High level of antibiotic production in a double polyphosphate kinase and phosphate binding protein mutant of *Streptomyces lividans*

Margarita Díaz[1], Laura Sevillano[1], Sergio Rico[1], Felipe Lombo[2], Alfredo F Braña[2], Jose A. Salas[2], Carmen Mendez[2] and Ramón I. Santamaría[1]*

[1]Instituto de Biología Funcional y Genómica /Departamento de Microbiología y Genética. Consejo Superior de Investigaciones Científicas (CSIC)/Universidad de Salamanca. Zacarías González sn. 37007-Salamanca, Spain.

[2]Area de Microbiología, Departamento de Biología Funcional, Facultad de Medicina, Instituto Universitario de Oncología del Principado de Asturias (IUOPA), Universidad de Oviedo, 33006 Oviedo, Asturias, Spain.

***Corresponding author**:

Ramón I. Santamaría

Instituto de Biología Funcional y Genómica. Zacarías González sn. E-37007 Salamanca, Spain

Telephone: +34-923-294899; Fax: +34-923-224876;

E-mail: santa@usal.es

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ABSTRACT

Phosphate metabolism regulates most of the life processes of microorganisms. In the present work we obtained and studied a *Streptomyces lividans ppk/pstS* double mutant, which lacks polyphosphate kinase (PPK) and the high-affinity phosphate binding protein (PstS), impairing at the same time the intracellular storage of polyphosphate and the intake of new inorganic phosphate from a phosphate limited media respectively. In some of the aspects analyzed, the *ppk/pstS* double mutant was more similar to the wild-type strain than the single *pstS* mutant. So, the double mutant was able to grow in phosphate-limited media, while the *pstS* mutant required the addition of 1 mM phosphate under the assay conditions used. The double mutant was able to incorporate more than one fourth of the inorganic phosphate incorporated by the wild-type strain, while phosphate incorporation was almost completely impaired in the *pstS* mutant. Noteworthy, under phosphate limitation conditions, the double *ppk/pstS* mutant showed a higher production of the endogenous antibiotic actinorhodin and the heterologous antitumor 8-demethyl-tetracenomycin (up to 10-fold with respect to the wild-type strain), opening new possibilities in the use of this strain for the heterologous expression of antibiotic pathways.

INTRODUCTION

In nature, microorganisms of the genus *Streptomyces*, a filamentous bacterium, grow in soil by hydrolysing different complex carbon sources. The changing conditions of this way of life have forced these microorganisms and others in similar habitats, to develop adaptive responses to different types of stress and nutritional deficiencies. One of these adaptive responses to the nutritional environment is mediated by the level of polyphosphate (poly(P)) (Rao & Kornberg, 1999, Manganelli, 2007). The poly(P) chain is a linear polymer of orthophosphate residues linked by high-energy bonds that is ubiquitous in all living organisms (Kulaev & Kulakovskaya, 2000). It constitutes a phosphate reservoir that is mobilized under Pi starvation conditions (Rao & Kornberg, 1996, Van Dien & Keasling, 1999). The enzyme polyphosphate kinase (PPK) synthesizes this polymer mainly from ATP and it is a homotetrameric protein that is associated with the outer membrane in *Escherichia coli* (Ahn & Kornberg, 1990). A second PPK (PPK2), described as widely conserved in bacteria, can synthesize poly(P) from GTP or ATP (Zhang*, et al.*, 2002). Polyphosphate also functions as a source of phosphate group for the phosphorylation of sugars, nucleotide diphosphate, and proteins, and its degradation is mainly carried out by phosphatases, although some kinases may use it as an ATP substitute, and even PPK and PPK2 can use it to generate ATP or GTP from the corresponding nucleotide diphosphate (Tzeng & Kornberg, 2000, Ishige*, et al.*, 2002).

To date, only one *ppk* gene has been studied in detail in *S. lividans* and it exerts a negative role in antibiotic production (Chouayekh & Virolle, 2002). Transcriptional studies of *ppk* have demonstrated that this gene is mainly expressed under conditions of Pi limitation, although a weak expression is also detectable with phosphate-rich medium. This expression is controlled by the two-component PhoR/PhoP system and by an unknown repressor that uses ATP as a corepressor (Ghorbel*, et al.*, 2006).

