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5 **Post-translational enzyme modification by the  
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**SHORT TITLE:** Role of the stand-alone PPTase of *P. chrysogenum*.

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50 **SYNOPSIS**

Nonribosomal peptide synthetases (NRPSs) and polyketide synthases (PKSs) require a post-translational phosphopantetheinylation to become active. This reaction is catalysed by the 4'-phosphopantetheinyl transferase (PPTase). The *ppt* gene of *Penicillium chrysogenum*, encoding a protein that shares 50% homology with the stand-alone large PPTases, has been cloned. This gene is present as a single copy in the genome of the wild type and high penicillin producing strains (containing multiple copies of the penicillin gene cluster). Amplification of the *ppt* gene produced increases in isopenicillin N and benzylpenicillin biosynthesis. A PPTase defective mutant (Wis54-PPT<sup>-</sup>) was obtained. It required lysine and lacked pigment and penicillin production, but it still synthesized normal levels of roquefortine. The biosynthesis of roquefortine does not appear to involve a PPTase-mediated modification of the synthesizing enzymes. The PPT<sup>-</sup> mutant did not require fatty acids, which indicates that activation of the fatty acid synthase occurs by a different PPTase. Complementation of Wis54-PPT<sup>-</sup> with the *ppt* gene, restored lysine biosynthesis, pigmentation and penicillin production, which evidences the wide range of processes controlled by this gene.

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Keywords: 4'-phosphopantetheinyl transferase, lysine, beta-lactam antibiotics, post-translational modification, roquefortine, secondary metabolism

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Abbreviations used: ACPS, acyl carrier protein synthase; ACV,  $\delta$ (L- $\alpha$ -aminoadipyl)-L-cysteinyl-D-valine; ACVS,  $\delta$ ( $\alpha$ -aminoadipyl)-cysteinyl-valine synthetase; ble, bleomycin and phleomycin resistance gene; CP, complex penicillin production medium; DP, defined production medium; FAS, fatty acid synthase; GATase, glutamine amidotransferase; HPLC, high performance liquid chromatography; IOD, integrated optical density; LB, Luria-Bertani broth medium; NRPS, nonribosomal peptide synthetase; PKS, polyketide synthases; ORF, open reading frame; Pgdh, glutamate dehydrogenase gene promoter; PPTase, 4'-phosphopantetheinyl transferase; RT-PCR, reverse transcription-PCR; Tcyc1, cytochrome c1 transcriptional terminator.

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## INTRODUCTION

*Penicillium chrysogenum* is a filamentous fungus which produces penicillins with an aromatic side chain, like benzylpenicillin or phenoxymethylpenicillin [1]. The pathway for penicillin formation has been largely elucidated [2]. It starts with the non-ribosomal biosynthesis of the tripeptide  $\delta$ (L- $\alpha$ -aminoadipyl)-L-cysteinyl-D-valine (ACV), synthesized by the large multienzyme  $\delta$ ( $\alpha$ -aminoadipyl)-cysteinyl-valine synthetase (ACVS), a typical non-ribosomal peptide synthetase (NRPS) encoded by the *pcbAB* gene [3-5]. The last steps include cyclization of the ACV tripeptide to isopenicillin N (catalyzed by the isopenicillin N synthase encoded by the *pcbC* gene) and substitution of the L- $\alpha$ -aminoadipyl side chain of isopenicillin N by aromatic acyl side chains (performed by the isopenicillin N acyltransferase encoded by the *penDE* gene) [6, 7]. Previous activation of the aromatic acid by a specific aryl-CoA ligase [8, 9] is required. The genes involved in the biosynthesis of  $\beta$ -lactam antibiotics are arranged in clusters in bacteria as well as in filamentous fungi [1, 10, 11]. In *P. chrysogenum*, the three penicillin biosynthetic genes are clustered together in a DNA region, which is amplified in tandem repeats in high producing strains, together with other ORFs [12, 13, 14]. Besides penicillin, *P. chrysogenum* and other filamentous fungi synthesize different secondary metabolites, such as roquefortine and the characteristic green pigment [15]. Multidomain enzymes such as polyketide synthases (PKSs) and nonribosomal peptide synthetases (NRPSs) are in charge of the biosynthesis of secondary metabolites [16-19]. A common feature to these enzymes is that they require a post-translational modification to become active. The apoprotein form (inactive) becomes active (holo form) after the covalent attachment of the 4'-phosphopantetheine moiety, which derives from coenzyme A [20]. Phosphopantetheine has been found to be present in ACV synthetase and in other peptide synthetases [21]. The binding site of this moiety is a serine, which is conserved in all acyl carrier and peptidyl carrier domains present in fatty acid synthases PKSs and NRPSs, respectively [22]. Phosphopantetheine is added to the apoprotein by an enzyme known as a 4'-phosphopantetheinyl transferase (PPTase) [20, 23]. In the case of the ACVS, the acyl carrier domain (thiolation) of each of the three modules bears a conserved serine residue that binds a thiol-containing phosphopantetheine co-factor [21, 24]. This co-factor may in turn, form the thioester with the activated amino acid, recognized by the cognate activation (A) domain of each module. Activation through phosphopantetheine addition is not only found in enzymes of secondary metabolism. Fungi have a unique pathway for the biosynthesis of lysine via the  $\alpha$ -aminoadipic acid. The  $\alpha$ -aminoadipate reductase catalyses the conversion of  $\alpha$ -aminoadipate into  $\alpha$ -aminoadipate semialdehyde [25-27]. This enzyme is synthesized as a catalytically inactive form, which is activated after the addition of a phosphopantetheinyl group by the PPTase [26, 28, 29]. PPTases have been classified into three major groups according to their substrate specificity. The first class includes the PPTases of bacterial acyl carrier protein synthases (ACPS-type), which are involved in the fatty acid synthesis [20] and in the activation of bacterial polyketide synthases [30]. The second type includes eukaryotic PPTases that constitute an integral domain of fungal type I fatty acid synthases (FASs) [31]. The third group, the Sfp-type PPTases, are twice the size of the ACPS-type enzymes and show a broader range of specificity. PPTases from this family are often associated with a biosynthetic pathway and are required for secondary metabolism in bacteria [20, 32] and for lysine biosynthesis in fungi [33].

Some strains of *P. chrysogenum* contain an amplification of the penicillin gene cluster (*pen*) region [12-14] and produce very large amounts of penicillin, thus requiring a high PPTase activity. This might be limiting for ACVS modification unless there is a gene duplication. Therefore, it was important to identify the gene encoding the PPTase of *P. chrysogenum* to elucidate the role of this protein in primary and secondary metabolism. In this work, we report the isolation and characterization of the gene encoding a stand-alone large (class III) PPTase of *P. chrysogenum* as well as the generation of overexpressing and lack-of-function mutants. Our results indicate that a single PPTase is involved in a wide spectrum of processes, including penicillin production, pigmentation and lysine biosynthesis, but not in fatty acid or roquefortine formation.

## EXPERIMENTAL

### Strains and culture conditions

*P. chrysogenum* NRRL-1951 (wild type) and Wis54-1255 strains, which contain a single copy of the penicillin gene cluster [12], were used as the parental strains in this work. The *P. chrysogenum* Wis54-1255 pyrG<sup>-</sup> strain (an uridine auxotroph derived from the Wis54-1255 strain) and the high penicillin producing strains AS-P-78 and AS-P-99 were used to study the gene copy number. The lysine auxotroph L2/lys1<sup>-</sup> strain, a derivative of the L2 mutant strain [34] disrupted in the *lys1* gene (F. Teves et al., unpublished data) was used as control strain of lysine auxotrophy, respectively. Uridine auxotrophs were grown in presence of 140 µg/ml of uridine. Selection of transformants with plasmids carrying the *ble* marker was made using 30 µg/ml phleomycin. Lysine auxotrophs were grown in the presence of 255 µg/ml of lysine (1.75 mM). Fungal spores were plated on complex Power medium [35] or Czapek minimal medium (30 g/l sucrose, 2g/l NaNO<sub>3</sub>, 0.5 g/l K<sub>2</sub>HPO<sub>4</sub>, 0.5 g/l MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.01 g/l FeSO<sub>4</sub>) and grown for seven days at 28°C. In some experiments, 2,5% (v/v) olive oil and 2,5% (v/v) corn oil were added to the Czapek minimal medium.

