# Is PhoR-PhoP partner fidelity strict? PhoR is required for the activation of the *pho* regulon in *Streptomyces coelicolor*

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**Running Title:** Characterization of a Δ*phoR* strain in *Streptomyces coelicolor*.

**Keywords:** *Streptomyces*, two-component regulatory systems, phosphate, sensor kinase.

**Abstract**

Two-component regulatory systems play a key role in the cell metabolism adaptation to changing nutritional and environmental conditions. The fidelity between the two cognate proteins of a two-component system is important since it determines whether a specific response regulator integrates the signals transmitted by different sensor kinases. Phosphate regulation in *Streptomyces coelicolor* is mostly mediated by the PhoR-PhoP two-component system. Previous studies elucidated the mechanisms that control phosphate regulation as well as the genes directly regulated by the response regulator PhoP (*pho* regulon) in this organism. However, the role of the histidine kinase PhoR in *Streptomyces coelicolor* had not been unveiled so far. In this work, we report the characterization of a non-polar Δ*phoR* deletion mutant in *S. coelicolor* that keeps its native promoter. Induction of the *phoRP* operon was dependent upon phosphorylation of PhoP but the Δ*phoR* mutant expressed *phoP* at a basal level. RT-PCR and reporter luciferase assays demonstrated that PhoR plays a key role in the activation of the *pho* regulon in this organism. Our results point towards a strict cognate partner specificity in terms of the phosphorylation of PhoP by PhoR thus corroborating the tight interaction between the two-components of this system.

## Introduction

*Streptomyces coelicolor* is a Gram-positive soil bacterium and as such, it must be able to adapt to the frequent sudden changes of the environment. One of the most widely used mechanisms exploited by bacteria in order to overcome rapid environmental changes is the use of two-component systems (TCSs). Bacterial two-component systems typically consist of a membrane histidine kinase (HK) which acts as a sensor protein and is able to auto-phosphorylate a conserved histidine residue in response to an environmental or nutritional signal and consequently transfer this phosphate group onto an aspartic acid residue on the response regulator (RR). As a result, a conformational change is produced in the response regulator which usually activates a C-terminal DNA binding domain thus regulating the expression of the target genes (Hakenbeck and Stock 1996). The *S. coelicolor* genome encodes 84 HKs and 80 RRs including 67 sensor-regulator pairs (Hutchings et al. 2004), which highlights the importance of signal sensing in this organism.

In a number of *Streptomyces* species phosphate regulation is mediated by the TCS PhoR-PhoP (Sola-Landa et al. 2003; 2005; Ghorbel et al. 2006; Mendes et al. 2007). The global phosphate response regulator PhoP and its target genes have been characterized in *S. coelicolor* over the last decade (reviewed in Martin et al. 2011). Genes under direct control of PhoP constitute the denominated *pho* regulon. Several PhoP operators have been characterized by protein-DNA binding assays (EMSA and DNase I footprinting) in numerous promoters (Apel et al. 2007; Rodriguez-García et al. 2007; 2009; Sola-Landa et al. 2005; 2008; Santos-Beneit et al. 2008; 2009 a,b; 2011). PhoP can act either as a positive regulator, by binding at the -35 region (or nearby), or as a repressor, when bound

to the -10 region (i.e. as a road-block for the RNA polymerase) (Santos-Beneit et al. 2008). Interestingly, PhoP acts not only as a specific regulator involved in controlling phosphate levels within the cell by regulating processes such as extracellular phosphate scavenging and transport, but it also seems to have a more global implication, regulating other primary and secondary metabolic genes involved in nitrogen and carbon metabolism (Rodríguez-García et al. 2009); hence the importance of understanding the upstream regulation of this complex response regulator.

Due to the organization of the *phoR-phoP* operon in *S.coelicolor*, where both genes share a common promoter from which they are likely to co-transcribe, since this is the case in the close relative *S. lividans* (Ghorbel et al. 2006), it had been difficult so far to study the role of the sensor kinase PhoR via a deletion mutant. In other organisms, where disruption of the sensor kinase was less complicated due to the genetic arrangement, in an operon in which the HK is located downstream of the RR, it has been shown that other proteins and/or small molecules are able to compensate for loss of PhoR. For example, in *E. coli*, the sensor kinase CreC (PhoM), which is involved in carbon metabolism, is able to phosphorylate PhoB (homologous RR to the *S. coelicolor* PhoP) in the absence of PhoR (Amemura et al. 1990). At the same time, it has also been observed that acetyl phosphate, an intermediate of carbon metabolism, was able to activate PhoB, thus indicating certain cross-talk between the phosphate and carbon metabolic pathways (McCleary and Stock 1994). Furthermore, other studies have revealed that at least other six different kinases present the ability to phosphorylate PhoB *in vivo* in *E. coli* (Fisher et al. 1995; Zhou et al. 2005). However, the *B. subtilis* PhoP-PhoR system, which belongs

to a more intricate multi-component network involved in phosphate signal transduction (Sun et al. 1996), seems to be very specific and although PhoR is able to phosphorylate a non-cognate partner YycF, the reciprocal cross-talk does not occur and PhoP seems unable to be phosphorylated by a kinase other than PhoR in this organism.