In previous work with *S. lividans* we described the increased accumulation of the PstS protein in a polyphosphate kinase-null mutant (*Δppk*) (Diaz*, et al.*, 2005). The PstS protein is a high-affinity phosphate-binding protein that forms part of the high-affinity phosphate transport system encoded by the *pst* operon. This operon, which is expressed under the control of PhoR/PhoP is induced under phosphate limitation and is also induced in the presence of an excess of certain carbon sources as fructose (Diaz*, et al.*, 2005, Sola-Landa*, et al.*, 2005) suggesting a dual carbon-phosphate regulation (Esteban*, et al.*, 2008). Recently, and in relation to this complex regulation, it has been reported that the sugar phosphates affect *Streptomyces* development through genes that are under the positive control of the two-component system PhoR/PhoP (Tenconi*, et al.*, 2012)

In the present work we studied an *S. lividans* double mutant -*Δppk/ΔpstS*- in order to check the viability of this mutant under phosphate-limited conditions. Differences were detected in comparison with the wild type or the single *ΔpstS* or *Δppk* mutants upon incubation on Asparagine-minimal solid medium (AMM) or in liquid R2YE under phosphate-limited conditions that suggested a cumulative effect of double mutation that partially suppresses the effects of separate single mutations. The most interesting feature of the double mutant was the overproduction of the pigmented antibiotic actinorhodin, when cultured in liquid R2YE under limited phosphate conditions. Additionally, when the double mutant was used as host to express the heterologous biosynthetic pathway for the antitumor compound 8-demethyl-tetramycin, a strong increase in production was obtained.

MATERIALS AND METHODS

Bacterial strains, plasmids and media. All strains and plasmids used are listed in Table 1. *Streptomyces* strains were grown at 30 °C on Solid Mannitol Soy Flour Agar medium (MSA), or R2YE (Kieser*, et al.*, 2000) for normal cultures and sporulation. Asparagine-minimal medium (AMM) (Martin & McDaniel, 1975, Sola-Landa*, et al.*, 2003) solidified with 3 % agarose and supplemented with different amounts of phosphate (from 0 to 5 mM sodium phosphate, pH 7) was used to study the growth of the different mutants. Cultures in liquid AMM media with different amounts of phosphate were done but very limited growth was obtained even for the wild type strain in all the concentrations assayed (data not shown). So, submerged cultures were normally carried out in YE medium (0.5 % yeast extract) supplemented with different amounts of the carbon source studied, normally fructose or glucose plus 2 mM $MgCl_2$. Other liquid media used were R2YE (the same as solid media without agar) supplemented with different amounts of sodium phosphate, pH 7. The *Streptomyces* culture conditions have been described previously (Fernández-Abalos*, et al.*, 2003).

E. coli was grown in Luria Broth (LB) at 37 °C, supplemented with kanamycin (25-50 µg ml^{-1}) when needed.

DNA manipulations and transformations of *S. lividans* and *E. coli*. Total DNA isolation (genomic + plasmid), transformation, and protoplast manipulation were done as indicated previously (Diaz*, et al.*, 2005). Intergeneric conjugation was used to transfer cosmids from *E. coli* to *S. lividans* as described in Gust et al. (Gust*, et al.*, 2003).

Phosphate uptake. Phosphate incorporation in *S. lividans* cultures was studied in cells grown in liquid YE + 5 % fructose for 60 h (30 ºC, 200 rpm). Cells were washed with 0.9 % NaCl and ^{32}P-labeled Na_2HPO_4 was added (2 x 10^5 cpm/ml). Phosphate uptake was measured after 2 minutes at 30 ºC with a liquid scintillation counter (Wallac 1409-001).

The phosphate uptake results were normalized to dry weight of the corresponding cells used in the assay.

Construction of *S. lividans* Δ*ppk*/Δ*pstS* mutant. Deletion of the *pstS* gene was accomplished using the REDIRECT technology (Gust, *et al.*, 2003). The *ppk::Ωhygro* mutant strain (TK24 derivative) (Chouayekh & Virolle, 2002) was used as a host to obtain the double mutant. A *pstS*-deletion cassette generated previously to delete the *pstS* gene, in *S. lividans* 1326 and in *S coelicolor* M145, was used (Diaz, *et al.*, 2005). The recombinant cosmid (SCD84 *pstS::acc(3)IV-oriT*) was introduced into *S. lividans* TK24 and the *S. lividans ppk::Ωhygro* mutant to obtain the *pstS* and the *pstS/ppk* null mutants, respectively, by intergeneric conjugation (*E. coli/Streptomyces*). Correct replacement was checked in Southern blot experiments.

Protein analysis. Total cell protein was obtained breaking the cells in a fast prep (MP-Biomedicals) and boiling the extract in SDS-polyacrylamide loading buffer for ten minutes. Protein electrophoresis was accomplished in denaturing polyacrylamide gels (SDS-PAGE), as described elsewhere (Ruiz-Arribas, *et al.*, 1995). Coomassie blue staining was done to visualize proteins. Western blot analyses of the proteins separated in SDS-PAGE were done as in Esteban et al. (Esteban, *et al.*, 2008). Anti-PstS antibodies were used as primary antibodies and horseradish peroxidase-conjugated secondary donkey-anti-rabbit antibody was used. The blot was developed with ECL reagents obtained from General Electric, used according to the manufacturers' instructions.