*P. chrysogenum* liquid cultures were initiated by inoculating fresh spores in complex medium CIM (20 g/l corn steep solids, 10 g/l yeast extract, 58 mM sucrose, 50 mM calcium carbonate, pH 5.7) or defined DP medium [35] without phenylacetate. After incubation at 25°C for 20 h in an orbital shaker (250 rpm), aliquots were inoculated in complex penicillin production medium CP (4 g/l potassium phenylacetate, 20 g/l pharmamedia, 50 g/l lactose, 0.03 M ammonium sulphate, 0.05 M calcium carbonate, pH 6.6) or defined DP medium with phenylacetate.

Competent cells of *Escherichia coli* DH5α strain were used for amplification and isolation of plasmid DNA. They were grown in Luria- Bertani Broth medium (LB) with ampicillin (100 µg/ml).

### Transformation of *P. chrysogenum* protoplasts

*P. chrysogenum* protoplasts were obtained and transformed as previously described [36,37]. Selection of transformant clones was achieved by complementation of the uridine auxotrophy or by resistance to phleomycin (final concentration of 30 µg/ml).

### Southern and Northern blotting

DNA and RNA isolation, Southern and Northern blotting were carried out as described before [8,13,38]. For hybridizations different probes were used; i) a 1284-bp *ppt* gene, ii) a 366-bp fragment located inside the *ppt* gene (see below), iii) a 524-bp *EcoRV* fragment, which includes the initial 96 bp from the *ppt* gene plus 428 bp of the 5' region, iv) a 429-bp fragment located inside the third exon of the *penDE* gene (from

nucleotide 727 to 1155), v) a fragment including the last 1209-bp of the *pcbAB* gene and vi) *β-actin* gene. The signal provided by the Northern blotting was quantified by densitometry using the “Gel-Pro Analyzer” software (Media Cybernetics). The transcript levels were determined comparing the intensity of the *ppt* mRNA signal to the *β-actin* mRNA signal (IOD/IOD *β-actin*).

### Cloning of the *ppt* gene

In order to generate a DNA probe for the isolation of the *P. chrysogenum ppt* gene, PCR was carried out using genomic DNA as template. Two oligonucleotides were designed based upon two conserved amino acid sequences present in the NpgA/CfwA protein (PPTase) of *A. fumigatus* and *A. nidulans*. A sense oligonucleotide, encoding the conserved motif FNVSHQ, was used as forward primer (Fppt): 5′-tcaacgtcagccaccaa-3′. As reverse primer, an oligonucleotide encoding the conserved motif WALKEA, was used (Rppt): 5′-cgcctctttgagcgcceca-3′. Thirty cycles of 1 min denaturation at 94°C, 1 min annealing at 55 °C and 1 min extension at 72°C, gave rise to the amplification of a 366-bp band. This band was used as probe to screen a *P. chrysogenum* NRRL-1951 genomic library [12] constructed in the vector λGEM-12 (Promega, Madison, WI, USA). One positive phage was isolated after three consecutive rounds of hybridisation. DNA was isolated from this clone and digested with different restriction enzymes, electrophoresed on 0.7% agarose gels, transferred by Southern blotting and probed with the 366-bp DNA fragment. Positive bands were sequenced and analysed using the blast algorithms available at <http://www.ncbi.nlm.nih.gov/BLAST/>.

### Intron analysis

Identification of introns was performed by RT-PCR using the “OneStep RT-PCR Kit” (Qiagen, Hilden, Germany) following the manufacturer’s instructions. Total RNA was extracted from mycelia grown for 48 h in CP using the “RNeasy Mini Kit” columns (Qiagen), following the manufacturer’s instructions. RNA was treated with RQ1 RNase-free DNase (Promega Corporation) following the manufacturer’s instructions. Oligonucleotides ppt1; 5′-atgtagacccagtggtc-3′ and ppt2; 5′-ttaaagcttgtaaatacc-3′ were used for this purpose. They were designed to amplify the whole *ppt* gene. The presence of introns was confirmed by sequencing.

### Plasmid constructions

Plasmid p43gdh-*ppt* was constructed to overexpress the *ppt* gene in *P. chrysogenum* as follows: The *ppt* gene was amplified using primers pptampLF; 5′-tcacaatgccatgtagacccagtggtc-3′ and pptampLR; 5′-cagcaagaaggcctttaaagcttgtaaatac-3′ and was cloned in the *NcoI-StuI* sites of pIBRC43 [39] between the *A. awamori* *gdh* gene promoter (a very efficient promoter in ascomycetes) and the *Saccharomyces cerevisiae cycl1* transcriptional terminator. Plasmid pKS-3188*ppt* contains the 3188-bp DNA fragment released after *SalI* digestion of the positive phage including the *ppt* gene. This fragment contains the *ppt* gene and flanking regions and it was inserted in the *SalI* restriction site of pBluescript KS+. For some experiments the 3188-bp fragment was amplified by PCR using primers 3188F; 5′-gtcgaccgaagtgttcggtt-3′ and 3188R; 5′-gtcgacgcgggattcagatgctc-3′.

Plasmid pBG, containing the *P. chrysogenum pyrG* gene, has been previously described [38]. Plasmid pJL43-RNAi-*ppt* is a derivative of the pJL43-RNAi (Ullán et al., unpublished data) and was made for the attenuation of the *ppt* gene. It was constructed as follows: a 1055-bp exon fragment of the *ppt* gene, spanning positions 145-1199, was amplified by PCR using primers atppt1; 5′-acgttggtccatggtacatggatgctc-3′ and atppt2;

5'-aatatcgacctcctccatggggagcca-3' and subcloned into the *NcoI* site of plasmid pJL43-RNAi.

#### Penicillin bioassays and HPLC analysis

240 The benzylpenicillin secreted to the culture medium by *P. chrysogenum* was quantified by HPLC and bioassay with *Micrococcus luteus* as test microorganism. Benzylpenicillin and isopenicillin N analyses by HPLC were carried out as previously described [38].

#### Roquefortine extraction and HPLC quantification

245 Roquefortine C was determined by HPLC. The mycelium from 72-hour cultures in complex penicillium production medium CP, was washed with NaCl 0.9% and extracted in Falcon<sup>TM</sup> tubes with 20 mL of dichloromethane. The extraction mixture was sonicated for 30 minutes and centrifuged at 4500 rpm for ten minutes at 4°C. The dichloromethane extract was transferred to a clean tube and evaporated to dryness in a  
250 rotary Vacuum Concentrator (RotaVapor-210, Buchi, Switzerland). The residues were re-dissolved in 150 µL of methanol, centrifuged and stored at -20°C. Chromatographic determinations were performed on a Waters HPLC-system (Waters Corporation, Milford, Massachusetts, USA) consisting of a 996 Photodiode Array Detector, a binary pump, and a Symmetry reversed-phase C<sub>18</sub> column (150 mm x 3.9 mm) of particle size  
255 5 µm (Waters Corporation) at room temperature. The mobile phase consisted of acetonitrile and water containing 0.04 % trifluoroacetic acid with a flow-rate of 0.7 mL/min. A linear gradient ranging from (70:30) to (20:80) water-acetonitrile was used for 25 minutes. After 5 minutes at (20:80) the eluent composition was changed to starting conditions. A standard of pure roquefortine C was kindly provided by A.  
260 Fernández (Biomar S.A.; León, Spain). A stock solution of roquefortine C was prepared in methanol and stored at -20°C. Working standards were prepared just before use by serial dilutions of the stock solution in methanol.

