, DRu-A is not contiguous with DRu-1. According to the model of Sola-Landa *et al.* (2008), we propose that DRu-1 and DRu-2 would form the core of the operator; binding of PhoP to the poorly conserved DRu-3 of E_U type (unstable extension) would be facilitated by cooperative interaction between PhoP monomers bound to DRu-2 and DRu-4 (E_S). Thus, DRu-3 and DRu-4 would be bound at the same time in a second stage. The two retarded complexes detected previously (Rodríguez-García *et al.*, 2007) might correspond to two PhoP monomers bound to the core (DRu-1 and DRu-2) and to four PhoP monomers bound together to DRu-1, DRu-2, DRu-3 and DRu-4. However, further binding to DRu-A would imply the existence of a third complex of lower migration, which was not observed.

Orthologous genes of *glpQ1* and *glpQ2* were found in other sequenced *Streptomyces* genomes. The operator structures described here are depicted in Fig. 4. These structures are conserved in the orthologous counterparts with the exception of the *S. griseus glpQ1* gene (SGR5973), which has a different operator structure. According to the

Effect of inorganic phosphate, G3P, serine and inositol on *glpQ1* and *glpQ2* expression

et al., 1987; Nilsson *et al.*, 1994). The effect of adding inorganic phosphate (P_i) or P_i plus glycerol on the expression of both *glpQ* genes was also tested. Thus, 15 mM inorganic phosphate (or G3P) or 15 mM P_i plus 15 mM glycerol was added to 36 h cultures of *S. coelicolor* M145 in MG-3.2 medium. At this time cultures were in the fast growth phase and luciferase activities of both promoters were still below the detection level. Maximum activities from both promoters were detected at 44 h or 48 h in the unsupplemented cultures, before the beginning of the secondary growth phase (Fig. 2). Therefore, luciferase activities were measured 8 and 12 h after the

Fig. 3. DNase I footprinting of GST-PhoP^{DBD} binding to the *glpQ1p* coding (a) and complementary (b) strands, and to the *glpQ2p* coding (d) and complementary (e) strands. The upper fluorogram of each panel corresponds to the control reaction without protein and the lower one to the protected reaction (with 1 μ M protein concentration for *glpQ1* and 2 μ M for *glpQ2*). The correspondence between fluorescence peaks and nucleotide bases was determined using sequencing reactions. The shaded peak areas were used to calculate the degree of protection. The main protected sites are boxed and the secondary protected sites are indicated by asterisks. The vertical arrows indicate DNase I hypersensitive sites. The position with respect to the translation start codon is indicated under the nucleotide sequence. (c) and (f) Summary of GST-PhoP^{DBD} protection results for the *glpQ1* and *glpQ2* promoters, respectively. The sequence protected by GST-PhoP^{DBD} in each strand is overlined or underlined. The 11 nt DRu that form the PhoP operator are boxed and the R_i value of each DRu is indicated below.

(a)

Organism Gene	Individual information content (bit) and walkers*				Core†	Structure‡	Distance to -10	-10 element§	Distance to ATG
	DRu-A	DRu-1	DRu-2	DRu-3					
<i>S. coelicolor</i> <i>glpQ1</i> (SCO1565)	8.4 	3.8 	6.9 	2.1 	10.8	E _s [1]CCE	7	TACGAT	49
<i>S. avermitilis</i> SAV6784	7.2 	5.8 	6.6 	1.9 	12.3	E _s [1]CCE	7	TACGAT	58
<i>S. griseus</i> SGR5973	-0.6 	9.6 	-4.4 	6.5 	11.6	E _s [2]CCC	50	TACGAT	102
<i>S. scabies</i> SCAB74351	2.3 	2.6 	8.5 	5.2 	11.1	E _s [1]CCE	7	TACGAT	29

(b)

