The high-affinity phosphate-binding protein PstS is accumulated under high fructose concentrations and mutation of the corresponding gene affects differentiation in Streptomyces lividans

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The secreted protein pattern of Streptomyces lividans depends on the carbon source present in the culture media. One protein that shows the most dramatic change is the high-affinity phosphate-binding protein PstS, which is strongly accumulated in the supernatant of liquid cultures containing high concentrations (>3%) of certain sugars, such as fructose, galactose and mannose. The promoter region of this gene and that of its Streptomyces coelicolor homologue were used to drive the expression of a xylanase in S. lividans that was accumulated in the culture supernatant when grown in the presence of fructose. PstS accumulation was dramatically increased in a S. lividans polyphosphate kinase null mutant (Δppk) and was impaired in a deletion mutant lacking phoP, the transcriptional regulator gene of the two-component phoR-phoP system that controls the Pho regulon. Deletion of the pstS genes in S. lividans and S. coelicolor impaired phosphate transport and accelerated differentiation and sporulation on solid media. Complementation with a single copy in a S. lividans pstS null mutant returned phosphate transport and sporulation to levels similar to those of the wild-type strain. The present work demonstrates that carbon and phosphate metabolism are linked in the regulation of genes and that this can trigger the genetic switch towards morphogenesis.

INTRODUCTION

Streptomyces inhabit soil in which plant debris is the main source of nutrients and which are rich in carbon and poor in nitrogen and phosphate (Hodgson, 2000). The sensing of these environmental nutritional conditions requires complex pathways that include specialized sensors that drive the transcription of specific sets (regulons) of genes. Among these sensors, the kinases, which form part of two-component systems, play a key role in controlling the expression of the regulons that permit rapid metabolic adjustments. It has been proposed that the number of environmental stimuli detected by an organism is directly linked to the number of sensor kinases present in the organism (Hutchings et al., 2004). Thus, that number would be smaller for organisms that are obligate pathogens than for free-living organisms, such as streptomycetes.

On the other hand, the variable availability of nutrients (for example the carbon source) may control the expression of a large number of genes, some of which may or may not be directly involved in their metabolism (Hodgson, 2000). To date, sugar-uptake mechanisms have only been described for a limited number of carbohydrates in Streptomyces (Bertram et al., 2004; Hurtubise et al., 1995; Schlösser et al., 1999; van Wezel et al., 1997, 2005). Glucose uptake is mediated by two proton symporters encoded by the glcP1 and glcP2 genes (van Wezel et al., 2005), and the glucose kinase (GlkA) responsible for glucose phosphorylation plays a key role as a global carbon regulator (Angell et al., 1994). In contrast, the HPr protein of the phosphotransferase system (PTS) does not have a general role in carbon regulation, as it does in other bacteria (Nothaft et al., 2003b), although the role of PTS proteins has been clearly demonstrated for the transport of N-acetylglucosamine and fructose, but not for glucose (Nothaft et al., 2003a, b; Wang et al., 2002).

Phosphate availability is also important in gene expression and differentiation. In Streptomyces, the production of secondary metabolites, among them antibiotics, is known to be under phosphate control. However, very little is known about the molecular mechanism(s) of this control.
(Chouayekh & Virolle, 2002; Gil & Campelo-Diez, 2003; Liras et al., 1990; Martin & Demain, 1980; Martin & McDaniel, 1975; Sola-Landa et al., 2003). In bacteria, phosphorus is generally obtained as inorganic phosphate, which is captured by two transport systems of high and low affinity, respectively. *Escherichia coli* and *Bacillus subtilis* are the organisms most studied in this sense. In both cases, high-affinity transport is implemented by a *Pst* (phosphate-specific transport) system, similar to ATP-binding cassette (ABC) transporters, and composed of the proteins *PstS*, *PstC*, *PstA* and *PstB* in *E. coli*, and the proteins *PstS*, *PstC*, *PstA*, *PstB1* and *PstB2* in *B. subtilis*. Their expression responds to the phosphate level in the medium and they are induced by phosphate starvation. A phosphate-regulated promoter located upstream from the most proximal gene (*pstS*) controls the transcription of the entire operon (Aguenas et al., 2002; Qi et al., 1997). The *pst* operon is part of the Pho regulon under the control of a two-component system composed of the proteins PhoB/PhoR in *E. coli* and PhoP/PhoR in *B. subtilis* (Hulett, 2002; Qi & Hulett, 1998; Torriani, 1990). The phosphorylated forms of the transcriptional factors PhoB or PhoP are the direct effectors of the activation or repression of a large number of genes through binding to a DNA sequence known as the ‘Pho box’, which does not have a canonical sequence for all micro-organisms. The second phosphate transporter system, composed of divalent metal transporters (Hantke, 2001; Harris et al., 2001; Hoffer et al., 2001), is a low-affinity one that is expressed constitutively and is functional at high inorganic phosphate concentrations. In *Streptomyces*, the functionality of both transport systems has been described (Licha et al., 1997) and the two-component PhoP-PhoR system has also been elucidated (Sola-Landa et al., 2003).

