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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ABSTRACT

The secreted protein pattern of *Streptomyces lividans* depends clearly 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 (higher than 3 %) of certain sugars such as fructose, galactose and mannose. The promoter region of this gene and that of its *S. 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 *Streptomyces coelicolor* impaired phosphate transport and accelerated differentiation and sporulation on solid media. Complementation in a single copy of *S. lividans pstS* null mutant returned phosphate transport and sporulation to levels similar to those of the wt 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.

Key words: *Streptomyces*, phosphate binding, phosphate transport, sugar stress, PstS, *pstS.*

INTRODUCTION

Streptomycetes inhabit soil where plant debris is the main source of nutrients 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 the two-component systems, play a key role in controlling the expression of the regulons that permits rapid metabolic adjustments. It has been proposed that the number of environmental stimuli detected by an organism would be 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 different 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.*, 2005; van Wezel *et al.*, 1997). 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 its 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; Nothaft *et al.*, 2003b; 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 system (**P**hosphate-**s**pecific **t**ransport), similar to ATP-binding cassette (ABC) transporters, and is composed of the proteins PstS, PstC, PstA and PstB in *E. coli*, and the proteins PstS, PstC, PstA, $PstB_1$ and $PstB_2$ 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 (Aguena *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 microorganisms. 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 that 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 been also elucidated (Sola-Landa *et al.*, 2003).

In a previous work we have described the morphological changes induced in *S. lividans* when grown in liquid medium in the presence of high concentration 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 *ppk*, a polyphosphate kinase-encoding gene, elicited overexpression of this protein. Analysis of *S. coelicolor* and *S. lividans pstS* null mutants phenotype revealed that spore differentiation was triggered earlier in these mutants (lacking the PstS protein) than in the corresponding wild-type strains.

MATERIALS AND METHODS

Bacterial strains, plasmids and media. *S. coelicolor* M145 and *S. lividans* 66 were used in all cloning experiments carried out in *Streptomyces*. *Escherichia 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.

*Streptomyce*s 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 $MgCl_2$, pH 7) (Ruiz-Arribas *et al.*, 1995) or in YE (0.5 %, yeast extract 5 mM $MgCl_2$, 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 was used (Hopwood, 1967). The composition of this modified medium, per litre, was: yeast extract, 2 g; L-asparagine. 0.5 g; $MgSO_4.7H_2O$, 0.2 g; $FeSO_4.7H_2O$, 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 of yeast extract per litre. Both media were supplemented with different amounts of the carbon source studied. Phosphate concentrations were determined by a modification the Malachite green-molybdate acid method (Lanzetta *et al.*, 1979) in which Sterox was replaced by Tween 20 (0.01%). 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}) were 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 (25μg 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,* and Sambrook (Kieser *et al.*, 2000; Sambrook *et al.*, 1989). Transference of cosmids from *E. coli* to *S. coelicolor* or *S. lividans* was carried out by intergeneric conjugation, as described in Gust et al. (Gust *et al.*, 2003).

DNA sequencing and analysis. The DNA sequences were determined in both strands using 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, inc) and analyses were done with the DNA Strider (Marck, 1988). Comparisons of DNA or protein sequences were carried out on-line (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 were used for DNA-pattern analysis (van Helden *et al.*, 2000)**.**

Construction and verification of Δ*pstS* mutants in *S. coelicolor* and *S. lividans.* The *pstS* gene, coding for 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 the *E.coli* BW25113/pIJ790 harbouring the SCD84 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 (26 nts each side) was obtained by PCR amplification. Plasmid pIJ773 (see Table 1) digested with *Hind*III/*EcoR*I was used as template and the oligonucleotides pRA1: (*TCC TGG AAG GAA CTC CCT CAA GTG AAG CTT CAG CGC ATG* ATT CCG GGG ATC CGT CGA CC) and pRA2: (*CTG TCG GCT CCG CGC GGA GGC CGG ACC GCA CTC GGG TCA* TGT AGG CTG GAG CTG 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 SCD84 cosmid was checked by restriction enzyme digestion. The new recombinant cosmid (SCD84 *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 *S. coelicolor* and *S. lividans* genomes was corroborated by Southern blot experiments.

