Phosphate-dependent regulation of the low- and high-affinity transport systems in the model actinomycete *Streptomyces coelicolor*

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The transport of inorganic phosphate (P_i) is essential for the growth of all organisms. The metabolism of soil-dwelling *Streptomyces* species, and their ability to produce antibiotics and other secondary metabolites, are strongly influenced by the availability of phosphate. The transcriptional regulation of the SCO4138 and SCO1845 genes of *Streptomyces coelicolor* was studied. These genes encode the two putative low-affinity P_i transporters PitH1 and PitH2, respectively. Expression of these genes and that of the high-affinity transport system *pstSCAB* follows a sequential pattern in response to phosphate deprivation, as shown by coupling their promoters to a luciferase reporter gene. Expression of *pitH2*, but not that of *pap-pitH1* (a bicistronic transcript), is dependent upon the response regulator PhoP. PhoP binds to specific sequences consisting of direct repeats of 11 nt in the promoter of *pitH2*, but does not bind to the *pap-pitH1* promoter, which lacks these direct repeats for PhoP recognition. The transcription start point of the *pitH2* promoter was identified by primer extension analyses, and the structure of the regulatory sequences in the PhoP-protected DNA region was established. It consists of four central direct repeats flanked by two other less conserved repeats. A model for PhoP regulation of this promoter is proposed based on the four promoter DNA–PhoP complexes detected by electrophoretic mobility shift assays and footprinting studies.

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INTRODUCTION

Streptomyces are soil-dwelling Gram-positive filamentous bacteria with complex regulatory networks that allow their adaptation to changing nutritional conditions (Chater & Bibb, 1997). *Streptomyces* species have a central role in soil ecology processes, and produce an impressive array of secondary metabolites with interesting biological properties and pharmacological activities (Bérdy, 2005). There are hundreds of genes in the sequenced genomes of *Streptomyces* that encode enzymes for the biosynthesis of secondary metabolites (Omura *et al.*, 2001; Bentley *et al.*, 2002). The biosynthesis of most secondary metabolites is negatively regulated by phosphate (Martín *et al.*, 1994).

Phosphorus is an essential component of bacterial nutrition and is one of the major constituents of the cell, making up ~1.5–2.1 % of the cell dry weight. Phosphorus plays an important role in cell metabolism and is a

constituent of nucleic acids, phospholipids, phosphate-containing lipopolysaccharides, nucleotide cofactors and some post-translationally modified proteins.

Expression of phosphate-regulated genes in *Streptomyces* species is modulated by the two-component system PhoR–PhoP (Sola-Landa *et al.*, 2003). PhoR is the membrane sensor kinase, and senses phosphate scarcity; PhoP is the response regulator, and binds DNA and controls the transcription of genes belonging to the so-called PHO regulon. PhoP has been shown to modulate the expression of primary and secondary metabolism genes, including the actinorhodin and undecylprodigiosin biosynthesis genes (Sola-Landa *et al.*, 2003; Ghorbel *et al.*, 2006). Binding of PhoP to the promoter regions of three different genes of the PHO regulon, *pstS*, *phoU* and *phoRP*, has been shown in both *Streptomyces coelicolor* (Sola-Landa *et al.*, 2005) and *Streptomyces natalensis* (Mendes *et al.*, 2007). The PhoP operator sequences of these genes, as well as those present in the promoter regions of *phoA* and *phoD* of *S. coelicolor* (Apel *et al.*, 2007), are composed of direct repeat units (DRus) of 11 nt. Novel operator sequences have been

Abbreviations: DRu, direct repeat unit; EMSA, electrophoretic mobility shift assay; R_i, information content; TMS, transmembrane segment; TSP, transcription start point.

recently described in *S. coelicolor* (Sola-Landa *et al.*, 2008). Analysis of the novel and the previously known operators (up to 19) by means of electrophoretic mobility shift assays (EMSAs), footprinting, and information theory studies, has revealed the structure of the PhoP-binding sites. Two or three well-conserved DRus form the core of the binding site, in which each DRu is bound by a protein monomer. Complex sites have adjacent DRus that are bound subsequently (Sola-Landa *et al.*, 2008).

The transport of phosphorus sources is essential for the growth of all living organisms. The preferred source of phosphorus in bacteria is inorganic phosphate (P_i), which can enter the cell with the aid of at least two different transport systems (as described below), although there are other phosphorus-containing compounds that can enter the cell intact, such as organophosphates and phosphonates. However, numerous organic phosphate compounds have to be hydrolysed before being transported into the cell, such as nucleotides, some sugar phosphates, and phospholipids (Martin & Demain, 1977, 1980). *S. coelicolor* and other *Streptomyces* species contain at least three phosphatases and a phosphodiesterase system (Apel *et al.*, 2007) to hydrolyse different organic phosphates.

P_i is taken up in bacteria mainly by two different transport systems, the high-affinity phosphate-specific transporter (Pst) and the low-affinity phosphate inorganic transporter (Pit). Pst is an ABC transporter that has an ATP-driven high-affinity P_i uptake. In *Escherichia coli* this transporter is composed of four proteins. These are the PstS (periplasmic phosphate-binding), PstA and PstC (integral membrane proteins), and PstB (ATP-binding) subunits. The *pstSCAB* operon belongs to the PHO regulon and has been identified in *E. coli* and in other bacterial species (Rao & Torriani, 1990; Nikata *et al.*, 1996). The *pstS* gene has also been studied in *S. coelicolor* (Sola-Landa *et al.*, 2005; Díaz *et al.*, 2005), in which its expression is subject to a strict phosphate control.

Pit is a low-affinity, high-velocity phosphate-uptake system and is dependent on the proton motive force for energy. The Pit transport system has been studied in *E. coli* (Hoffer *et al.*, 2001; Harris *et al.*, 2001; van Veen *et al.*, 1994a; van Veen, 1997), *Acinetobacter johnsonii* (van Veen *et al.*, 1993, 1994b, c; van Veen, 1997) and *Sinorhizobium meliloti* (Voegelé *et al.*, 1997; Bardin & Finan, 1998; Bardin *et al.*, 1998; Yuan *et al.*, 2006a). In *E. coli*, Pit is the major P_i uptake system when P_i is in excess (Rosenberg *et al.*, 1977, 1979) and consists of a single transmembrane protein (Elvin *et al.*, 1986). Divalent cations such Mg^{2+} and Ca^{2+} are essential for Pit activity in both *E. coli* (Russell & Rosenberg, 1980) and *A. johnsonii* (van Veen *et al.*, 1994b), since phosphate is symported with a proton, as a soluble and neutral metal complex ($MeHPO_4$; van Veen *et al.*, 1994a, c; van Veen, 1997).