In a previous work, we described the morphological changes induced in *S. lividans* when grown in liquid medium in the presence of high concentrations of glucose and fructose (Santamaría et al., 2002).

The present work reports the differences in the secreted protein pattern of *S. lividans* 66 under these different culture conditions. The most marked deviation was observed in the expression of the high-affinity phosphate-binding protein (PstS), which was accumulated in the presence of fructose and other carbon sources. Mutation of *phoP*, the transcriptional regulator of the two-component PhoP-PhoR system involved in the Pho regulon, impaired the expression of *pstS*, while a mutation in *ppt*, a polyphosphate kinase-encoding gene, elicited overexpression of the PstS protein. Analysis of the *S. coelicolor* and *S. lividans* *pstS* null mutant phenotypes revealed that spore differentiation was triggered earlier in these mutants (lacking the PstS protein) than in the corresponding wild-type strains.

**METHODS**

**Bacterial strains, plasmids and media.** *S. coelicolor* M145 and *S. lividans* 66 were used in all cloning experiments carried out in *Streptomyces* strains. *E. coli* DH5α was routinely used for sub-cloning and the isolation of plasmids. *E. coli* BW25113/pIJ790 and *E. coli* ET12567/pUZ8002 were used for gene replacement, using REDIRECT technology (Gust et al., 2003). The cloning vectors used are described in Table 1.

*Streptomyces* strains were grown and sporulated on solid R2YE and mannitol soya flour agar medium (MSA) at 28 °C (Kieser et al., 2000). Submerged cultures were carried out in YES medium (0.5 % yeast extract, 10-3 % sucrose, 5 mM MgCl2, pH 7) (Ruiz-Arribas et al., 1995) or in YE (0.5 % yeast extract, 5 mM MgCl2, pH 7) supplemented with different amounts of the carbon source studied. When 1 % glucose was used, the medium was denominated YEG and was used for phosphate-uptake measurements. For low-phosphate liquid-medium studies, a modification of the minimal medium described by Hopwood (1967) was used. The composition of this modified medium, per litre, was: yeast extract, 2 g; l-asparagine, 0.5 g; MgSO4.7H2O, 0.2 g; FeSO4.7H2O, 0.01 g; pH 7.0-7.2. A modification of asparagine-minimal medium was also used (Martin & McDaniel, 1975; Sola-Landa et al., 2003). This medium was modified by the addition of 2 g yeast extract l⁻¹. Both media were supplemented

**Table 1. Plasmids**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Characteristics</th>
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<tbody>
<tr>
<td>pIJ773</td>
<td>pBluescript KS(+) derivative. Template plasmid containing the apramycin resistance</td>
<td>Gust et al. (2003)</td>
</tr>
<tr>
<td>pNX24</td>
<td>pN702GEM3 derivative. yxaA promoter controlling yxaA expression</td>
<td>Adham et al. (2001)</td>
</tr>
<tr>
<td>pNUF2</td>
<td>pNX24 derivative. <em>pstS</em> promoter from <em>S. coelicolor</em> controlling yxaA expression</td>
<td>This study</td>
</tr>
<tr>
<td>pNUF3</td>
<td>pNX24 derivative. <em>pstS</em> promoter from <em>S. lividans</em> controlling yxaA expression</td>
<td>This study</td>
</tr>
<tr>
<td>pNUF4</td>
<td>pNUF2 derivative. Transcriptional terminator <em>mmrt</em> inserted upstream from the <em>pstS</em> promoter from <em>S. coelicolor</em> controlling yxaA expression</td>
<td>This study</td>
</tr>
<tr>
<td>pNUF5</td>
<td>pNUF3 derivative. Transcriptional terminator <em>mmrt</em> inserted upstream from the <em>pstS</em> promoter from <em>S. lividans</em> controlling yxaA expression</td>
<td>This study</td>
</tr>
<tr>
<td>pNUF6</td>
<td>pNUF4 derivative. The <em>pstS</em> promoter from <em>S. coelicolor</em> controls <em>pstS</em> expression</td>
<td>This study</td>
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<tr>
<td>pNUF7</td>
<td>pNUF5 derivative. The <em>pstS</em> promoter from <em>S. lividans</em> controls <em>pstS</em> expression</td>
<td>This study</td>
</tr>
<tr>
<td>pKC796Hyg</td>
<td>Integrative plasmid derived from pKC796 (Kuhstoss et al., 1991) in which apramycin resistance has been replaced by hygromycin resistance</td>
<td>This study</td>
</tr>
<tr>
<td>pINTUF5</td>
<td>pKC796Hyg derivatized containing <em>pstS</em> gene and promoter from <em>S. lividans</em></td>
<td>This study</td>
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with different amounts of the carbon source studied. Phosphate concentrations were determined by a modification of the malachite green/molydate method (Lanzetta et al., 1979) in which Sterox was replaced by Tween 20 (0-10%). The culture conditions were as described previously (Fernández-Abalos et al., 2003). Apramycin (50 μg ml⁻¹), neomycin (15 μg ml⁻¹), thiostrepton (5-10 μg ml⁻¹) or hygromycin (50-100 μg ml⁻¹) was added when necessary.