Alkaline phosphatase assay. Alkaline phosphatase activity was measured following the method described by Moura et al. (Moura, *et al.*, 2001). In summary, 50 µL of sample was added to 50 µL of 25 mM Tris-HCl buffer pH 8 containing 10 mM *p*-nitrophenyl phosphate (PNPP) and 0.4 mM $CaCl_2$ and incubated at 37 ºC for ten minutes. The

reaction was stopped by adding 1 mL of 0.5 M Na_2CO_3 and absorbance was measured at 410 nm. The growth rates of all the strains tested were similar (data not shown).

Generation of an integrative version of 8-demethyl-tetracenomycin C cosmid clone cos16F4. Cosmid clone cos16F4 is a pKC505 derivative (Kieser*, et al.*, 2000) that contains most of the genes from the elloramycin gene cluster from *Streptomyces olivaceus* Tü2353 and is responsible for the biosynthesis of 8-demethyl-tetracenomycin C aglycon (Ramos*, et al.*, 2008).

In order to convert this replicative (low copy number) and apramycin-resistant cosmid into an integrative one, a 6.9-kb SpeI DNA fragment from pFL1139 was cloned into the unique XbaI restriction site of cos16F4, which is located at its multiple cloning site. This 6.9-kb DNA fragment contained the conjugative *oriT* (for conjugation from *E. coli*), the tetracycline resistance cassette, the site-specific recombination *attP* site, the *int* integrase gene from ΦC31, and the *ermE* erythromycin resistance cassette (for selection in *Streptomyces*). pFL1139 is a pBluescriptSK derivative that contains the *ermE* cassette cloned as an EcoRV-StuI 1.7 kb DNA fragment into the unique EcoRI site (blunt-ended) of pFL1138. pFL1138 is a pBluescriptSK derivative that contains a 5.2 kb DraI-BsaI DNA fragment from pIJ787 (kindly provided by Dr. Bertold Gust, Universität Tübingen, Germany) cloned at the pBluescriptSK SmaI site.

HPLC analysis and quantification of 8-demethyl-tetracenomycin C. Liquid cultures (10 ml in R2YE with different phosphate concentrations see below) were incubated at 28º C for 5 days and then extracted with 1 volume of ethyl acetate and the organic layer was dried *in vacuo*. The dry extracts were finally resuspended in methanol. These extracts were analyzed by reversed phase chromatography in an Acquity UPLC device with a BEH C18 column (1.7 mm, 2.1 x 100 mm, Waters) and equipped with a DAD (Waters 2996). The two mobile phase solvents were acetonitrile and 0.1% trifluoroacetic acid (in

water). Samples were chromatographed using this elution programme: 10% acetonitrile for 1 min, followed by a linear gradient from 10% to 80% acetonitrile over 7 min at a flow rate of 0.5 ml/min and a column temperature of 30º C. Detection and spectral characterization of the peaks were performed by photodiode array detection and Empower software (Waters), extracting two-dimensional chromatograms at 280 nm. The peaks corresponding to 8-demethyl-tetracenomycin C eluted at 4.04 min and were quantified by area integration as comparison with pure 8-demethyl-tetracenomycin C. Actinorhodin was quantified using the standard spectrophotometric method (Kieser*, et al.*, 2000).

Enzymes and reagents. The products used were purchased from Bio-Rad, Boehringer Mannheim, Invitrogen, Merck, Panreac, Promega, Quiagen or Sigma, and were used following the manufacturers' guidelines.

RESULTS AND DISCUSSION

The double mutant *ppk/pstS* restores deficiencies of the *pstS* mutant growth under phosphate-limited conditions.

We have previously reported the over-accumulation of the PstS protein in the *S. lividans* *Δppk* mutant (Diaz*, et al.*, 2005). In order to check the effect that the join deletion *ppk-pstS* has in the growth of *S. lividans* under limited phosphate conditions a double mutant, *ppk/pstS,* was generated in *S. lividans* TK24. The *S. lividans ppk* mutant (Chouayekh & Virolle, 2002) was used as a host to delete the *pstS* gene with the Redirect technology (Gust*, et al.*, 2003). The apramycin cassette was used to replace the *pstS* gene, as described in Diaz *et al*. (Diaz*, et al.*, 2005). In order to obtain isogenic strains a single *pstS* mutant was also generated in the *S. lividans* TK24 strain that was the parental strain of the *ppk* mutant. DNA-DNA hybridization and PCR analyses were used to corroborate *pstS* gene replacement in both mutants (data not shown). The absence of PstS protein in the cells (Fig. 1A) and in the culture supernatant (not shown) of these *ΔpstS* and Δ*ppk*/*pstS* mutants was also corroborated by SDS-PAGE and Western blot with *anti*-PstS antibodies.

