

ORIGINAL ARTICLE

Self-control of the PHO regulon: the PhoP-dependent protein PhoU controls negatively expression of genes of PHO regulon in *Streptomyces coelicolor*

Seomara Martín-Martín¹, Antonio Rodríguez-García¹, Fernando Santos-Beneit², Etelvina Franco-Domínguez¹, Alberto Sola-Landa¹ and Juan Francisco Martín³

Phosphate control of the biosynthesis of secondary metabolites in *Streptomyces* is mediated by the two component system PhoR–PhoP. Linked to the *phoR*–*phoP* cluster, and expressed in the opposite orientation, is a *phoU*-like encoding gene with low identity to the *phoU* gene of *Escherichia coli*. Expression of this *phoU*-like gene is strictly dependent on PhoP activation. We have isolated a PhoU-null mutant and used transcriptomic and RNA-sequencing (RNA-seq) procedures to identify its transcription start site and regulation. RNA-seq studies identified two transcription start sites, one upstream of *phoU* and the second upstream of the *mptA* gene. Whereas transcription of PhoU is entirely dependent on PhoP, expression of the downstream *mptA* gene is only partially dependent on PhoP activation. The *phoU* mutant grows more slowly than the parental strain, sporulates poorly and the spores lack pigmentation. Production of actinorhodin and undecylprodigiosin decreased in the *phoU* mutant, indicating that PhoU has a positive modulating effect on production of these antibiotics. Indeed, transcriptional studies of expression of the *actII-ORF4* and *redD* genes indicated that the PhoU protein activates expression of these antibiotic regulators. Using the *glpQ1* promoter as *in vivo* reporter of the activity of the PHO regulon genes, we observed that expression of *glpQ1* is negatively modulated by PhoU. These results were confirmed by reverse transcription-PCR studies of three genes of the PHO regulon; that is, *glpQ1*, *pstS* and *phoR*. In conclusion, PhoU acts as a negative modulator of expression of the PHO regulon genes and as *phoU* expression is strictly dependent on PhoP activation, this mechanism appears to work as a feed-back control mechanism (self-regulation).

The Journal of Antibiotics (2018) 71, 113–122; doi:10.1038/ja.2017.130; published online 1 November 2017

INTRODUCTION

Organic or inorganic phosphate is a very important phosphorus source for *Streptomyces* species and other bacteria.¹ Phosphate forms part of DNA, RNA, phospholipids, phosphoproteins, nucleotides and other high-energy molecules and plays key roles in the respiratory chain² and in regulation of enzyme activity by protein phosphorylation/dephosphorylation.³

In *Streptomyces* species, both primary and secondary metabolism are controlled by the two-component system PhoR–PhoP (orthologous of PhoR–PhoB in *Escherichia coli*). PhoR is a membrane-bound sensor histidine kinase that is able to autophosphorylate at His²¹⁵.^{4,5} The phosphorylated PhoR interacts with the cognate response regulator PhoP (a transcriptional factor member of the OmpR family) and transfers its phosphate group from PhoR to the receiver domain (Asp⁵³) of PhoP (designated PhoP-P) that changes its configuration and binds to specific sequences (PHO boxes) in the promoter regions or, in a few cases, inside the open reading frames (ORFs).^{6–10} PhoR

also has phosphatase activity and may dephosphorylate PhoP-P.¹¹ The phosphorylated/dephosphorylated PhoP ratio is a key parameter in control of the *pho* regulon.

The structure and topology of the direct repeat units that conform each PHO box in *Streptomyces coelicolor* and their relevance in the control of expression of PhoP-regulated genes has been studied in detail.^{6,7,10,12–17} In most cases, the phosphorylated PhoP acts as a positive regulator of the genes containing PHO boxes in their promoter regions but, in some cases, PhoP-P binds PHO boxes in the –10 promoter region or inside the ORFs and exerts negative regulation,¹⁴ apparently by a ‘road block’ mechanism that prevents movement of the RNA polymerase on the DNA.⁸

In *Streptomyces* species, a PhoU-like protein of 229 amino acids (hereafter designated as PhoU) is encoded by a gene located adjacent to the PhoR–PhoP operon that is expressed divergently from the *phoRP* transcript.⁶ Surprisingly, the *Streptomyces* PhoU-like protein has little conservation (28.1% identical residues) with that of *E. coli*¹⁸

¹Instituto de Biotecnología de León (INBIOTEC), León, Spain; ²Centre for Bacterial Cell Biology, Institute for Cell and Molecular Biosciences, Medical School, Newcastle University, Newcastle upon Tyne, UK and ³Área de Microbiología, Departamento de Biología Molecular, Facultad de Ciencias Biológicas y Ambientales, Universidad de León, Campus de Vegazana s/n, León, Spain

Correspondence: Professor JF Martín, Área de Microbiología Departamento de Biología Molecular, Facultad de Ciencias Biológicas y Ambientales, Universidad de León, Campus de Vegazana s/n, 24071 Leon, Spain.

E-mail: jf.martin@unileon.es

Received 30 May 2017; revised 17 September 2017; accepted 26 September 2017; published online 1 November 2017

Table 1 Bacterial strains, plasmids and primers used in this study

Strain	Features	Reference
<i>S. coelicolor</i> M145	Parental strain	26
<i>S. coelicolor</i> Δ <i>phoU</i>	Interrupted <i>phoU</i> gene, Neo ^r	This work
<i>S. coelicolor</i> INB201	Deleted in <i>phoP</i> gene, Am ^r	14
<i>E. coli</i> XL10-Gold	<i>recA1</i> , <i>relA1</i> , <i>gyrA96</i> , <i>thi-1</i> , <i>supE44</i> , <i>endA</i> , Tetr Δ (<i>mcrA</i>)183 Δ (<i>mcrCB</i> - <i>hsdSMR</i> - <i>mrr</i>)173, <i>lac</i> , Hte [F' <i>proAB</i> <i>lacIqZ</i> Δ M15 Tn10 (Tetr) Amy Cam ^r]	40
<i>E. coli</i> ET12567 [pUZ8002]	<i>dam dcm</i> mutant, Neo ^r Cm ^r	26
Plasmid	Features	Reference
pLUX- <i>glpQ1</i>	<i>Bam</i> HI- <i>Nde</i> I pFS- <i>glpQ1</i> fragment cloned into pLUXAR-neo, Am ^r Neo ^r	15
pUC19	Cloning vector, Ap ^r	28
pAV11b	Integrative plasmid (ϕ iBT1) Hyg ^r	31
pAV11b-N	Derivative of pAV11b, Hyg ^r	This work
pHZ1351	Highly unstable Sti ⁺ vector, useful for gene replacement in <i>Streptomyces</i>	26
pBluescript KS(+)	Cloning vector, Ap ^r	Stratagene
pBS <i>phoRP</i>	<i>phoR</i> - <i>phoP</i> genes, cloned into pBluescript KS+ (<i>Eco</i> RI- <i>Sma</i> I)	4
pHZ <i>phoU</i>	pHZ1351 with disrupted <i>phoU</i> gene, Kn ^r	This work
pCOM <i>phoU</i> -b	Integrative plasmid with <i>phoU</i> gene and Hyg ^r	This work
Oligonucleotides	Sequence (5'-3')	Features
SM27	CGTCTGCAGGCATCAACGGCGCACTGG	PCR <i>phoU</i> gene
SM28	TCTCTCTAGACAGGTTAGGCACGGCGAA	PCR <i>phoU</i> gene
SM40	GACCTGGTGGACGAAGTGTG	qPCR <i>redD</i> (SCO5877)
SM41	ACGCTCGTTGAGCACTTTCC	qPCR <i>redD</i> (SCO5877)
SM42	AATCACCGATGCGGGATGTG	qPCR <i>actII-ORF4</i> (SCO5085)
SM43	GCTGGGTCAGGACCAATTCC	qPCR <i>actII-ORF4</i> (SCO5085)
SM13	ACGGCGTGCAGATCAACTAC	qPCR <i>pstS</i> (SCO4142)
SM14	CCGTCCTTGCAGACCTTCTTC	qPCR <i>pstS</i> (SCO4142)
SM15	GGCGGGACGGAGAGATACG	qPCR <i>phoR</i> (SCO4229)
SM16	CTCACGTTGGCGACGAAGTC	qPCR <i>phoR</i> (SCO4229)
SM38	AAGGACGGTCACCTCGTCTG	qPCR <i>glpQ1</i> (SCO1565)
SM39	TGAGTTCCGGCAGCGTGAAG	qPCR <i>glpQ1</i> (SCO1565)
SM17	CACTACGTGGGCGTGACCTTC	qPCR SCO1638
SM18	CCTGGTCCCAGCCCTTGATGAC	qPCR SCO1638

and the *phoU* gene is located in a different cluster. In *E. coli*, *phoU* is integrated in the *pstSCAB-phoU* cluster, whereas in *Streptomyces* it is linked to the PhoR – PhoP genes. Although the role of PhoU in *E. coli* is well studied,^{19,20} the precise role of the PhoU-like protein in *Streptomyces* is still obscure.^{18,21} In *Streptomyces lividans* and *S. coelicolor*, downstream of the *phoU*-like gene is *mtpA*; a gene encoding a metalloprotein of the metallothioneine family, related to the *spx* gene of *Bacillus subtilis* that appears to have a role in homeostasis of disulfide bonds protecting the cell against oxidative stress.¹⁸