E. coli was grown in Luria Broth (LB) at 37 °C, supplemented with ampicillin (100 μg ml⁻¹), apramycin (50 μg ml⁻¹), chloramphenicol (25 μg ml⁻¹) or kanamycin (25-50 μg ml⁻¹) when needed.

Microscopic techniques. Scanning electron microscopy was performed on pieces of agar obtained from the corresponding plates and coated with gold (Trujillo et al., 2005). Samples were examined in a Zeiss DSM 940 electron microscope and all the images were recorded digitally.

DNA manipulations and transformations. Streptomyces and E. coli total genomic and plasmid DNA, transformation and protoplast collection were accomplished as indicated by Kieser et al. (2000) and Sambrook et al. (1989). Transfer of cosmids from E. coli to S. coelicolor or S. lividans was carried out by intergeneric conjugation, as described by Gust et al. (2003).

DNA sequencing and analysis. The DNA sequences were determined in both strands with a Perkin Elmer ABI Prism 377 DNA sequencer, using several oligonucleotides designed from the DNA sequences collected. Manipulation was accomplished with the Gene Construction Kit (GCK) (Textco) and analyses were done with the DNA Strider (Marck, 1988). Comparisons of DNA or protein sequences were carried out online (http://www2.ebi.ac.uk/) with FASTA (Pearson & Lipman, 1988) and BLAST (Altschul et al., 1997). CLUSTAL W (Thompson et al., 1994) was used for sequence alignment. RSA-Tools was used for DNA-pattern analysis (van Helden et al., 2000).

Construction and verification of ΔpstS mutants in S. coelicolor and S. lividans. The pstS gene, encoding the PstS phosphate-binding protein, corresponds to SCO4142 from the S. coelicolor genome. The whole ORF was replaced by the apramycin gene-oriT fragment [acc(3)IV-oriT] in E. coli BW25113/p[p]T790 harbouring the SC84 cosmid, using the REDIRECT technology (Gust et al., 2003). The deletion cassette with [acc(3)IV-oriT] DNA in the middle of the pstS flanking sequences (39 nt each side) was obtained by PCR amplification. Plasmid pJ773 (see Table 1) digested with HindIII/EcoRI was used as template and the oligonucleotides pRA1 (TCC TGG AAG GAA CTC CCA AAG TGT AAG CTT CAG CGC ATG ATT CCG GGG ATC ATC CGA CC) and pRA2 (CTG TCG CCT CCG GCC GGA GGC CGG ACC GCA CTC GGG TCA TGT AGG CGT GAG CGT CTT C) were used as primers in the PCR reaction [pstS flanking sequences in italics and the sequence that anneals the template (acc(3)IV-oriT) underlined]. Correct replacement of the pstS gene by the deletion cassette [acc(3)IV-oriT] in the SC84 cosmid was checked by restriction enzyme digestion. The new recombinant cosmids [SC84 pstS::acc(3)IV-oriT] was then introduced into S. lividans 66 and S. coelicolor M145 to obtain the pstS null mutants by intergeneric conjugation (Gust et al., 2003). The correct replacement of pstS in the S. coelicolor and S. lividans genomes was confirmed by Southern blot experiments.