## RESULTS AND DISCUSSION

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#### Cloning of the *ppt* gene from a *P. chrysogenum* genomic library

To clone the *ppt* gene of *P. chrysogenum*, two primers (Fp<sub>ppt</sub> and R<sub>ppt</sub>) corresponding to conserved internal sequences from PPTases of the phylogenetically related fungi *A. fumigatus* and *A. nidulans*, were designed. They encode the conserved motifs FNVSHQ and WALKEA, respectively, which are present in the NpgA/CfwA protein (PPTase) of  
270 *A. fumigatus* and *A. nidulans*. A 366-bp band was amplified, sequenced and compared to the NCBI sequences using the BLAST algorithms. Significant similarities of the PCR product with PPTases (NpgA/CfwA) of *A. fumigatus*, *Neosartorya fischeri*, *A. clavatus* or *A. nidulans*, were found. Therefore, the 366-bp fragment was used as probe to isolate  
275 the complete *ppt* gene from a *P. chrysogenum* NRRL-1951 genomic library in the Lambda phage vector [12]. One positive phage was isolated after three consecutive rounds of hybridisation and its DNA was analysed by Southern blotting with a probe containing the 366-bp DNA fragment (see supplementary material S1). One of the positive bands (the 3188-bp band released after the *SalI* digestion), was subcloned into a  
280 *SalI*-digested pBluescript KS+, thus generating pKS-3188*ppt*. Sequence of the 3188-bp region was identical to the sequence 4 reported in the patent application *P. chrysogenum* WO 2005/040369 (Accession No. CS086376), and revealed the presence of a 1284-bp ORF in the center of this region. Upstream of it, a 424-bp ORF (incomplete and present in antisense orientation) showed a strong similarity with a glutamine amidotransferase  
285 subunit (PdxT) of *A. terreus* (e-value: 4e-49; Positives: 80%) and with a glutamine

amidotransferase (GATase\_I) involved in pyridoxine biosynthesis of *A. fumigatus* (e-value: 4e-49; Positives: 81%) or *N. fischeri* (e-value: 1e-48; Positives: 81%).

In order to detect the presence of introns within the 1284-bp ORF, RT-PCR assays were carried out using primers ppt1 and ppt2. One 48-bp intron was detected spanning nucleotides 28-75 (data not shown). Sequences of the 5' and 3' intron splice sites were the consensus "GTGAGT" and "CAG", respectively. This ORF encoded a PPTase (see below) and the gene was named *ppt* gene (for phosphopantetheinyl transferase).

The predicted protein sequence encoded by the cDNA of the *ppt* gene, contained 412 amino acids with an estimated molecular mass of 45,8 kDa. This protein was 51% homologous (38% identity, e-value 3e-64) to the NpgA/CfwA protein (PPTase) of *A. fumigatus*, 51% homologous to the PPTase of *N. fischeri* (38% identity, e-value 3e-62), 50% homologous (38% identity, e-value: 4e-59) to the *npgA* protein (PPTase) of *A. nidulans*, 48% homologous (33% identity, e-value 5e-23) to the PPTase of *Gibberella zeae*, 46% homologous (33% identity, e-value 1e-18) to the PPTase of *Magnaporthe grisea* and 41% homologous (28% identity, e-value 8e-16) to hypothetical protein related to the *npgA* protein of *Neurospora crassa*. The protein product encoded by the *ppt* gene of *P. chrysogenum* closely resembles the stand-alone large size (class III) PPTases from several filamentous fungi and it shared conserved regions such as "LASQLLK", "FNVSHQA", "VGIDV" and "RLFYSIWALKEAYLKMTGDGLLASWIKDLEF", which are present in the PPTases from several filamentous fungi (Fig. 1) and in the PPTases of other organisms, such as *Bacillus subtilis*, *Homo sapiens*, *Arabidopsis thaliana* or *Saccharomyces cerevisiae* [40]. This class of fungal PPTases includes the PPTase encoded by the *A. nidulans npgA* gene, which has been shown to be necessary for penicillin [40], lysine [41] and siderophore biosynthesis [42].

### **The *ppt* gene is present as a single copy in the genome of wild type and high penicillin producing strains of *P. chrysogenum***

It is well known that many high penicillin-producing strains contain multiple copies of the *pen* gene cluster [12]. To determine the *ppt* gene copy number, genomic DNA from the *P. chrysogenum* NRRL-1951 strain was isolated, digested with different restriction enzymes and hybridised with a probe containing the *ppt* gene. As shown in figure 2 only one band was detected with most of enzymes. Only *ApaI* (~6000 bp and ~700 bp) and *EcoRI* (~1600 bp and ~1100 bp) gave rise to two hybridisation signals because they cut inside the *ppt* gene. These results indicate that the *ppt* gene is present as a single copy in the genome of the wild-type strain of *P. chrysogenum*. The same hybridisation pattern was obtained when the genomic DNA of the high penicillin-producing strains *P. chrysogenum* AS-P-78 and AS-P-99, was digested with the same restriction enzymes (data not shown). These results evidence that only one copy of this gene exists in the genome of the low and high penicillin producing strains, fact that is consistent with the finding that the *ppt* gene is not located inside the 56.8-kb amplified region containing the *pen* gene cluster [13, 14].

### **Amplification of the *ppt* gene in *P. chrysogenum* Wis54-1255. Overexpression of the *ppt* gene leads to increased levels of penicillin**

Overexpression of the *ppt* gene was achieved using plasmid p43gdh-*ppt*. This plasmid was co-transformed together with plasmid pBG (which contains the *pyrG* gene as selectable marker) into the *P. chrysogenum* Wis54-1255 PyrG<sup>-</sup> strain. Transformants were selected by complementation of the uridine auxotrophy. Ectopic integration of the full *Pgdh-ppt-Tcyc1* cassette in four transformants was confirmed by Southern blotting

(see supplementary material S2). One of these transformants (T11) was randomly selected for further studies. Northern blottings were carried out with RNA extracted at 48 h and 72 h from cultures of the Wis54-1255 strain and transformant T11 to confirm the overexpression of the *ppt* gene. As observed in figure 3A, transformant T11 showed approximately 3-fold higher steady-state levels of the *ppt* transcript than the parental strain and therefore, it was named Wis54-PPT<sup>+</sup> strain.

HPLC analyses of the penicillin produced in cultures in DP medium indicated that the specific benzylpenicillin production (per mg of dry weight) of the Wis54-PPT<sup>+</sup> strain at 72 hours was up to 30% higher than that of Wis54-1255 at the same time grown under identical culture conditions (Fig. 3B). Isopenicillin N (an intermediate of the penicillin pathway) production was also determined by HPLC in the Wis54-PPT<sup>+</sup> strain cultured in complex CP medium without phenylacetic acid (to optimize isopenicillin N production). Isopenicillin N levels in the transformant T11 were two-fold higher than in the Wis54-1255 strain (Fig. 3C). This result indicates that overexpression of the *ppt* gene leads to an increase in the initial steps of antibiotic production; i.e. ACV formation or cyclization of ACV to isopenicillin N. Since the 4'-phosphopantetheine moiety is present in the ACVS [21] and it is necessary for the activation of this multidomain enzyme, an increase of the ACVS activity as a consequence of the *ppt* gene overexpression (giving rise to increased levels of the tripeptide ACV and therefore, to higher levels of antibiotic production), is more likely.

#### **Characterization of mutants defective in PPTase activity. The *ppt* gene is essential for lysine biosynthesis but not for fatty acids formation**

Plasmid pJL43RNAi-*ppt* (see Experimental Procedures) was used to generate loss-of-function mutants in the PPTase. After transformation, selection of transformants was done through phleomycin resistance. Since the *A. nidulans* PPTase has been reported as essential for lysine biosynthesis [41], those phleomycin resistant transformants that showed reduced growth on minimal medium or lysine auxotrophy, were selected. Only one clone out of 100 was completely auxotroph for lysine and full integration of the *PgpdA-ppt-reversePpcbC* cassette was confirmed by Southern blotting (see supplementary material S3). This transformant showed a reorganization of the *ppt* locus. This fact was confirmed by Southern blotting (Fig. 4A), which was carried out using genomic DNA digested with *SaI*I. A 524-bp *EcoRV* fragment (absent in the *PgpdA-ppt-reversePpcbC* attenuation cassette, thus giving information only about the internal *ppt* gene) was used as probe. Results evidenced that the 3188-bp *SaI*I fragment, which includes the endogenous *ppt* gene, had been reorganized as a 2100-bp band. This generates the deletion of the last ~191 bp of the *ppt* gene giving rise to a disrupted mutant, which was named Wis54-PPT<sup>-</sup>. Lysine auxotrophy of the *ppt* disrupted mutant was confirmed growing the Wis54-1255 strain, the Wis54-PPT<sup>-</sup> strain, and the lysine auxotroph L2/*lys1*<sup>-</sup> strain (as control) in Czapek minimal medium supplemented with 1.75 mM lysine (Fig. 4B). As observed in this figure, addition of lysine is completely essential for growth of the Wis54-PPT<sup>-</sup> strain. This indicates that the *ppt* gene encodes the PPTase in charge of activation of the  $\alpha$ -amino adipate reductase, which catalyses the conversion of  $\alpha$ -amino adipic acid into  $\alpha$ -amino adipate semialdehyde in the fungal lysine biosynthetic pathway [27].