The *phoU*-*mtpA* genes seem to be transcribed as a single transcript from the PhoP-regulated promoter of *phoU* but there is also evidence for a possible promoter located between these two genes based on conventional promoter-probe studies.^{18,21} To clarify the control of expression of these two genes in this study, we have applied transcriptomic approaches.

In *E. coli*, PhoU serves as a modulator of the phosphate starvation stress response¹⁹ and it appears to play a role in the coupling of the PstSCAB complex to the PhoR sensor kinase.^{20,22,23} PhoU does not exist in *B. subtilis* and therefore is not an essential protein for bacterial phosphate metabolism; its ‘coupling’ effect may vary in different bacteria.²⁴ Therefore, it is of great interest to clarify the role of the

PhoU-like protein in *S. coelicolor*. In this bacterium the PhoR–PhoP system has been already shown to differ from that of *E. coli* in relation to ‘partner fidelity’.²⁵ Thus, the main objective of this study was to elucidate the role of the PhoU protein in the control of the *pho* regulon. We show, using both promoter probe and quantitative reverse transcription-PCR (RT-PCR) analyses, that PhoU acts as a negative regulator of PhoP upregulated genes. The negative effect of PhoU may serve to compensate for the strong positive effect exerted by PhoP-P on expression of *phoU* and other *pho* regulon genes, thus establishing a balance in the control of the *pho* regulon.

MATERIALS AND METHODS

Bacterial strains and plasmids

The bacterial strains, plasmids and oligonucleotides used in this work are listed in Table 1. *S. coelicolor* strains M145²⁶ and its derivatives *S. coelicolor* Δ *phoP*¹⁵ and *S. coelicolor* Δ *phoU* were manipulated according to standard procedures.^{26,27} *E. coli* ET12567 [pUZ8002] was used for intergeneric conjugative transfer of plasmid DNA into *Streptomyces* strains.²⁸

The *S. coelicolor* Δ *phoU* mutant was obtained for this work. The mutant was constructed by interruption of *phoU* gene with kanamycin resistance cassette using pHZ1351 system.⁴ The *phoU* gene was cloned into pBluescript KS(+)²⁹

from a 3 kb *EcoRI*–*Ecl136II* fragment of D46 cosmid³⁰ and a 1.7 kb fragment *EcoRI*–*NruI* of pBS*phoRP*,⁴ obtaining pBS*phoU*. The disruption of *phoU* gene (kanamycin resistance gene in the opposite orientation) was derived from *Eco72I*-fragment pBS*phoU* and 1.3 kb *Bam*HI fragment pTC192-km, thus obtaining pBS*phoU*intKn. Finally, plasmid pHZ*phoU*, which contains DNA inserts from *SpeI*–*XhoI* pBS*phoU*intKn and *Ecl136II* pHZ1351 fragments, was transferred into *S. coelicolor* M145. Candidate mutants were validated by Southern blot methodology (data not show).

The complementation of the mutant strain was performed with integrative conjugative plasmids. The parental plasmid pAV11b-N is a derivative of pAV11b,³¹ in which 24 bp were deleted by *AvrII*, *Acc65I* digestion, end-filling and ligation. The *phoU* gene, with its promoter, was amplified by PCR. The primers SM27 and SM28 amplified a 976 bp fragment corresponding to the *phoU* gene from –219 position (regarding ATG translation start triplet). The *PstI* (SM27) and *XbaI* (SM28) cloning sites were introduced via the primer sequences. First, the *XbaI/PstI* fragments were subcloned into pUC19 for validation of sequence and, then *Bam*HI/*AseI* fragments were cloned into pAV11b-N for conjugation in *Streptomyces*. The final plasmid was pCOM*phoU*-b.

Culture conditions

S. coelicolor cultures were grown in defined liquid MG-3.2 medium (containing, as carbon and nitrogen sources, 50 g l⁻¹ starch and 60 mM glutamate and a growth-limiting amount of 3.2 mM phosphate),¹⁴ or in complex liquid R5 medium²⁶ with either 370 or 40 μM phosphate. Baffled flasks (0.5 l, 100 ml of medium) were inoculated with spores (10⁶ ml⁻¹) and incubated at 30 °C, 300 r.p.m., for reproducible and dispersed growth.¹⁴ Plates of solid media TBO, TSA and R5 were inoculated with a suspension of 10⁸ spores and incubated at 30 °C. TBO medium was used to obtain spore preparations.³² Supplements of kanamycin (50 μg ml⁻¹), hygromycin (100 μg ml⁻¹) or apramycin (50 μg ml⁻¹) were added when needed.

Luciferase assay

The reporter luciferase activity was measured in a Luminoskan luminometer (Labsystems, Helsinki, Finland) as follows. Culture samples were immediately cooled in ice until luminiscence readings were made. For measurement, 250 μl of 0.1% n-decanal was added to 500 μl of sample and the light emission was read after 20 s of integration time.

Antibiotic, growth and phosphate assays

The phosphate concentration of culture supernatants was measured using the malachite green assay.³³ Antibiotic assays were performed as described by Kieser *et al.*²⁶ For growth measurements, culture samples (2 ml) were centrifuged, washed twice with MilliQ (Merk, Kenilworth, NJ, USA) water and the dry weight was determined after desiccation at 80 °C for 3 days.

Transcriptomic data

A differential RNA-sequencing (dRNA-seq) analysis³⁴ was carried out by Vertis Biotechnologie AG (Freising, Germany) using an Illumina HiSeq 2000 platform (San Diego, CA, USA). This approach discriminates primary and processed 5' ends (identification of transcriptional start sites (TSSs)). Studies with two complementary DNA libraries were performed, one library from untreated total bacterial RNA and the other enriched for primary transcripts by terminator exonuclease treatment that degrades 5'P but not 5'PPP RNA. Total RNA was isolated using NucleoSpin miRNA kit (Macherey-Nagel, Düren, Germany) ref. 740971.50. The sequenced RNA sample was a pool of RNA samples extracted from *S. coelicolor* M145 and INB201 (*ΔphoP*) cultures in MG-3.2 medium. The sampling comprised the late exponential phase (36–40 h, 6 samples), the response to phosphate depletion from the medium (40.5–45.5 h, 9 samples) and the response to 10 mM phosphate addition to the cultures after phosphate depletion; 40.5–45.5 h (13 samples). Reads were quality trimmed and mapped with BBTtools (<http://jgi.doe.gov/data-and-tools/bbttools/>). Coverage values were calculated with BEDtools³⁵ and visualized in the IGB browser.³⁶