Cloning of pstS promoters from S. coelicolor and S. lividans. The intergenic regions between SCO4142 (pstS gene) and SCO4143 (possible MutT-like protein) in S. coelicolor and the equivalent region from S. lividans were cloned by PCR using oligonucleotides based on the S. coelicolor sequence of this region: MRG-27 (reverse, TAA TAA CAT ATG GCC CTG AAG CTT CAT TG CGA AGG GAG) and MRG-28 (forward, TTT TTA GAT CAT ATG GCC ACC GGG ACC GGG CCC T). The NdeI or BglII (underlined) restriction sites were included in MRG-27 and MRG-28, respectively, for further cloning.

Amplification was carried out in an MJ Research thermocycler as described previously (Fernández-Abalos et al., 2003), using genomic DNA from S. coelicolor M145 and S. lividans 66 as template. Both PCR products (about 350 bp) were purified by agarose gel electrophoresis and digested with NdeI and BglII. The amplified bands were cloned in plasmid pNX24 (Table 1), digested with the same restriction enzymes and transformed into E. coli DH5α (under kanamycin selection), yielding plasmids pNUF2 and pNUF3 (Table 1). In these plasmids, the pstS promoter regions control the expression of the xysA xylanase gene from Streptomyces halstedii JI84, used as reporter (Adham et al., 2001). In a second step (detailed data not shown), the transcriptional terminator mntR (T1) was introduced upstream from the pstS promoter region to yield the final plasmids pNUF4 and pNUF5 (Table 1), which were introduced by transformation into S. lividans 66.

Cloning of the pstS ORFs from S. coelicolor and S. lividans. The complete ORFs of the pstS genes from S. coelicolor and S. lividans were amplified by PCR, using oligonucleotides based on the pstS gene from S. coelicolor: MRG-33 (forward: TTTT CAT ATG AAC CCG CGG GCC CTC GC) and MRG-34 (reverse: TTT TTC TAG ATC AGC TCA GGC CCG AGA TGG TC) including an NdeI or Xbal restriction site (underlined), respectively, for further cloning.

Amplification was carried out in an MJ Research thermocycler as described previously (Fernández-Abalos et al., 2003). The PCR products (1130 bp) were purified by agarose gel electrophoresis and digested with NdeI and XbaI. The amplified ORFs were placed under the control of their corresponding promoters using the plasmids pNUF4 and pNUF5, digested with the same enzymes removing the xylanase ORF. Thus, the amplified band from S. coelicolor was cloned into pNUF4 downstream from the S. coelicolor pstS promoter, yielding plasmid pNUF6, while the band amplified from S. lividans DNA was cloned in pNUF5 downstream from the S. lividans pstS promoter, yielding plasmid pNUF7 (Table 1). These plasmids were introduced into S. lividans 66 for pstS overproduction analysis.

pstS integrative plasmids. To perform complementation studies, an integrative plasmid (pINTUF5) containing one copy of the pstS gene was obtained. The pstS gene with its promoter region was isolated from pNUF7 by digestion with PvuII/XbaI and cloned in EcoRV/XbaI sites of a hygromycin-resistance plasmid, called pKC796Hyg (Table 1). These plasmids (pINTUF5 and the empty vector pKC796Hyg) were introduced into S. lividans ΔpstS by protoplast transformation, and the integrated strains were selected for apramycin and hygromycin resistance.

Phosphate uptake. The uptake of phosphate was measured as described by Sola-Landa et al. (2003), with slight modifications. S. lividans cultures were grown in liquid YEG medium for 40 h (28 °C, 200 r.p.m.). The cells collected were washed twice with 0-9% NaCl and transferred to asparagine minimal medium without any inorganic phosphate (Martin & McDaniel, 1975). After stabilization of the cell suspension for 6 h at 28 °C, 32P-labelled NaHPO₄ (Amersham Biosciences) was added (2 × 10⁶ c.p.m. ml⁻¹). Phosphate uptake was measured after 15 min at 30 °C. Cells were recovered by filtration through Whatman GF/C filters and washed twice with 0-9% NaCl, and the radioactivity from the filter was quantified in a liquid scintillation counter (Wallac 1409-001).

Protein analysis and enzyme assays. Electrophoresis in denaturing polyacrylamide gels (SDS-PAGE) was performed as described...
elsewhere (Ruiz-Arribas et al., 1995). The N-terminal end of the protein was determined using an Applied Biosystems Protein Sequenator.