E. coli contains two functional *pit* genes, *pitA* and *pitB* (Hoffer *et al.*, 2001; Harris *et al.*, 2001). Two or more paralogues can also be found in many other genomes. PitA

and PitB have similar transport characteristics (Hoffer *et al.*, 2001) and have 90% similar amino acid sequences. However, the regulation of the two genes appears to be different. Thus, *pitA* is expressed constitutively, while *pitB* appears to be repressed under conditions of phosphate limitation (Elvin *et al.*, 1986; Harris *et al.*, 2001).

The *pit* gene(s) have not been studied in *Streptomyces* species, but their characterization is important to establish their role in phosphate transport and their effect on the biosynthesis of secondary metabolites and on the ecology of these bacteria in soil. This work describes two paralogous *pit* genes in *S. coelicolor*, and elucidates their pattern of regulation by means of both *in vivo* assays, using wild-type *S. coelicolor* and Δ *phoP* mutant strains, and DNA-binding PhoP-footprinting analysis.

METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this work are listed in Table 1. *S. coelicolor* strains M145 (Kieser *et al.*, 2000) and INB101 (Δ *phoP*) (Rodríguez-García *et al.*, 2007) were manipulated according to standard procedures (Kieser *et al.*, 2000). TBO medium (Higgins *et al.*, 1974) was used to obtain spore preparations. *E. coli* DH5 α was the general cloning host. Cloning procedures were performed as described by Sambrook *et al.* (1989).

The *pap-pitH1* and *pitH2* promoter regions were amplified by PCR using total DNA as template, as follows. The primers FSC21 (5'-TGGTGGATCCCGCGTCCTCCAATAACGG) and FSC22 (5'-CAGAGGTACCATATGGTGGGGTCTCGGGGTGCATGGGTTC) amplified a 232 bp fragment encompassing the promoter region of *pap-pitH1* from -210 to -3 (positions from the ATG translation start triplet). The *Bam*HI (FSC21) and *Kpn*I/*Nde*I (FSC22) cloning sites (underlined) were introduced via the primer sequences. Primers FSC15 (5'-CTTCGGATCCTCCGGCCCGCAATAC) and FSC16 (5'-GAGAGGTACCATATGTACCCAGGCAATCGTTTCG) were used to amplify a 270 bp fragment encompassing the promoter region of *pitH2* from positions -250 to -3 (positions from the translation start) flanked by the above-mentioned cloning sites. *Bam*HI-*Kpn*I fragments were cloned into pBluescript II SK+ to obtain pFS-*pitH1* and pFS-*pitH2*, respectively. The inserts of both plasmids were checked by sequencing.

The *pstS* promoter region was cloned from pGEM-P*pstS* (Sola-Landa *et al.*, 2005). *Bam*HI-*Nde*I fragments of 222 bp from pFS-*pitH1*, 260 bp from pFS-*pitH2*, and 403 bp from pGEM-P*pstS* were cloned into the promoter-probe vector pLUXAR+ (Rodríguez-García *et al.*, 2007), yielding pLUX-*pitH1*, pLUX-*pitH2* and pLUX-*pstS*, respectively. In order to introduce the pLUX plasmids into the *S. coelicolor* INB101 strain (which is apramycin resistant), the *neo* gene was inserted to construct derivatives with neomycin resistance (Table 1).

Culture conditions. *S. coelicolor* cultures were performed in defined MG (maltose and glutamate) medium containing starch (Scharlau; 50 g l⁻¹) and glutamate (60 mM) (Doull & Vining, 1989). Aliquots (100 ml) of MG medium in 500 ml baffled flasks were inoculated with 10⁶ spores ml⁻¹ and incubated at 30 °C, 300 r.p.m. (25.4 mm orbit diameter) for reproducible and dispersed growth. For the phosphate-replete and the phosphate-limited conditions, cultures contained 18.5 and 3.2 mM potassium phosphate, respectively (MG-18.5 and MG-3.2 media; experimentally determined concentrations). The potassium concentrations in MG-18.5 and MG-3.2 were equalized by adding KOH to MG-3.2, instead of NaOH alone, when

Table 1. Bacterial strains and plasmids used in this work

Strain or plasmid	Characteristics	Reference or source
Strains		
<i>S. coelicolor</i> A3(2) M145	Wild-type	Kieser <i>et al.</i> (2000)
<i>S. coelicolor</i> A3(2) INB101	Δ <i>phoP</i> , Am ^r	Rodríguez-García <i>et al.</i> (2007)
<i>E. coli</i> DH5 α	F' Φ 80 <i>dLacZ</i> Δ M15	Hanahan (1983)
<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	Stratagene
pFS- <i>pitH1</i>	PCR product carrying <i>pitH1</i> promoter cloned into pBluescript II SK +, Amp ^r	This work
pFS- <i>pitH2</i>	PCR product carrying <i>pitH2</i> promoter cloned into pBluescript II SK +, Amp ^r	This work
pLUXAR +	Integrative promoter-probe vector, <i>luxAB</i> genes, Am ^r	Rodríguez-García <i>et al.</i> (2007)
pLUX- <i>pitH1</i>	<i>Bam</i> HI- <i>Nde</i> I pFS- <i>pitH1</i> fragment cloned into pLUXAR +, Am ^r	This work
pLUX- <i>pitH2</i>	<i>Bam</i> HI- <i>Nde</i> I pFS- <i>pitH2</i> fragment cloned into pLUXAR +, Am ^r	This work
pLUX- <i>pstS</i>	<i>Bam</i> HI- <i>Nde</i> I pGEM-P <i>pstS</i> fragment cloned into pLUXAR +, Am ^r	This work
pLUX- <i>pitH1</i> -Neo	pLUX- <i>pitH1</i> with the <i>neo</i> gene inserted, Am ^r -Neo ^r	This work
pLUX- <i>pitH2</i> -Neo	pLUX- <i>pitH2</i> with the <i>neo</i> gene inserted, Am ^r -Neo ^r	This work

the pH was adjusted (sodium ions in both media are in excess, ~60–75 mM). Samples were taken after 36, 40, 44, 48, 60, 70 and 90 h of incubation.

