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

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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). 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.

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 phospholipid 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 (Tommassen *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 monocistrionically 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).
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 GPDP-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 operon 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 (ΔphoP, Santos-Beneit et al., 2009) were manipulated according to standard procedures (Kieser et al., 2000). *E. coli* DH5α 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’-TGGATCCACACGGCCCGGATCGAAG-3’) and FSC20 (5’-GGTACCATATGTACTCCTCGCGTCGAAC-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). BamHI (FSC19) and KpnI/NdeI (FSC20) cloning sites (underlined) were introduced into the primer sequences. Primers FSC17 (5’-CCGGGATCCCTCGCGATTCTCCTGATG-3’) and FSC18 (5’-CCGAGGTACCATATGACTCCTCGCGTGAAC-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 BamHI–KpnI fragments were cloned into pBluescript II SK+ , yielding pFS-glpQ1 and pFS-glpQ2, respectively. The inserts of the plasmids were checked by sequencing. BamHI–NdeI 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 10⁶ 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 + KH₂PO₄; 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-PhoPDBD protein (Sola-Landa et al., 2005) as described previously (Santos-Beneit et al., 2009). Final protein concentrations were 1 μM and 2 μM 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. Electrophorograms 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**

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<th>Strain or plasmid</th>
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<td><strong>Strains</strong></td>
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<td>Santos-Beneit et al. (2009)</td>
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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 RNasey Midi kit (Qiagen). RNA concentration and quality were checked using a NanoDrop ND-1000 (Thermo Fisher Scientific) and a 2100 Bioanalyzer (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://strepdbs.streptomyces.org.uk). For the analysis of the PhoP-binding sites, we calculated the individual information content (the Ri value; Schneider, 1997a) of each 11 nt direct repeat unit (DRu) using model 1 of the PhoP-binding site (an R 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 (Rodriguez-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 cytoplasmic proteins, and that the four other genes (SCO1090, SCO1419, SCO3976, and SCO5661) encode cytosolic 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 ΔphoP mutant) in our previous transcriptomic analysis (Rodriguez-García et al., 2007; Fig. 1). The binding of PhoP to these two promoter regions was demonstrated by electrophoretic mobility shift assay (EMSA) (Rodriguez-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 ΔphoP mutant strains grown in 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 ΔphoP 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**

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**Fig. 1.** Transcriptomic profiles of the seven *S. coelicolor* GDPD-encoding genes in the wild-type and ΔphoP mutant backgrounds from the microarray studies of Rodriguez-García et al. (2007). The samples were taken from phosphate-replete MG cultures immediately before the shiftdown (*t₀*), and 7.5 h after phosphate shiftdown (*t₁₅₅*). (a) Fold changes were calculated from transcription values at *t₀* (*Mₙ* values, see below). Grey lines, wild-type strain; black lines, ΔphoP mutant strain. Only glpQ1 (SCO1565) and glpQ2 (SCO1968) showed significant changes (*A₀* profiles). (b) *Mₙ* values (normalized log₂ of Cy3/Cy5 intensities) and standard deviations of four biological replicates. The Cy5 signal corresponded to the labelled cDNA and the Cy3 signal to the labelled gDNA as the common reference.
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 are 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 (Pi) below 0.1 mM, which triggers the pho response, as is seen for the phoS gene (Rodriguez-Garcia 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<sup>DBD</sup> 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<sup>DBD</sup> protein at 1 μM produced 63 % ± 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 % ± 13 %) than the following sequence of 15 nt (34 % ± 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<sub>S</sub>; Sola-Landa et al., 2008). Since we observed three retarded DNA–protein complexes in the EMSA analysis (Rodriguez-Garcia 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<sup>DBD</sup> 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 μM 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 % ± 8 % in the coding strand, and 42 % ± 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<sub>S</sub> type (unstable extension) would be facilitated by cooperative interaction between PhoP monomers bound to DRu-2 and DRu-4 (E<sub>S</sub>). Thus, DRu-3 and DRu-4 would be bound at the same time in a second stage. The two retarded complexes detected previously (Rodriguez-Garcia 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
classification of Sola-Landa et al. (2008) these operators belong to class III (operators of complex structure).

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

GPDP hydrolyses glycerophosphodiester into G3P and the corresponding alcohol (Larson et al., 1983). We tested the effect of adding G3P on glpQ1 and glpQ2 expression because G3P is the final product in all glycerophosphodiester hydrolysis reactions. Moreover G3P induces the expression of both *E. coli* and *B. subtilis* glpQ genes (Larson 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<sup>DBD</sup> binding to the glpQ1<sub>p</sub> coding (a) and complementary (b) strands, and to the glpQ2<sub>p</sub> 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 μM protein concentration for glpQ1 and 2 μM 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<sup>DBD</sup> protection results for the glpQ1 and glpQ2 promoters, respectively. The sequence protected by GST-PhoP<sup>DBD</sup> 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.
**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 DRus that are included in the model I matrix). The walker limits are 2 bits, which is also the top of the sine wave, and $\sim3$ 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 DRus 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 DRus. § 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\textsubscript{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\textsubscript{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\textsubscript{i} plus glycerol or P\textsubscript{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\textsubscript{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 Diaz 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 ΔphoP 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

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**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\textsubscript{i}, P\textsubscript{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.
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 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 streptomycete 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; Diaz 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...
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 (GkA) 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 (Diaz 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.

REFERENCES


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