Cloning of *pstS* promoters from *S. coelicolor* and from *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 GCG CTG AAG CTT CAC TTG AGG GAG) and MRG-28 (forward: TTT TTA GAT CTC AGC CCC GGG ACC GGG CCC T). The *Nde*I or *Bgl*II (underlined) restriction sites were included in MRG-27 and MRG-28, respectively, for further cloning.

Amplification was carried out in a MJ Research thermocycler as described previously (Fernández-Abalos *et al.*, 2003), using genomic DNA from *S. coelicolor* M145 and from *S. lividans* 66 as template. Both PCR products (about 370 bp) were purified by agarose gel electrophoresis and digested with *Nde*I and *Bgl*II. The amplified bands were cloned in plasmid pNX24 (Table 1), digested with the same restriction enzymes and transformed in *E. coli* DH5α (under km selection), affording plasmids pNUF2 and pNUF3 (Table 1). In these

plasmids, the *pstS* promoter regions control the expression of the xylanase *xysA* gene from *S. halstedii* JM8 used as reporter (Adham *et al.*, 2001). In a second step (detailed data not shown), the transcriptional terminator *mmrt* (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 from *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 CGG CGG GCC CTC GC) and MRG-34 (reverse: TTT TTC TAG ATC AGC TCA GGC CCG AGA TGG TC) including an *Nde*I or *Xba*I restriction sites (underlined), respectively, for further cloning.

Amplification was carried out in a 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 *Nde*I and *Xba*I. 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, affording 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 *pstsS* gene was obtained. The *psts* gene with its promoter region was isolated from pNUF7 by digestion with *Pvu*II/*Xba*I and cloned in *EcoR*V/*Xba*I sites of a hygromycin-resistance plasmid, called pKC796Hyg (Table 1). These

plasmids (pINTUF5 and the empty vector pKC796Hyg) were introduced into *S. lividans* Δ*pstS* by protoplast transformation and the integrated strains were selected for apramycin and hygromycin resistance.

Phosphate uptake. The uptake of phosphate was performed as described by Sola-Landa et al. (Sola-Landa *et al.*, 2003) with slight modifications. *S. lividans* cultures were grown in liquid YEG medium for 40 h (28 ºC, 200 rpm). The cells collected were washed twice with NaCl 0,9 % and transferred to asparagine minimal medium- without any inorganic phosphate (Martin & McDaniel, 1975). After stabilization of the cell suspension for 6 h at 28 ºC, ^{32}P-labeled Na_2HPO_4 (Amersham Biosciences) was added (2 x 10^5 cpm/ml). Phosphate uptake was measured after 15 minutes at 30 ºC. Cells were recovered by filtration through Whatman GF/C filters, 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 amino-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 Data Bank with accession number AJ698727.

RESULTS

PstS is accumulated in the supernatant of *S. lividans* grown in the presence of high fructose concentrations.

The effect of high concentrations of fructose and glucose on 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 in YE media supplemented with high concentration of glucose (5%) or fructose (5%). When grown for 96 hours in liquid media containing high concentration 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 in 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 between 3 to 10 % fructose, reaching a maximum at 5 % (Fig. 1C). High concentrations of other carbon sources (5 %), such as galactose or mannose, were also able to induce this accumulation of the 35-kDa-protein in the culture supernatants, but the same concentration of fructooligosaccharides was not (data not shown).