The recent availability of molecular tools to facilitate the construction of non-polar deletion mutants in *Streptomyces* (Gust et al. 2003; Fedoryshyn et al. 2008; Fernández-Martínez et al. 2011) has allowed us to construct and characterize for the first time a *S. coelicolor* Δ*phoR* mutant strain in which *phoP* is transcribed from the native *phoRP* promoter, a key step in order to assess the fidelity of the PhoR-PhoP system in this organism. In this work, we demonstrate that the expression of genes of the *pho* regulon is not induced in the absence of PhoR thus pointing towards the specificity of the phosphorylation of PhoP by its cognate partner PhoR in *S. coelicolor*. On the other hand, this work also presents a study of both phenotypic characterization and antibiotic production of the Δ*phoR* strain under different conditions compared to both the Δ*phoP* and the wild-type M145 strains.

## Results

### Construction of a non-polar *S. coelicolor* Δ*phoR* mutant

In order to characterize Δ*phoR* and assess the fidelity of the PhoR-PhoP two-component system, a non-polar deletion of *phoR*, to allow normal transcription of *phoP* was constructed (See diagram if Fig. 1.C.). The *phoR* gene is located on cosmid 7H10 (Fernández-Martínez et al. 2011). pIJ774 (Khodakaramian et al. 2006) was used as the

template in a PCR since it contains the *loxP* sites flanking the apramycin resistance cassette, thus allowing its posterior removal using the Cre-*loxP* system. To amplify pIJ774,primersCAR01(TGGGGCCGGACGGTTCGCCGTGCCTAACCTGGAGACAT GATTCCGGGGATCCGTCGACC) and phoRrev (AGTCCGGCGCGGTCGGACCGCC GCTGCGCGCGGTCGCGGTGTAGGCTGGAGCTGCTTC) were used to generate the recombination substrate for replacement of *phoR* in cosmid 7H10.

The above PCR product was transformed into *E. coli* BW25113/pIJ790 (expressing the λ-Red recombination functions) already containing cosmid 7H10 as described in Gust et al. 2003. Apramycin resistant transformants were selected, and the recombinant cosmid was identified by PCR and restriction endonuclease analysis (data not shown) and conjugated into *S. coelicolor* M145 via *E. coli* ET12567 (pUZ8002). Selected exconjugants were screened for $Kn^S$/$Apra^R$ and the double-crossover confirmed by PCR.

To remove the resistance marker, pUWLCRE, containing a synthetic *cre* recombinase (Fedoryshyn et al. 2008), was transformed into *E. coli* ET12567 (pUZ8002) and conjugated into the above *S. coelicolor* strain containing the apramycin cassette flanked by the *loxP* sites instead of the *phoR* sequence. Around 15% of the colonies obtained were apramycin sensitive and after two rounds of plating on non-selective (without thiostrepton) medium, pUWLCRE was also lost. PCR amplification of a resulting Δ*phoR* clone and posterior sequencing indicated a successful removal of the pIJ774 cassette marked only by a "scar" sequence thus obtaining a non-polar *phoR* deletion which allows transcription of *phoP* from its native *phoRP* promoter.

**Phenotypic characterization of *S. coelicolor* Δ*phoR* showed no morphological differences respect to the wild-type strain M145**

It has been previously reported (Sola-Landa et al. 2003) that a *S. lividans* Δ*phoP* strain is unable to grow at very low inorganic phosphate concentrations on solid medium. To assess the effect of low inorganic phosphate concentrations in our mutants respect to the parental strain, *S. coelicolor* M145, Δ*phoP* and Δ*phoR* were grown on MM-agarose containing asparagine as the sole carbon source and adding 0, 10 μM, 20 μM, 50 μM and 100 μM final concentration of inorganic phosphate ($KH_2PO_4$). Fig. 1 shows how both M145 and Δ*phoR* are able to grow with minimal phosphate concentrations (20 μM), whilst Δ*phoP* is only able to grow when at least 100 μM inorganic phosphate is present in the medium. These results indicate that whereas PhoP is strictly required for phosphate utilization at very low Pi concentrations, the sensor kinase PhoR is not so strictly required (see below).

The three strains were also grown on several media to assess their morphological development under different conditions. Although the phenotype of *S. coelicolor* Δ*phoP* has been studied in defined liquid medium with different inorganic phosphate concentrations (Pi), its phenotype on complex media on solid agar plates had not been characterized so far. The results showed that on MS, TBO and TSA all strains were able to develop normally completing the life cycle at a similar growth rate (data not shown). On the other hand, when grown on R5 agar plates, Δ*phoP* started to produce actinorhodin much earlier than the wild-type and Δ*phoR* strains. Fig. 2 shows how, after 72 hours of growth Δ*phoP* starts to produce actinorhodin whilst the other strains do not. After 7 days (data not shown) the levels of actinorhodin production among the three strains looked

very similar, indicating that Δ*phoP* is not overproducing this antibiotic but simply initiating its production earlier under the conditions tested. We also decided to try different concentrations of added Pi to the R5 medium and observed that as expected, there is an inverse proportional relationship between Pi concentration in the medium versus actinorhodin production observed in all strains (Fig. 2).

Furthermore, when the three strains are grown on liquid MG containing 3.2 mM inorganic phosphate final concentration (phosphate limiting conditions), Δ*phoR* is able to reach nearly wild-type growth levels up to 70 hours as shown by the dry weight per ml values (Fig. 3) whilst Δ*phoP* is severely impaired in growth in this medium, as previously reported (Santos-Beneit et al. 2008). Hence, Δ*phoR* seems to be able to utilize minimal concentrations of phosphate in order to develop only slightly delayed respect to the wild-type strain whereas the Δ*phoP* mutant cannot.