Enzymes and reagents were purchased from Boehringer Mannheim, Promega, Bethesda Research Laboratories, Pharmacia, Sigma, Merck, Panreac, Bio-Rad, Santa Cruz and Ambion, and were used following the manufacturers’ guidelines.

The sequence of the S. lividans pstS gene has been deposited in the EMBL Database with accession number AJ698727.

RESULTS

PstS accumulates in the supernatant of S. lividans grown at high fructose concentrations

The effect of high concentrations of fructose and glucose on the morphological differentiation of S. lividans on solid and in liquid media has been reported previously (Santamaría et al., 2002). The present work was started after the observation that the pattern of proteins secreted by S. lividans 66 was very different when the microorganism was grown in YES or YE media supplemented with a high concentration of glucose (5 %) or fructose (5 %). When grown for 96 h in liquid media containing high concentrations of fructose, supernatants of S. lividans accumulated large amounts of a protein of about 35 kDa that was not observed in the presence of glucose or in the presence of both carbon sources at the same concentrations (Fig. 1A). The total cellular protein pattern did not show such striking differences under any of the culture conditions studied (Fig. 1B). This accumulation of the 35 kDa protein was dependent on the fructose concentration in the culture media and was detected at between 3 and 10 % fructose, reaching a maximum at 5 % (Fig. 1C). High concentrations (5 %) of other carbon sources, such as galactose or mannose, were also able to induce this accumulation of the 35 kDa protein in the culture supernatant, but the same concentration of fructooligosaccharides was not able to do so (data not shown).

The N-terminus sequence SNIKCDDA of this fructose-abundance protein purified from the supernatant was identical to residues 42–49 from the S. coelicolor PstS protein (SCO4142). In the previous 41 residues present in the encoded PstS, there is a sequence – AVSGALALTAC – from amino acid 12 to 22, showing high similarity to the prokaryotic membrane lipoprotein attachment site (Prosite Family pattern PS 0013). This suggests that the protein might be a lipoprotein and that the greater part of the molecule is located on the outer surface of the cell membrane, as occurs in other Gram-positive bacteria, such as Mycobacterium (Espitia et al., 1992). However, cell extracts from cultures grown in the presence of fructose never displayed the accumulation of PstS observed in the corresponding supernatant (lanes YE+5 % F in Fig. 1A, B). The extracellular location of the PstS protein has also been described in Bacillus species, but no such accumulation has been described previously in the presence of a carbon source.

In other systems, such as E. coli and B. subtilis, expression of this gene responds to the phosphate concentration of the medium, being expressed at low phosphate concentrations (lower than 50 and 160 μM, respectively) and repressed

![Fig. 1. PstS accumulation under different culture conditions. (A and B) Coomassie blue R-stained SDS-PAGE of proteins of S. lividans 66 grown for 4 days in liquid YE media with glucose (G), fructose (F) or with both (G+F). (A) 100 μl of supernatants; (B) cell extracts from 20 μl of culture; (C) supernatants of S. lividans 66 under different fructose concentrations (100 μl of a 4-day-old culture); (D) supernatants of S. lividans 66 grown in the presence of 5 % fructose and different phosphate concentrations (added as sodium phosphate) (100 μl of a 4-day-old culture).]
at high phosphate concentrations (Aguena et al., 2002; Antelmann et al., 2000). We studied the effect of the phosphate concentration on S. lividans PstS supernatant accumulation using different liquid media. The initial phosphate concentration in YE medium (without extra phosphate added) was 2 mM and decreased to lower than 100 μM after 4 days of S. lividans growth. No accumulation of extracellular PstS was observed, even when the culture was maintained for up to 10 days. Furthermore, modifications of two minimal media low in phosphate (200 μM, without extra phosphate added) were used; the phosphate concentration fell to 30 μM after 4 days of S. lividans growth, but no PstS accumulation was observed in the supernatant. The addition of 5% fructose to these minimal media induced S. lividans extracellular PstS accumulation, although to a lower extent than that obtained in YE supplemented with the same carbon source (data not shown). The addition of different amounts of phosphate (from 5 mM to 50 mM sodium phosphate) to YE medium containing 5% fructose prevented PstS accumulation, showing that high phosphate concentrations in the medium impair PstS accumulation in the presence of high fructose concentrations (Fig. 1D). Intracellular protein levels of cells grown in the presence of fructose and phosphate were also studied by SDS-PAGE, but no accumulation of PstS— at the Coomassie blue-stained gel level— was observed under any of the conditions used (data not shown).

