The two-component PhoR-PhoP system controls both primary metabolism and secondary metabolite biosynthesis in *Streptomyces lividans*

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The biosynthesis of most secondary metabolites in different bacteria is strongly depressed by inorganic phosphate. The two-component *phoR-phoP* system of *Streptomyces lividans* has been cloned and characterized. PhoR showed all of the characteristics of the membrane-bound sensor proteins, whereas PhoP is a member of the DNA-binding OmpR family. Deletion mutants lacking *phoP* or *phoR-phoP*, were unable to grow in minimal medium at low phosphate concentration (10 μM). Growth was fully restored by complementation with the *phoR-phoP* genes. Both *S. lividans* Δ*phoP* and Δ*phoR-phoP* deletion mutants were unable to synthesize extracellular alkaline phosphatase (AP) as shown by immunodetection with anti-AP antibodies and by enzymatic analysis, suggesting that the PhoR-PhoP system is required for expression of the AP gene (*phoA*). Synthesis of AP was restored by complementation of the deletion mutants with *phoR-phoP*. The biosynthesis of two secondary metabolites, actinorhodin and undecylprodigiosin, was significantly increased in both solid and liquid medium in the Δ*phoP* or Δ*phoR-phoP* deletion mutants. Negative phosphate control of both secondary metabolites was restored by complementation with the *phoR-phoP* cluster. These results prove that expression of both *phoA* and genes implicated in the biosynthesis of secondary metabolites in *S. lividans* is regulated by a mechanism involving the two-component PhoR-PhoP system.

Phosphate control of the biosynthesis of antibiotics and many other types of secondary metabolites is a well known phenomenon (1–5), although the molecular mechanism by which this control is exerted is unknown (6, 7). Expression of genes encoding enzymes for the biosynthesis of secondary metabolites is negatively regulated by phosphate, and formation of the corresponding transcripts occurs only under phosphate-limiting conditions (8–10); but, surprisingly, nothing is known about the molecular mechanism of phosphate control of expression of the corresponding biosynthetic genes (11).

In *Escherichia coli* and *Bacillus subtilis* the genes belonging to the *pho* regulon, including the alkaline phosphatase (AP) gene (*phoA*) and the phosphate-specific transport (*pst*) genes, are regulated by a two-protein system consisting of a phosphate-sensor protein, PhoR, and a transcriptional activator protein, PhoB (named PhoP in *B. subtilis*; refs. 12–14). The sensor kinase PhoR is self-phosphorylated under phosphate starvation conditions (forming PhoR-P) that transfer its phosphate group to dephosphorylated PhoB. The phosphorylated PhoB transcriptional factor (PhoB-P) activates the expression of ≈30 different genes by binding to the *pho* boxes located upstream of the phosphate-regulated genes (12). Expression of *phoA* and other members of the *pho* regulon occurs under phosphate limitation when the transcriptional activator is available in its phosphorylated form.

An important question is whether the control of the biosynthesis of secondary metabolites in actinomycetes is exerted by the same mechanism as the control of AP and other genes involved in phosphate metabolism, or whether control of secondary metabolism proceeds by an entirely different molecular mechanism.

Recently, we cloned the AP gene (*phoA*, a member of the *pho* regulon) of *Streptomyces griseus* IMRU 3570 (the producer of the polyene macrolide candicidin) as a model to study the phosphate control of primary metabolism in actinomycetes (15), but this strain is not amenable to genetic manipulation. It was, therefore, of great interest to elucidate the presence in the model actinomycetes *Streptomyces lividans* and *Streptomyces coelicolor* A3 (2) of a two-component signal transduction system similar to *phoR-phoB* of *E. coli* and its possible involvement in the control of the biosynthesis of either primary metabolites, secondary metabolites, or both.