S. coelicolor microarrays were obtained from Agilent (Santa Clara, CA, USA) as a custom design. Two time series were done with *S. coelicolor* M145 and INB201 (*ΔphoP*) strains. Briefly, both strains were cultured in flasks containing defined MG-3.2 medium and samples were taken from 36 to 41 h of culture (13 samples) and stabilized with RNAProtect Bacteria reagent (Qiagen Venlo, Netherlands). The time span included the point when phosphate becomes depleted from the medium and the *pho* regulon is activated. Total RNA was purified from the culture samples by acid phenol–chloroform extraction and ethanol precipitation. RNA was labeled with Cy3 using the Label IT reagent (Mirus Bio, Madison, WI, USA). Genomic DNA (strain M145) was labeled with Cy5 by Exo-Klenow polymerase (BioPrime kit, Invitrogen (Carlsbad, CA, USA)). Transcriptional values (Mg) were calculated as the normalized log ratio Cy3/Cy5, as indicated previously.³⁷

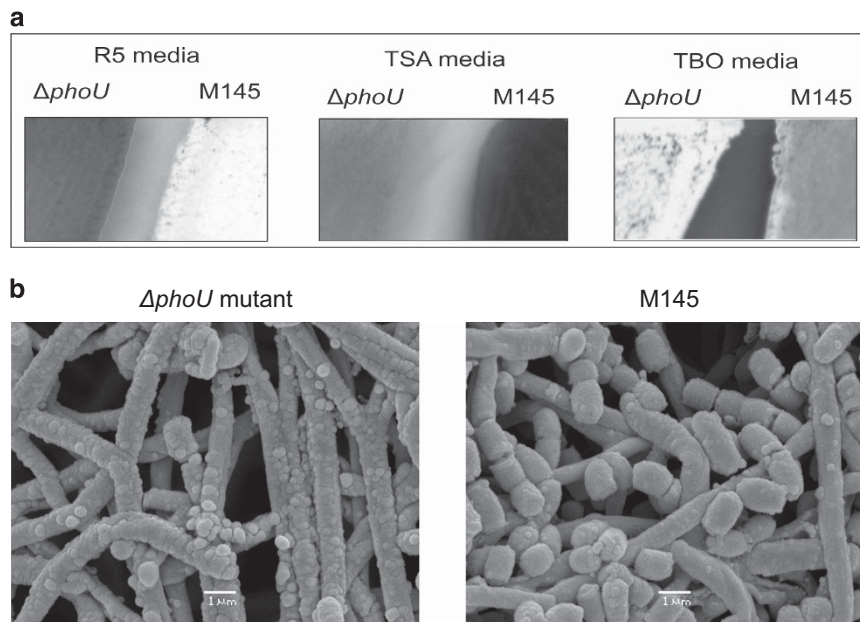


Figure 1 (a) Phenotypic effect of the mutants on growth, sporulation and pigmentation on different solid media: R5, TSA and TBO (left to right). Plates are shown after 3 days of growth at 30 °C. (b) Scanning electron microscopy of spores of the parental (M145) and *phoU* mutant strains after growth on TBO for 7 days at 30 °C. A full colour version of this figure is available at the *Journal of Antibiotics* journal online.

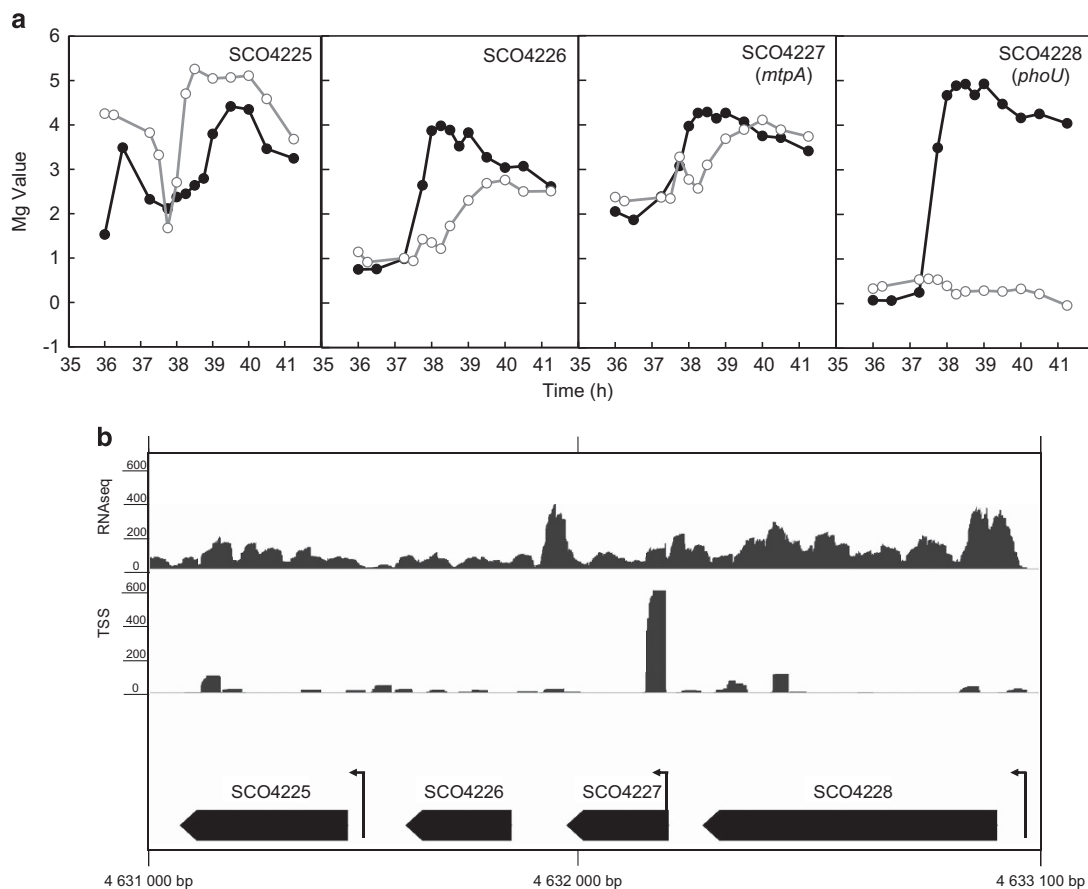


Figure 2 (a) Microarray transcription profiles of SCO4225–SCO4228 genes from cultures of M145 (black line) and $\Delta phoP$ (gray line) strains in MG-3.2 medium. Horizontal axis corresponds to the time (h) after inoculation in the cultures. The vertical axis shows the normalized log transcription values (i.e., \log_2 abundance). Note the strong dependence of *phoU* on a functional PhoP regulator. (b) *S. coelicolor* transcription start sites (TSSs) revealed by differential RNA-sequencing (dRNA-seq) in SCO4228–SCO4225 region. Coverage graphs (i.e., number of reads mapped at each nucleotide position) are shown for the control library (normal RNA-seq, upper graph) and for the library generated from the same RNA treated with *Terminator* exonuclease (bottom graph) that is enriched in primary 5' ends. Determined three TSSs are shown as arrows on annotated genes SCO4228, SCO4227 and SCO4225.

Reverse transcription quantitative PCR (RT-qPCR)

RT-qPCR was performed in 96-well format with a Stratagene MX3005P (Agilent) and the Brilliant II SYBR Green qPCR Master Mix (Agilent). Complementary DNA was obtained from 0.5 μg of RNA with AffinityScript reverse transcriptase and random primers (Agilent). PCR cycling consisted of a single incubation at 95 °C for 5 min, followed by 40 cycles of 95 °C for 30 s and 65 °C for 1 min. A final melting curve analysis (95 °C for 1 min, 55 °C for 30 s and 95 °C for 30 s) was always included.

The quantification of gene expression was performed by MxPro QPCR Software (Agilent Technologies) according to the $\Delta\Delta Ct$ method.³⁸ The *S. coelicolor* gene SCO1638 was chosen for the reference (housekeeping) assay, as analyses of our microarray data with NormFinder³⁹ revealed this gene as having one of the most stable transcription profiles in our culture conditions (not shown). This was confirmed with a subsequent geNorm⁴⁰ analysis of RT-qPCR data.

Electron microscopy

Scanning electron microscopy of sporulated mycelium was performed with a JSM-6480 LV scanning electron microscope (JEOL, Tokyo, Japan). *S. coelicolor* strains were cultured in TBO agar for 7 days at 30 °C. Samples were fixed with glutaraldehyde and osmium tetroxide and dehydrated with increasing concentrations of ethanol. Subsequently, samples were critical-point dried in a CPD 030 critical-point dryer (Bal-Tec, Liechtenstein, Balzers, Liechtenstein) using liquid CO₂. Finally, the samples were coated with a gold layer in a Balzers Union (Liechtenstein) SCD 004 sputter coater.