The N-terminus sequence –SNIKCDDA- of this fructose-abundant 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 this protein might be a lipoprotein mainly 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 Figs. 1A and 1B). The extracellular location of the PstS protein has also been described in *Bacillus,* 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 in low-phosphate (lower than 50 and 160 µM respectively) and repressed 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. Additionally, modifications of two minimal media low in phosphate (200 µM, without extra phosphate added) were used; the phosphate concentration was lowered to 30 µM after four 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 of 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 on presence of fructose and phosphate were also studied by SDS-PAGE but no accumulation of PstS -at 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 Materials and 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, it is 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 *S. avermitilis* and from *S. 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. 3A and B). 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 A and B). 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 Materials and 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 corroborated 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 ºC, 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 and 4D). This acceleration also led to faster sporulating colonies, with more abundant spores than in the wt strain. The same effect was observed on MSA medium, where sporulation was even faster (36-48h) and more abundant (Fig. 4C and 4D). The acceleration of sporulation was corroborated under scanning electron microscopy, observing that after 36 h at 28 ºC on MSA the spores were clearly visible in the mutants while only normal mycelium was observed in the corresponding wt strains (Fig. 4E). Overproduction of actinorhodin was observed in the *S.*

coelicolor Δ*pstS* mutant when this microorganism was grown on R2YE but not on MSA (Fig. 4C). The overproduction of actinorhodin was not observed on *S. lividans* Δ*pstS* mutant in any of the conditions used.

To confirm that the *pstS* gene indeed encoded a functional high-affinity phosphate-binding protein, PstS, the incorporation of ^{32}P-labeled 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 demonstrates the functionality of the PstS protein in phosphate transport.

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

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

To check that the *pstS* null mutant phenotypes were only due to the lack of PstS protein in these mutants, complementation studies were performed. One copy of the *pstS* gene was introduced into the *S. lividans pstS* null mutant by transformation with the integrative plasmid pINTUF5. Integration of this copy of the *pstS* gene occurred at the phage ΦC31 integration site, distant from the *pst* operon in the genome. This ectopic integration of *pstS* rescued the incorporation of ^{32}P-labeled phosphate up to levels slightly lower than those of the *S. lividans* wild-type strain. As expected, the strain transformed with the integrative empty plasmid used as a control, pKC796Hyg, had a similar degree of ^{32}P-labeled phosphate incorporation to the one obtained with *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 the level obtained in the wt strain (Fig. 5B).

With respect to sporulation on solid MSA medium, the *S. lividans ΔpstS*/pINTUF5 integrated strain behaved like the wild-type strain, the differentiation process in these strains being slower than in the *S. lividans pstS* null mutant and in the control strain *S. lividans ΔpstS*/pKC796Hyg (Fig 5C). This result pointed out the ability to restore the mutant phenotypes by the single copy of the *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 *S. coelicolor* and *S. lividans pstS* promoters, obtaining the multicopy plasmids pNUF6 and pNUF7. Both plasmids were introduced into *S. coelicolor* M145 and *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 *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 been reported previously in *Rhodobacter sphaeroides* f. sp. *Denitrificans* (Matsuzaki *et al.*, 2003).

PstS overexpression has been described in several organisms under different stress conditions (Runyen-Janecky & Payne, 2002); (Atalla & Schumann, 2003), (Duwat *et al.*, 1999; Rallu *et al.*, 2000). This multiresponse of *pstS* induction suggests that the gene is one of the many emergency genes able to respond to multistress conditions that permit rapid adaptation of the microorganism to different habitats, perhaps because these conditions lead to a depletion of the internal phosphate pool and hence the need for external phosphate to be captured. This phosphate depletion may induce the expression of *pstS* promoter more than 100-fold in *E. coli*, and more than 5000-fold in *B. subtilis* (Qi *et al.*, 1997; Wanner, 1993). In *S. lividans* we failed to detect any phosphate-limited medium able to support growth and able to originate PstS accumulation at the same level that the presence of the mentioned carbon sources does. However, the effect of phosphate on the expression of this gene is clear because the addition of 5mM inorganic phosphate impaired PstS production in the presence of the inducer carbon sources. Since PstS was not accumulated in the presence of glucose, even if fructose was present, a catabolite repression response might also be regulating the expression of *pstS*. This hypothetical repression has been suggested previously for *R. sphaeroides* (Matsuzaki *et al.*, 2003).