Experimental Procedures

Bacterial Strains and Plasmids. The bacterial strains and plasmids used are listed in Table 1. The *phoR-phoP* genes of *S. coelicolor* were cloned from cosmid D8A (16) and those of *S. lividans* were cloned by PCR (see below). The *phoR-phoP* cluster from *S. coelicolor* was subcloned in a 2.9-kb *Eco*RI–*Stu*I fragment into the *Eco*RI–*Sma*I site of pBluescript KS+. This plasmid, named pBS*phoRP*, was digested with *Eco*RV–*Eco*72I and religated, obtaining plasmid pBS*phoP* (Fig. 1). pBSΔ*phoRP* is a derivative of pBS*phoRP* in which a 1,651-bp *Sal*I–*Sph*I fragment from *phoR* and *phoP* genes was deleted and replaced by the *aphII* (kanamycin resistance) gene in the opposite orientation (Fig. 1). Similarly, pBSΔ*phoP* was derived from pBS*phoP* by deleting a 434-bp *Nru*I–*Sph*I fragment from the *phoP* gene and replacing it by the *aphII* gene. Plasmids pHZΔ*phoRP* and pHZΔ*phoP* contain the DNA inserts from pBSΔ*phoRP* and pBSΔ*phoP*, respectively, cloned into the recombination-prone unstable vector pHZ1351 (17). The plasmid pIJ*phoRP* contains pBS*phoRP* cloned into the positive selection vector pIJ699 (17).

***S. lividans* Deletion Mutants.** The *S. lividans* Δ*phoP* and Δ*phoRP* mutants were constructed by gene replacement using pHZΔ*phoP* and pHZΔ*phoRP* plasmids, respectively. Protoplasts of *S. lividans* 1326 were transformed and kanamycin-resistant thiostrepton-sensitive transformants were selected. For complementation studies the Δ*phoP* and Δ*phoRP* mutants were transformed with pIJ*phoRP* yielding *S. lividans phoP*$^+$ and *phoRP*$^+$ strains, respectively.

Culture Conditions. All strains were grown in solid asparagine-minimal medium (18) supplemented with KH_2PO_4 in low (10 μM; phosphate starvation conditions) or high concentration (5 mM) and in R5 solid medium with low (0.37 mM) or high (1.85 mM) phosphate concentrations (17).

Abbreviation: AP, alkaline phosphatase.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AJ544582).

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Table 1. Bacterial strains, cosmids, and plasmids

Strains/plasmids	Characteristics*	Ref.
Strains		
Streptomyces lividans JI 1326	Wild type	John Innes†
S. lividans Δ*phoP*	JI 1326, *phoP*::*aphII*	This work
S. lividans Δ*phoRP*	JI 1326, *phoRP*::*aphII*	This work
S. lividans phoP$^+$	Δ*phoP*, harboring the plasmid pIJ*phoRP*	This work
S. lividans phoRP$^+$	Δ*phoRP*, harboring the plasmid pIJ*phoRP*	This work
E. coli DH5α	F′ ϕ80 *dlacZ* ΔM15 Δ*(lacZYA-argF) U169 recA1 endA1 hsdR17* (r$^-$ m$^+$) *supE44 medλ*$^-$ *thi-1 gyrA relA1*	33
Plasmids/cosmids		
D8A	41.8-kb DNA fragment from *S. coelicolor* cloned into Supercos-1	16
pBluescript KS+	Cloning vector, ColE1 origin, Amp^r	Stratagene
pBS*phoP*	*Eco*72I (1,690)–*Stu*I (2,887) fragment carrying *phoP* gene, cloned into pBluescript KS+ (*Eco*RV–*Sma*I)	This work
pBS*phoRP*	*Eco*RI (from the cosmid D8A)–*Stu*I (2,887) fragment carrying *phoR-phoP* genes, cloned into pBluescript KS+ (*Eco*RI–*Sma*I)	This work
pBSΔ*phoP*	Like pBS*phoP*, with *aphII* (*Sph*I–*Ecl*136II) insertion in *Sph*I (2,433)–*Nru*I (1,999)	This work
pBSΔ*phoRP*	Like pBS*phoRP*, with *aphII* (*Sph*I–*Sal*I) insertion in *Sph*I (2,433)–*Sal*I (782)	This work
pGEM-T-easy	Vector system for the cloning of PCR products	Promega
pHZ1351	Highly unstable Sti$^+$ vector, useful for gene replacement in *Streptomyces*	17
pHZΔ*phoP*	Insert from pBSΔ*phoP* (*Sac*I–*Hind*III), cloned into pHZ1351 (*Sac*I–*Hind*III)	This work
pHZΔ*phoRP*	Insert from pBSΔ*phoRP* (*Sac*I–*Hind*III), cloned into pHZ1351 (*Sac*I–*Hind*III)	This work
pIJ699	High-copy number cloning vector for *Streptomyces*	17
pIJ*phoRP*	*Streptomyces–E. coli* bifunctional plasmid, with pBS*phoRP* cloned into pIJ699	This work