**Cloning of the promoter region of pstS from S. coelicolor and S. lividans 66**

The *pstS* promoter regions from *S. coelicolor* and *S. lividans* were cloned as described in Methods. The sequences of the regions of both species displayed several differences. The intergenic region of *S. lividans* was 28 bp longer than that of *S. coelicolor* (329 versus 301 bp). The sequence ACTCACCCCCGC is repeated three times in the *S. coelicolor* promoter and, with some discrepancies, is

![Fig. 2. pstS promoter analysis. (A) CLUSTAL W alignment of the pstS promoters (intergenic regions) of several Streptomyces species: *S. avermitilis, S. griseus, S. coelicolor* and *S. lividans*. The 12 bp sequence repeated six and eight times (with some mismatches) in *S. coelicolor* and *S. lividans* is underlined. The sequence GTTCAN 6 GTTCA is shaded. * Indicates identity of nucleotides. (B) *S. lividans* 66 expression of the xylanases Xys1L and Xys1S [a processed form of Xys1L (Ruiz-Arribas et al., 1997)] under the *S. coelicolor* (pNUF4) and *S. lividans* (pNUF5) *pstS* promoters. The media used were: YE, YE+5% fructose (+F) and YE+5% fructose +10 mM phosphate (+F+Pi).](http://mic.sgmjournals.org)
repeated eight times in the *S. lividans* promoter and six times in *S. coelicolor*. Comparison of the *S. lividans* and *S. coelicolor* *pstS* promoters with the DNA database revealed that the above sequence was absent in another two *pstS* promoters sequenced from *Streptomyces avermitilis* and *Streptomyces griseus*. Nevertheless, all four promoters maintained several conserved regions that could be involved in their regulation (Fig. 2A).

The functionality of the *S. coelicolor* and *S. lividans* intergenic regions was demonstrated by generating two transcriptional fusions between the cloned *pstS* promoters and the ORF of the xylanase gene *xysA* from *S. halstedii* JM8 (Ruiz-Arribas *et al.*, 1997) (see Table 1). Xylanase accumulation was clearly observable in the supernatants of media containing 5% fructose (Fig. 2B). This protein was more abundant when its expression was under the control of the *S. lividans* promoter. In both cases, xylanase production was impaired when 10 mM sodium phosphate was also added to the media, confirming the results described for *PstS* expression (Fig. 2B).

**PstS is overexpressed in a ppk mutant and its expression is controlled by the PhoR-PhoP system**

Since intracellular phosphate results from the transport of extracellular phosphate and from the mobilization of intracellular polyphosphate, we decided to study the expression of *PstS* in a null mutant in the gene encoding polyphosphate kinase (*ppk*), which is responsible for polyphosphate accumulation inside the cells (Chouayekh & Virolle, 2002); *PstS* was overproduced in this mutant (Fig. 3). This result clearly indicates that the need for phosphate in this mutant is met by the overexpression of other genes involved in phosphate uptake, such as the one encoding the high-affinity phosphate-binding protein *PstS*.

The two-component PhoR-PhoP system controls primary and secondary metabolism in *S. lividans*, the Pho regulon being the core of this control (Sola-Landa *et al.*, 2003). By studying *PstS* expression in the transcriptional activator PhoP mutant (Ghorbel & Virolle, 2003), we observed that the absence of PhoP protein impaired the synthesis of *PstS*, even in cultures with fructose (Fig. 3). The use of anti-*PstS* antibodies confirmed this result (Fig. 3B).

**Deletion of *pstS* and phenotypic effects on solid media**

*PstS* null mutants of *S. coelicolor* M145 and *S. lividans* 66 were obtained by REDIRECT technology (Gust *et al.*, 2003), as described in Methods. The correct *pstS* replacement in both genomes was checked by Southern blot (data not shown).

Liquid cultures of these mutants in YE+5% fructose medium revealed no *PstS* accumulation in the supernatant (Fig. 4A, lanes 2 and 4). Western blotting with anti-*PstS* antibodies confirmed the absence of this protein in the mutants (Fig. 4B, lanes 2 and 4).