RESULTS

Phenotypic studies in solid medium and sporulation: the *phoU* mutant is defective in spore pigment

A *phoU* mutant of *S. coelicolor* was isolated by insertion of a kanamycin resistance cassette, as indicated in the Materials and Methods. To determine the phenotype of the *phoU* mutant, it was grown on R5, TBO and TSA media (Figure 1a). The *S. coelicolor phoU* mutant showed delayed growth in all media tested. Moreover, sporulation of this mutant was delayed in comparison with that of the parental strain in the three media. Noteworthy, the *phoU* mutant sporulated weakly and its spores remained white after 2 weeks, although the spores were viable (Figure 1b). Therefore, PhoU is required for the formation of the polyketide pigment of the spores (see Discussion). Complementation of the *phoU* mutant with the wild-type *phoU* allele restored the sporulation of the mutant and, partially, the spore pigmentation

Transcriptomic analysis of the *phoU*-*mptA* region

The *S. coelicolor phoU* gene (SCO4228) has its own promoter activated by PhoP when the phosphate concentration in the medium is low.^{6,12} These previous results are confirmed and extended in this article by the transcriptomics results of the genes in this region (SCO4228–SCO4225). The microarray profile in MG-3.2 showed that the transcription of *phoU* is totally dependent on PhoP (Figure 2a). In

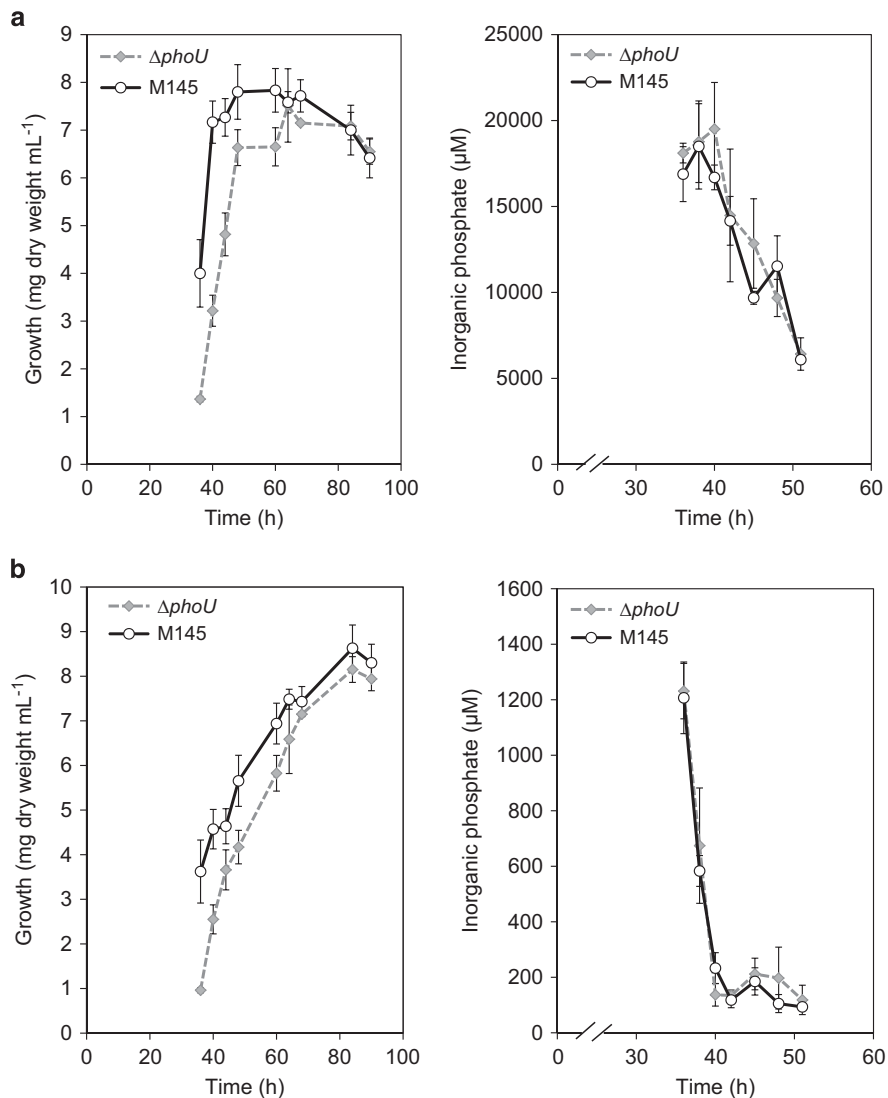


Figure 3 Growth and phosphate uptake rate by the parental strain M145 (white circles) and *phoU* mutant (gray diamonds) strains in MG-18.5 medium (a) and MG-3.2 medium (b). Error bars correspond to the s.e.m. for three biological replicates.

contrast, the downstream genes SCO4227 (*mptA*) and SCO4226 (hypothetical protein) share a profile that indicates only a partial transcriptional activation by PhoP. A clear promoter at the 5' end of the SCO4227 coding sequence was deduced from the results of the dRNA-seq assay (see below) (Figure 2b). This promoter appears to be independent of PhoP, as no conserved PHO boxes are shown upstream and it would account for the transcription of the SCO4227–SCO4226 genes in the strain INB201 ($\Delta phoP$) (Figure 2a). Both these genes would form a transcriptional unit as the downstream gene SCO4225, encoding a membrane integral protein, behaved differently and appears to contain its own promoter (Figure 2a).

Analysis of the TSSs was based on RNA-seq approach (see Materials and Methods). The TSS of the *phoU* PhoP-dependent promoter was previously identified by primer extension.⁶ The dRNA-seq approach revealed the same TSS with a minor difference of 2 nt with respect to the data of Sola-Landa *et al.*⁶ (2005) (genomic coordinates 4633052) (Figure 2b). The transcription start point of *mptA* was identified at

genome position 4632207 (Figure 2b). A less clear TSS is suggested for the SCO4225 gene at coordinates 4631506.

phoU disruption delays growth and phosphate uptake

The growth of *S. coelicolor phoU* mutant was compared with that of the parental *S. coelicolor* M145 strain in both phosphate-replete (MG-18.5) and phosphate-limited (MG-3.2) media. Results of several experiments (see also below studies in R5 medium) showed that the *phoU* mutant grows more slowly than the parental strain M145 in MG-18.5; although it reaches finally a similar biomass (dry weight) to that of the parental strain (Figure 3). Similarly, growth of the *phoU* mutant in phosphate-limited (MG-3.2) medium was also delayed with respect to the parental strain and the rate of phosphate utilization by this mutant was slightly lower than that of other strains (Figure 3b). The effect of the *phoU* mutation slightly reducing the uptake of phosphate is consistent with the modulating effect of the PhoU protein on other genes of the *pho* regulon, particularly with the *pstS* transport system. In conclusion, PhoU is required for optimal