Organism Gene	Individual information content (bit) and walkers*					Core†	Structure‡	Distance to -10	-10 element§	Distance to ATG
	DRu-A	DRu-1	DRu-2	DRu-3	DRu-4					
<i>S. coelicolor</i> <i>glpQ2</i> (SCO1968)	1.3 	2.5 	3.7 	-8.0 	3.9 	6.3	E _s [2]CCE _u E _s	6	CACAGT	48
<i>S. avermitilis</i> SAV6271	-6.3 	-1.7 	1.2 	-5.6 	1.5 	-0.5	E _s [2]CCE _u E _s	6	CACTCT	48
<i>S. griseus</i> SGR5556	-5.7 	6.8 	3.0 	-1.4 	4.1 	9.8	E _s [2]CCE _u E _s	6	CACTCT	48
<i>S. scabies</i> SCAB69411	-4.2 	-1.4 	8.1 	-3.6 	7.6 	6.8	E _s [2]CCE _u E _s	6	CACTCT	87

Fig. 4. Individual information analysis of the PhoP-binding sites using the sequence walker method (Schneider, 1997b) and the model I weight matrix of Sola-Landa *et al.* (2008) for the *glpQ1* (a) and *glpQ2* (b) operators. * Boxes contain the individual information content (R_i , bits) of each 11 nt DRu. The height of the letters represents the R_i contribution of each position to the total information content. Letters extending downward represent unfavourable protein–DNA contacts (negative R_i values; a grey letter background indicates $R_i < -3$ bits; a black background indicates that the base is not found at that position in any of the 37 core DRu that are included in the model I matrix). The walker limits are 2 bits, which is also the top of the sine wave, and -3 bits at the bottom; 0 bits is at the middle of the walker. The sine wave depicts the accessibility of a face of the DNA double helix (10.6 bases of helical pitch) to a globular protein. The maximum R_i for each base is 2 bits when that base is contacted through the major groove (Schneider, 1996). Here the wave maximum is located at position 4 of each DRu. † The value comprises the sum of the R_i values of the DRu that form the core of the operator, which are indicated as 'C' in the operator structure. ‡ Each letter symbolizes the functional class of each DRu according to model I of Sola-Landa *et al.* (2008). Numbers in square brackets indicate the separation in base pairs between contiguous DRu. § The -10 element has been identified experimentally only for the *S. coelicolor glpQ1* and *glpQ2* genes.

addition (44 and 48 h of culture time). Compared to the unsupplemented cultures, *glpQ1* and *glpQ2* were barely expressed when P_i was added to the medium (down to 2 % and 1.5 % at 44–48 h, respectively, in *glpQ1*; down to 2.5 % and 9 % at 44–48 h in *glpQ2*; Fig. 5a, b). These results are in agreement with an expected PhoP-dependent activation. Both promoter activities were also severely inhibited by G3P (down to 27 % and 6 % at 44–48 h in *glpQ1*; down to 9 % and 17 % at 44–48 h in *glpQ2*) and by P_i plus glycerol (down to 13 % and 3 % at 44–48 h in *glpQ1*; down to 22 % and 16 % at 44–48 h in *glpQ2*). These results show that the effect of adding G3P is very similar to those of adding P_i plus glycerol or P_i alone, which strongly indicates that the G3P in the medium is hydrolysed by secreted phosphatases and used mainly as a phosphate source in *S. coelicolor*. The fact that the only two GDPDs activated by the *pho* regulon in this bacterium are secreted supports this hypothesis.

Phosphatidylinositol and phosphatidylserine are two of the major lipids of biological membranes (Divecha & Irvine,

1995). In addition to G3P, we also tested if other products GDPD hydrolysis inhibit *glpQ1p* and *glpQ2p* activities. Besides G3P, GDPD decomposes glycerophosphoserine and glycerophosphoinositol, yielding serine and inositol, respectively. To test the effect of these compounds 15 mM serine or inositol was added to 36 h cultures of *S. coelicolor* M145 in MG-3.2 medium, as above. The *glpQ1p* activities were reduced more than twofold when serine or inositol was added (Fig. 5a). In contrast, *glpQ2p* activities did not significantly change (Fig. 5b). In summary, serine and inositol exert a repressive effect on *glpQ1* but not on *glpQ2* expression.