These results clearly indicate that deletion of PhoR has no effect over morphological differentiation in *Streptomyces coelicolor*, at least under the conditions tested. There are three hypotheses able to explain this phenotype: the first one is that the response regulator PhoP might be phosphorylated by a different kinase in the absence of PhoR; secondly, PhoP could be able to strongly bind to the DNA as an unphosphorylated form; or finally, the constitutive levels of unphosphorylated PhoP present in the Δ*phoR* cells at any time may be sufficient to overcome, even in a weak manner, the effect of low phosphate levels. Further molecular analyses were carried out to try to discriminate amongst these three hypotheses.

**Actinorhodin production in a Δ*phoR* strain is similar to that of Δ*phoP***

Actinorhodin production in a Δ*phoP* background has been previously characterized (Santos-Beneit et al. 2009a) in MG defined liquid medium. It has been observed that under low Pi concentrations, the Δ*phoP* strain produces significantly lower levels of actinorhodin respect to the wild-type M145 strain. To assess the effect of the deletion of PhoR over actinorhodin production, M145, Δ*phoP* and Δ*phoR* were grown in liquid MG with 3.2 mM Pi. Fig. 4 shows how actinorhodin production in all three strains starts after 44 to 46 hours, when Pi in the medium is depleted. As previously described, Δ*phoP* produces significantly lower levels of actinorhodin than the wild-type strain. Interestingly, Δ*phoR* is also unable to reach the production levels of the wild-type strain, showing levels of actinorhodin production very similar to those of the Δ*phoP* strain. Therefore, in contrast to the phenotypic characterization analysis, where the phenotype of Δ*phoR* appears to be similar to the M145 wild-type strain, Δ*phoR* seems to behave like the Δ*phoP* strain in terms of actinorhodin production.

**Expression of the *pho* regulon genes is greatly reduced in a Δ*phoR* background**

Since Δ*phoR* seems to behave similarly to the wild-type in terms of phenotype and growth under phosphate limiting conditions but appears to be similar to Δ*phoP* in terms of actinorhodin production, it was of upmost interest to assess the expression of the *pho* regulon genes in a Δ*phoR* background. In order to further investigate this, RT-PCR of several members of the *pho* regulon such as *glpQ1*, *pstS*, and *phoU* as well as *phoR* and *phoP* were carried out and compared to the housekeeping gene *hrdB* used as a control. Fig. 5 shows that interestingly, expression of the well known *pho* regulon genes *glpQ1*,

*pstS* and *phoU* is greatly reduced in a Δ*phoR* and undetectable in a Δ*phoP* background showing that the *pho* regulon is clearly under-expressed in the absence of PhoR respect to the wild-type strain. As expected, expression of *phoR* is absent in Δ*phoR* and expression of *phoP* is absent in the Δ*phoP* strain. Interestingly, *phoP* expression is greatly reduced in a Δ*phoR* background, indicating that in the absence of PhoR, unphosphorylated PhoP is unable to activate full induction from its own promoter, as occurs with the other *pho* regulon genes tested. This result again highlights the role of PhoR-mediated phosphorylation in the response to phosphate limitation.

Another observation was the fact that low levels of *phoR* expression are still detectable in Δ*phoP*, i. e. in the absence of the PhoP activator. This is the only transcript, from a *pho* regulon member, that we were able to detect in this strain and indicates certain level of basal expression of the *phoR-P* operon even in the absence of PhoP. This is to be expected, since PhoR and PhoP should always be present in wild type cells at low basal levels in order to be ready to rapidly activate the signaling cascade leading to adaptation to limiting phosphate conditions when required. Moreover, transcriptomic data (E. Wellington and STREAM, Sysmo EU project, personal communication) clearly reproduced all the results obtained by RT-PCR, including the weak induction of *phoR* expression in Δ*phoP* when phosphate levels are scarce.

To further corroborate this result one of the PhoP-dependent genes, *glpQ1*, was used as a model for report (luciferase) studies. The promoter region of *glpQ1* fused to the *luxAB* reporter genes, which had been previously integrated into the *attP* φC31 site of M145 and Δ*phoP* (Santos-Beneit et al. 2009b) was also integrated in a Δ*phoR* background. Fig. 6

shows how expression of *glpQ1* in M145 is much higher than in Δ*phoR*, therefore validating the RT-PCR results. On the other hand, barely detectable levels of *glpQ1* expression were observed in the Δ*phoP* mutant.

Thus, in a M145 background, as expected, the *pho* regulon genes are highly induced after the depletion of phosphate in the medium. In a Δ*phoP* background, there is no activation whatsoever of the *pho* regulon and in a Δ*phoR* background, the *pho* regulon is activated about 5-fold more than in Δ*phoP* but the activation is significantly reduced compared to that in the wild-type. Since the expression of genes of the *pho* regulon is severely compromised in a Δ*phoR* background it seems unlikely that PhoP might be phosphorylated by a different histidine kinase *in vivo*, at least with high affinity. If the unphosphorylated form of PhoP could be able to bind in a strong manner to the PHO boxes, operator sequences which PhoP recognizes, then expression levels of the *pho* regulon genes should be normal in the Δ*phoR* mutant. Our results exclude that possibility; hence, the more plausible hypothesis is that the basal constitutive levels of unphosphorylated PhoP are responsible for the phenotype of Δ*phoR*.