It was also observed that growth was slower in the Wis54-PPT<sup>-</sup> strain than in the rest of strains. Since the *A. nidulans* PPTase, encoded by the *npgA* gene, is essential for siderophore biosynthesis [42], it is likely that the *P. chrysogenum* PPTase is involved in the maintenance of the intracellular pools of iron through siderophore biosynthesis. However, the lack-of-function mutant Wis54-PPT<sup>-</sup> was able to grow in lysine-

supplemented minimal medium without addition of siderophores. This indicates that although siderophores have been described in *P. chrysogenum* [43,44], those might not be essential, unlike the NRPS-mediated siderophore biosynthesis in *A. nidulans*, which has been shown to be completely necessary for growth [45].

390 A limitation in fatty acid biosynthesis could be another explanation to the growth defects observed in the Wis54-PPT<sup>-</sup> strain. To test this, the parental Wis54-1255 strain, the Wis54-PPT<sup>-</sup> mutant, and the L2/lys1<sup>-</sup> strain were grown on Czapek minimal medium supplemented with 2,5% (v/v) olive oil plus 2,5% (v/v) corn oil. Addition of oils to the minimal medium did not restore the growth rate of the Wis54-PPT<sup>-</sup> strain  
395 (see supplementary material S4). This indicates that there must be other PPTases different from that encoded by the *ppt* gene in charge of the activation of FAS in *P. chrysogenum*. In *S. cerevisiae* the subunit  $\alpha$  of FAS is capable of self-pantetheinylation [31]. Probably the same mechanism occurs in *P. chrysogenum*, where a class of PPTase controls lysine biosynthesis and other class, present as an integral domain of fungal type  
400 I FASs, controls fatty acids biosynthesis. This may explain the lack of requirement of fatty acids in the Wis54-PPT<sup>-</sup> mutant.

#### **The *ppt* gene is involved in penicillin and green pigment formation, but it is not required for roquefortine C biosynthesis**

405 The Wis54-PPT<sup>-</sup> mutant took 10 days to develop conidiophores and spores in Power medium, unlike the Wis54-1255 strain, which sporulated in about 5 days. White cotton-like conidiophores, unlike the characteristic green conidiophores of the Wis54-1255 strain, were formed in this mutant (see supplementary material S5). The conidia remained colourless due to the inability to synthesize the green pigment, apparently a polyketide-derived compound. It has been reported that the *A. nidulans* wA gene encodes all or part of a PKS involved in the formation of a green pigment present in  
410 conidia [46]. The lack of green pigment formation in the *ppt* knockout mutant indicates that the PPTase encoded by the *ppt* gene is also involved in PKSs activation, specially in the activation of the PKS wA-encoded ortholog of *P. chrysogenum*.

415 The role of the *ppt* gene on secondary metabolism was evident when the penicillin production was tested using sporulated agar plugs (Fig. 5). As shown in this figure, penicillin biosynthesis was completely abolished in the Wis54-PPT<sup>-</sup> strain. The same results were obtained using filtrates from submerged cultures in complex CP medium (see supplementary material S5). The lack of antibiotic production was not due to a reorganization of the penicillin biosynthetic cluster in the Wis54-PPT<sup>-</sup> strain, as we confirmed by Southern blotting (see supplementary material S6). Instead, it is likely accounted by the absence of activity of the ACVS, since this NRPS requires phosphopantetheine-mediated activation of the constituent peptidyl carrier domain to catalyse the biosynthesis of the tripeptide ACV [5,21,24,47]. In addition, the  
420 isopenicillin N synthase, which is a nonheme Fe<sup>2+</sup>-dependent oxygenase [48] may have a reduced activity in the Wis54-PPT<sup>-</sup> mutant, since the availability of iron might become limiting because of a defect in the siderophore biosynthesis.

In addition to  $\beta$ -lactam antibiotics, *P. chrysogenum* is able to produce other secondary metabolites, such as the alkaloid roquefortine C [15]. The biosynthesis of this  
430 mycotoxin is poorly known; it is believed to be formed by a NRPS (the gene encoding this NRPS has not been cloned yet) that uses dimethylallyltryptophan as one of the precursors [49]. When the roquefortine C biosynthesis was analysed by HPLC in the Wis54-PPT<sup>-</sup> mutant and in the Wis54-1255 strain, no differences were found between these two strains (see supplementary material S7), which suggests that the biosynthesis  
435 of roquefortine may not require post-translational modification by the PPTase.

Alternatively, the presence of a second PPTase involved in the activation of the NRPS in charge of the biosynthesis of this alkaloid, cannot be excluded.

#### 440 **Complementation of the Wis54-PPT<sup>-</sup> strain with the *ppt* gene restores lysine biosynthesis, normal growth rate, pigmentation and penicillin production**

In order to confirm the role of the *P. chrysogenum* PPTase in lysine biosynthesis and secondary metabolism, the Wis54-PPT<sup>-</sup> strain was complemented with plasmid pKS-3188*ppt* (see Experimental Procedures). Integration of the 3188-bp region, which includes the *ppt* gene, was confirmed by PCR (Fig. 6A). Transformants were selected 445 by their ability to grow in presence of phleomycin (a characteristic of the Wis54-PPT<sup>-</sup> strain) and lysine prototrophy restoration was confirmed in one of the transformants (randomly selected) (Fig. 6B). The transformant showed a growth rate similar to that of the Wis54-1255 strain. In addition, the green pigmentation of conidiophores and penicillin production were completely restored in the complemented transformant (see 450 supplementary material S8).

All these data, taken together, indicate that the *P. chrysogenum* stand-alone large size (class III) PPTase encoded by the *ppt* gene is in charge of the post-translational activation of the  $\alpha$ -amino adipate reductase, ACVS and the NRPS involved in the green 455 pigment formation, evidencing that this protein is involved in the post-translational control of both primary and secondary metabolism.

#### 460 **ACKNOWLEDGEMENTS**

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## REFERENCES

- 1 Aharonowitz, Y., Cohen, G., and Martín, J.F. (1992) Penicillin and cephalosporin biosynthetic genes: structure, regulation, and evolution. *Annu. Rev. Microbiol.* **46**, 461-495  
470
- 2 Martín, J.F., Gutiérrez, S., Fernández, F.J., Velasco, J., Fierro, F., Marcos, A.T. and Kosalkova, K. (1994) Expression of genes and processing of enzymes for the biosynthesis of penicillins and cephalosporins. *Antonie van Leeuwenhoek* **65**, 227-243
- 475 3 Díez, B., Gutiérrez, S., Barredo, J.L., van Solingen, P., van der Voort, Lucia, H.M., and Martín, J.F. (1990) The cluster of penicillin biosynthetic genes. *J. Biol. Chem.* **265**, 16358-16365
- 480 4 Byford, M.F., Baldwin, J.E., Shiau, C.Y., and Schofield, C.J. (1997) The mechanisms of ACV synthetase. *Chem. Rev.* **97**, 2631-2650
- 5 Martín, J.F. (2000)  $\alpha$ -aminoadipyl-cysteinyl-valine synthetases in  $\beta$ -lactam producing organisms. From Abraham's discoveries to novel concepts of non-ribosomal peptide synthesis. *J. Antibiot.* **53**, 1008-1021
- 485 6 Álvarez, E., Cantoral, J.M., Barredo, J.L., Díez, B., and Martín, J.F. (1987) Purification to homogeneity and characterization of the acyl-CoA: 6-APA acyltransferase of *Penicillium chrysogenum*. *Antimicrob. Agents Chemother.* **31**, 1675-1682
- 490 7 Martín, J.F., Ingolia, T.D., and Queener, S.W. (1990) Molecular genetics of penicillin and cephalosporin antibiotic biosynthesis. In *Molecular Industrial Mycology* (Leong, S.A. and Berka, R. eds.), pp. 149-195, Marcel Dekker, New York
- 495 8 Lamas-Maceiras, M., Vaca, I., Rodríguez, E., Casqueiro, J., and Martín, J.F. (2006) Amplification and disruption of the phenylacetyl-CoA ligase gene of *Penicillium chrysogenum* encoding an aryl-capping enzyme that supplies phenylacetic acid to the isopenicillin N acyltransferase. *Biochem. J.* **395**, 147-155
- 9 Wang, F.Q., Liu, J., Dai, M., Ren, Z.H., Su, C.Y., and He, J.G. (2007) Molecular cloning and functional identification of a novel phenylacetyl-CoA ligase gene from *Penicillium chrysogenum*. *Biochem. Biophys. Res. Commun.* **360**, 453-458
- 500 10 Martín, J.F. and Liras, P. (1989) Organization and expression of genes involved in the biosynthesis of antibiotics and other secondary metabolites. *Annu. Rev. Microbiol.* **43**, 173-206
- 505 11 Martín, J.F. (1998) New aspects of genes and enzymes for beta-lactam antibiotic biosynthesis. *Appl. Microbiol. Biotechnol.* **50**, 1-15.
- 12 Fierro, F., Barredo, J.L., Díez, B., Gutiérrez, S., Fernández, F.J. and Martín, J.F. (1995) The penicillin gene cluster is amplified in tandem repeats linked by conserved hexanucleotide sequences. *Proc. Natl. Acad. Sci. USA.* **92**, 6200-6204
- 510 13 Fierro, F., García-Estrada, C., Castillo, I., Rodríguez, R., Velasco-Conde, T., and Martín, J.F. (2006) Transcriptional and bioinformatic analysis of the 56.8 kb DNA region amplified in tandem repeats containing the penicillin gene cluster in *Penicillium chrysogenum*. *Fungal Genet. Biol.* **43**, 618-629