The effect of the several concentrations of phosphate (from 0 μM to 5 mM) on the different mutants was studied on AMM solid medium. 100 viable spores from each strain were deposited in a drop of 5 μl of water onto the surface of the medium and incubated at 30 ºC for several days and the growth of all the strains was monitored. The growth of the single *pstS* mutant was the most affected. After three days of incubation, this mutant was unable to grow on any of the phosphate concentrations used while all the other strains grew well (data not shown). Longer incubations (4-6 days) permitted the growth of the single *pstS* mutant in media containing 1 mM phosphate and higher while all the other strains (including the double *ppk/pstS* mutant) were able to grow even in the absence of

added phosphate (Fig. 1B). All the strains tested grew perfectly well in other complex media, such as R2YE and MSA (Fig. 1B).

These results suggested that another way to obtain and capture phosphate might be activated in the double mutant. At least two possibilities may explain these results: first, an increase the extracellular phosphatase level and/or second, an increase in phosphate incorporation. To study this we used the media YE containing 5 % fructose and 2 mM $MgCl_2$ that was used in our previous work on *pstS* gene (Diaz*, et al.*, 2005). Phosphatase activity was similar in the wild type strain and in the *pstS* mutant in all the times assayed. However, phosphatase activity increased up to 2.5 times in the *ppk* and in the *ppk/pstS* double mutant at 60 hours cultures (Fig. 2A). Inorganic phosphate incorporation was studied in the different strains with ^{32}P-labeled phosphate uptake As described previously for *S. lividans* 1326 *ΔpstS* (Diaz*, et al.*, 2005) a striking reduction in phosphate uptake was observed for *S. lividans* TK24 *ΔpstS* , which was only able to incorporate 5.2 % of the amount taken up by the wt strain. The *ppk* mutant was able to incorporate 82 % of the amount incorporated by the wt strain while the *pstS/ppk* double mutant was able to uptake 28 % of the phosphate incorporated by the wt strain (5.3-fold the amount from the *pstS* mutant) (Fig 2B). Both, higher phosphatase activity and an increase in phosphate uptake, may explain the previous observation that this *Δppk*/*pstS* double mutant grew better than the single *ΔpstS* mutant under limited phosphate conditions.

This opens the possibility that another high affinity phosphate transport system could be activated in the double mutant. Although a putative orthologous *pst* operon is present in *S. lividans* 1326 and in *S. coelicolor* (ORFs: SCO6814, SCO 6815 and SCO 6816) that operon is missing in *S. lividans* TK24 genome (Lewis*, et al.*, 2010). So, up to now we do not have a clear candidate that may be the responsible for the increase of phosphate transport in the double mutant under low phosphate concentrations.

Because the *ppk* mutant of *S. lividans* displays a higher expression of the PhoP regulator (Ghorbel*, et al.*, 2006) and a higher expression of the complete *pst* operon (data not shown) higher phosphate transport would be expected in this mutant. However, under our experimental conditions, the incorporation of radioactive phosphate was slightly lower in this strain than in the wild type strain, indicating the existence of another level of regulation, perhaps triggered by a saturation of the concentrations of intracellular phosphate that was not processed into polyphosphate in this mutant. *S. lividans* has another putative functional polyphosphate kinase encoded by the SSPG_07441.1 ORF, which is identical to *SCO0166* from *S. coelicolor*. That protein, classified as a putative regulator, in both databanks, shares 64 % identity and 77 % similarity with the PPK2A (NCgl0880) and 53 % identity and 71 % similarity with the PPK2B (NCgl2620) from *Corynebacterium glutamicum*. (Lindner*, et al.*, 2007). The protein encoded by *SCO0166* also shares high similarity (60 % identity and 72 % similarity) with *Pseudomonas aeruginosa* PPK2, whose activity has been demonstrated experimentally (Zhang*, et al.*, 2002, Rao*, et al.*, 2009). Future studies addressing the activity of the putative *Streptomyces* PPK2 may clarify the role of this enzyme in phosphate storage and uptake.

The *pstS/ppk* double mutant expresses higher amounts of endogenous actinorhodin and of heterologous 8-demethyl-tetracenomycin C than the other strains under low phosphate concentrations.

During the study of the growth of the different strains on solid AMM with different phosphate concentrations (see above), the production of the blue-coloured antibiotic actinorhodin was detected on plates containing 250 and 500 μM phosphate for the *ppk*/*pstS* double mutant and on plates with phosphate concentrations of 500 μM and 1

mM for the *ppk* mutant, while higher concentrations impaired antibiotic production in both strains (Fig. 1B).