When parental *S. coelicolor* and *S. lividans* strains and the corresponding Δ*pstS* mutants were inoculated on solid R2YE media and incubated at 28 °C, differentiation was accelerated in the mutant strains. Aerial mycelia of Δ*pstS* mutants were observed after 48 h, while the wild-type required 72 h for the same event to occur (Fig. 4C, D). This acceleration also led to faster-sporulating colonies, with more abundant spores than in the wild-type strain. The same effect was observed on MSA medium, where sporulation was even faster (36–48 h) and more abundant (Fig. 4C, D). The acceleration of sporulation was confirmed under scanning electron microscopy, observing that after 36 h at 28 °C on MSA, the spores were clearly visible in the mutants while only normal mycelium was observed in the corresponding wild-type strains (Fig. 4E). Overproduction of actinorhodin was observed in the *S. coelicolor* Δ*pstS* mutant when this micro-organism was grown on R2YE, but not on MSA (Fig. 4C). The overproduction of actinorhodin was not observed in an *S. lividans* Δ*pstS* mutant under any of the conditions used.

To confirm that the *pstS* gene indeed encoded a functional high-affinity phosphate-binding protein, *PstS*, the incorporation of 32P-labelled phosphate was quantified in cells of *S. lividans* wild-type and in the corresponding *pstS* null mutant strain. A dramatic reduction in the uptake of inorganic phosphate was observed in the mutant strain in comparison with the uptake in the parental strain, as expected (Fig. 5A). This result clearly demonstrated the functionality of the *PstS* protein in phosphate transport.

**Cloning of the *pstS* ORFs from *S. coelicolor* M145 and *S. lividans* 66 and complementation studies**

The *pstS* ORFs from *S. coelicolor* and *S. lividans* were amplified by PCR (see Methods) and the DNA sequences of
both fragments were obtained. CLUSTAL W analysis of both sequences failed to detect any difference in either ORF. This conservation contrasts with the differences observed in the promoter region of both genes.

To check that the \textit{pstS} null mutant phenotypes were only due to the lack of PstS protein in these mutants, complementation studies were performed. One copy of the \textit{pstS} gene was introduced into the \textit{S. lividans} \textit{pstS} null mutant by transformation with the integrative plasmid \textit{pINTUF5}. Integration of this copy of the \textit{pstS} gene occurred at the phage W\textit{C31} integration site, distant from the \textit{pst} operon in the genome. This ectopic integration of \textit{pstS} restored the

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**Fig. 4.** \textit{pstS} null mutant phenotype. (A) Coomassie blue R-stained SDS-PAGE of supernatant of \textit{S. coelicolor} wild-type (lane 1), \textit{S. coelicolor} \textit{ΔpstS} mutant (lane 2), \textit{S. lividans} wild-type (lane 3) and \textit{S. lividans} \textit{ΔpstS} mutant (lane 4) grown on \textit{YE+5\% fructose} (100 \textmu l of supernatant of 4-day cultures). The molecular mass is indicated in kDa on the right. (B) Western blot with anti-PstS antibodies of the same samples (5 \textmu l of supernatant). (C and D) Effect of \textit{pstS} deletion (\textit{ΔpstS}) on \textit{S. coelicolor} (C) and \textit{S. lividans} (D) grown on \textit{R2YE} or on \textit{MSA}. The plates were incubated at 28°C for 48 h. (E) Scanning electron micrographs of the parental strains (wt) and the corresponding \textit{ΔpstS} mutants. Bars, 2 \textmu m. The MSA plates used to prepare the samples were incubated at 28°C for 36 h.

**Fig. 5.** Complementation studies. (A) Uptake of 32P-labelled phosphate after 15 min at 30°C in the wild-type \textit{S. lividans} (wt), the \textit{ΔpstS} deletion mutant (\textit{ΔpstS}), the complemented transformant \textit{ΔpstS} (\textit{ΔpstS}/\textit{pINTUF5}) and the integrative negative control strain \textit{ΔpstS} (\textit{ΔpstS}/\textit{pKC796Hyg}). (B) Western blot with anti-PstS antibodies of 5 \textmu l of supernatant of the indicated strain. (C) MSA plate inoculated with different strains of \textit{S. lividans} to observe the effect of \textit{ΔpstS} complementation (\textit{ΔpstS}/\textit{pINTUF5}). \textit{ΔpstS}/\textit{pKC796Hyg} is the mutant \textit{ΔpstS} transformed with the empty vector; wt is \textit{S. lividans} 1326. The plate was incubated at 28°C for 48 h.
incorporation of $^{32}$P-labelled phosphate to levels slightly lower than those of the \textit{S. lividans} wild-type strain. As expected, the strain transformed with the integrative empty plasmid used as a control, pKC796Hyg, had a degree of $^{32}$P-labelled phosphate incorporation similar to that obtained with the \textit{pstS} null mutant (Fig. 5A). Western blot analyses with anti-PstS antibodies confirmed that the production of PstS by the strain transformed with plasmid pINTUF5 was slightly lower than that of the wild-type strain (Fig. 5B).