- 14 van den Berg, M.A., Westerlaken, I., Leeflang, C., Kerkman, R., and Bovenberg, R.A. (2007) Functional characterization of the penicillin biosynthetic gene cluster of *Penicillium chrysogenum* Wisconsin54-1255. *Fungal Genet. Biol.* **44**, 830-844
- 515 15 Keller, N.P., Turner, G., and Bennett, J.W. (2005) Fungal secondary metabolism - from biochemistry to genomics. *Nat. Rev. Microbiol.* **3**, 937-947
- 16 Von Dohren, H., Keller, U., Vater, J., and Zocher, R. (1997) Multifunctional Peptide Synthetases. *Chem. Rev.* **97**, 2675-2706
- 17 Marahiel, M.A., Stachelhaus, T., and Mootz, H.D. (1997) Modular Peptide Synthetases Involved in Nonribosomal Peptide Synthesis. *Chem. Rev.* **97**, 2651-2674
- 520 18 Metz, J.G., Roessler, P., Facciotti, D., Levering, C., Dittrich, F., Lassner, M., Valentine, R., Lardizabal, K., Domergue, F., Yamada, A., Yazawa, K., Knauf, V., and Browse, J. (2001) Production of polyunsaturated fatty acids by polyketide synthases in both prokaryotes and eukaryotes. *Science* **293**, 290-293
- 525 19 Cox, R.J. (2007) Polyketides, proteins and genes in fungi: programmed nanomachines begin to reveal their secrets. *Org. Biomol. Chem.* **5**, 2010-2026
- 20 Lambalot, R.H., Gehring, A.M., Flugel, R.S., Zuber, P., LaCelle, M., Marahiel, M.A., Reid, R., Khosla, C., and Walsh, C.T. (1996) A new enzyme superfamily - the phosphopantetheinyl transferases. *Chem. Biol.* **3**, 923-936
- 530 21 Baldwin, J.E., Bird, J.W., Field, R.A., O'Callaghan, N.M., Schofield, C.J., and Willis, A.C. (1991) Isolation and partial purification of ACV synthetase from *Cephalosporium acremonium* and *Streptomyces clavuligerus*: evidence for the presence of phosphopantothenate in ACV synthetase. *J. Antibiot.* **44**, 241-248
- 535 22 Martín, J.F. and Gutiérrez, S. (1992) Molecular genetics of fungal secondary metabolites. In *Applied Molecular of filamentous Fungi*. (Kinghorn J.R., and Turner G., eds.), pp. 214-252, Blackie and Son, Ltd., Glasgow
- 23 Walsh, C.T., Gehring, A.M., Weinreb, P.H., Quadri, L.E., and Flugel, R.S. (1997) Post-translational modification of polyketide and nonribosomal peptide synthases. *Curr. Opin. Chem. Biol.* **1**, 309-315
- 540 24 Kleinkauf, H. and von Döhren, H. (1995) The nonribosomal peptide biosynthetic system- on the origins of structural diversity of peptides, cyclopeptides and related compounds. *Antonie van Leeuwenhoek* **67**, 229-242
- 25 Bhattacharjee, J.K. (1985) Alpha-Amino adipate pathway for the biosynthesis of lysine in lower eukaryotes. *Crit. Rev. Microbiol.* **12**, 131-151
- 545 26 Ehmman, D.E., Gehring, A.M., and Walsh, C.T. (1999). Lysine biosynthesis in *Saccharomyces cerevisiae*: mechanism of alpha-amino adipate reductase (Lys2) involves posttranslational phosphopantetheinylation by Lys5. *Biochemistry* **38**, 6171-6177.
- 550 27 Casqueiro, J., Gutiérrez, S., Bañuelos, O., Fierro, F., Velasco, J. and Martín, J.F. (1998) Characterization of the *lys2* gene of *Penicillium chrysogenum* encoding  $\alpha$ -amino adipic acid reductase. *Mol. Gen. Genet.* **259**, 549-556
- 28 Garrad, R.C., and Bhattacharjee, J.K. (1992) Lysine biosynthesis in selected pathogenic fungi: characterization of lysine auxotrophs and the cloned LYS1 gene of *Candida albicans*. *J. Bacteriol.* **174**, 7379-7384

- 555 29 Guo, S., Evans, S.A., Wilkes, M.B., and Bhattacharjee, J.K. (2001) Novel posttranslational activation of the LYS2-encoded alpha-amino adipate reductase for biosynthesis of lysine and site-directed mutational analysis of conserved amino acid residues in the activation domain of *Candida albicans*. *J. Bacteriol.* **183**, 7120-7125
- 560 30 Suo, Z., Tseng, C.C., and Walsh C.T. (2001) Purification, priming, and catalytic acylation of carrier protein domains in the polyketide synthase and nonribosomal peptidyl synthetase modules of the HMWP1 subunit of yersiniabactin synthetase. *Proc. Natl. Acad. Sci. USA.* **98**, 99-104
- 565 31 Fichtlscherer, F., Wellein, C., Mittag, M., and Schweizer, E. (2000) A novel function of yeast fatty acid synthase. Subunit alpha is capable of self-pantetheinylation. *Eur. J. Biochem.* **267**, 2666-2671
- 32 Mofid, M.R., Finking, R., and Marahiel, M.A. (2002) Recognition of hybrid peptidyl carrier proteins/acyl carrier proteins in nonribosomal peptide synthetase modules by the 4'-phosphopantetheinyl transferases AcpS and Sfp. *J. Biol. Chem.* **277**, 17023-17031
- 570 33 Mootz, H.D., Schörgendorfer, K., and Marahiel, M.A. (2002) Functional characterization of 4'-phosphopantetheinyl transferase genes of bacterial and fungal origin by complementation of *Saccharomyces cerevisiae* lys5. *FEMS Microbiol. Lett.* **213**, 51-57
- 575 34 Esmahan, C., Álvarez, E., Montenegro, E., and Martín, J.F. (1994) Catabolism of lysine in *Penicillium chrysogenum* leads to formation of  $\alpha$ -amino adipic acid, a precursor of penicillin biosynthesis. *Appl. Environ. Microbiol.* **60**, 1705-1710
- 35 Casqueiro, J., Bañuelos, O., Gutiérrez, S., Hijarrubia, M.J., and Martín, J.F. (1999) Intrachromosomal recombination between direct repeats in *Penicillium chrysogenum*: gene conversion and deletion events. *Mol. Gen. Genet.* **261**, 994-1000
- 580 36 Cantoral, J.M., Díez, B., Barredo, J.L., Alvarez, E., and Martín, J.F. (1987) High frequency transformation of *Penicillium chrysogenum*. *Bio/Technology* **5**, 494-497
- 37 Díez, B., Álvarez, E., Cantoral, J.M., Barredo, J.L., and Martín, J.F. (1987) Isolation and characterization of pyrG mutants of *Penicillium chrysogenum* by resistance to 5'-fluorotic acid. *Curr. Genet.* **12**, 277-282
- 585 38 García-Estrada, C., Vaca, I., Lamas-Maceiras, M., and Martín, J.F. (2007) In vivo transport of the intermediates of the penicillin biosynthetic pathway in tailored strains of *Penicillium chrysogenum*. *Appl. Microbiol. Biotechnol.* **76**, 169-82
- 590 39 Cardoza, R.E., Moralejo, F.J., Gutiérrez, S., Casqueiro, J., Fierro, F., and Martín, J.F. (1998) Characterization and nitrogen-source regulation at the transcriptional level of the *gdhA* gene of *Aspergillus awamori* encoding an NADP-dependent glutamate dehydrogenase. *Curr. Genet.* **34**, 50-59
- 595 40 Keszenman-Pereyra, D., Lawrence, S., Twfieg, M.E., Price, J., and Turner, G. (2003) The *npgA/cfwA* gene encodes a putative 4'-phosphopantetheinyl transferase which is essential for penicillin biosynthesis in *Aspergillus nidulans*. *Curr. Genet.* **43**, 186-190
- 41 Márquez-Fernández, O., Trigos, A., Ramos-Balderas, J.L., Viniegra-Gonzalez, G., Deising, H.B., and Aguirre, J. (2007) Phosphopantetheinyl transferase CfwA/NpgA is required for *Aspergillus nidulans* secondary metabolism and asexual development. *Eukaryot. Cell* **6**, 710-720