**Discussion**

PhoP, the RR of the TCS PhoR-PhoP, appears to be a key global regulator in *S. coelicolor*, not only on account of its major role in phosphate regulation but also due to its indirect involvement in nitrogen and carbon metabolism via cross-talk with their main regulators (Rodríguez-García et al. 2009; Santos-Beneit et al. 2009a). Previous research had been mostly centred in the study of the downstream processes of this complex signalling pathway, i.e. how the absence of PhoP affects the regulation of metabolism and

which are its targets (Sola-Landa et al. 2005, 2008; Apel et al. 2007; Rodriguez-García et al. 2007; 2009; Santos-Beneit et al. 2008; 2009 a,b). However, a strain in which *phoP* is being constitutively expressed in the absence of PhoR-mediated activation was not available in this organism. This Δ*phoR* strain has now been obtained for the first time and this has allowed us to gain an insight on the upstream regulation of this signalling cascade.

The fidelity of TCSs involved in the phosphate starvation response has been characterized in other organisms resulting in distinct outcomes. For example in *E. coli*, PhoB can be phosphorylated by several other SKs and/or molecules in the absence of PhoR (Amemura et al. 1990; Fisher et al. 1995; McCleary and Stock 1994; Zhou et al. 2005). The promiscuity shown by PhoB in *E. coli* is probably due to the need to generate interconnected networks that potentially lead to cross-talk and cross regulation, particularly between carbon and phosphate regulatory cascades. This promiscuity can be beneficial or even necessary when simultaneously integrating the response to multiple signals. On the other hand *B. subtilis* PhoP seems to require specific phosphorylation by PhoR, indicating a tighter control over this RR (Sun et al. 1996).

In this work, we have established that in *S. coelicolor*, contrary to *E. coli* but similar to *B. subtilis*, PhoR appears to be the only specific SK for PhoP, giving a good example of TCS fidelity amongst signal transduction pathways. Some signals require a unique response, thus promoting exclusivity within particular signalling cascades. The recent attainment of crystallized structures for a number of SK-RR complexes has given us an insight into how this specificity is achieved, mainly through conserved contact residues within the primary sequence of both proteins (reviewed in Szurmant and Hoch 2010).

This information will be used to generate algorithms which hopefully will help to predict the TCS networks of entire organisms, thus allowing us to gain a global view of their interactions. How the response to the same nutrient can be specific or not depending on the organism is probably related to their respective regulatory mechanisms and adaptive advantages.

Our results reveal that PhoR is necessary for the complete induction of the *pho* regulon. However, certain level of expression of the *pho* regulon was detected in a *S. coelicolor* Δ*phoR* background. This could be due to weak binding of the unphoshporylated PhoP to its target sequences or feeble non-specific phosphorylation of PhoP by other SKs. It is known that unphosphorylated PhoP is still able to interact with the PHO boxes *in vitro* (Sola-Landa et al. 2005) and it seems likely that unphosphorylated PhoP is therefore responsible for the residual levels of activation of the *pho* regulon.

Another interesting observation was the fact that certain level of expression of the *phoR* transcript was detectable in a *S .coelicolor* Δ*phoP* background. In *B. subtilis*, the well characterized *phoPR* promoter region contains five different starts which respond to different environmental stimuli, some of them constitutive but enhanced by PhoP~P (phosphorylated PhoP) binding (Paul et al. 2004). Constitutive low expression of the *S. coelicolor phoRP* promoter region in a similar manner is very plausible since a basal level of PhoR-PhoP ready to be activated when phosphate is scarce must be available within the cell.

Finally, even though only basal levels of expression of the *pho* regulon members were observed in a Δ*phoR* background, the mutant strain showed only minor phenotypic defects in laboratory culture media, either in terms of growth or development. Therefore,

the low expression level of the genes of the *pho* regulon together with the expression of genes involved in phosphate regulation which are independent of PhoP seems sufficient to cope with phosphate starvation with respect to morphological differentiation. However, significant differences were observed in terms of actinorhodin production in MG defined medium. Some of the *pho* regulon members might be responsible for down-regulation of actinorhodin production in the Δ*phoR* strain as in the case of Δ*phoP*.

In summary, this article provides an insight into the puzzling subject of partner fidelity between cognate proteins, a question that has raised considerable interest. The PhoR-PhoP system of *S. coelicolor*, as we have demonstrated in this work, shows a high degree of fidelity and no crosstalk in terms of receiving signals from other pathways, such as carbon metabolim, in contrast to some similar systems in other bacteria. Further work in other *Streptomyces* species will help unveil whether this fidelity of the phosphate starvation response TCS is a common trend within the genus.

## Experimental Procedures

### Bacterial strains and growth conditions

*S. coelicolor* A3(2) derivatives and *E. coli* strains used in this work are listed in Table 1. *E. coli* strains were cultured using standard procedures (Sambrook et al. 1989). Strains, plasmids and cosmids used in this work are listed in Table 1. *S. coelicolor* strains were grown on MS agar plates (mannitol soy flour), TBO agar plates, TSA agar plates, R5 agar plates, MM-agarose plates (modified from Kieser et al. 2000) with different phosphate concentrations and no additional carbon source and MG-3.2 liquid medium for phosphate

limiting conditions (Santos-Beneit et al. 2008). Construction of the non-polar deletion of *phoR* (Δ*phoR* strain) is detailed in the results section.

**Reverse transcriptase PCR (RT-PCR)**

RNA from *S. coelicolor* M145, Δ*phoP* and Δ*phoR* was extracted at 42 and 45 hours of growth on MG-3.2 medium using RNAProtect Bacterial Reagent (Qiagen) to stabilize RNA. Samples were disrupted with 2 cycles of 30 s at speed 6.5 in a Ribolyser instrument using the lysing matrix B (BIO 101) and RNA was extracted using an RNeasy mini kit (Qiagen) as described in Tunca et al. 2007. RNA concentration and quality was checked using NanoDrop ND-1000 (Thermo Fisher Scientific).