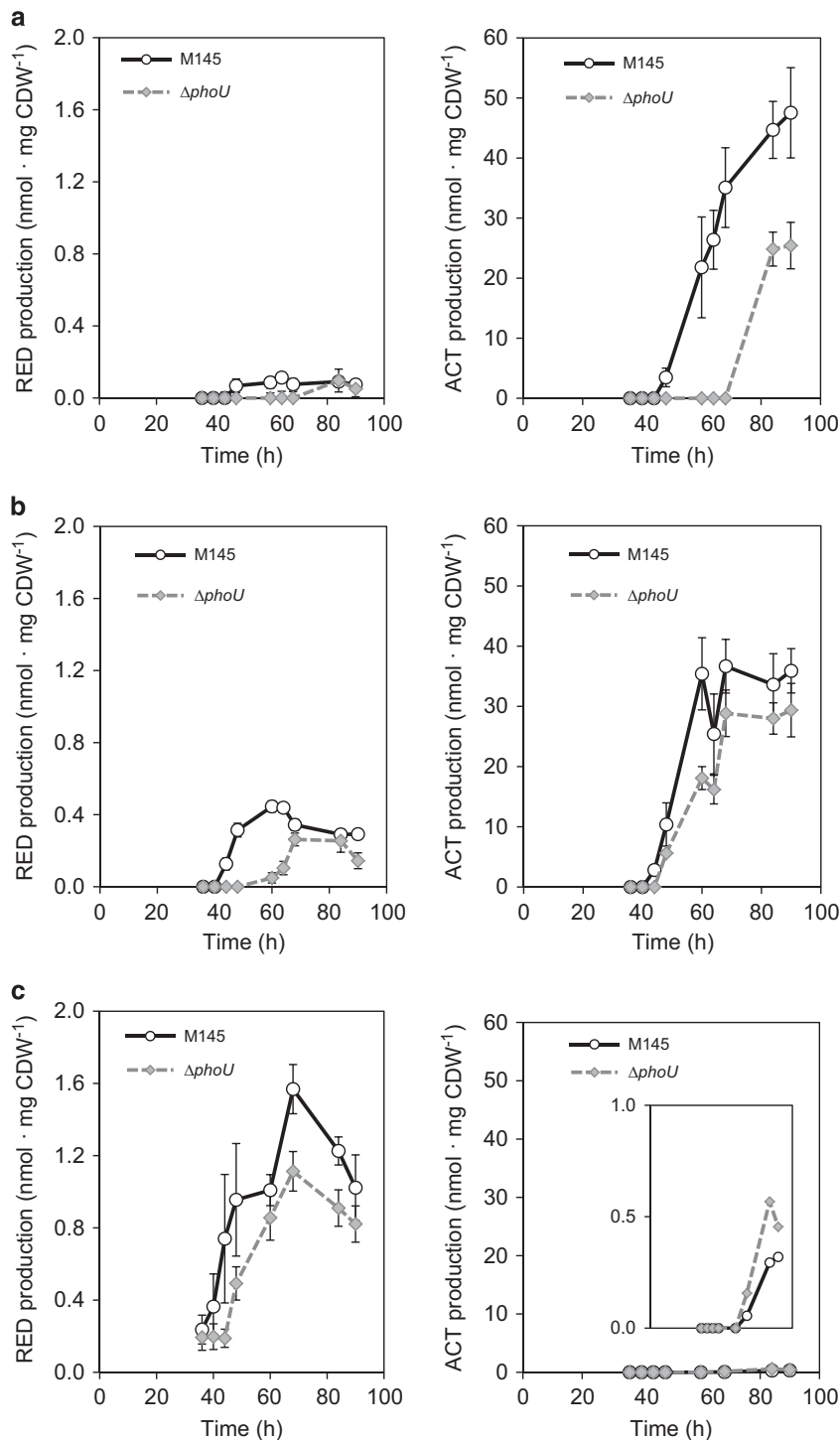


Figure 4 Production of actinorhodin (ACT) and undecylprodigiosin (RED) by the parental strain M145 (white circles) and the *phoU* mutant (gray diamonds) strain in MG-18.5 medium (a), MG-3.2 medium (b) and R5 medium (370 μM phosphate) (c). Error bars correspond to the s.e.m. for three biological replicates.

phosphate uptake in *S. coelicolor* and this role is independent of the amount of extracellular phosphate.

Antibiotic production in complex R5-370 medium and defined MG medium by the *phoU* mutant

In order to quantify the effect of the mutation on antibiotic production in submerged cultures, the parental strain and the mutant *phoU* complemented strain were grown in phosphate-limited MG-3.2

medium and medium R5-370 (R5 medium with 370 μM phosphate concentration), a complex medium that has been optimized by us for antibiotic production.

Growth of the *phoU* mutant was again slower than that of the parental during the first 60 h of culture. However, the final biomass was similar in this mutant and in the other strains. Antibiotic actinorhodin (ACT) and undecylprodigiosin (RED) production in liquid cultures was delayed and lower in the *phoU* mutant than that of

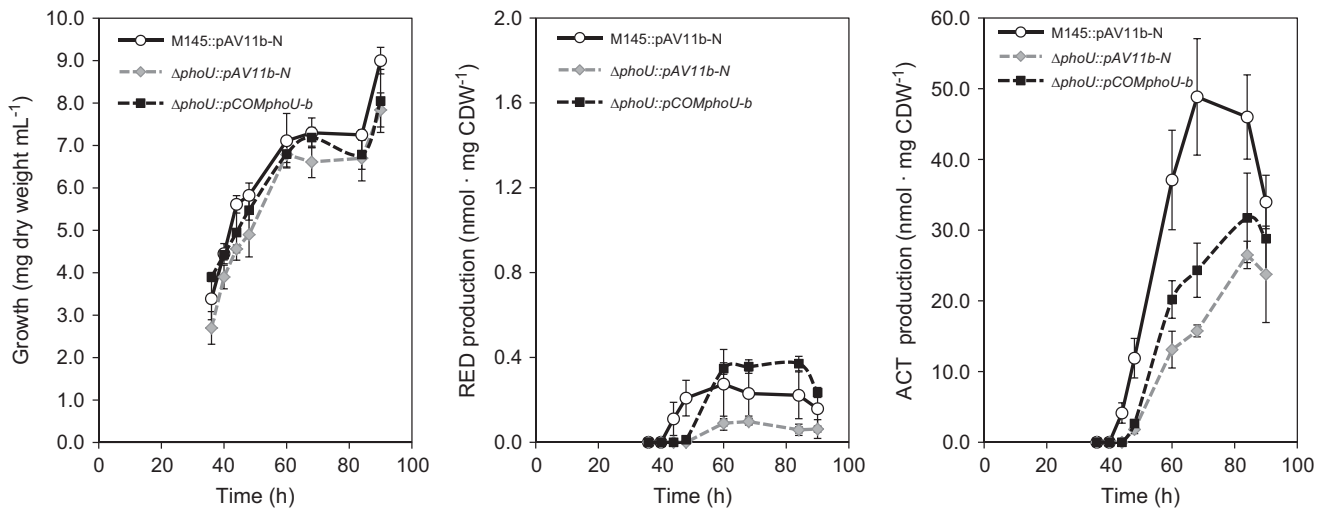


Figure 5 Growth and production of actinorhodin (ACT) and undecylprodigiosin (RED) by the parental strain M145::pAV11b-N (white circles), the *phoU*::pAV11b-N mutant (gray diamonds) and the complemented *phoU*::pCOM*phoU-b* (black squares) strains in MG-3.2 medium. Error bars correspond to the s.e.m. for four biological replicates.

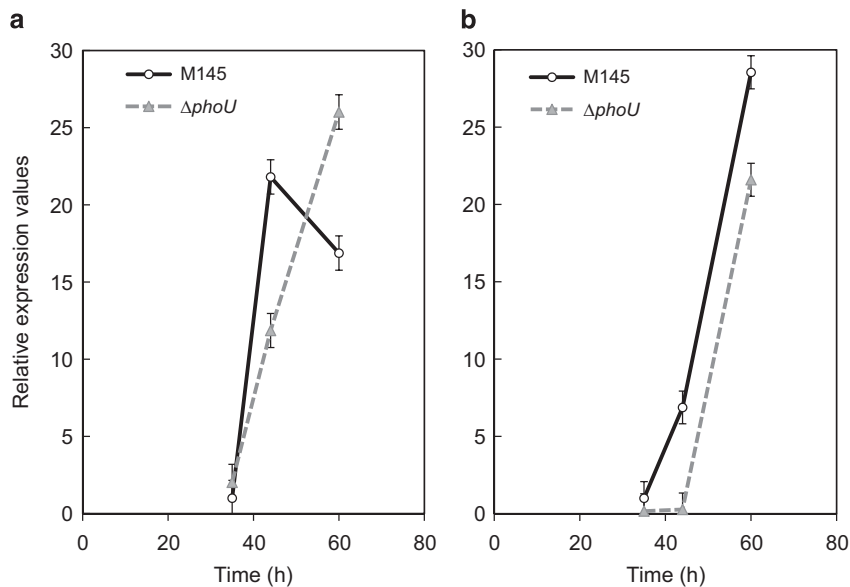


Figure 6 Validation of genes involved in antibiotic production by reverse transcription quantitative PCR (RT-qPCR). Quantitative RT-PCR of genes is indicated below each panel. Error bars were calculated by measuring the s.d. among technical replicates of each sample. (a) *SCO5877*, encoding RedD, the undecylprodigiosin regulator and (b) *SCO5085*, encoding ActII-ORF4, the actinorhodin activator. Parental M145 (white circles) and *phoU* mutant (gray triangles) strains.

the M145 strain in both defined and complex media (Figures 4a–c). The respective *phoU* complemented strain was grown in MG-3.2 medium and the growth and RED and ACT production were partially restored (Figure 5). Therefore, PhoU modulates ACT and RED biosynthesis through its effect on phosphate regulation. As shown below, PhoU modulates negatively the *pho* regulon and this includes genes involved in antibiotic biosynthesis.