Analysis of the *S. coelicolor* and *S. lividans pstS* promoter sequences allowed us to detect the repetition of the sequence –ACTCACCCCCGC- (with some discrepancies) in these promoters that might play some role in regulation. This sequence is not present in any other

promoter region of the *S. coelicolor* genome. Comparison of all known *Streptomyces pstS* promoters revealed the conservation of several sequences, among which the sequence -GTTCAN$_6$GTTCA- presents similarity to the consensus "Pho boxes" of *E. coli* and *B. subtilis* –CT(G/T)TCATA(A/T)A(A/T)CTGTCA(C/T)- and TT(A/T/C)ACAN5+/-2TT(A/T/C)ACA respectively (Blanco *et al.*, 2002; VanBogelen *et al.*, 1996; Wanner, 1996; Qi *et al.*, 1997; Liu *et al.*, 1998). Analysis of the *S. avermitilis* and *S. coelicolor* genomes with the Regulatory Sequence Analysis Tools (RSAT) (http://rsat.ulb.ac.be/rsat/) permitted us to observe that this sequence is located upstream from 16 and 21 ORFs respectively. Most of these ORFs encode hypothetical proteins and at least one quarter of these ORFs are the same in both organisms. Interestingly, one of them is the putative low-affinity phosphate transport protein that corresponds to SCO1845 in *S. coelicolor* and SAV6965 in *S. avermitilis*. The putative role of these sequences in control of the expression of the *pstS* gene and the complete *pst* operon must be demonstrated experimentally in future work

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

S. coelicolor and *S. lividans* PstS proteins are 100 % identical and share 79 % identity with the PstS protein from *S. avermitilis* and 68 % with the protein from *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 *Mycobacterium tuberculosis* (Lefevre *et al.*, 1997). These three proteins share a high degree of similarity among one another but their expression seems to respond differentially to the habitat. One of them, PstS-1, is one of the most important immunodominant antigens of *M. tuberculosis* (Chang *et al.*, 1994; D'Souza *et al.*, 2002). Although there is no clear duplication of the *pstS* gene in the *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 *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 *pstS*.

One plausible hypothesis of 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, would elicit a dramatic decrease in inorganic phosphate inside cells and activation of the Pho-regulon. The requirement of extra phosphate to phosphorylate the high concentration of internalised sugar would trigger PstS expression in order for inorganic phosphate molecules to be captured from the environment.

Acknowledgments

This research was partially supported by a Grant from the European Union-Ministerio de Ciencia y Tecnología (FD1997-1134-C03-01). We thank Dr. M. Virolle for the gift of the strains *S. lividans ΔppK* and *ΔphoP*. Dr. F. Leal is thanked for his comments and S. Andres and A. Saura for their excellent technical work. Thanks are also due to N. Skinner for supervising the English version of the m.s.

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Figure legends

Figure 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), with 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 four-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 four-day old culture).

Figure 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_6GTTCA 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).

Figure 3: **PstS production regulation.** Production of PstS protein by three different *S. lividans* strains cultured on YE + 5 % fructose: *S. lividans* TK24 (wt); *S. lividans Δppk* (*Δppk*) and *S. lividans ΔphoP* (*ΔphoP*). A.- Coomassie-Blue-R-stained gel (100 μl supernatant). B.- Anti-PstS polyclonal antibodies Western blot (5 μl supernatant).

Figure 4: *pstS* null mutant phenotype. A.-, Coomassie-Blue-R-stained SDS-PAGE of supernatant of *S. coelicolor* wt (lane 1); *S. coelicolor ΔpstS* mutant (lane 2); *S. lividans* wt (lane 3) and *S. lividans ΔpstS* mutant (lane 4) grown on YE + 5 % fructose (100 μl of

supernatant of 4-day cultures). The MW is indicated in kDa on the right. B.-, Western blot with anti-PstS antibodies of the same samples (5 μl of supernatant). C and D.-, Effect of *pstS* deletion (*ΔpstS*) on *S. coelicolor(*C) and *S. lividans* (D) grown on R2YE or on MSA. The plates were incubated at 28 ºC for 48 h. E.- Scanning electron micrographs of the parental strains (wt) and the corresponding *ΔpstS* mutants. The bar is 2 μm. The MSA plates used to prepare the samples were incubated at 28 ºC for 36 h.