*Numbers in parentheses represent positions in the sequence of cosmid D8A of *S. coelicolor.*
†Collection of microorganisms of the John Innes Institute.

For studies on the production of secondary metabolites and on AP secretion, seed cultures were made in 100 ml of YED medium [(10 g/liter yeast extract/10 g/liter D-glucose (pH 7.0)] in 500-ml baffled flasks inoculated with ≈10^7 spores. Five milliliters of a seed culture grown for 30 h at 30°C and 250 rpm were centrifuged, washed twice with NaCl 0.9%, and used to inoculate 100 ml of R5 liquid medium (17).

Thiostrepton was added at final concentrations of 5 and 50 μg/ml in liquid and solid media, respectively, to the cultures of *S. lividans phoP*$^+$ and *phoRP*$^+$ strains.

DNA Procedures. Genomic DNA was isolated by standard procedures (17). For cloning the *S. lividans phoR-phoP* genes, a 2.7-kb DNA fragment was amplified by PCR using two primers based in *S. coelicolor* genome sequence: O1, 5′-GGTCCATCGCGTCGTCGTCCTGCTC-3′, and O2, 5′-GGTCCCGCCCTTCGCCGTGTCC-3′. This fragment was cloned into pGEM-T-easy (Promega) vector and sequenced by using pUC/M13 forward and reverse primers in an ABI PRISM 310 genetic analyzer (Perkin–Elmer).

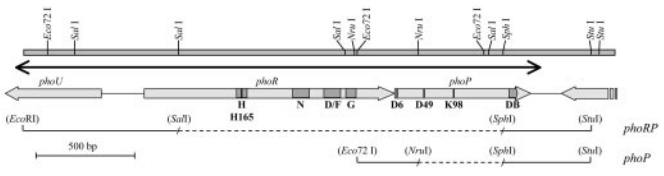

Fig. 1. Restriction map and organization of the DNA region containing the *phoR-phoP* cluster of *S. lividans*. The same organization was found in the genome of *S. coelicolor* A3(2). The double-headed arrow indicates the sequenced DNA fragment. Thick arrows show the size and orientation of the *phoU*, *phoR*, and *phoP* genes. The H, N, D/F, and G boxes in *phoR* and the DNA-binding (DB) domain in *phoP* are indicated as shaded regions, and the highly conserved residues His-165 in *phoR* and Asp-6, Asp-49, and Lys-98 in *phoP* are shown as a vertical solid line. Horizontal solid lines represent DNA fragments cloned into different plasmids. The discontinuous line indicates the deleted DNA fragment replaced by *aphII*. *phoRP* refers to the DNA insert into plasmids pBS*phoRP*, pBSΔ*phoRP*, pHZΔ*phoRP*, and pIJ*phoRP*. *phoP* refers to the insert in plasmids pBS*phoP*, pBSΔ*phoP*, and pHZΔ*phoP*.