With respect to sporulation on solid MSA medium, the \textit{S. lividans} \textit{Δ pstS} pINTUF5 integrated strain behaved like the wild-type strain, the differentiation process in these strains being slower than in the \textit{S. lividans} \textit{pstS} null mutant and in the control strain \textit{S. lividans} \textit{Δ pstS} pKC796Hyg (Fig. 5C). This result highlighted the ability to restore the mutant phenotypes of the single copy of the \textit{pstS} gene integrated in the mutant genome and ruled out the possibility of a polar effect.

Overproduction of the PstS protein was studied by cloning the corresponding ORF under the control of \textit{S. coelicolor} and \textit{S. lividans} \textit{pstS} promoters, obtaining the multicopy plasmids pNUF6 and pNUF7. Both plasmids were introduced into \textit{S. coelicolor} M145 and \textit{S. lividans} 66 by transformation. No clear phenotype was observed either on solid media or in liquid media when PstS was overexpressed in both strains, in spite of the high amount of protein produced detected in SDS-PAGE gels (data not shown).

**DISCUSSION**

In our study with \textit{S. lividans} supernatants, a striking change in the degree of extracellular accumulation of the high-affinity phosphate-binding protein PstS was observed in the presence of high concentrations of fructose, galactose and mannose. No such accumulation has been described previously, in the presence of carbon sources, in other systems, although the induction of PstS expression by 1% malate has previously, in the presence of carbon sources, in other systems, such as \textit{S. coelicolor} and \textit{S. lividans} 66, and \textit{S. coelicolor} M145 and \textit{S. lividans} \textit{pstS} promoter sequences, among which the sequence GTTCAN6GTTCA seems to respond differently to the habitat. One of them, the putative low-affinity phosphate transport protein that corresponds to SCO1845 in \textit{S. coelicolor} and SAV6965 in \textit{S. avermitilis}. The putative role of these sequences in the control of the expression of the \textit{pstS} gene and the complete \textit{pst} operon must be demonstrated experimentally in future work.

The deletion of \textit{pstS} in \textit{S. coelicolor} led to the overproduction of actinorhodin when grown on solid R2YE media. The overproduction of antibiotics in \textit{pstS} null mutants has also been described in other organisms, such as \textit{Serratia}, which displays a high level of prodigiosin and carbapenem production (Slater et al., 2003). A plausible explanation for this induction is that this mutation mimics low-phosphate conditions because, as demonstrated here, this mutation is impaired in phosphate transport. An acceleration of differentiation was also observed in this mutant, indicating that phosphate limitation may be responsible for this phenotype. A phosphate downshift, achieved by adding calcium ions, has been described elsewhere as being responsible for the submerged sporulation of different species of \textit{Streptomyces} (Daza et al., 1989).

The \textit{S. coelicolor} and \textit{S. lividans} \textit{PstS} proteins are 100% identical and share 79% identity with the \textit{PstS} protein from \textit{S. avermitilis} and 68% with the protein from \textit{S. griseus}, respectively. At the same time, they share about 42–43% identity with three different putative phosphate receptors – PstS-1, PstS-2 and PstS-3 – from \textit{Mycobacterium tuberculosis} (Lefevre et al., 1997). These three proteins share a high degree of similarity among one another, but their expression seems to respond differently to the habitat. One of them,
PstS-1, is one of the most important immunodominant antigens of \textit{M. tuberculosis} (Chang et al., 1994; D’Souza et al., 2002). Although there is no clear duplication of the \textit{pstS} gene in the \textit{S. coelicolor} genome, a second phosphate-binding protein was identified by sequence similarity. That protein corresponds to SCO2428 and is a putative secreted protein with a length of 522 amino acids that shares 23\% identity with the protein studied in this work, corresponding to ORF SCO4142. It is possible that this protein could somehow mimic or partially complement the function of PstS in the \textit{pstS} null mutant, permitting the cells to survive. The functionality of this protein will be further studied by obtaining the corresponding single and double null mutants with \textit{pstS}.

One plausible hypothesis from the present work would be that the activation of carbohydrate metabolism in general, produced by an excess availability of certain carbon sources in the culture medium, elicits a dramatic decrease in inorganic phosphate inside cells and activation of the Pho regulon. The requirement of extracellular phosphate to phosphorylate the high concentration of internalized sugar would trigger PstS expression in order for inorganic phosphate molecules to be captured from the environment.

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