Figure 5: Complementation studies. A**.-** Uptake of ^{32}P-labeled phosphate after 15 minutes at 30 ºC in the wild-type *S. lividans* (wt), the Δ*pstS* deletion mutant (Δ*pstS*), the complemented transformant Δ*pstS* (Δ*pstS*/pINTUF5), and the integrative negative control strain Δ*pstS* (Δ*pstS*/pKC796Hyg). B.- Western blot with anti-PstS antibodies of 5 μl of supernatant of the indicated strain. C.- MSA plate inoculated with different strains of *S. lividans* to observe the effect of *ΔpstS* complementation (Δ*pstS*/pINTUF5). Δ*pstS*/pKC796Hyg is the mutant Δ*pstS* transformed with the empty vector; wt is *S. lividans* 1326. The plate was incubated at 28 ºC for 48 hours.

1 **Table 1: Plasmids**

PLASMID	CHARACTERISTICS	REFERENCE
pIJ773	pBluescript KS(+) derivative. Template plasmid containing the apramycin resistance gene *aac(3)IV* and the *oriT* of plasmid RP4.	(Gust *et al*., 2003)
pNX24	pN702GEM3 derivative. *xysA* promoter controlling *xysA* expression.	(Adham *et al*., 2001)
pNUF2	pNX24 derivative. *pstS* promoter from *S. coelicolor* controlling *xysA* expression	This study
pNUF3	pNX24 derivative. *pstS* promoter from *S. lividans* controlling *xysA* expression	This study
pNUF4	pNUF2 derivative. Transcriptional terminator *mmrt* inserted upstream from the *pstS* promoter from *S. coelicolor* controlling *xysA* expression	This study
pNUF5	pNUF3 derivative. Transcriptional terminator *mmrt* inserted upstream from the *pstS* promoter from *S. lividans* controlling *xysA* expression	This study
pNUF6	pNUF4 derivative. The *pstS* promoter from *S. coelicolor* controls *pstS* expression	This study
pNUF7	pNUF5 derivative. The *pstS* promoter from *S. lividans* controls *pstS* expression	This study
pKC796Hyg	Integrative plasmid derived from pKC796 (Kuhstoss *et al*., 1991) in which apramycin resistance has been replaced by hygromycin resistance	This study

pINTUF5	pKC796Hyg derivatived containing *pstS* gene and promoter from *S. lividans*	This study

1

2

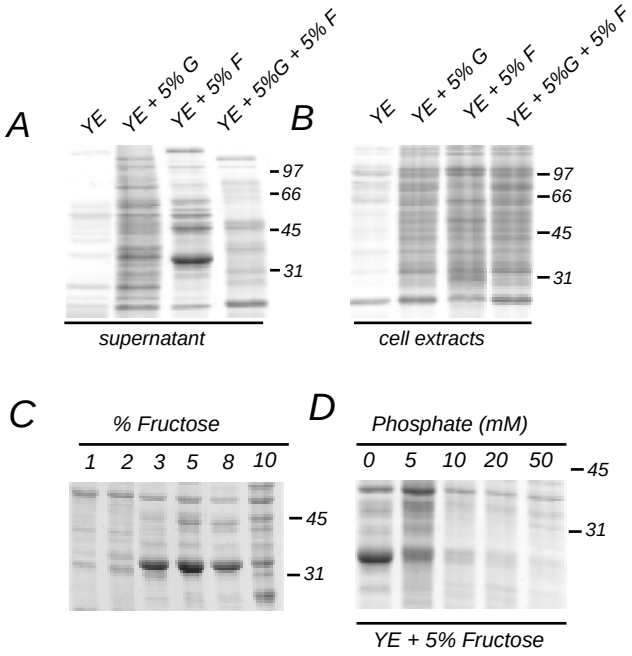

Fig. 1 Díaz et al.