Gene expression analysis by RT-PCR was carried out using the SuperScript One-Step RT-PCR system with Platinum *Taq* (Invitrogen) as described in Santos-Beneit et al. 2008. Platinum *Taq* was used as a control to check the lack of DNA contamination from the samples. The primers used in this study are listed in Table 2.

**Luciferase assay**

The *luxAB* activity driven from the *glpQ1* promoter was determined in a SIRIUS Luminometer V3.2 (Berthold Technologies). The samples for the luciferase assay were kept on ice and measured as follows: 500 µL of each sample were mixed with 250 µL of n-decanal 0.1 % by injection. The measurement integration time is of 20 seconds after a 5 second delay-time step. Measuring units are expressed as RLU (relative light units)/s.

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460 **Tables**

**Table 1.** Strains, plasmids and cosmids used in this study.

| Strain, cosmid or plasmid | Description | Source or reference |
|---|---|---|
| **Strains** | | |
| ***S. coelicolor* A3 (2)** | | |
| M145 | Prototrophic SCP1$^-$ SCP2$^-$ Pgl$^+$ | Kieser *et al*., (2000) |
| INB201 | Δ*phoP*::*apra* | Santos-Beneit et al, (2009a) |
| M145Δ*phoR* | M145 *phoR::loxP scar* | This work |
| ***E. coli*** | | |
| JM109 | F' *traD36 proA*$^+$*B*$^+$ *lacI*$^q$ Δ*(lacZ)M15/*Δ*(lac-proAB) glnV44 e14- gyrA96 recA1 relA1endA1 thi hsdR17* | Promega Corp. Yanisch-Perron *et al*., (1985) |
| DH5α | *fhuA2* Δ*(argF-lacZ)U169 phoA glnV44 Φ80* Δ*(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17* | Hanahan, D., (1983) |
| ET12567 (pUZ8002) | *Dam13*::Tn*9 dcm6 hsdM hsdR recF143* 16 *zjj201*::Tn*10 galK2 galT22 ara14 lacY1xyl5 leuB6 thi1 tonA31 rpsL136 hisG4 tsx78 mtli glnV44*, containing the non-transmissible *oriT* mobilizing plasmid, pUZ8002 | Flett *et al*., (1997) |
| BW25113/pIJ790 | K12 derivative*:* Δ*araBAD,* Δ*rhaBAD* containing pIJ790 (λ-RED (*gam*, *bet*, *exo*), *cat*, *araC*, *rep101*$^{ts}$) | Datsenko and Wanner (2000) and Gust et al. (2003) |
| **Cosmids** | | |
| 7H10 | *S. coelicolor* pWEB$^{TM}$ based cosmid containing *phoR* | Fernández-Martínez et al. (2011) |
| **Plasmids** | | |
| pLUX-*glpQ1* | BamHI-NdeI pFS-*glpQ1* fragment cloned into pLUXAR-neo | Santos-Beneit et al. (2009b) |
| pUWLCRE | Replicative vector for actinomycetes containing replicon pIJ101,*oriT* and cre(a) gene under *ermE* promoter, ampicillin and thiostrepton resistant | Fedoryshyn et al. (2008) |
| pIJ774 | pBluescript II SK$^-$derivative containing *aac(3)IV* flanked with *loxP* sites | Khodakaramian et al. (2006) |

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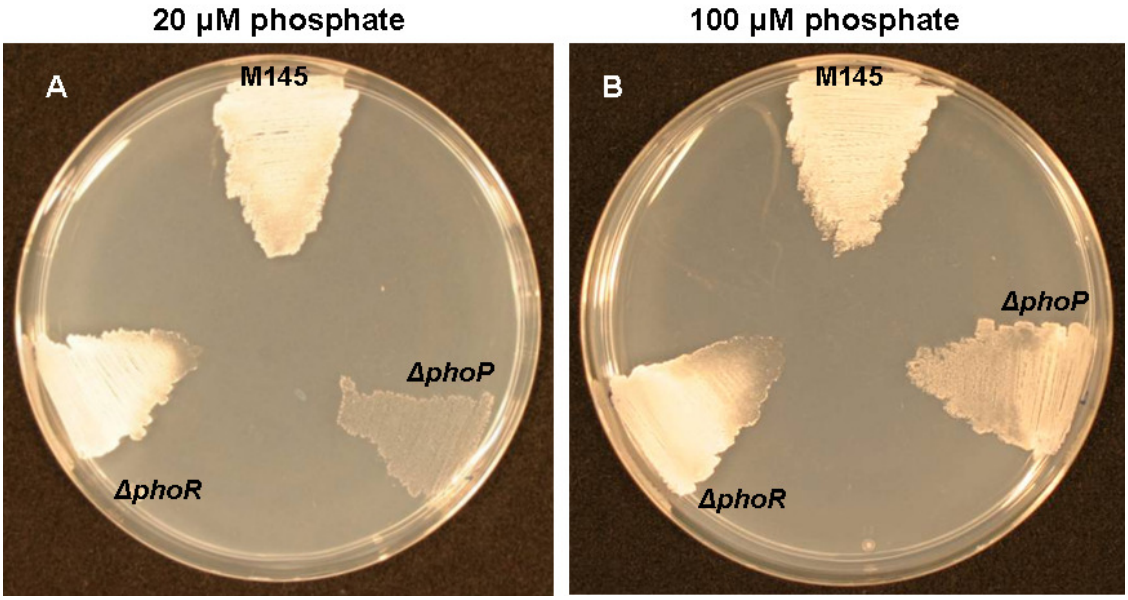
**Table 2.** RT-PCR primers used in this study