- 600 42 Oberegger, H., Eisendle, M., Schrettl, M., Graessle, S., and Haas, H. (2003) 4'-  
phosphopantetheinyl transferase-encoding *npgA* is essential for siderophore  
biosynthesis in *Aspergillus nidulans*. *Curr. Genet.* **44**, 211-215
- 43 Charlang, G., Ng, B., Horowitz, N.H., and Horowitz, R.M. (1981) Cellular and  
605 extracellular siderophores of *Aspergillus nidulans* and *Penicillium chrysogenum*.  
*Mol. Cell. Biol.* **1**, 94-100
- 44 Charlang, G., Horowitz, R.M., Lowy, P.H., Ng, B., Poling, S.M., and Horowitz,  
N.H. (1982) Extracellular siderophores of rapidly growing *Aspergillus nidulans* and  
*Penicillium chrysogenum*. *J. Bacteriol.* **150**, 785-787
- 45 Eisendle, M., Oberegger, H., Zadra, I., and Haas, H. (2003) The siderophore system  
610 is essential for viability of *Aspergillus nidulans*: functional analysis of two genes  
encoding l-ornithine N 5-monooxygenase (*sidA*) and a non-ribosomal peptide  
synthetase (*sidC*). *Mol. Microbiol.* **49**, 359-375
- 46 Mayorga, M.E., and Timberlake, W.E. (1992) The developmentally regulated  
615 *Aspergillus nidulans* *wA* gene encodes a polypeptide homologous to polyketide and  
fatty acid synthases. *Mol. Gen. Genet.* **235**, 205-212
- 47 Aharonowitz, Y., Bergmeyer, J., Cantoral, J.M., Cohen, G., Demain, A.L., Fink, U.,  
Kinghorn, J., Kleinkauf, H., MacCabe, A., Palissa, H., et al. (1993 ) Delta-(L-alpha-  
aminoadipyl)-L-cysteinyl-D-valine synthetase, the multienzyme integrating the four  
620 primary reactions in beta-lactam biosynthesis, as a model peptide synthetase.  
*Biotechnology* **11**, 807-810
- 48 Bainbridge, Z.A., Scott, R.I. and Perry, D. (1992) Oxygen utilization by  
isopenicillin N synthase from *Penicillium chrysogenum*. *J. Chem. Technol.*  
*Biotechnol.* **55**, 233-238
- 49 Steiner, U., Ahimsa-Müller, M.A., Markert, A., Kucht, S., Gross, J., Kauf, N.,  
625 Kuzma, M., Zych, M., Lamshöft, M., Furmanowa, M., et al. (2006) Molecular  
characterization of a seed transmitted clavicipitaceous fungus occurring on  
dicotyledoneous plants (Convolvulaceae). *Planta* **224**, 533-544

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## FIGURE LEGENDS

650 **Figure 1. Alignment of the conserved motifs present in the *P. chrysogenum* PPTase to different PPTases from several filamentous fungi.**

655 npgA (PPTase) from *A. nidulans* (Accession No. AAF12814) (npgAAnidu); PPTase NpgA/CfwA from *Aspergillus fumigatus* (Accession No. XP\_755193) (npgAAfum); protein NpgA related to null pigmentation protein from *Neurospora crassa* (Accession No. CAE76613) (npgANcras); hypothetical protein MGCH7\_ch7g840 from *Magnaporthe grisea* (Accession No. XP\_001522742) (PPTaseMgri) and hypothetical protein FG08779.1 from *Gibberella zeae* (Accession No. XP\_388955) (PPTaseGzea). These proteins were aligned to the *P. chrysogenum* PPTase (PPTasePchr) using the ClustalX program. Note the strong conservation of amino acids 224-254.

660 **Figure 2. Southern blot analysis of the *ppt* gene copy number in *P. chrysogenum*.**

665 DNA from the *P. chrysogenum* NRRL-1951 wild type strain was digested with several restriction enzymes (A: *Apa*I; B: *Bam*HI; E: *Eco*RI; H: *Hind*III; K: *Kpn*I; N: *Nco*I; P: *Pst*I; S: *Sac*I; Sa: *Sal*I; X: *Xho*I). The *P. chrysogenum ppt* gene was used as probe. The  $\lambda$ -*Hind*III molecular mass marker is indicated as M.

665 **Figure 3. Amplification of the *ppt* gene in *P. chrysogenum*.**

670 **A)** Northern blottings performed with samples taken after 48 h and 72 h from cultures of the transformant T11 (PPT<sup>+</sup>) and the Wis54-1255 strain (Wis54) grown in CP medium. The filter was hybridised to a probe containing a fragment of the *ppt* gene. As a control of RNA levels loaded in the gel, a probe with the  $\beta$ -actin gene was used. The steady-state levels of the pptase transcripts of transformant T11 (PPT<sup>+</sup>) and Wis54-1255 strain were determined by densitometry. Transformant T11 was referred hereafter as Wis54-PPT<sup>+</sup> strain. **B)** Representative HPLC chromatogram of the filtrates obtained from one of the liquid cultures (72 hours) of the Wis54-PPT<sup>+</sup> and Wis54-1255 strains under penicillin production conditions. Three different experiments with duplicate flasks were done. Potassium benzylpenicillin (PenG) was used as control. **C)** Isopenicillin N specific production of the Wis54-PPT<sup>+</sup> and Wis54-1255 strains after 24 h, 48 h and 72h of growth in CP medium without the addition of phenylacetic acid. Three different experiments with duplicate flasks were done and isopenicillin N levels were determined by HPLC. Three determinations were done for each value. Vertical bars indicate the standard deviation.

680 **Figure 4. Disruption of the *ppt* gene leads to lysine auxotrophy.**

685 **A)** Southern blotting performed with total DNA digested with *Sal*I and probed with a 524-bp *Eco*RV fragment (grey box), which includes the first 96 bp of the *ppt* gene plus 428 bp of the 5' region. Note the size reduction of the 3188-bp band containing the *ppt* gene in the Wis54-PPT<sup>-</sup> strain. The  $\lambda$ -*Hind*III molecular mass marker is indicated as M. **B)** Growth of Wis54-1255, the lysine auxotroph L2/lys1<sup>-</sup> and Wis54-PPT<sup>-</sup> strains in Czapek minimal medium with or without addition of 1.75 mM lysine.

690 **Figure 5. Role of the *ppt* gene in penicillin production.**

695 Bioassay of sporulated agar plugs showing that benzylpenicillin biosynthesis is completely abolished in the Wis54-PPT<sup>-</sup> strain grown on solid medium. The Wis54-1255 strain was used as control.