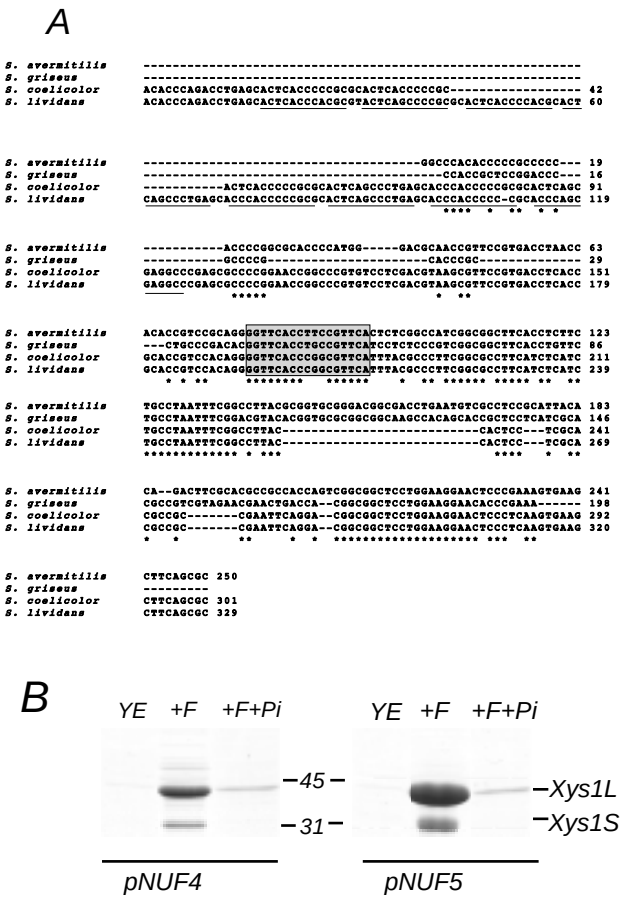

Fig. 2 Díaz et al.

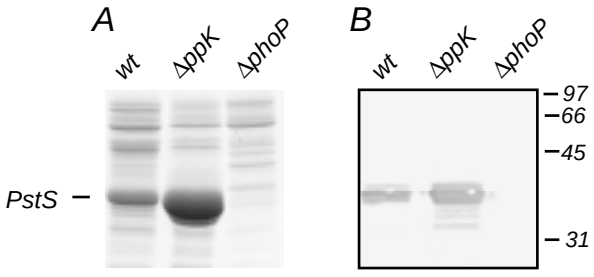

Fig. 3 Díaz et al.