The effect of the different phosphate concentrations on actinorhodin production by all four strains was also studied and quantified in liquid R2YE medium with three different amounts of added phosphate: medium without phosphate (R2-P); medium supplemented with the normal amount of phosphate (0.37 mM), and medium with a higher amount of phosphate (1.85 mM). Actinorhodin production by the *ppk* and *ppk*/*pstS* mutants was clearly observed when grown in R2-P and in normal R2 after 4 days of culture. Higher production was obtained in the *ppk*/*pstS* mutant in both conditions (Fig. 3A). However, the addition of a high phosphate concentration (R2+1.85 mM P) blocked antibiotic production in these strains.

Overproduction of actinorhodin by the *ppk* mutant under phosphate-limiting conditions was described previously (Chouayekh & Virolle, 2002). These authors reported that the expression of *actII-ORF4* increased drastically in the *ppk* mutant and originates an increase in actinorhodin production. Although this strain has a functional *pst* operon that permits a phosphate incorporation almost similar to the wild-type strain the incapacity to accumulate polyphosphate may originates a phosphate starvation under low phosphate culture concentrations. This starvation is increased in the *ppk*/*pstS* double mutant on which a limitation on phosphate transport is observed when compared with the phosphate incorporation on the *ppk* mutant. This phosphate famine might explain the higher actinorhodin production of the double mutant Δ*ppk*Δ*pstS* compared to the single one Δ*ppk*.

The effect of phosphate on antibiotic production was also studied in the ability of these strains to produce heterologous compounds. The integrative cosmid cos16F4iE, which directs the biosynthesis of the polyketide antitumor 8-demethyl-tetracenomycin C, was

introduced into all the strains and the production of this antitumor agent was carried out in R2-P or in R2+1.85 mM P. Production of the antitumor agent was quantified by HPLC, with the observation that it was higher under phosphate limitation: three-fold higher than in media with the phosphate supplement. The best producer under both conditions was the *pstS/ppk* double mutant, which attained a production of about 9.7 μg/ml under phosphate limitation and 3 μg/ml under an excess of phosphate. These yields represent over 10-fold more antibiotic than that obtained with the wild-type strain and about 3-fold more than that obtained with the *ppk* single mutant. These results open the future possibility of using the Δ*ppk*/Δ*pstS* strain as a host for the industrial production of metabolites of interest.

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Figure legends

Figure 1: PstS expression and strains growth in AMM with different amount of phosphate. A) Western blot to detect cell-bound PstS in the indicated strains (3 µg of total protein were loaded per lane) using anti-PstS. B) Growth of the different strains in minimal medium (AMM) supplemented with the indicated amount of sodium phosphate buffer, pH 7. The growth of these strains in R2YE and MSA are also included as controls.

Figure 2: Extracellular phosphatase and phosphate transport A) Extracellular phosphatase activity (µmol PNP/mL) of the different strains: *wt* (◆), *ΔpstS* (■), *Δppk* (▲) *Δppk*/*pstS* (X). B) Uptake of ^{32}P-labeled phosphate after 2 minutes at 30 ºC of the indicated strains. The results were normalized to dry weight of the corresponding cells used in the assay. The results presented are the means of three independent experiments.

Figure 3: Antibiotic production by the different strains A) Histogram showing the production of actinorhodin in R2YE with different amounts of phosphate (P): without phosphate (■); supplemented with the normal amount of phosphate (0.37 mM) (■), and with a higher amount of phosphate (1.85 mM) (□). B) Histogram showing the production of 8-demethyl-tetracenomycin C from an integrated plasmid in all the different strains. The cultures were carried out in R2YE without phosphate (■) and in the same medium supplemented with 1.83 mM phosphate (□). The results presented are the means of two independent experiments.