Luciferase assay, and growth and phosphate determination.

luxAB gene expression was determined in a Luminoskan luminometer (Labsystems). Culture samples (1 ml) were taken, spun down (4 °C, 10 min) and kept frozen until all samples from an experiment were available to be processed simultaneously; then the cells were resuspended in 1 ml NaCl (0.9%, w/v), incubated at 25 °C for 15 min and measured in triplicate, as described in Rodríguez-García *et al.* (2007).

For dry weight determination, culture samples (2 ml) were washed twice with MilliQ water and dried for 4 days at 80 °C.

The phosphate concentration of MG medium and culture supernatants was measured using the malachite green assay (Lanzetta *et al.*, 1979).

RT-PCR. RNAs from *S. coelicolor* wild-type and Δ *phoP* strains were isolated using RNeasy Mini Spin columns (Qiagen). Before RNA isolation, one volume of each culture was treated with two volumes of RNAProtect Bacteria reagent (Qiagen) to provide immediate stabilization of RNA. Cell lysis and phenol pre-purification were carried out prior to column purification as described previously (Rodríguez-García *et al.*, 2007). RNA preparations were treated on-column with DNase I (Qiagen), and the eluted solution with the DNA-free kit (Ambion) to eliminate possible chromosomal DNA contamination. RNA concentration and quality were checked using a NanoDrop ND-1000 (Thermo Fisher Scientific) and RNA nano labchips in a 2100 Bioanalyser (Agilent).

Gene expression analysis by RT-PCR was performed with the SuperScript One-Step RT-PCR system with Platinum *Taq* (Invitrogen). Primers CAR65 (5'-ATTTACGAACGGTTTCCAC) and CAR66 (5'-GATGCCCATGGTCTTCTGG) were used to amplify 542 bp of the *pitH2* coding region; primers CAR67 (5'-CGTACACCAACGGTTTCCAC) and CAR68 (5'-ATGCCCATCGTCTTCTGC) were used to amplify 541 bp of *pitH1*. The synthesis of a common transcript *pap-pitH1* was detected with the primer pair CAR73 (5'-ACCGCGAGGACATCTACAAC) and CAR74 (5'-ACCAGGTGATGAGGTTCCAG). RT-PCR cycling conditions were

as follows: 50 °C for 60 min, 94 °C for 2 min; 11 cycles of 94 °C for 30 s, 65 °C for 30 s with touchdown of 1 °C per cycle, 72 °C for 60 s; 23–29 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 40 s; finally 72 °C for 10 min. Total RNA (200 ng), 1.6 mM MgSO₄ (final concentration), and 5 % DMSO were added in all reactions. Platinum *Taq* was used in the control reactions to check for the absence of DNA contamination.

DNA-protein binding assays. DNA-binding analyses were performed by EMSA with the GST-PhoP^{DBD} protein (PhoP DNA-binding domain fused to glutathione-S-transferase), as described previously (Sola-Landa *et al.*, 2005). The probes used were the *Bam*HI-*Kpn*I fragments of 222 and 260 bp obtained by PCR of the promoter regions of *pap-pitH1* and *pitH2* and purified after agarose-gel electrophoresis (GFX columns; GE Healthcare). Probes were labelled at both ends with DIG (DIG Oligonucleotide 3'-End Labeling kit, Roche Applied Science).

DNase I footprinting assays. DNase I footprinting assays based on the fluorescent label procedure (Rodríguez-García *et al.*, 1997) were performed as described in Sola-Landa *et al.* (2005). DNA probes were obtained by PCR using pFS-*pitH2* as template. For analysis of the coding strand, a fluorescently-labelled universal primer and a non-labelled reverse primer were used; for the complementary strand, the labelled primer was the reverse primer. The labelled probes (424 and 419 bp for the coding and complementary strand, respectively) were purified by agarose electrophoresis (GFX columns). DNase I footprinting was performed by incubating 0.28 pmol of the DNA probe with different concentrations of GST-PhoP^{DBD} protein for 30 min at 30 °C. Nuclease digestions were carried out for 1 min at 30 °C. The reaction products were resolved in an ALF DNA sequencer (GE Healthcare) and analysed with the Fragment Manager program.

Primer extension analysis. For primer extension analysis, RNA samples were isolated as described above, but with the RNeasy Midi kit (Qiagen) and the on-column DNase treatment only. Total RNA concentration and quality were checked both spectrophotometrically and by denaturing agarose gel electrophoresis. Transcription start sites were determined by the fluorescent primer extension procedure (Altermann *et al.*, 1999; Fekete *et al.*, 2003), modified as follows. Primers LUX-FAM + 47 [6-carboxyfluorescein- (6-FAM) 5'-

GATAGCTCAGGTGGCTGATAAG] and LUX-FAM+135 (6-FAM 5'-GTGGTGCTCTAGCAACCAAAC), both obtained from MWG-Biotech, were complementary to the coding region of *luxAB* genes of the pLUXAR+ vector (nucleotides +25 to +47 and +114 to +135 with respect to the translation start, respectively). These primers (20–40 pmol) were hybridized with total RNA (20–30 µg) in a 10 µl final volume by heating to 90 °C for 2 min and cooling to 30 °C at a rate of 2 °C min⁻¹. Then, cDNA synthesis was performed with either Superscript II or Superscript III reverse transcriptase (Invitrogen). The primer extension reaction (30 µl) contained 10 mM DTT, 500 µM dNTPs (NEB), 1.5 µg actinomycin D, 10 U SUPERase-In (RNase inhibitor; Ambion), and 200 U reverse transcriptase in 1 × First Strand buffer (Invitrogen). The samples were incubated at 42 °C for 5 or 15 h. Then, 10 µl NaOH (1 M) was added and the RNA was lysed by heat treatment (70 °C, 10 min). A 10 µl volume of HCl (1 M) was added to neutralize the solution; the cDNA samples were phenolized and precipitated with ethanol. The pellets were dissolved in a solution of 9.6 µl Hi-Di formamide (Applied Biosystems) and 0.4 µl GeneScan LIZ-500 internal size standard (Applied Biosystems). Samples were heated to 95 °C for 5 min, placed immediately on ice for 5 min, and loaded onto an ABI PRISM 3130 sequencer (Applied Biosystems). To determine the product size accurately, sequencing reactions were performed with the same primer as the reverse transcription step and with the Thermo Sequenase Primer Cycle Sequencing kit (GE Healthcare). Reactions contained 400 ng plasmid DNA as template, and 2 pmol of the corresponding primer. Cycling conditions were 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 60 s, for a total of 60 cycles. The EDTA/ethanol-precipitated products of the four sequencing reactions were combined with the GeneScan LIZ-500 size standard and analysed on the DNA sequencer. Electrophoretograms were aligned according to the size standards using the GeneMapper 3.7 software (Applied Biosystems).