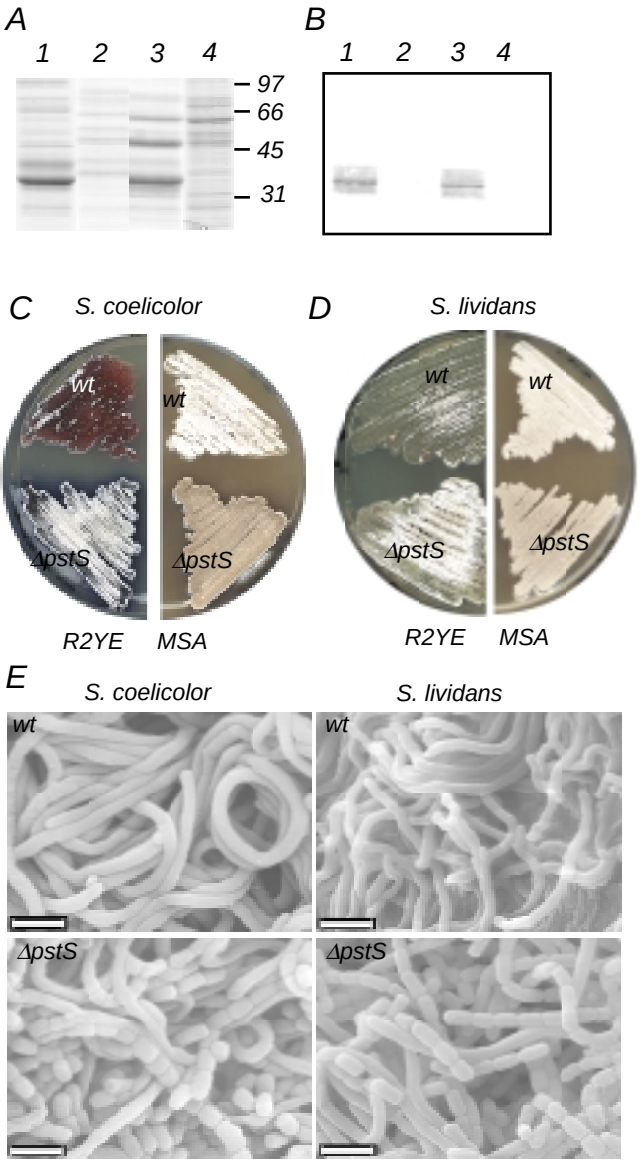

Fig. 4 Díaz et al.

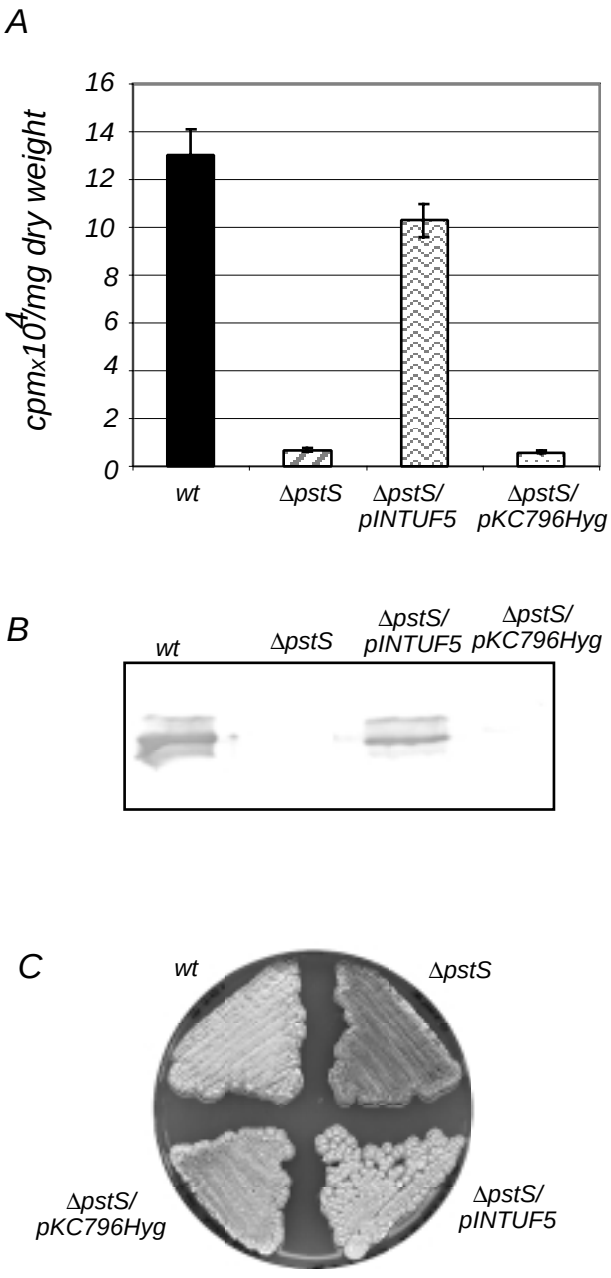

Fig. 5 Díaz et al.