1 Table 1: Bacterial strains

Strain	Genotype	Comments	Reference
Streptomyces lividans TK24	*str-6* SLP2$^-$ SLP3$^-$	parental strain	(Kieser, *et al.*, 2000)
Streptomyces lividans Δppk	*str-6* SLP2$^-$ SLP3$^-$ *Δppk*	Polyphosphate kinase-defective mutant	(Chouayekh & Virolle, 2002)
Streptomyces lividans ΔpstS	*str-6* SLP2$^-$ SLP3$^-$ *ΔpstS*	Mutant defective in the high-affinity phosphate protein PstS.	This Work
Streptomyces lividans ΔpstS/Δppk	*str-6* SLP2$^-$ SLP3$^-$ *ΔpstS/Δppk*	Mutant defective in the high-affinity phosphate protein PstS and in the Polyphosphate kinase, Ppk.	This Work
Escherichia coli DH5α	F$^-$, ϕ80d*lac*ZΔM15, Δ(*lac*ZYA-argF)U169, *rec*A1, *end*A1, *hsd*R17(rk$^-$, mk$^+$), *sup*E44, λ-, *thi*-1, *gyr*A, *rel*A1	Cloning, plasmid isolation	(Hanahan & Meselson, 1983)
E. coli BW25113/pIJ790	*E. coli* K12 derivative *ΔaraBAD, ΔrhaBAD*	Gene replacement	(Datsenko & Wanner, 200
E. coli ET12567/pUZ8002	*dam, dcm, hsdS, cat, tet*	*E. coli/S. lividans* conjugation	(MacNeil, *et al.*, 1992)