Bioinformatic analysis. The phylogenetic studies were done using the BLAST tree option on the NCBI Web BLAST service with the Fast Minimum Evolution method (Desper & Gascuel, 2002) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Kanehisa *et al.*, 2006). Comparative genomics was done using the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING; von Mering *et al.*, 2007). The percentages of sequence similarity between two proteins were calculated using the Needleman-Wunsch global alignment of the European Molecular Biology Open Software Suite (EMBOSS; Rice *et al.*, 2000). The putative transmembrane segments (TMSs) were determined using the TopPred server (Claros & von Heijne, 1994) and the TMPred server (Hofmann & Stoffel, 1993). Prediction of signal peptides was done with the SignalP server (Bendtsen *et al.*, 2004).

To identify and analyse putative binding sites of the response regulator PhoP, the information theory programs delila, makebk, encode, rseq, dalvec, ri, scan and lister (Schneider & Stephens, 1990; Schneider, 1996, 1997) were used. Model 1 of Sola-Landa *et al.* (2008) provided the information content matrix to assign the information content (Ri) values.

RESULTS

Two putative low-affinity phosphate transporters are encoded in the *Streptomyces coelicolor* genome

Homology searches identified two putative low-affinity phosphate transport genes in the *S. coelicolor* genome, SCO4138 and SCO1845 (Bentley *et al.*, 2002); they are designated in this work as *pitH1* and *pitH2*, respectively,

following the annotation of the homologous genes of *Streptomyces avermitilis* (Ikeda *et al.*, 2003). The PitH1 and PitH2 proteins show 39 and 33 % amino acid sequence similarity, respectively, to the transporter PitA of *E. coli* and contain the Pfam motif characteristic of the phosphate transporter family (PF01384 PHO4). Their sizes and similarities with homologous proteins are shown in Fig. 1. Both PitH1 and PitH2 contain putative TMSs.

In *S. coelicolor*, both *pitH1* and *pitH2* are clustered with genes encoding other proteins that may participate in phosphate transport. Firstly, *pitH1* lies 8 bp downstream of a gene, SCO4137, that encodes a conserved protein containing a motif known as DUF47 (Pfam PF01865.7; Fig. 1). In *Sinorhizobium meliloti*, the homologous genes of SCO4137-*pitH1* are considered to form an operon (*orfA-pit*). The *orfA* gene is annotated as *pit*-accessory, although the possible role of the OrfA protein in P_i transport has not yet been investigated (Voegele *et al.*, 1997; Bardin *et al.*, 1998). In this work the SCO4137 protein is referred as Pit-accessory protein (Pap). On the other hand, downstream of *pitH2* there is a gene (SCO1846) that encodes a membrane protein. The amino acid sequences of homologous proteins in other bacteria are poorly conserved, as expected for membrane proteins, but in all cases they contain two putative TMSs (Fig. 1). Comparative genomics showed that whereas the *pap-pitH1* genetic organization is widespread in bacteria, the distribution of the *pitH2*-SCO1846 gene organization is confined to actinobacteria.

The upstream sequences of *pitH2* and *pap-pitH1* were selected for study of their transcriptional regulation by phosphate-limitation. Interestingly, bioinformatics analysis identified putative PhoP-binding sequences (PHO boxes) in the promoter region of *pitH2*, but no PHO boxes were found in the upstream region of *pap-pitH1*. The promoter of the *pstS* gene, which encodes the phosphate-binding transport protein, was also included in this study as a positive control PhoP-activated gene (Sola-Landa *et al.*, 2005).

Phosphate-limited and phosphate-replete cultures of wild-type and Δ *phoP* mutant *S. coelicolor* strains

In order to study phosphate regulation, P_i-limited and P_i-replete conditions were defined in initial experiments. Cultures of *S. coelicolor* wild-type and Δ *phoP* mutant strains were carried out in MG medium with initial P_i concentrations of 2.1, 3.2 and 18.5 mM (media designated MG-2.1, MG-3.2 and MG-18.5, respectively). The growth of both cultures was quite similar in MG-18.5 (Fig. 2a). Indeed, in MG-18.5, the P_i concentration in the medium was in excess throughout the time-course of the culture (always higher than 4 mM in both strains; Fig. 2a). For this reason, the MG-18.5 medium was chosen as the P_i-replete condition. On the other hand, an initial P_i concentration of 2.1 mM supported only poor growth of the Δ *phoP* mutant, which can be explained by the lack of both PhoP-dependent phosphate transport and a nutrient-limitation

Fig. 1. Map of the organization of *pitH1* and *pitH2* genes of *S. coelicolor*. Below are tables summarizing the sequence similarity (including identical and functionally conserved amino acids) of the *S. coelicolor* (Sco) Pit proteins with Pit proteins of *S. avermitilis* (Sma), *Acidothermus cellulosilyticus* (Ace), *Sinorhizobium meliloti* (Sme), *B. subtilis* (Bsu), *Leifsonia xyli* (Lxx), *Mycobacterium tuberculosis* (Mtc) and *C. glutamicum* (Cgb), the number of amino acids (aa) of the Pit proteins, the number of deduced TMSs and the separation (in nt) between the genes.

response; however, the growth of the wild-type strain was also severely reduced (results not shown). In contrast, MG-3.2 medium allowed a substantial biomass increase in the Δ *phoP* mutant during the first 44 h, although the growth did not follow the diauxic pattern of the wild-type (Fig. 2b). About 85 % of the initial P_i was utilized in the first 40 h (corresponding with the rapid growth), and after 44 h it was mostly spent (coinciding with the end of the rapid growth phase), falling below 0.1 mM in both wild-type and Δ *phoP* mutant cultures (Fig. 2b). The growth of the wild-type in MG-3.2 was also reduced as compared to the phosphate-replete condition (Fig. 2a, b). Therefore, MG-3.2 was chosen as the P_i -limited condition.