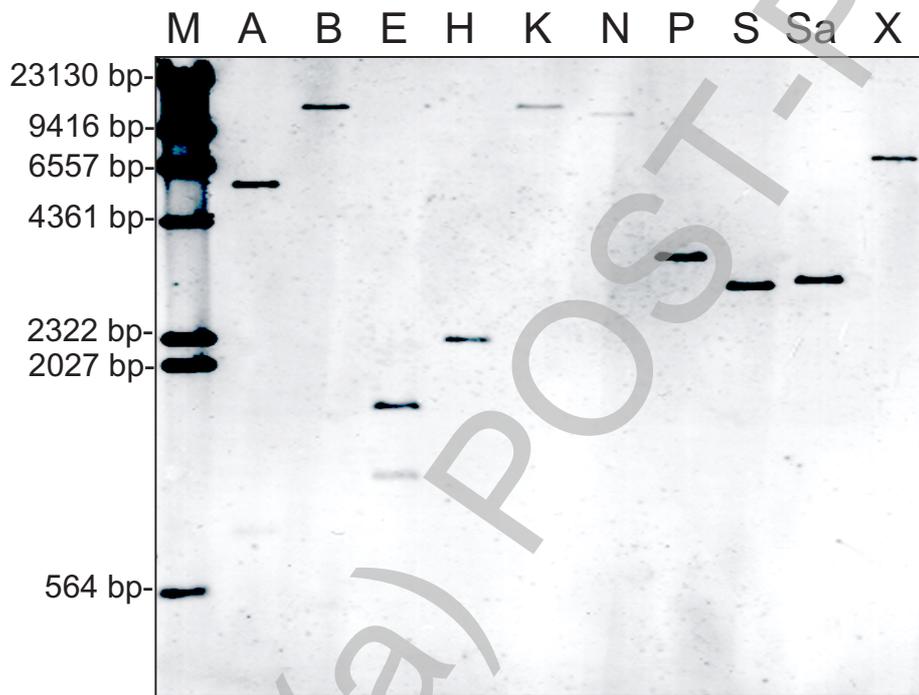
**Figure 6. Complementation of the Wis54-PPT<sup>-</sup> strain with the *ppt* gene.**

700 A) Electrophoresis of the PCR product obtained after amplification of the 3188-bp region, which includes the *ppt* gene. The PCR was carried out using DNA extracted from the complemented (Com), the Wis54-PPT<sup>-</sup> (PPT<sup>-</sup>) and Wis54-1255 (Wis) strains and the primers specified in Experimental Procedures. Note the integrity of the 3188-bp band in the complemented and Wis54-1255 strains and the lack of amplification in the Wis54-PPT<sup>-</sup> strain as a consequence of the *ppt* gene disruption. The authenticity of this band was confirmed by sequencing the PCR product. B) Growth of one randomly-selected transformant (Comp) obtained after complementation of the Wis54-PPT<sup>-</sup> strain with the 3188-bp *ppt* region in Czapek minimal medium without lysine addition. 705 The Wis54-1255, Wis54-PPT<sup>-</sup> and L2/lys1<sup>-</sup> strains were included as controls.

PPTasePchr : 73 LASQLLK 79 114 FNVSHQA 120 146 VGIDV 150  
 NpgAAnidu : 58 LASNLLK 64 115 FNVSHQA 121 145 VGIDI 149  
 NpgAAfum : 56 LASNLLK 62 110 FNVSHQA 116 156 VGIDI 160  
 NpgANcras : 3 LASALLK 9 51 FNVSHQA 57 83 VGVDV 87  
 PPTaseMgri : 63 LASHLLK 69 108 FNVTHQA 114 135 VGIDV 139  
 PPTaseGzea : 62 LASALLK 68 106 FNISHQA 112 129 VGVDV 133

PPTasePchr : 224 RLFYSIWALKEAYLKMTGDGLASWIKDLEF 254  
 NpgAAnidu : 209 RLFYTYWALKEAYIKMTGELLAPWLRELEF 239  
 NpgAAfum : 221 RLFYTYWALKEAYIKMTGELLAPWLRELEF 251  
 NpgANcras : 157 RLFYALWALREAYVKLTGELLAEWIGELEF 187  
 PPTaseMgri : 204 RLFYTLWCLREAYVKMTGELLASWICELEF 234  
 PPTaseGzea : 188 RLFYALWCLREAYVKMTGDALLASWIKDLEM 218

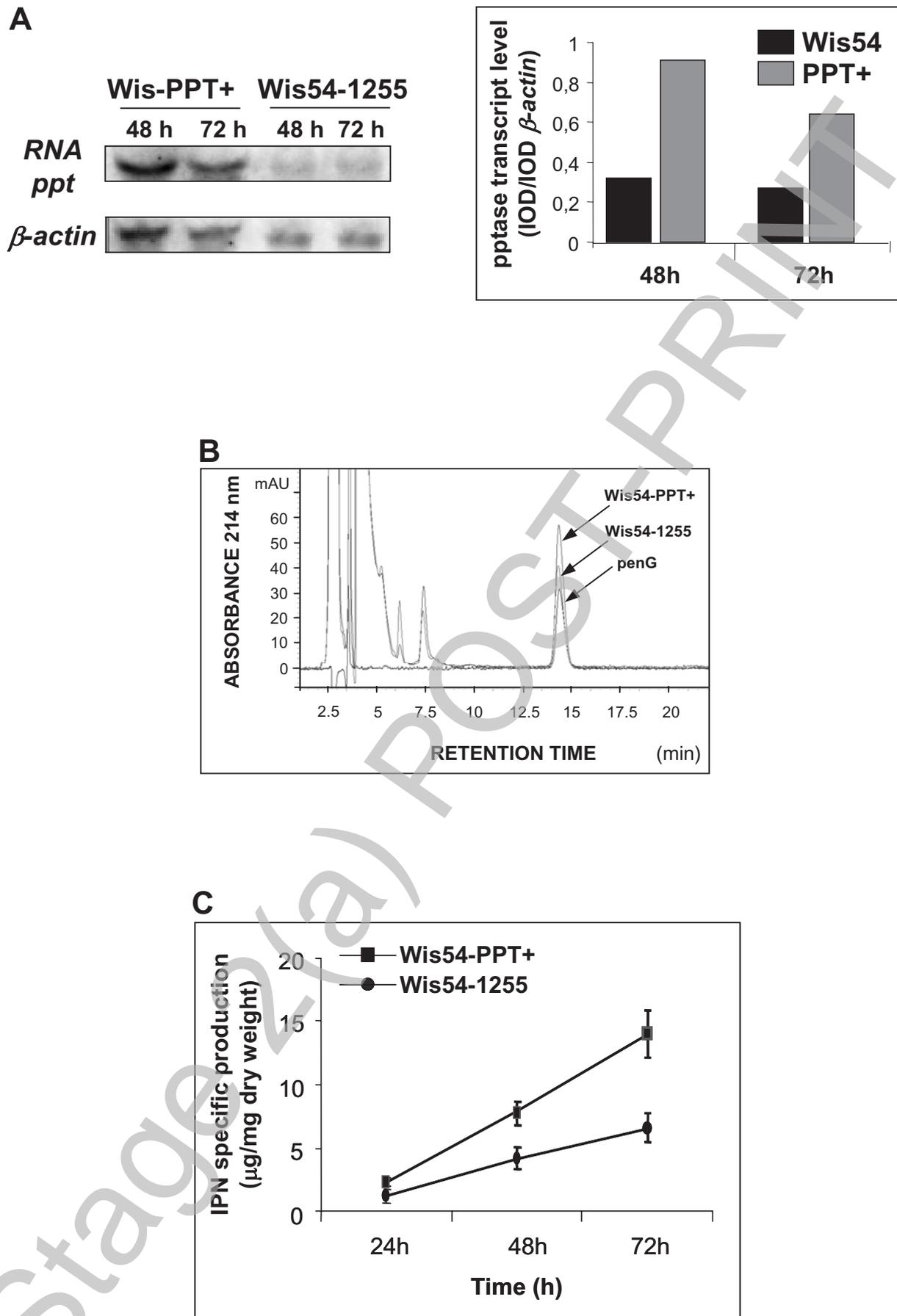
Figure 1. García-Estrada et al.