2

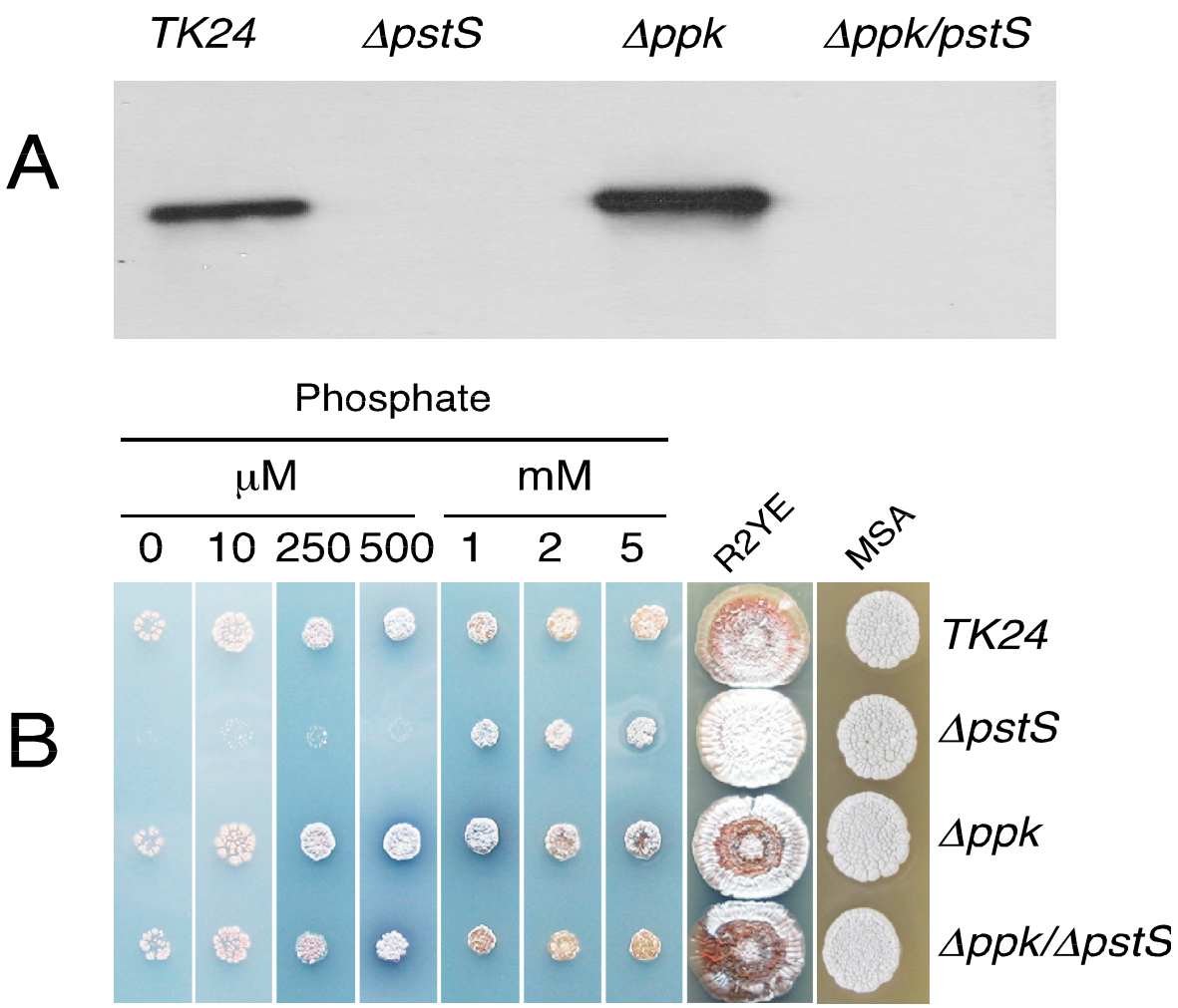


Figure 1, Diaz et al

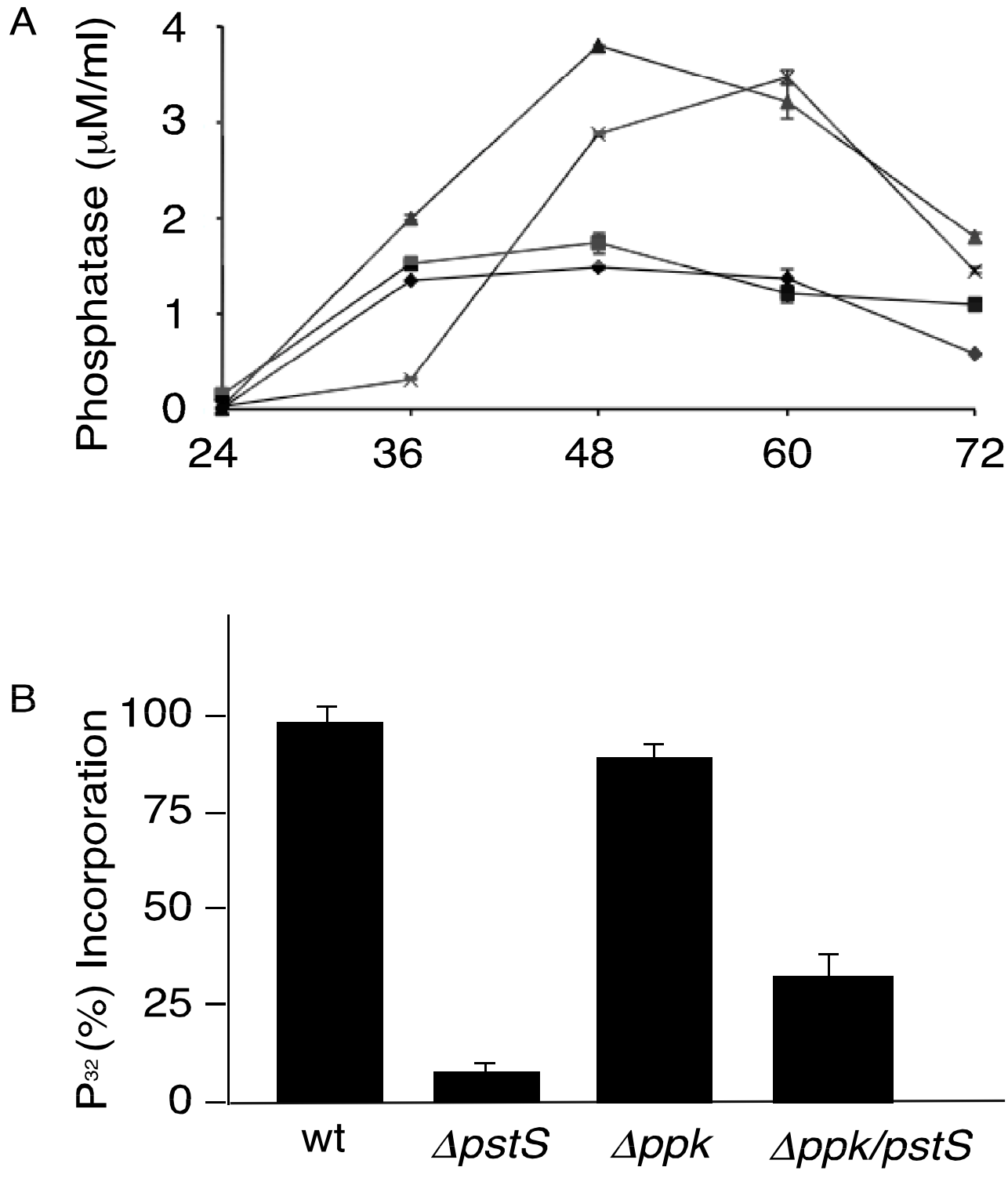
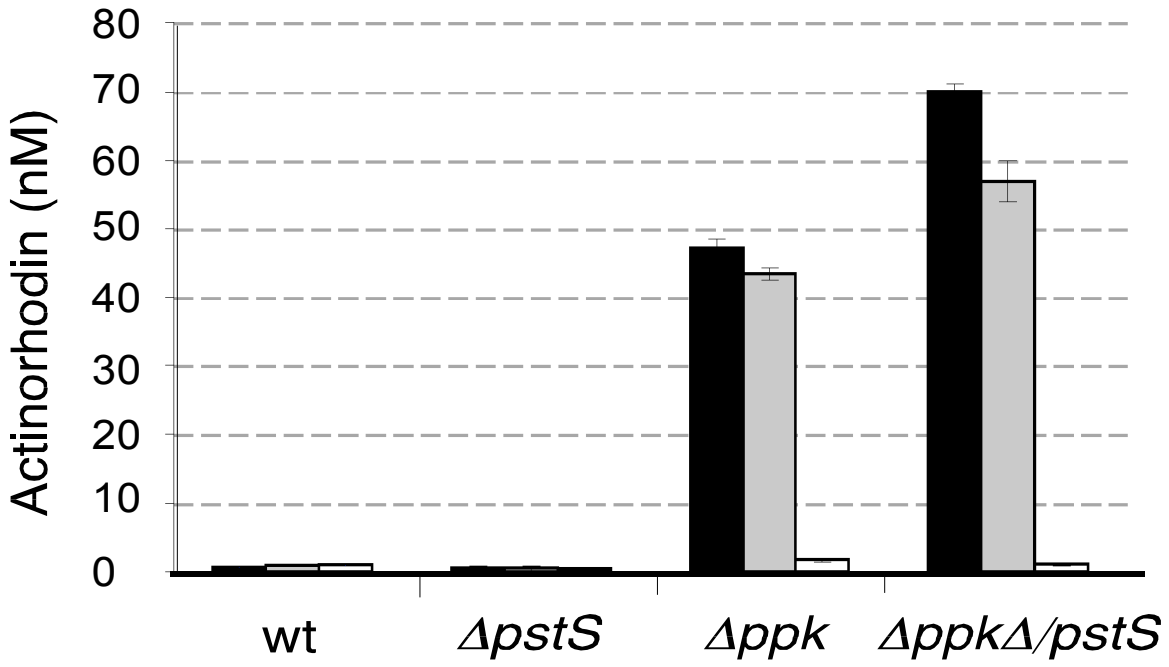