Quantification of expression of regulatory genes *actII-ORF4* and *redD*

In order to validate the effect of *phoU* mutation on ACT and RED production, we analyzed the expression of antibiotic biosynthesis regulatory genes, including *SCO5085* (*actII-ORF4*) and *SCO5877* (*redD*) by RT-qPCR. The expression profile of these genes in the wild-type and *phoU* mutant strains was studied in samples taken after

35, 44 and 60 h of cultivation in MG-3.2 medium. Results of this study showed that the *actII-ORF4* regulatory gene of ACT had a lower expression level in the *phoU* mutant than in the parental strain at all times studied (Figure 6b). These results explain the modulation effect exerted by *phoU* on ACT production.

In the mutant *phoU* the *redD* gene was activated later; it showed a lower level of expression than wild-type strain at 44 h. However, at later times *redD* was activated and showed a higher level of expression than the parental strain (Figure 6a).

Activation of the *glpQ1* promoter as an ‘*in vivo*’ monitor of the PhoP-mediated response

Plasmid pLUX-*glpQ1*, containing the *glpQ1* promoter coupled to *luxAB* genes, was used previously to determine the *glpQ1* promoter activity in Pi shift-down experiments, because expression of this gene

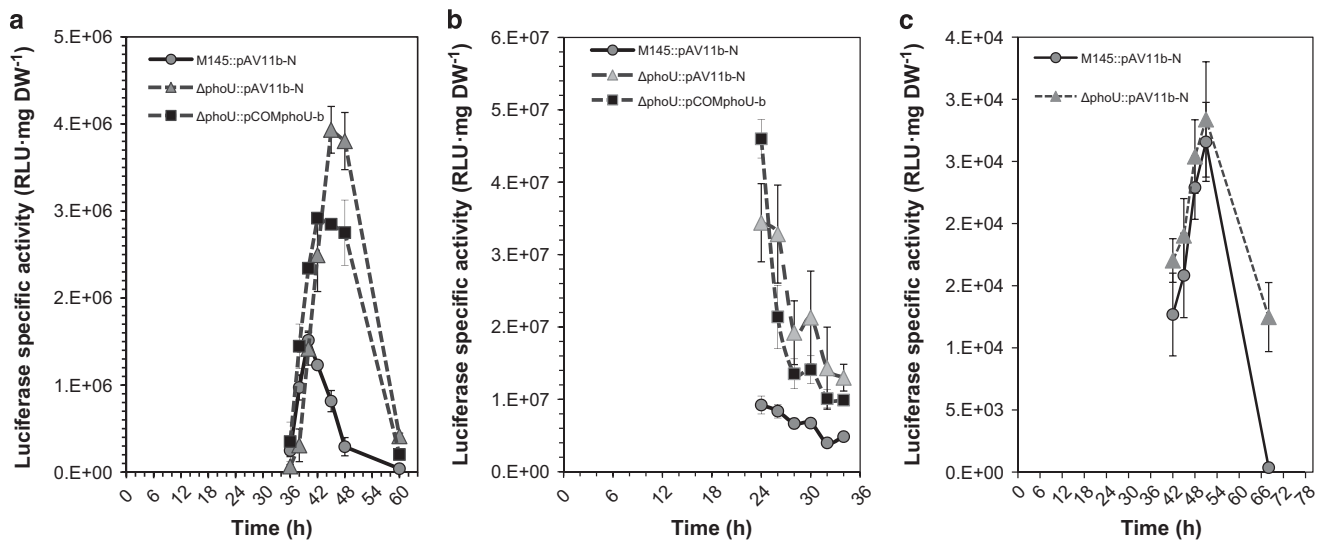


Figure 7 Expression of the *glpQ1* promoter coupled to the *luxAB* reporter genes in *S. coelicolor* M145 and mutants derived from it. M145::pAV11b-N (circles), *phoU*::pAV11b-N mutant (triangles) and the complemented *phoU*::pCOM*phoU*-b strain (squares) in MG-3.2 (a), R5 40 μ M (b) and MG-18.5 (c) medium.

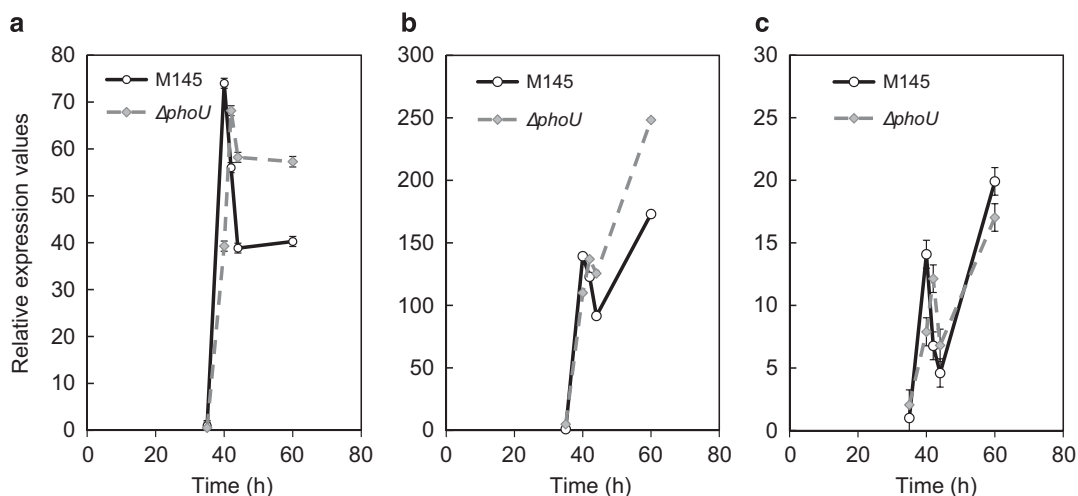


Figure 8 Validation of the luciferase results by reverse transcription quantitative PCR (RT-qPCR). Quantitative RT-PCR of genes: (a) SCO1565 (*glpQ1*), (b) SCO4142 (*pstS*) and (c) SCO4229 (*phoR*). Parental M145 (white circles) and *phoU* mutant (gray diamonds) strains. Error bars were calculated by measuring the s.d. among technical replicates of each sample.

is totally and specifically dependent upon PhoP and serves to monitor the *pho* response *in vivo*.¹⁶ The strict dependence of the *glpQ1* promoter in PhoP activation has been confirmed recently (M. Ordóñez-Robles, unpublished data). However, the effect of the PhoU in the expression of the *pho* regulon genes may be distinct for different PhoP-regulated promoters. In order to elucidate the molecular basis of the role of *phoU* gene on the *pho* regulon, plasmid pLUX-*glpQ1* was introduced into the parental and in the *phoU* mutant strains. Cultures for the reporter luciferase analysis were made in media containing a limiting phosphate concentration, that is, MG-3.2 and R5-40 (R5 medium supplemented with 40 μ M phosphate is growth limiting). The disruption of *phoU* caused consistently higher *glpQ1* promoter activities throughout most of the time course of the cultures in both media. When promoter activities were compared, the *glpQ1* promoter activity in the *phoU* mutant was more than 2.5 or 5 times (depending on the medium) higher than that of the parental strain transformed

with the same construction (Figure 7). This higher expression of *glpQ1* promoter in the mutant points to an important negative role of the PhoU protein in the control of the *pho* regulon. To compensate for this negative effect, the cell might reduce *phoU* transcription by lowering PhoP phosphorylation, establishing a *pho* regulon balance under Pi limitation.