Expression of the *glpQ1* and *glpQ2* *phoP*-dependent genes is also influenced by the carbon source

It is well known that expression of *pho* regulon genes depends on the P_i concentration in the medium, but to our knowledge the effect of carbon sources on the expression of these genes has not been investigated in *Streptomyces*, with the exception of the works of Díaz *et al.* (2005) and Esteban *et al.* (2008). A possible link between phosphate and carbon source regulation is of great interest.

The effects of different carbon sources on the promoter activity of the *glpQ1* and *glpQ2* genes were investigated. For this purpose glucose, fructose, maltose and glycerol were added to 36 h *S. coelicolor* M145 cultures in MG-3.2 medium at a final concentration of 2 % (w/v) and the promoter activity was measured at 44 h and 48 h. The addition of these carbon sources to the cultures at 36 h had no significant effect on the growth rates; in all cases the cultures were still at the transition growth phase when the promoter activity was determined and dry weights (44 h and 48 h) were similar (data not shown).

Of the four carbon sources added, only maltose produced a significant effect on *glpQ1p* (Fig. 6a). At 48 h the promoter activity of the maltose-supplemented culture was reduced to 45 % of the control. In contrast, *glpQ2p* activities were significantly increased when glucose, fructose or glycerol was added: the values of the reporter activities were twofold higher than those of maltose-supplemented or unsupplemented cultures (Fig. 6b). None of these carbon sources reversed the lack of expression from *glpQ2p* in the $\Delta phoP$ mutant background (data not shown). Thus, even in the presence of these inducing carbon sources the *glpQ2* gene depends on PhoP to be expressed. In other words, the positive effect of glucose, fructose and glycerol on *glpQ2* gene expression requires the presence of the PhoP activator protein and the effect of these carbon sources appears to be complementary to that of PhoP.

Characterization of the *glpQ1* and *glpQ2* promoters

The transcription start points (TSPs) of *glpQ1* and *glpQ2* were determined by primer extension using RNA samples

Fig. 5. Effect of the addition to the medium of phosphate, phosphate plus glycerol, G3P, serine and inositol on the promoter activity of *glpQ1* (a) and *glpQ2* (b). Cultures were grown in MG-3.2 medium at 300 r.p.m. and 30 °C and supplemented at 36 h with 15 mM P_i , P_i +glycerol, G3P, serine or inositol. Cultures were done in triplicate and samples were taken at 44 and 48 h of culture. The control cultures (no addition of any compound) represent the values at 44 and 48 h of *glpQ1* and *glpQ2* expression shown in Fig. 2. Error bars, SD.

Fig. 6. Effect of the addition to the medium of different carbon sources on the promoter activity of *glpQ1* (a) and *glpQ2* (b). Cultures were grown in MG-3.2 medium at 300 r.p.m. and 30 °C and supplemented at 36 h with 2% (w/v) maltose, glycerol, fructose or glucose. Cultures were done in triplicate and samples were taken at 44 and 48 h of culture. Control cultures were the same as in Fig. 5. Error bars, SD.

extracted from control MG-3.2 cultures at the time of maximum promoter activities (44 h). RNAs were also extracted from glucose- and maltose-supplemented cultures (as in the expression studies, glucose and maltose addition was done at 36 h and samples were taken at 44 h). As shown above, glucose induced the expression of *glpQ2*, while maltose had no effect. In contrast, *glpQ1* expression was unaffected by glucose, but repressed by maltose. Thus, it was investigated if the carbon regulation relies on the use of different TSPs.

Extension products of the *glpQ1p-luxAB* fusion gene with sizes of 74 nt and 164 nt, primed with LUX-FAM + 47 and LUX-FAM + 135, respectively, were clearly detected (Fig. 7a). When compared with the sequencing reactions, the same TSP was deduced from both primer extension reactions, which corresponded to a guanine located 32 bp upstream of the ATG codon (Fig. 7a). The same TSP was determined with the three sugar conditions (control, glucose or maltose) used. Although an extra signal of apparent size 68 nt was detected with the LUX-FAM + 47 primer in the maltose condition, we discarded the idea that

it represented a TSP because the corresponding product was not detected with the other primer.