Figure 2, Diaz et al

A



B

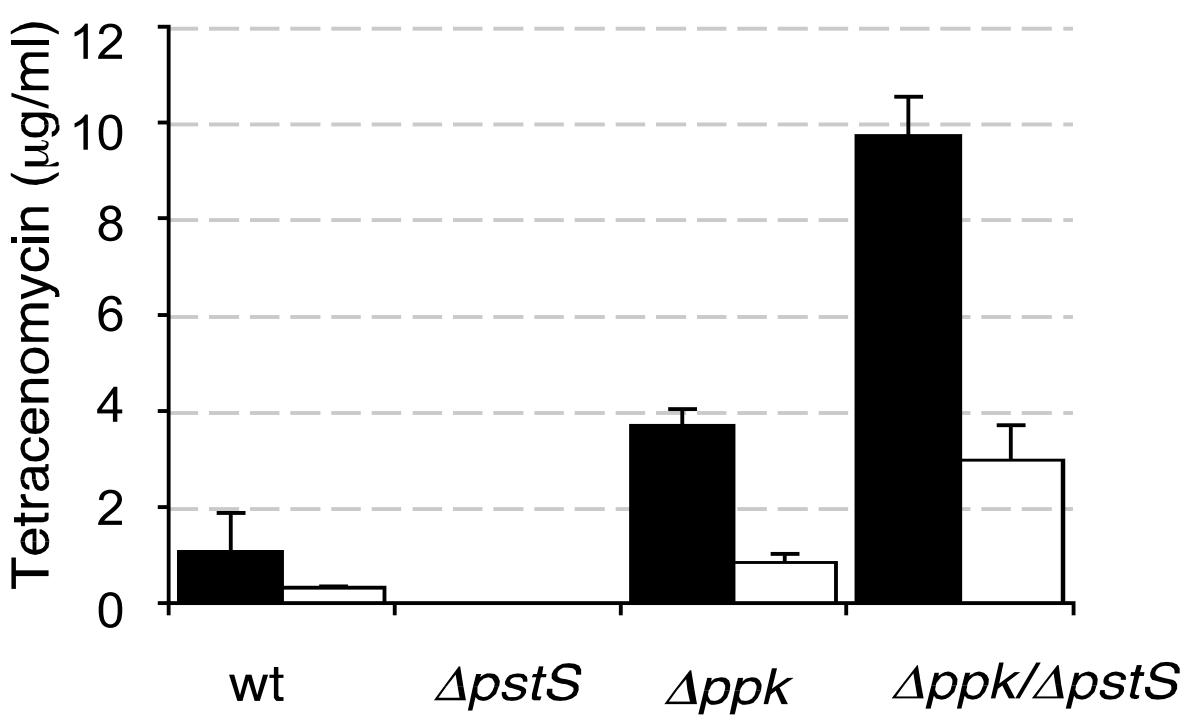


Figure 3, Diaz et al