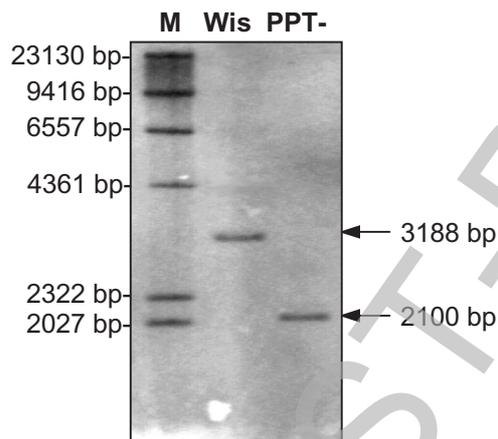
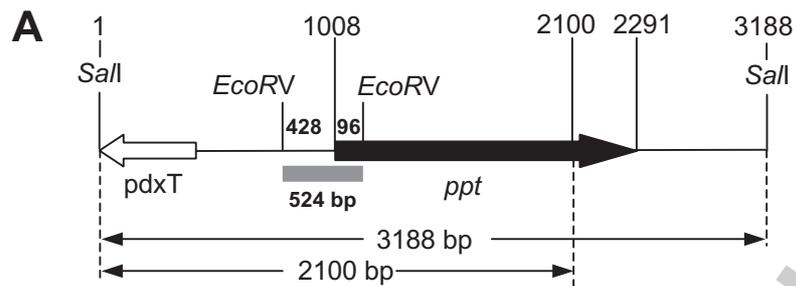
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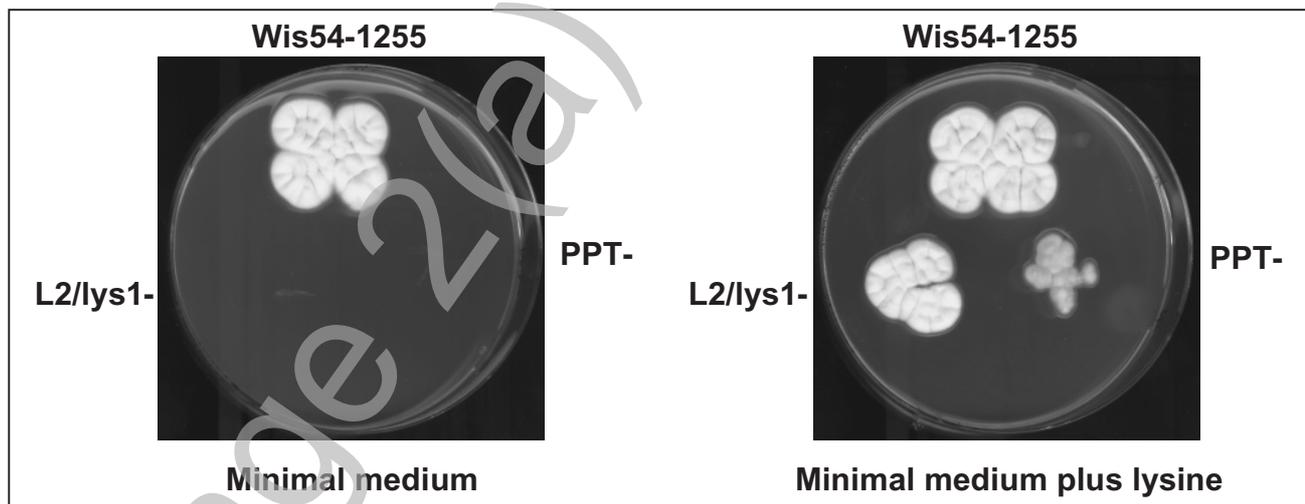
**Figure 2. García-Estrada et al.**



**Figure 3. García-Estrada et al.**



**B**



**Figure 4. García-Estrada et al.**

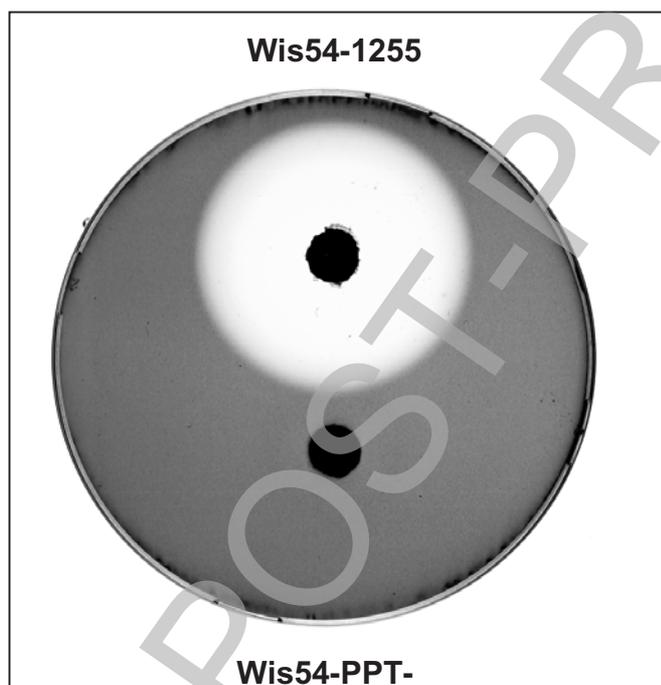
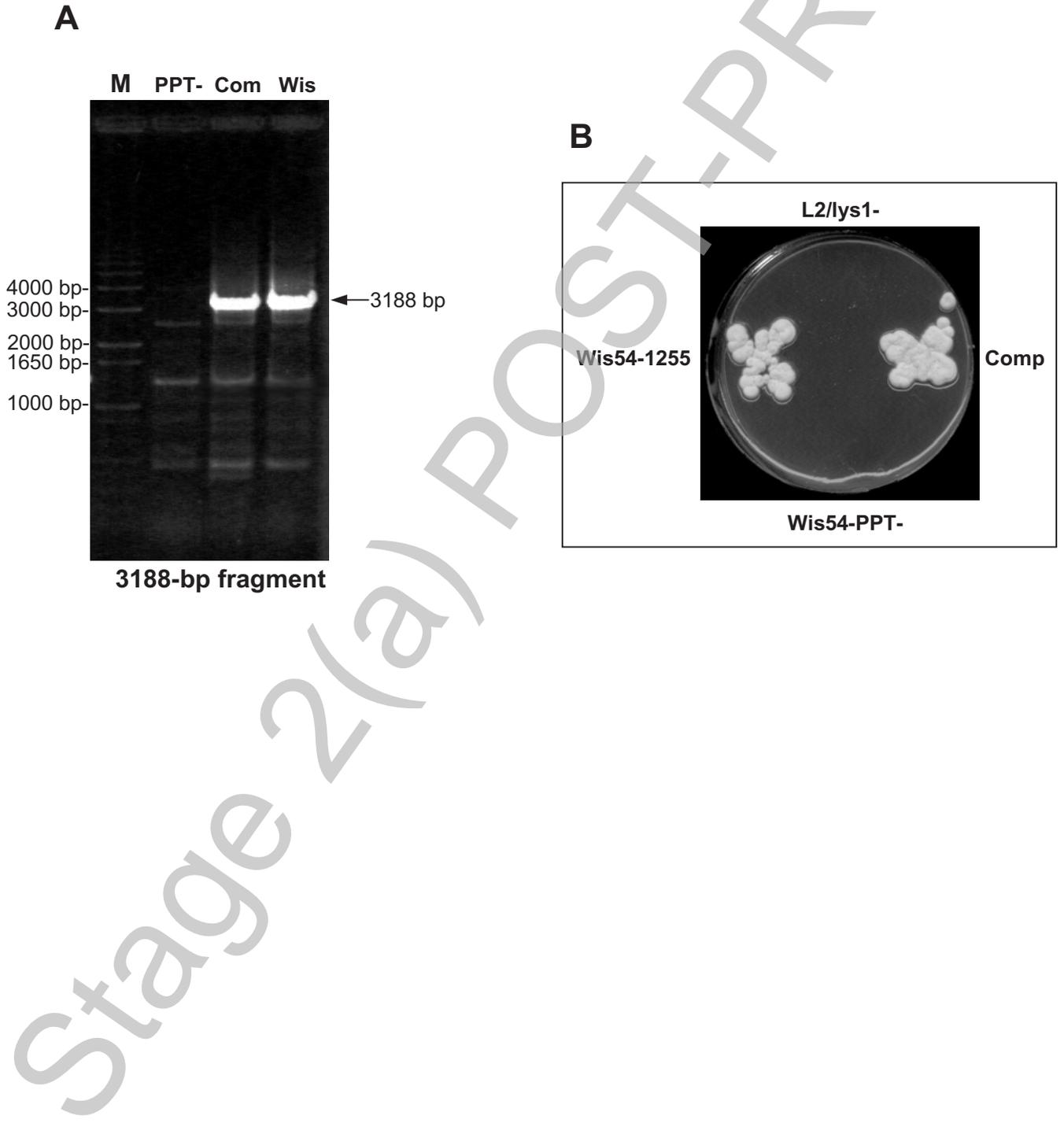


Figure 5. García-Estrada et al.



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**Figure 6. García-Estrada et al.**