The extension products of the *glpQ2p-luxAB* fusion gene were 74 nt and 164–165 nt in size for the LUX-FAM + 47 and LUX-FAM + 135 primers, respectively (Fig. 7b). A double peak of 164 and 165 nt (different in only one nucleotide) is observed with the LUX-FAM + 135 primer, and a single peak is detected with the LUX-FAM + 47 primer, suggesting the existence of a unique TSP. The 74 and 164 nt products correspond to a cytosine located 32 bp upstream of the ATG codon (Fig. 7b). The same TSP was determined using RNA samples from glucose- and maltose-supplemented cultures. We concluded that the glucose and maltose regulation of *glpQ1* and *glpQ2* genes is not executed from different promoters.

To identify promoter elements based on the identified TSPs, we used the frequency matrices of Strohl (1992), which include 29 streptomyces promoters that are recognized by the major sigma subunit (i.e. vegetative promoters). Clear sequences were found for the –10 element (Fig. 7), but both the *glpQ1* and *glpQ2* promoters lacked obvious –35 elements.

DISCUSSION

Streptomyces species constitute some of the most proficient producers of naturally occurring therapeutic molecules such as antibiotics, immunosuppressants and antitumour agents (Von Döhren & Gräfe, 1997; Challis & Hopwood, 2003). The regulation of biosynthesis of these compounds is influenced by phosphate, carbon and nitrogen sources (Martin & Demain, 1980; Doull & Vining, 1989). However, very little is known about the interaction between the metabolic pathways of these nutrients in *Streptomyces*.

There are some articles describing a cross-regulation between the carbon and phosphate sources (Wanner *et al.*, 1988; Sage & Vasil, 1997; Díaz *et al.*, 2005; Oh *et al.*, 2007), but only a few of them describe this regulation at the molecular level (Kasahara *et al.*, 1991; Puri-Taneja *et al.*, 2006; Esteban *et al.*, 2008). The involvement of carbon catabolite regulation (CCR) in the expression of the *phoPR* operon of *B. subtilis* provides an interesting link between the carbon regulation and the *pho* regulon (Puri-Taneja *et al.*, 2006). This operon is repressed by direct binding of CcpA (a transcriptional regulator mediating CCR) to a *cre* box consensus sequence localized upstream of the PhoP operator. On the other hand, the *ugp* operon of *E. coli* is induced under both phosphate- and carbon-limiting conditions from two separate promoters. The downstream promoter has multiple copies of the *E. coli* consensus PHO box and the upstream promoter has a consensus sequence for the promoters activated by the cAMP–CRP complex, which is formed only under starvation of preferred carbon sources, such as glucose (Kasahara *et al.*, 1991).

In *S. coelicolor* CCR is independent of the phosphotransferase system, which plays a central role in CCR in other

(a) *glpQ1* **(b) *glpQ2***

Fig. 7. Primer extension analysis of *glpQ1* (a) and *glpQ2* (b) promoters using fluorescent capillary electrophoresis. Primer extension reactions were done with both the LUX-FAM + 47 and LUX-FAM + 135 primers. In the upper fluorograms, the black-filled peaks represent the extension products (FAM-labelled cDNA) and the unfilled grey traces represent the LIZ-500 standards that were included in each sample. Standard sizes are marked on the upper axis, and the product sizes above the corresponding peak. The expanded lower fluorograms correspond to the four sequencing reactions (A, C, G, T) obtained with the Thermo Sequenase kit using the respective primers. Size standards were also included and the apparent molecular sizes were determined using GeneMapper software. The nucleotide corresponding to the extension fragment is indicated in bold. The box below summarizes the promoter elements of each gene. The translation start triplet is in boldface. The TSP (+1) is indicated with a bigger letter in bold and with an arrow above. The -10 element is shown in bold and underlined. The DRU that form the PhoP operator are boxed. The putative ribosome-binding site (RBS) is boxed.