The *pitH2* and *pstS* promoters, but not the *pap-pitH1* one, are strongly induced under P_i -limited conditions

To investigate how expression of *pap-pitH1*, *pitH2* and *pstS* responds to P_i concentration in the growth medium, their

promoter regions were amplified, sequenced and cloned in the pLUXAR(+) promoter-probe plasmid, as described in Methods. Derivative plasmids were introduced by conjugation into both *S. coelicolor* wild-type and Δ *phoP* mutant strains. The reporter activity was quantified in liquid cultures of phosphate-replete (MG-18.5) or phosphate-limited (MG-3.2) media (Fig. 3).

In the wild-type strain under conditions of phosphate limitation (MG-3.2), the activities of *pstS*, *pitH2* and *pap-pitH1* promoters followed a pattern of sequential induction that could be correlated with the decrease in P_i concentration of the medium (compare Figs 2b and 3a). As shown in Fig. 3(a), the maximum activity of *pitH2p* was reached at 40 h, when the P_i concentration had decreased to 0.5 mM; until 44 h of culture this was the major activity. This time (44 h) marked the end of the transition growth phase, as well as the maximum of *pap-pitH1p* activity, which showed a small delay with respect to the peak of *pitH2p* activity. Later, the *pstSp* activity became increasingly predominant. In fact, this is the only one of these promoter activities that can be

Fig. 2. Growth (○) and residual P_i concentration (△) for *S. coelicolor* M145 (wild-type) and INB101 ($\Delta phoP$ mutant) grown on MG-18.5 (a) and MG-3.2 (b). In all cases the dashed line represents the $\Delta phoP$ mutant strain. *n*, Number of independent experiments represented in each curve. Error bars, SEM.

detected up to 70 h. This is not surprising, since the Pst transporter is the high-affinity system that accounts for P_i transport under conditions of low concentration (Rao & Torriani, 1990). The highest induction of *pstSp* correlated with the drop of P_i below 0.1 mM (44–48 h; Figs 2b and 3a).

In MG-18.5, in which P_i is in excess throughout the course of growth (Fig. 2a), the pattern of expression changed dramatically. Firstly, *pstS* promoter activity remained at very low levels throughout the culture (Fig. 3b). Secondly, the activity of *pitH2p* was constant until the late growth phase, and lacked the high levels reached in MG-3.2 at 40 and 44 h (compare Fig. 3a, b). The predominant activity in cultures in

MG-18.5 was that of *pap-pitH1p* throughout most of the culture. Indeed, its values increased 1.3–5.8-fold, as compared with that of the MG-3.2 culture, throughout the growth course. In conclusion, the *pitH2* and *pstS* promoters are induced by P_i limitation, while the *pap-pitH1* promoter is more active under P_i -replete conditions.

Expression of *pitH2*, but not that of *pap-pitH1*, is *phoP*-dependent

The transcriptional regulator PhoP controls expression of the *pstSCAB* transporter gene cluster by binding to the

Fig. 3. Promoter activity of the exconjugants containing pLUX-*pitH2* ($\diamond$), pLUX-*pitH1* ($\square$) and pLUX-*pstS* ($\blacktriangle$) derived from the wild-type M145 grown in MG-3.2 (a) and MG-18.5 (b), and from the Δ *phoP* mutant INB101 grown in MG-3.2 (c) and MG-18.5 (d). The same scale is used in the four panels for comparison. In all cases the dashed line represents the Δ *phoP* mutant strain. *n*, Number of independent experiments represented in each curve. Error bars, SEM.

PHO box located in the *pstS* promoter (Sola-Landa *et al.*, 2005). In order to study whether the expression of *pap-pitH1* and *pitH2* genes depends on the PhoP response regulator, reporter expression studies were performed with the *S. coelicolor* Δ *phoP* mutant (Rodríguez-García *et al.*, 2007). Results (Fig. 3c, d) clearly showed that expression of *pitH2* was dependent upon the PhoP activator. In contrast,

expression from the *pap-pitH1* promoter was not affected by the lack of PhoP in the Δ *phoP* mutant (Fig. 3c, d). Indeed, the *pap-pitH1* activity was higher in the mutant than in the wild-type strain in MG-3.2 (1.7 times higher when the maximum values are compared). This result may reflect a mechanism of adaptation of *pap-pitH1* to the lack of expression in the Δ *phoP* mutant of the other PhoP-

dependent transporters, Pst and PitH2. In the $\Delta phoP$ mutant grown in MG-18.5, the *pap-pitH1* promoter showed a similar behaviour to that in the wild-type strain, although the values at 36 and 60 h were higher in the mutant than in the wild-type strain.

Transcriptional analysis of *pitH2* and *pap-pitH1*

In order to confirm the promoter-probe results, RT-PCR was carried out as described in Methods using total RNA isolated from 40 h MG-3.2 cultures of both wild-type and $\Delta phoP$ mutant strains. Amplification of the *pitH2* transcript was clearly detected in the wild-type RNA; in contrast, only a low-intensity band was present in the $\Delta phoP$ mutant reaction, even after 40 cycles of PCR (Fig. 4a). The presence of a low level of *pitH2* transcript in the $\Delta phoP$ mutant correlates well with the very low, but significant, activity of the *pitH2* promoter at 40 h in the $\Delta phoP$ mutant (see Fig. 3c). On the other hand, both wild-type and mutant RNA gave rise to amplification products corresponding to *pitH1* and *pap-pitH1* transcripts (Fig. 4a). As expected, these results indicate that *pap* and *pitH1* form a bicistronic transcript and are PhoP-independent.

The expression profile of *pitH2* in the wild-type strain was studied in samples taken after 36, 40, 44 and 48 h of culture in MG-3.2 (Fig. 4b). Amplification of the cDNA from *pitH2* transcripts is higher at the earlier times (36 and 40 h) than the later ones (44 and 48 h). Interestingly, maximum luciferase activities from the *pitH2p-luxAB* fusion were observed ~4 h later (Fig. 3a).

PhoP binds to the *pitH2* promoter but not to the *pap-pitH1* promoter

In order to test whether the PhoP protein binds to the *pitH2* and *pap-pitH1* promoters *in vitro*, the PhoP DNA-binding domain (PhoP^{DBD}) fused to GST was used in binding assays, as described in Sola-Landa *et al.* (2005). The promoter DNA-PhoP interaction was studied by EMSA. A protein concentration of 0.25 μ M was sufficient to produce up to four complexes with the *pitH2p* DNA fragment (Fig. 5, arrows). As the protein concentration increased, the amount of unshifted probe decreased. However, no shifted bands were observed with the *pap-pitH1* promoter under the same experimental conditions.