Assay and Immunodetection of AP. The AP activity in culture supernatants was measured as described (15). In addition, AP was observed by Western blot analysis. The proteins in liquid R5 culture filtrates of 72 h were precipitated with ammonium sulfate at a final concentration of 80% saturation at 4°C (15). The protein pellet was collected by centrifugation, resuspended in 10 mM Tris·HCl (pH 8.0), applied to a desalting Sephadex G-25 column, and eluted with the same buffer. Samples were analyzed by SDS/PAGE and blotted to a PVDF membrane. The AP protein band was detected with anti-AP rabbit antibodies raised against pure *S. griseus* AP (15).

Actinorhodin and Undecylprodigiosin Determination. Actinorhodin and undecylprodigiosin were extracted and determined spectrophotometrically as described by Kieser *et al.* (17).

Phosphate Uptake. *S. lividans* cultures were grown in liquid YED medium for 20 h (30°C, 250 rpm). The collected cells were washed twice with NaCl 0.9% and transferred to asparagine-minimal medium without inorganic phosphate. After stabilization of the cell suspension for 6 h, ^{32}P-labeled Na_2HPO_4 (Amersham Biosciences) was added (2×10^5 cpm/ml). The uptake of phosphate was quantified as described (1).

Results

Cloning of the *phoU-phoR-phoP* Genes of *S. lividans*. The *S. lividans phoU-phoR-phoP* region was cloned by PCR using oligonucleotides O1 and O2 as primers. Sequence analysis of a 2.7-kb region of the *S. lividans* cloned DNA fragment revealed that this region has the same organization as the homologous region described in the *S. coelicolor* genome. The *S. lividans phoR-phoP* cluster is arranged in a head-to-tail organization and *phoU* is located in opposite orientation (Fig. 1). The three genes appear to be expressed from a bidirectional promoter region both in *S.*