| Primers | Sequence (5'-3') |
|---|---|
| hrdB-Dir-RT | ACGCCCCGGCCCAGCAGGTC |
| hrdB-Rev-RT | CAGGTGGCGTACGTGGAGAACTTGT |
| glpQ1RT_F | TGGTTCACCGAGGACTTCAC |
| glpQ1RT_R | GAAGTAGGTGGGGTGCTTGA |
| phoP3 | GACCCATATGCTCGTCCTCGA |
| phoRcrecheckR | GAGAAGGGCTTGGTGACGTA |
| phoR_RT_F | GACACCGTCCTCTCCGTACT |
| phoR_RT_R | CTCGACCTGCCGTATCTCTC |
| phoU_RT_F | ACCACGAGGAACTTGATTCG |
| phoU_RT_R | GAGGTTGTCGACCTTCTGGT |
| pstS_RT_F | CTTCGACAGCAAGATCACCA |
| pstS_RT_R | CCCTCGTACTTCCAGTCGTC |

**FIGURES**

**Fig. 1. Phenotypic characterization of *S. coelicolor* M145, Δ*phoP* and Δ*phoR* after 7 days on MM+agarose. A.** Final inorganic phosphate concentration of 20µM**. B.** Final inorganic phosphate concentration of 100 µM. Δ*phoP* is unable to grow at low phosphate concentrations whilst both M145 and Δ*phoR* are able to sporulate in the same medium. **C.** Diagram showing the construction of the Δ*phoR* strain; for details refer to the main text.

**Fig. 1**



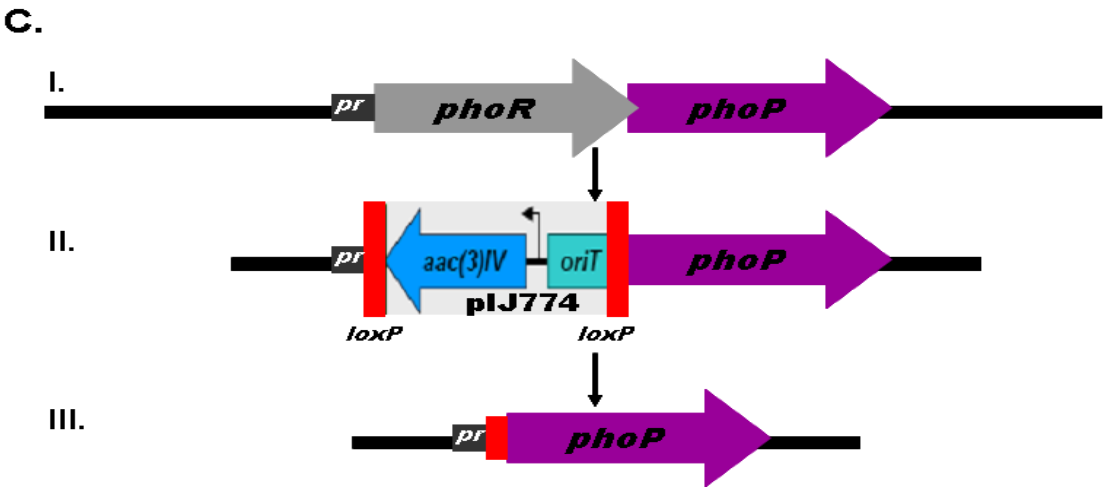



**Fig. 2. Phenotypic characterization of *S. coelicolor* M145, Δ*phoP* and Δ*phoR* after 72 h on R5 with different added Pi concentrations. A.** Front plate images of M145, Δ*phoP* and Δ*phoR* grown for 72 hours on R5 containing the following added Pi concentrations: **I.** 0 mM added Pi; **II.** 0.36 mM added Pi; **III.** 1.8 mM added Pi. **B.** Back plate images of the corresponding added Pi concentrations.