Validation studies by the quantitative RT-PCR chain reaction confirms the negative regulation exerted by PhoU on the *pho* regulon

In order to validate the luciferase reporter results and to extend the study to other PhoP-regulated genes, we analyzed by RT-qPCR the expression of three representative genes of the *pho* regulon; that is, *phoR* (SCO4229), *glpQ1* (SCO1565) and *pstS* (SCO4142). The expression profile of these genes in the parental and mutant strains was determined in samples taken after 35, 40, 42, 44 and 60 h of

cultivation in MG-3.2 medium. The gene SCO1638 of *S. coelicolor* (encoding a peptidyl-prolyl *cis*–*trans* isomerase) was chosen as the reference (housekeeping) gene as analyses of our microarray data with the geNorm program (not shown) highlighted this gene as having the more stable expression in these experimental conditions. Data on gene expression profile of *glpQ1* by RT-qPCR are shown in Figure 8 and agree with the profile obtained by the *luxAB* reporter method; *glpQ1* was activated at the same time in both strains but at later times (44 and 60 h) the expression in the *phoU* mutant strain of *glpQ1* was higher than in the parental strain; that is, PhoU exerts a negative modulation on the *pho* regulon. Similarly, the *pstS* gene showed a higher expression in the *phoU* mutant than in the parental strain at later times (44 and 60 h), thus extending the luciferase results to other genes of the Pho regulon. Finally, *phoR* expression does not seem to change significantly in the *phoU* mutant, perhaps because in addition to its regulation by PhoU, the *phoRP* operon is regulated by other interacting proteins.²³ These results indicated that PhoU has a distinct effect on *phoP*-regulated promoters and this may be because of a different degree of interaction of PhoP with those promoters, depending on their structure, not only to a simple effect of PhoU on the phosphorylation balance of PhoP.

DISCUSSION

It is well established that the phosphate starvation response is controlled by the two-component system PhoR–PhoP in *Streptomyces* species.^{4,5} PhoP-P is able to bind the *pstS* and *phoU* promoters in phosphate-limiting conditions.⁶

In this article, as shown in Figure 2, we proved that expression of *phoU* is entirely dependent on PhoP activation. Similarly, in *S. lividans*, *phoU* was totally PhoP-P dependent.²⁰ On the other hand, we have confirmed using transcriptomic studies that expression of SCO4227–SCO4226 is partially PhoP independent.

Based on the transcriptomic and RNA-seq analysis performed in this work we conclude that promoters of the genes *phoU* and *mtpA* drive the overlapping transcription of genes SCO4228 to SCO4226 and it is likely that the promoters respond to different transcriptional factors; for example, the *phoU* promoter is clearly dependent on PhoP, whereas *mtpA* is not.

Two TSSs were clearly identified upstream of *phoU* and *mtpA*. This agrees with the recently published dRNA-seq data.⁴¹ The *mtpA* TSS lies three codons inside the annotated coding sequence, implying that the bioinformatically deduced annotation is wrong. The next possible translational start codon in the open reading frame is a ATG codon at position 4632132. In favor of the alternative translation start is the existence of an upstream, well-located, putative ribosome-binding sequence (AGGAGG). In fact, the *S. lividans* TK24 homolog region is annotated (accession AIJ14424.1) as coding for a shorter protein containing 31 identical amino acid residues.

In *S. coelicolor*, our results show that PhoU is a negative modulator of PhoP- mediated activation of the Pho regulon. The PhoU negative control of the PhoP-P-mediated activation prevents uneconomical *pho* regulon activation. However, contrary to *E. coli*, disruption of *phoU* in *S. coelicolor* has a moderate effect (as shown in RT-qPCR studies), but disruption of *phoU* does not produce a fully constitutive activation of the *S. coelicolor* *pho* regulon. The stronger PhoU effect under Pi-limitation conditions matches with the PhoP-P-dependent expression profile of *phoU* in *S. coelicolor* (Figure 2). In summary, *phoU* expression is activated by PhoP-P in Pi-limited conditions. The synthesized PhoU protein, in turn, represses PhoP-P modulation, thus preventing this system to be out of control.

Interestingly, in the *phoU* mutant in phosphate-repleted conditions (MG-18.5) transcription from the *glpQ1* promoter is upregulated during the growth phase (up to 42 h) as compared with the parental strain (Figure 7). In the phosphate-limited MG-3.2 medium the *phoU* mutant also has an upregulation of the *glpQ1* promoter throughout the entire culture time (Figure 7). As expression of *glpQ1* is totally dependent on the phosphorylated form of PhoP, we conclude that the PhoU protein contributes to maintain the response regulator in its inactive unphosphorylated form, probably by favoring the phosphatase/kinase ratio of PhoR on the PhoP, as proposed in *E. coli*.²²

It is well known that expression of antibiotic and pigment biosynthetic genes is regulated by PhoP.^{1,10} Indeed, as observed in this study, PhoU modulates expression of actinorhodin and undecylprodigiosin and also spores pigment. It is known that PhoP regulates expression of secondary metabolite biosynthetic genes at transcriptional and post-transcriptional levels.^{5,8,9} Therefore, PhoU affects expression of antibiotic and pigment biosynthetic genes through different regulatory proteins (for example, AfsS–AfsR) by a cascade mechanism.^{42,43}

DEDICATION

Dedicated to Prof. H. Umezawa for his friendship.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This article was supported by CICYT Grants BIO2010-16094 and ERA-IB PIM2010EEI-00677 of the Ministry of Economy and Competitiveness, Madrid, Spain. Seomara Martín-Martín and Etelvina Franco received PhD fellowships from the Ministry of Science and Innovation (Madrid, Spain) and the Diputación de León (León), respectively.

- 1 Martín, J. F. Phosphate control of the biosynthesis of antibiotics and other secondary metabolites is mediated by the PhoR-PhoP system: an unfinished story. *J. Bacteriol.* **186**, 5197–5201 (2004).
- 2 Wanner, B. (1996). Molecular Biology. Phosphorous assimilation and control of the phosphate regulon. In: Neidhardt FC *et al.* (eds). in *Escherichia coli* and *Salmonella*: Cellular. DC. 1357-1381 (American Society for Microbiology, Washington).
- 3 Manteca, A., Ye, J., Sánchez, J. & Jensen, O. N. Phosphoproteome analysis of *Streptomyces* development reveals extensive protein phosphorylation accompanying bacterial differentiation. *J. Proteome Res.* **10**, 5481–5492 (2011).
- 4 Sola-Landa, A., Moura, R. S. & Martín, J. F. The two-component PhoR-PhoP system controls both primary metabolism and secondary metabolite biosynthesis in *Streptomyces lividans*. *Proc. Natl Acad. Sci. USA* **100**, 6133–6138 (2003).
- 5 Martín, J., Sola-Landa, A. & Rodríguez-García, A. Two Component Systems in Bacteria. (eds Gross R and Beier D) 315–331 (Horizon Sci Press, Norfolk, 2012b).
- 6 Sola-Landa, A., Rodríguez-García, A., Franco-Domínguez, E. & Martín, J. F. Binding of PhoP to promoters of phosphate-regulated genes in *Streptomyces coelicolor*: identification of PHO boxes. *Mol. Microbiol.* **56**, 1373–1385 (2005).
- 7 Sola-Landa, A., Rodríguez-García, A., Apel, A. K. & Martín, J. F. Target genes and structure of the direct repeats in the DNA-binding sequences of the response regulator PhoP in *Streptomyces coelicolor*. *Nucleic Acids Res.* **36**, 1358–1368 (2008).
- 8 Martín, J. F. *et al.* Transcriptomic studies of phosphate control of primary and secondary metabolism in *Streptomyces coelicolor*. *Appl. Microbiol. Biotechnol.* **95**, 61–75 (2012a).
- 9 Allenby, N. E. E., Laing, E., Bucca, G., Kierzek, A. M. & Smith, C. P. Diverse control of metabolism and other cellular processes in *Streptomyces coelicolor* by the PhoP transcription factor: genome-wide identification of *in vivo* targets. *Nucleic Acids Res.* **40**, 9543–9556 (2012).
- 10 Martín, J. F., Rodríguez-García, A. & Liras, P. The master regulator PhoP coordinates phosphate and nitrogen metabolism, respiration, cell differentiation and antibiotic biosynthesis: comparison in *Streptomyces coelicolor* and *Streptomyces avermitilis*. *J. Antibiot.* **70**, 534–541 (2017).
- 11 Carmany, D. O., Hollingsworth, K. & McCleary, W. R. Genetic and bio-chemical studies of phosphatase activity of PhoR. *J. Bacteriol.* **185**, 1112–1115 (2003).
- 12 Rodríguez-García, A., Barreiro, C., Santos-Beneit, F., Sola-Landa, A. & Martín, J. F. Genome-wide transcriptomic and proteomic analysis of the primary response to