Characterization of the *pitH2* and *pap-pitH1* promoters

The transcription start site of the *pitH2* promoter was determined by primer extension, using RNA from a 42 h culture in MG-3.2 of the *S. coelicolor* [pLUX-*pitH2*] exconjugant that corresponds to conditions of high promoter activity (Fig. 3a). Extension products with an apparent size of 76 and 166 nt, primed with LUX-FAM+47 and LUX-FAM+135, respectively, were observed clearly (Fig. 6). The same transcription start

Fig. 4. RT-PCR analysis of the *pitH2*, *pitH1* and *pap-pitH1* transcripts in *S. coelicolor* M145 and *S. coelicolor* INB101 ($\Delta phoP$). The analysis was carried out as described in Methods. (a) The expected RT-PCR products 1, 2 and 3 of the *pitH2* and *pap-pitH1* genes are depicted above. Below, RT-PCR results (40 cycles) using total RNA from wild-type and $\Delta phoP$ mutant cultures in MG-3.2 (40 h) as template. Lanes: M, 1 kb plus DNA ladder; 1, amplification of a 542 bp fragment internal to *pitH2*; 2, amplification of a 541 bp fragment internal to *pitH1*; 3, amplification of a 697 bp fragment covering both *pap* and *pitH1* coding sequences. The absence of contaminating DNA in the RNA samples was assessed using Platinum *Taq* (data not shown). (b) RT-PCR amplification of the *pitH2* transcript. Template total RNAs were isolated at 36, 40, 44 and 48 h from a wild-type MG-3.2 culture. Lanes: C, control reactions with Platinum *Taq*; RT, reverse transcriptase reactions. Upper panel, 34 cycles of amplification; lower panel, 37 cycles.

Fig. 5. EMSAs of the *pitH2* and *pap-pitH1* promoters. Control, probe without protein. Other lanes, increasing concentrations (0.25, 0.5, 1 and 2 μM) of the GST-PhoP^{DBD} protein (DNA-binding domain). For *pap-pitH1*, only 0.25 and 2 μM protein concentrations were used. Note the lack of binding to the *pap-pitH1* promoter.

point (TSP) was deduced from both products when compared with the respective sequencing reactions. This corresponds to a guanine located 32 bp upstream of the ATG codon (Fig. 6). Sequences resembling the -10 and -35 consensus (Strohl, 1992) were located at the proper distance from the TSP, centred at positions -7 and -33, respectively. These -10 and -35 boxes overlap the PhoP-binding site (Fig. 8). Although the distance between the

-10 and -35 boxes (20 nt) is longer than usual (16–18 nt), this feature seems to be a characteristic of the PhoP-dependent activation mechanism of this and other genes (see Discussion).

Following the research of Strohl (1992), the *pap-pitH1* upstream sequences TTAGCA-N18-TAGCAT constitute plausible -35 and -10 promoter boxes (located at

Fig. 6. Primer extension analysis of the *pitH2* promoter using automated fluorescent capillary electrophoresis. Primer extension reactions were done with both the LUX-FAM + 47 (a) and LUX-FAM + 135 (b) primers. In the upper fluorograms, the black-filled peaks represent the extension products (FAM-labelled cDNA) and the grey traces represent the LIZ-500 standards that were included in each sample. Standard sizes are marked on the upper axis, and fluorescence intensities are indicated as arbitrary units on the y axis. The expanded lower fluorograms correspond to the four sequencing reactions (A, C, G and 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. Each black-filled peak corresponds to the sequencing product with the same apparent size as the respective extension fragment. In both primer extension reactions the same base (the C shown in bold type; note that this is the non-coding strand) was established as the transcription start site of the *pitH2* gene.

positions -61 and -37, respectively, from the translation start codon). However, we have failed to obtain clear extension products with the *pap-pitH1* promoter, despite exhaustive trials. Retrotranscription reactions were done with five different RNA samples from wild-type and mutant cultures using two different enzymes (Superscript II and Superscript III, both from Invitrogen), two different primers (LUX-FAM + 47 and LUX-FAM + 135), varying the amounts of RNA (5–200 µg) and primer (5–50 pmol), varying reaction time and temperature (5–15 h; 42–50 °C), and with or without DMSO (5 %) or actinomycin D. The lack of detected extension products also occurs in the *Sinorhizobium meliloti* wild-type strain carrying only one copy of the *orfA-pit* gene region (Bardin *et al.*, 1998), and suggests that the *pap-pitH1* transcript may be rapidly degraded, thus precluding retrotranscription.

Analysis of the PhoP-binding sequence in the *pitH2* promoter

To determine the PhoP-binding sequence, DNase I footprinting of the *pitH2* promoter region in the presence and absence of PhoP^{DBD} was performed as described previously (Rodríguez-García *et al.*, 1997; Sola-Landa *et al.*, 2005). PhoP^{DBD} was found to protect a 69 bp region in the *pitH2* promoter. The protected sequence was of 53 nt in the *pitH2* coding strand and 64 nt in the complementary strand; 48 nt were coincident in both strands (Figs 7 and 8). Full protection of the coding strand was achieved at 0.5 µM GST-PhoP^{DBD}, and the protected region was not enlarged by increasing protein concentration (Fig. 7a). The protection of the complementary strand showed the same requirement for GST-PhoP^{DBD} protein (0.5 µM), and the protected sequence was not enlarged by increasing protein concentration (Fig. 7b). However, when a protein concentration of 0.26 µM was used, the protected region was shortened in the upstream and downstream regions (see the marked traces of the fluorograms in Fig. 7b), suggesting that the core region of the protected region has a higher affinity for PhoP than the adjacent nucleotide sequences (see below).

The information theory (Schneider, 1996, 1997) analysis of this *pitH2* region revealed six DRus of 11 nt in length using the published matrix model I (Sola-Landa *et al.*, 2008). The central sequence of the *pitH2* protected region contains four consecutive DRus (DRu-1, DRu-2, DRu-3 and DRu-4), and the flanking sequences contained two additional repeats, DRu-A and DRu-B. Both DRu-A and DRu-B are separated from the central repeats by 1 and 2 bp, respectively (Figs 7 and 8). The individual *R_i* values indicate that three of the DRus in the *pitH2* operator (DRu-1, DRu-3 and DRu-4) are well conserved, whereas DRu-2 is poorly conserved (Fig. 8). The DRu-A and DRu-B sequences, in contrast, show a negative *R_i* that reflects a poor sequence conservation (Fig. 8). The binding of PhoP to these poorly conserved DRus can be explained by a cooperative interaction between consecutive protein

monomers at high protein concentration. We have proposed elsewhere (Sola-Landa *et al.*, 2008), for these complex operator structures, that at lower PhoP concentrations only DRu-3 and DRu-4 are bound, and these form the core of the site. Higher PhoP concentrations would allow the sequential binding to DRu-1-DRu-2, DRu-A and DRu-B (Fig. 8; see Discussion). This explains the appearance of up to four retarded complexes in the EMSA assays (Fig. 5).