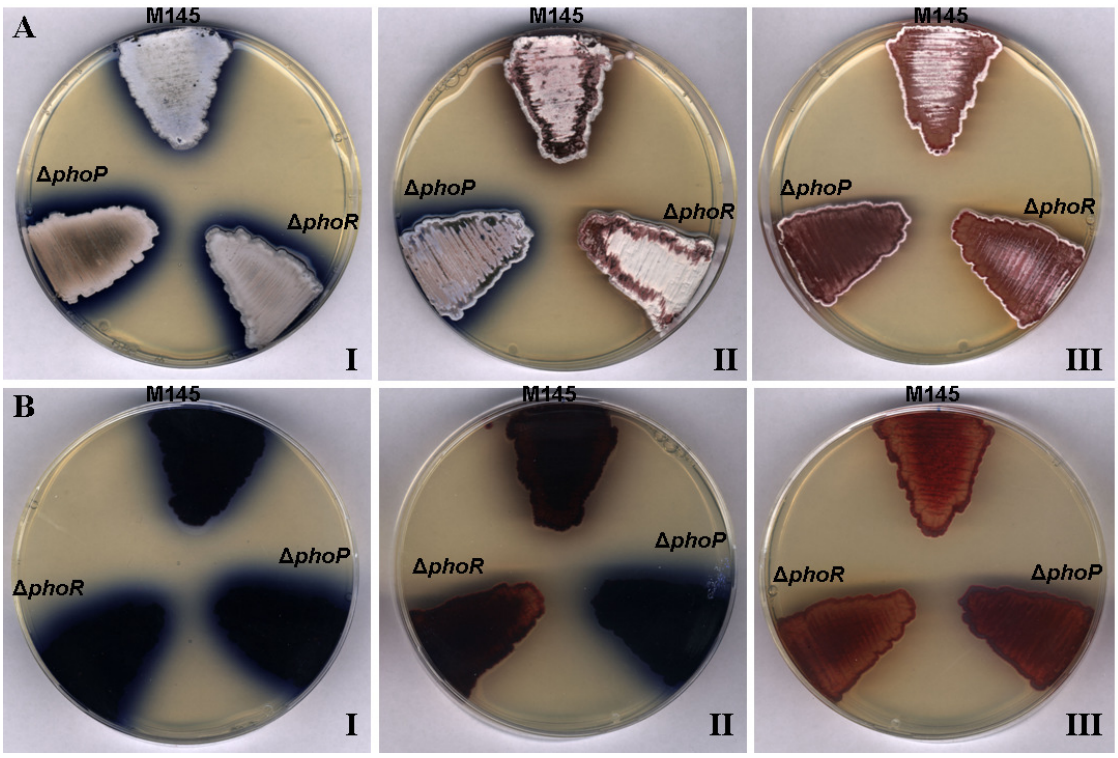
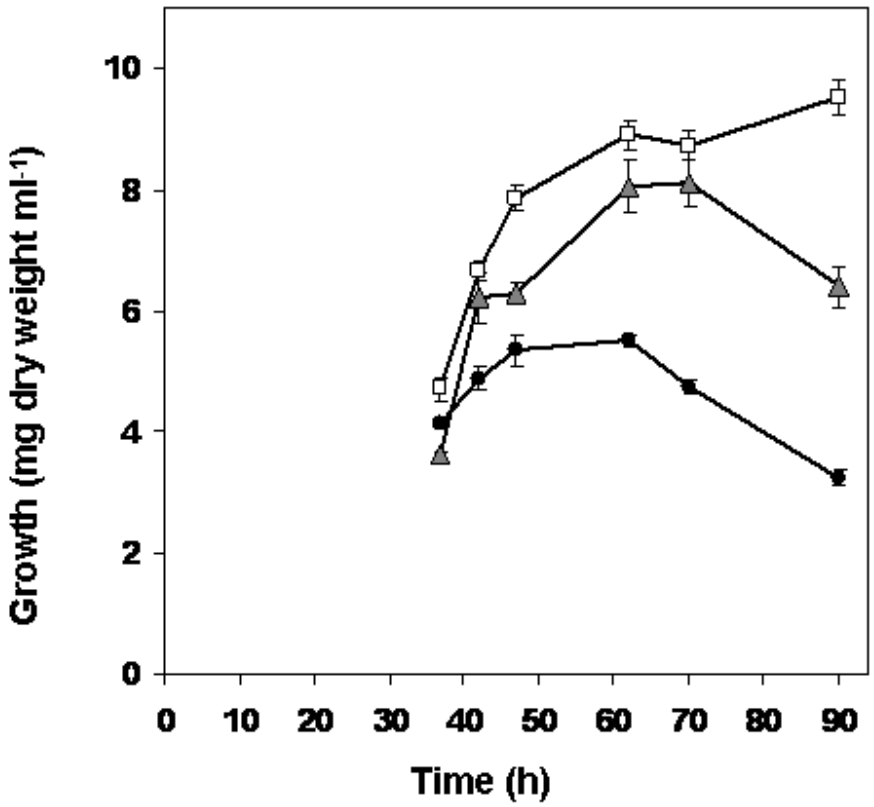


**Fig. 3. Growth by dry weight determination of M145, Δ*phoR* and Δ*phoP* in MG-3.2 medium.** Legend: strain M145 (white squares), Δ*phoR* (grey triangles) and Δ*phoP* (black circles). Vertical error bars correspond to the standard error of the mean of four biological replicates. The results show how M145 and Δ*phoR* are able to reach normal growth whilst Δ*phoP* is severely impaired.

**Fig. 3**



595 **Fig. 4. Actinorhodin production of M145, Δ*phoR* and Δ*phoP* in MG-3.2 medium.** Strains M145 (white squares), Δ*phoR* (grey triangles) and Δ*phoP* (black circles). Vertical error bars correspond to the standard error of the mean of four biological replicates. The results show how Δ*phoP* and Δ*phoR* are unable to reach normal actinorhodin yield levels with respect to the M145 wild-type strain under the conditions tested.