- phosphate limitation in *Streptomyces coelicolor* M145 and in a DeltaphoP mutant. *Proteomics* **7**, 2410–2429 (2007).
- 13 Rodríguez-García, A., Sola-Landa, A., Apel, K., Santos-Beneit, F. & Martín, J. F. Phosphate control over nitrogen metabolism in *Streptomyces coelicolor*: direct and indirect negative control of *glnR*, *glnA*, *glnII* and *amtB* expression by the response regulator PhoP. *Nucleic Acids Res.* **37**, 3230–3242 (2009).
 - 14 Santos-Beneit, F., Rodríguez-García, A., Franco-Domínguez, E. & Martín, J. F. Phosphate-dependent regulation of the low and high-affinity transport systems in the model actinomycete *Streptomyces coelicolor*. *Microbiology* **154**, 2356–2370 (2008).
 - 15 Santos-Beneit, F., Rodríguez-García, A., Sola-Landa, A. & Martín, J. F. Cross-talk between two global regulators in *Streptomyces*: PhoP and AfsR interact in the control of *afsS*, *pstS* and *phoRP* transcription. *Mol. Microbiol.* **72**, 53–68 (2009a).
 - 16 Santos-Beneit, F., Rodríguez-García, A., Apel, A. K. & Martín, J. F. Phosphate and carbon source regulation of two PhoP-dependent glycerophosphodiester phosphodiesterase genes of *Streptomyces coelicolor*. *Microbiology* **155**, 1800–1811 (2009b).
 - 17 Sola-Landa, A., Rodríguez-García, A., Amin, R., Wohlleben, W. & Martín, J. F. Competition between the GlnR and PhoP regulators for the *glnA* and *amtB* promoters in *Streptomyces coelicolor*. *Nucleic Acids Res.* **41**, 1767–1782 (2013).
 - 18 Ghorbel, S., Kormanec, J., Artus, A. & Virolle, M. J. Transcriptional studies and regulatory interactions between the *phoR-phoP* operon and the *phoU*, *mtpA*, and *ppk* genes of *Streptomyces lividans* TK24. *J. Bacteriol.* **188**, 677–686 (2006).
 - 19 Hsieh, Y. J. & Wanner, B. L. Global regulation by the seven-component Pi signaling system. *Curr. Opin. Microbiol.* **13**, 198–203 (2010).
 - 20 Gardner, S., Johns, K. D., Tanner, R. & McCleary, W. R. The PhoU protein from *Escherichia coli* interacts with PhoR, PstB, and metals to form a phosphate-signaling complex at the membrane. *J. Bacteriol.* **196**, 1741–1752 (2014).
 - 21 Darbon, E. *et al.* Transcriptional and preliminary functional analysis of the six genes located in divergence of *phoR/phoP* in *Streptomyces lividans*. *Appl. Microbiol. Biotechnol.* **95**, 1553–1566 (2012).
 - 22 Rice, C. D., Pollard, J. E., Lewis, Z. T. & McCleary, W. R. Employment of a promoter-swapping technique shows that PhoU modulates the activity of the PstSCAB2 ABC transporter in *Escherichia coli*. *Appl. Environ. Microbiol.* **75**, 573–582 (2009).
 - 23 Gardner, S. G. *et al.* Genetic analysis, structural modeling, and direct coupling analysis suggest a mechanism for phosphate signaling in *Escherichia coli*. *BMC Genet.* **16**, S2 (2015).
 - 24 Cheng, C., Wakefield, J. Y., Tauschek, M. & Robins-Browne, R. M. Genome-Wide analysis of the *pho* regulon in a *pstCA* mutant of *Citrobacter rodentium*. *PLoS ONE* **7**, 50682 (2012).
 - 25 Fernández-Martínez, L. T., Santos-Beneit, F. & Martín, J. F. Is PhoR-PhoP partner fidelity strict? PhoR is required for the activation of the *pho* regulon in *Streptomyces coelicolor*. *Mol. Genet. Genomics* **287**, 565–573 (2012).
 - 26 Kieser, T., Bibb, M. J., Buttner, M. J., Chater, K. F. & Hopwood, D. A. *Practical Streptomyces Genetics* (The John Innes Foundation, Norwich, UK, 2000).
 - 27 Greener, A. & Jerpseth, B. Highly transformable bacterial cells and methods for producing the same. US Patent US6706525 B1 (2006).
 - 28 MacNeil, D. *et al.* Analysis of *Streptomyces avermitilis* genes required for avermectin biosynthesis utilizing a novel integration vector. *Gene* **111**, 61–68 (1992).
 - 29 Yanisch-Perron, C., Vieira, J. & Messing, J. Improved M13 phage cloning vectors and host strains: nucleotide sequencing of the M13mp18 and pUC9 vectors. *Gene* **33**, 103–119 (1985).
 - 30 Redenbach, M. *et al.* A set of ordered cosmids and a detailed genetic and physical map for the 8 Mb *Streptomyces coelicolor* A3(2) chromosome. *Mol. Microbiol.* **21**, 77–96 (1996).
 - 31 Khaleel, T., Younger, E., McEwan, A. R., Varghese, A. S. & Smith, M. A phage protein that binds phiC31 integrase to switch its directionality. *Mol. Microbiol.* **80**, 1450–1463 (2011).
 - 32 Higgins, C. E., Hamill, R. L., Sands, T. H., Hoehn, M. M. & Davis, N. E. Letter: the occurrence of deacetoxycephalosporin C in fungi and streptomycetes. *J. Antibiot. (Tokyo)* **27**, 298–300 (1974).
 - 33 Lanzetta, P. A., Alvarez, L. J., Reinach, P. S. & Candia, O. A. An improved assay for nanomole amounts of inorganic phosphate. *Anal. Biochem.* **100**, 95–97 (1979).
 - 34 Sharma, C. M. *et al.* The primary transcriptome of the major human pathogen *Helicobacter pylori*. *Nature* **464**, 250–255 (2010).
 - 35 Quinlan, A. R. & Hall, I. M. BEDTools: a flexible suite of utilities for comparing genomic features. *Bioinformatics* **26**, 841–842 (2010).
 - 36 Freese, N. H., Norris, D. C. & Loraine, A. E. (2016) Integrated genome browser: visual analytics platform for genomics. *Bioinformatics* **32**, 2089–2095.
 - 37 Yagüe, P. *et al.* Transcriptomic analysis of liquid non-sporulating *Streptomyces coelicolor* cultures demonstrates the existence of a complex differentiation comparable to that occurring in solid sporulating cultures. *PLoS ONE* **9**, e86296 (2014).
 - 38 Livak, K. J. & Schmittgen, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* **25**, 402–408 (2001).
 - 39 Andersen, C. L., Jensen, J. & Orntoft, T. F. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Res.* **64**, 5245–5250 (2004).
 - 40 Vandesompele, J. *et al.* Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* **7**, RESEARCH0034 (2002).
 - 41 Jeong, Y. *et al.* The dynamic transcriptional and translational landscape of the model antibiotic producer *Streptomyces coelicolor* A3(2). *Nat. Commun.* **7**, 11605 (2016).
 - 42 Martín, J. F. & Liras, P. Engineering of regulatory cascades and networks controlling antibiotic biosynthesis in *Streptomyces*. *Curr. Opin. Microbiol.* **13**, 263–273 (2010).
 - 43 Liu, G., Chater, K. F., Chandra, G., Niu, G. & Tan, H. Molecular regulation of antibiotic biosynthesis in *Streptomyces*. *Microbiol. Mol. Biol. Rev.* **77**, 112–143 (2013).