DISCUSSION

Phosphate deprivation in bacteria triggers the PHO regulon, which includes a series of extracellular enzymes involved in phosphate scavenging from organic phosphates. In *S. coelicolor* these include at least a phytase gene, two glycerophosphodiester phosphodiesterase genes (Rodríguez-García *et al.*, 2007) and four phosphatase genes (Apel *et al.*, 2007; Sola-Landa *et al.*, 2008). As a parallel strategy to survive P_i starvation, *Streptomyces* cells may consume phosphorous storage material, such as polyphosphates, nucleotides and teichoic acids. The *ppk* gene, which encodes an enzyme that catalyses the reversible polymerization of the γ phosphate of ATP into polyphosphate, is under the positive control of the PhoR-PhoP system in *Streptomyces lividans* (Ghorbel *et al.*, 2006). Moreover, two PhoP-binding sites have been found in a cluster of genes putatively involved in the biosynthesis of cell wall polysaccharides, so that phosphate-free polymers could substitute for phosphate-rich polymers in the cell wall (Rodríguez-García *et al.*, 2007).

Inorganic phosphate transport (Pit) proteins are found in all groups of living organisms, including bacteria, archaea, yeast, fungi, plants and animals. *pit* genes encoding proteins from 195 to 763 aa in length are found in different organisms (Saier *et al.*, 1999). In some bacteria the Pit system has been proposed to have a role in heavy metal tolerance; thus, in the presence of heavy metals, metal phosphates are transported out of the cell by Pit (Keasling, 1997; Beard *et al.*, 2000; Álvarez & Jerez, 2004).

Three P_i transport systems have been identified in the genome of *S. coelicolor*. Of these three, PitH1 is the major transporter when P_i is in excess, whereas PitH2 and Pst work when P_i is limited. In *E. coli* there are two *pit* genes as well, although PitA and PitB share greater sequence similarity (90 %) than PitH1 and PitH2 in *S. coelicolor* (53 %), and have the same topological structures (10 TMS; Harris *et al.*, 2001). Moreover, the *S. coelicolor* *pit* genes are clustered with distinct accessory genes (Fig. 1), which is not the case for *E. coli*. On the other hand, in *Acinetobacter johnsonii*, *Sinorhizobium meliloti* and other bacteria, there is only one *pit* gene.

The *pit* genes can also be classified according to their regulation. We have described the regulation of the *pap-pitH1* and *pitH2* genes above. The *pitH2* gene is PhoP-dependent, while the *pap-pitH1* one is not. In *E. coli* the

Fig. 7. Footprinting of the coding (a) and the complementary (b) strands of the *pitH2* promoter region using GST-PhoP^{DBD}. The upper fluorograms correspond to the control DNA (without protein) and to the protected reaction (with increasing concentrations of protein). The lower fluorograms correspond to the full nucleotide sequence used as the molecular mass marker. The protected nucleotide sequence is indicated in bold type, and the direct repeats that form the PHO operator are boxed. The letters a–e represent the main peaks that decrease as the protein concentration increases in the complementary strand. Note that the protection is hardly detected between peaks a and b, and not detected between peaks c and e when the lowest protein concentration (0.26 μM) is used.

pitA promoter is constitutive, but the *pitB* promoter appears to be repressed by PhoB (the PhoP orthologue) (Elvin *et al.*, 1986; Harris *et al.*, 2001). Both patterns of regulation were also found in different organisms with a

single *pit* gene. In *A. johnsonii*, the *pit* gene is expressed constitutively (van Veen *et al.*, 1993; van Veen, 1997), whereas in *Sinorhizobium meliloti* it is repressed by PhoB (Bardin & Finan, 1998; Bardin *et al.*, 1998; Yuan *et al.*,

Fig. 8. Promoter region of *pitH2*. DRUs are indicated by shaded boxes. The number below each DRU is the *Ri* value. The region protected by PhoP^{DBD} in DNase footprinting assays is underlined in the corresponding strand. The -10 and -35 hexanucleotides are boxed. The TSP is indicated by a bent arrow and bold type. Nucleotides showing homology with the 16S RNA, which could form a ribosome-binding site, are framed with a box labelled RBS. The start codon is shown in bold type. The bars with roman numerals represent the expected DRUs that participate in each shifted band of the EMSA experiment.

2006a, b). In *Corynebacterium glutamicum* the *pit* gene has also been proposed to be repressed by PhoR, the response regulator of the two-component PhoS–PhoR (Schaaf & Bott, 2007). Similarly, the *Myxococcus xanthus* *pit* gene is repressed in phosphate-starved cells. This repression is not observed in a mutant in *phoP4*, which is one of the four response regulators involved in the PHO response in this organism (Whitworth *et al.*, 2008). In contrast, PhoP positively regulates *pitH2* of *S. coelicolor*. To our knowledge, this is the first *pit* gene to be described as PhoP-upregulated in bacteria, suggesting that the mechanism of action of the phosphate response regulator may be positive in some bacteria and negative in others.

As shown in this work, in the Δ *phoP* mutant only the *pap-pitH1* promoter is active, but both wild-type and mutant strains take up P_i from the medium with similar efficiency (Fig. 2). Gebhard *et al.* (2006) proposed that the loss of one or two P_i transport systems can be compensated for by increased activity of the remaining systems. It is reasonable to assume that the higher *pitH1* expression in the *S. coelicolor* Δ *phoP* mutant serves to compensate for the lack of the other two transport systems (Fig. 3). Additionally, another *pst* operon could exist in *S. coelicolor* (SCO6816–SCO6814), as suggested by Díaz *et al.* (2005). Two specific ABC P_i transport systems have been described in various bacteria, such as *Sinorhizobium meliloti* (Voegelé *et al.*, 1997; Yuan *et al.*, 2006a) and some species of mycobacteria (Braibant *et al.*, 1996; Gebhard *et al.*, 2006).