600

**Fig. 4.**

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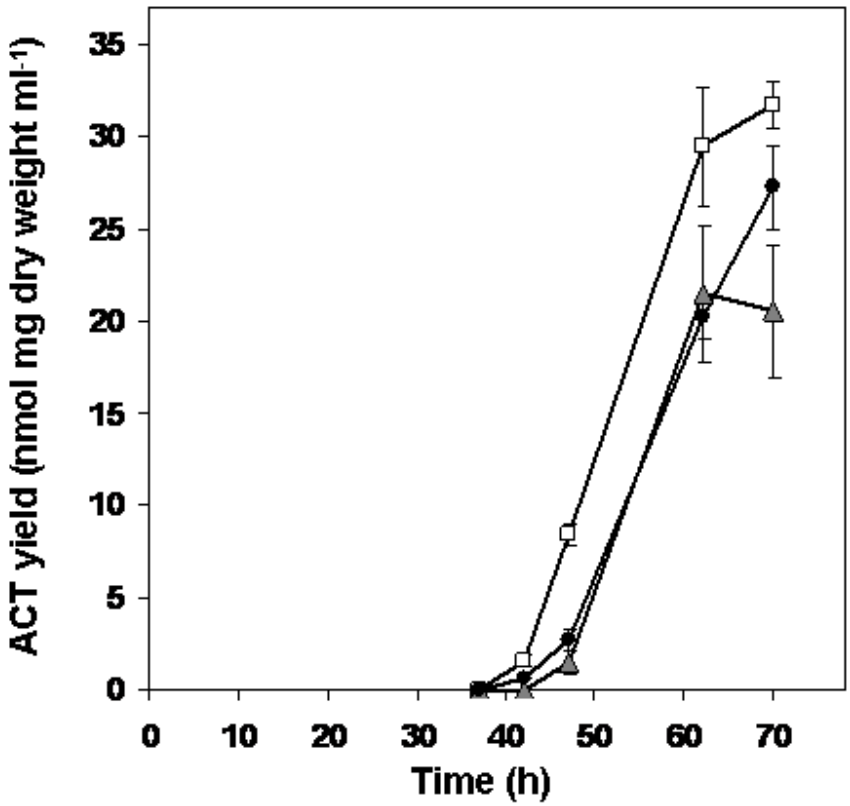


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**Fig. 5. RT-PCR for *hrdB* (as a constitutive control), *phoR*, *phoP*, *phoU*, *glpQ1* and *pstS*.** Controls without reverse transcriptase were carried out for all genes to validate the absence of DNA from the samples (data not shown). Expression of all genes under phosphate limiting conditions appears to be well-expressed in M145 at both time points. On the other hand, in a Δ*phoR* background only basal expression of the genes is detected whilst in a Δ*phoP* background detection is not visible thus indicating that PhoR is required to activate the *pho* regulon.

**Fig. 5**

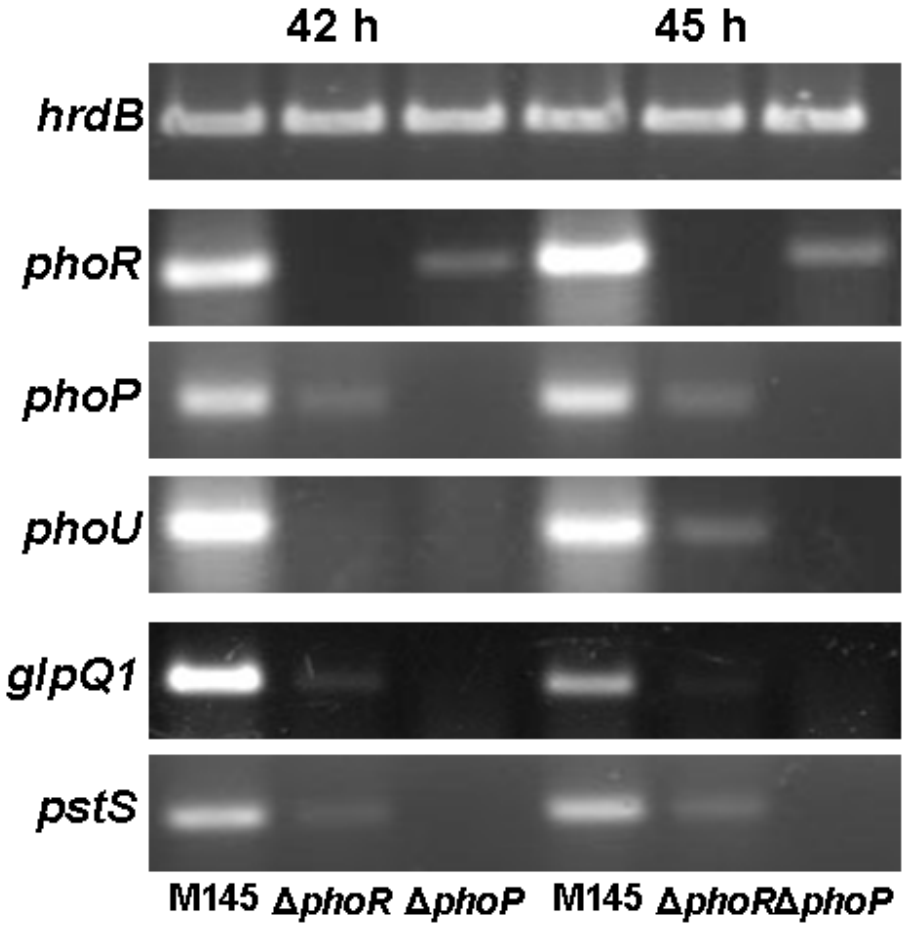

**Fig. 6. Promoter activity of *S. coelicolor* exconjugants containing pLUX-*glpQ1* in M145, Δ*phoR* and Δ*phoP* in MG-3.2 medium.** Legend: strain M145 (white bars), Δ*phoR* (grey bars) and Δ*phoP* (black bars). RLU (relative light units). Vertical error bars correspond to the standard error of the mean of four biological replicates (two replicates of two different exconjugants per strain). Promoter activity of *glpQ1* appears to be higher in Δ*phoR* than Δ*phoP* (top graph, amplification of the Y axis for Δ*phoR* and Δ*phoP*) although is far superior in a M145 background (bottom graph).

**Fig. 6**