bacteria (van Wezel *et al.*, 2007). Instead, glucose kinase (GlcA) is the key player of CCR (Angell *et al.*, 1994). In this work we have investigated the carbon regulation of two *pho* genes (*glpQ1* and *glpQ2*). Both genes are regulated by the carbon source but in a different manner. Thus, carbon sources exerting CCR in *S. coelicolor* such as glucose, fructose and glycerol (Kwakman & Postma, 1994) induce the expression of *glpQ2* but not that of *glpQ1*. However, *glpQ1* expression is reduced when maltose, serine or inositol is added to the medium, while *glpQ2* expression is not affected. A similar induction by high concentrations of certain sugars has been described for the *Streptomyces lividans* *pstS* gene (Díaz *et al.*, 2005; Esteban *et al.*, 2008).

Glycerophosphodiesterases are used as phosphate or carbon sources according to the requirements of the cell (Brzoska & Boos, 1989). In this work, we have studied the effect of different phosphate and carbon sources on the expression of two GDPD-encoding genes. The *E. coli* *ugpQ* and *B. subtilis* *glpQ* genes, also encoding GDPDs, are regulated by the carbon and phosphate sources from different promoters (Kasahara *et al.*, 1991; Antelmann *et al.*, 2000). Our primer extension analyses showed that phosphate and carbon regulation of *S. coelicolor* *glpQ1* and *glpQ2* genes is not executed from different promoters. Both genes are regulated by PhoP in a similar manner but they are subject to a different control by the carbon source, so these two *glpQ* genes appeared to be under the control of additional regulatory systems which may be specific for the expression of each gene.

Recently, the structure of the direct repeat units (DRu) in the DNA-binding sequences of the response regulator PhoP in *S. coelicolor* has been reported (model I; Apel *et al.*, 2007; Sola-Landa *et al.*, 2008). According to this model the PhoP operator structures are divided into three classes: class I (simple operators with two well-conserved DRu), class II (operators with three conserved DRu) and class III (operators of complex structure). In this study using DNase I footprinting experiments we have defined two new PhoP-binding sites, corresponding to the *glpQ1* and *glpQ2* genes. Both *glpQ1* and *glpQ2* have multiple DRu and belong to the class III PhoP operators. The structures of these operators are slightly different; however, the distances to the -10 element, TSP and translation start triplet are similar, suggesting a common mechanism of activation by PhoP. In fact, two identical features – (i) overlapping of the PhoP-binding sites with the -35 region and (ii) the exact location of the -10 element and TSP with respect to the translation start triplet – were found in the *glpQ1* and *glpQ2* promoters and also in that of *pitH2*, another *pho* regulon gene encoding a low-affinity phosphate transporter (Santos-Beneit *et al.*, 2008).

Bacterial GDPDs display broad catalytic activity on glycerophosphodiesterases (Larson *et al.*, 1983). This study shows that inositol and serine affect *glpQ1* expression but not that of *glpQ2*, so it could be hypothesized that GlpQ1 has more hydrolytic activity for glycerophosphoinositol

and glycerophosphoserine than GlpQ2. In contrast, the expression of *glpQ2* is significantly increased when preferred carbon sources such as glucose, fructose or glycerol are added to the medium. This could indicate a role for GlpQ2 in the link between carbon and phosphate assimilation. Interestingly, seven mammalian GDPDs homologous to bacterial GDPDs have been identified (Zheng *et al.*, 2003). Mammalian GDPDs are involved not only in phospholipid metabolism, but also in numerous physiological functions, such as signal transduction, cytoskeleton regulation and motor neuron differentiation (Zheng *et al.*, 2003; Nogusa *et al.*, 2004; Rao & Sockanathan, 2005). In view of the variety of GDPDs in *Streptomyces*, it will be interesting to determine if some of them (e.g. GlpQ2) also play roles in signal transduction.

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