The Δ *phoP* mutant did not grow after 44 h in phosphate-limited MG-3.2 medium, when the P_i concentration dropped below 0.1 mM (Fig. 2b). This is probably due to the fact that the Δ *phoP* mutant is defective in the expression of PhoP-dependent genes. The mutant strain has an altered expression of genes involved in central metabolism and protein synthesis, which are PhoP-upregulated (Rodríguez-García *et al.*, 2007).

Although transcription from *pitH2* and *pstS* promoters is PhoP-dependent, the two genes showed different patterns

of expression. Firstly, sequential activation and decay of the reporter activity at distinct levels of residual P_i are evident from the results. The first gene induced in MG-3.2 medium was *pitH2*, encoding the low-affinity transporter (Fig. 3a). It makes sense that when the P_i in the medium falls, the bacterium first responds with high induction of a low-affinity transporter (*pit*), since this system is energetically less expensive to the cell than the *pst* system. The expression of *pst* occurs when the P_i in the medium becomes scarce, which is consistent with the higher affinity of this transporter.

In phosphate-replete cultures the *pitH2* promoter was constantly active from 36 to 48 h, while *pstS* promoter activity was nearly absent (Fig. 3b). We propose two mechanisms to explain the differences between *pitH2* and *pstS* expression: (i) the different structure of the PhoP-binding sites, and (ii) the interaction of PhoP with specific sigma factors. These mechanisms, which can act together, are based on the operator structures and might involve the participation of alternative sigma factors.

The differences in structure of the PhoP-binding sites in the promoters of *pitH2* and *pstS* provide mechanisms to explain their expression patterns. The PHO box of *pstS* is composed of two well-conserved DRUs located upstream of the promoter elements (Sola-Landa *et al.*, 2005). In contrast, EMSA, footprinting and information content analysis of the *pitH2* operator revealed a complex structure of six DRUs. It is proposed, following the model of Sola-Landa *et al.* (2008), that phosphorylated PhoP (PhoP~P) binds first to the most conserved DRu-3 and DRu-4 sequences, which form the core of the binding site. Increasing concentrations of PhoP~P would result in the consecutive occupancy of DRu-1 and DRu-2. Since the last DRU shows a negative *Ri* (Fig. 8), the binding here of a protein monomer can be explained by protein–protein interactions, as proposed previously for the *phoRP* operator (Sola-Landa *et al.*, 2005). Higher concentrations of PhoP~P would allow the binding of DRu-A and DRu-B,

although the relevance to the control of gene expression of the binding of PhoP to this sequence may be minor. The functionality of a DRu separated by 2 bp has already been demonstrated for the *phoD* promoter (Apel *et al.*, 2007) and the SCO1196 operator (Sola-Landa *et al.*, 2008). This stoichiometry matches the number of EMSA complexes observed (Figs 5 and 8).

The position of these DRus in relation to the promoter elements accounts for the dual role, positive and negative, of PhoP, as occurs in the *phyC* gene of *Bacillus amyloliquefaciens* (Makarewicz *et al.*, 2006). The main features of the *phyC* promoter are: (i) one PHO box overlapping the -35 element; (ii) an improper spacing with the -10 element (21 bp); and (iii) a direct repeat overlapping the -10 element. Makarewicz *et al.* (2006) have proved that while binding of PhoP at -35 is essential for activation of the promoter, binding of PhoP at -10 suppresses promoter activity. Similarly, the PhoP operator core in the *pitH2* promoter overlaps the -35 region (Fig. 8); the separation from the -10 element is 20 bp, which is larger than those of standard *Streptomyces* promoters (16–18 bp; Strohl, 1992), and DRu-B overlaps the -10 region of the *pitH2* promoter (Fig. 8). This is a characteristic feature of some promoters regulated by dual activator/repressor proteins (Hidalgo & Demple, 1997; Makarewicz *et al.*, 2006). With this model in mind, PhoP~P oligomerization on the DNA up to DRus A, 1, 2, 3 and 4 would result in the activation of the *pitH2* promoter. However, the binding of PhoP~P to DRu-B would cause repression. The repression at the highest PhoP~P concentrations is in agreement with the rapid activity drop of the *pitH2* promoter from 40 to 48 h, coincident with the rise in *pstSp* activity (Fig. 3a).

The homologous *pitH2* gene in *S. avermitilis* (SAV6419) also has a PhoP-binding site in its promoter region with a similar structure, which supports our proposed model. In this species, there are two highly conserved DRus with R_i values of 9.8 and 11.6 bits, respectively. As in *S. coelicolor*, downstream of these DRus, separated by 2 bp, there is another DRu with an R_i of 0.1 bits. This DRu should be the functional homologue of *S. coelicolor* DRu-B in the *pitH2* promoter.

The earlier activation of the *pitH2* gene with respect to the *pstS* gene can be also explained if its promoter is recognized by a sigma factor, active in the first phase of growth, that interacts with PhoP, whereas *pstS* is triggered by PhoP in combination with a phosphate-limitation-responsive sigma factor. This mechanism is supported by the finding that the *Bacillus subtilis* PhoP is able to interact with promoters of at least three different sigma factors σ^A , σ^E and σ^M (Paul *et al.*, 2004; Minnig *et al.*, 2005). The sequencing of the *S. coelicolor* genome has identified a plethora of genes that encode sigma-factors, including four homologues (*hrdA*, *hrdB*, *hrdC* and *hrdD*) of the principal sigma factors, nine homologues of *B. subtilis* stress-response σ^B , and 51 extracytoplasmic function sigma factors (Bentley *et al.*, 2002; Hahn *et al.*, 2003). Therefore, it will not be surprising

if one or more sigma factors are dedicated to the phosphate-limitation stress response. Moreover, the sigma factor genes *hrdB* and *hrdD* of *Streptomyces griseus* are expressed differentially under P_i -rich or P_i -starved conditions, respectively (Marcos *et al.*, 1995).

Finally, the *pitH2* promoter activity in phosphate-replete cultures (Fig. 3b) indicates that unphosphorylated PhoP can bind and activate this promoter. *In vitro* DNA binding of the unphosphorylated PhoP (the PhoP form existing under high-phosphate conditions) proteins of *S. coelicolor* (Sola-Landa *et al.*, 2005) and *B. subtilis* has been reported, although in *B. subtilis*, the phosphorylated protein is required for full activation (Liu *et al.*, 1998; Eder *et al.*, 1999). Also, in *S. coelicolor*, the full *pitH2* promoter activity is achieved under conditions of phosphate limitation, i.e. when PhoP is phosphorylated.

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