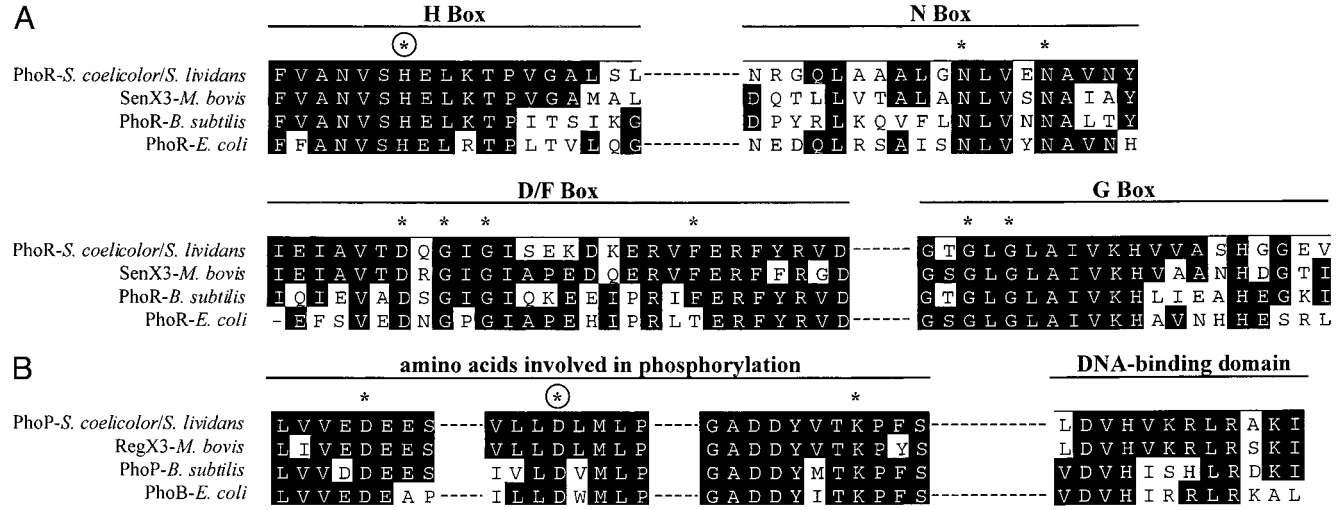

Fig. 2. (*A*) Conserved motifs in the H box, N box, D/F box, and G box of *S. lividans* PhoR as compared with the homologous boxes of sensor proteins of two-component systems of *M. bovis*, *B. subtilis*, and *E. coli*. Identical amino acids are shown as white on black letters. Many differences in the four sequences correspond to functionally conserved amino acids. (*B*) Conserved amino acids involved in phosphorylation of PhoP of *S. lividans* or *S. coelicolor* and in the DNA-binding domain. Asterisks indicate essential amino acids for phosphorylation. Encircled asterisks show the amino acid (H or D) that is phosphorylated.

lividans and in *S. coelicolor*. This particular two-component system has been designated PhoR-PhoP (see below) by using the *B. subtilis* designation, rather than that of *E. coli*, to avoid confusion, because the *phoB* name has previously been used to nominate one of the four AP genes (*phoA*, *phoB*, *phoC*, and *phoD*) occurring in *S. coelicolor* (15). The nucleotide sequence and deduced amino acid sequences for the PhoR and PhoP proteins were very similar in *S. lividans* and *S. coelicolor*. All of the amino acid changes in *S. lividans* with respect to the *S. coelicolor* sequence, Leu-24 to Pro, Ala-62 to Ser, Gly-86 to Ser, and Ala-384 to Pro, were found in PhoR. No amino acid changes were observed in the *S. lividans* PhoP with respect to the *S. coelicolor* homologous protein. The separation of *phoR* and *phoP* genes was only 5 bp in both *Streptomyces* species, strongly suggesting that both genes are cotranscribed.

The *phoR* product of *S. lividans* is a 426-aa protein with a deduced molecular mass of 45.4 kDa. It contains a hypothetical transmembrane domain in its amino-terminal region that may serve as an anchor to the cytoplasmic membrane. The deduced protein shows all of the characteristics of the sensor proteins of the PhoR family, including the H box (residues His-159 to Leu-176, His-165 being the putative phosphorylation site) and motifs N (residues Asn-267 to Tyr-285), D/F (residues Ile-307 to Asp-334) and G (residues Gly-343 to Val-362) (ref. 19; Fig. 2).

The *phoP* gene product is a protein of 223 aa with a deduced molecular weight of 24.7 kDa and belongs to the OmpR family (20). It shows the conserved amino acids in the amino-terminal region of PhoB(PhoP)-like proteins including Asp-6, Asp-49 (the putative phosphorylation site), and Lys-98 (21). The carboxyl terminal region includes a DNA-binding motif (residues 190–201 in the *S. lividans* PhoP protein; Fig. 2).

Deletion of the *phoP* and *phoR-phoP* Genes. Two different deletion mutants, Δ*phoP* and Δ*phoRP*, were constructed by *in vivo* gene replacement in *S. lividans* (Fig. 3). The Δ*phoP* mutant was obtained by using the construction pHZΔ*phoP*. After recombination, transformants resistant to kanamycin and sensitive to thiostrepton were selected. The recombination was confirmed by Southern hybridization using probes corresponding to *phoR-phoP* and *aphII* (Fig. 3). The DNA fragment deleted from *phoP* in the Δ*phoP* mutant removed most of the conserved motifs of PhoP (except Asp-6), including the putative phosphorylation site Asp-49 (Fig. 1).

Similarly, a 1,651-bp DNA fragment was removed from the *phoR-phoP* region by *in vivo* recombination with plasmid pHZΔ*phoRP*. The deleted region in the Δ*phoRP* mutant comprised all motifs of the PhoR protein involved in the self-

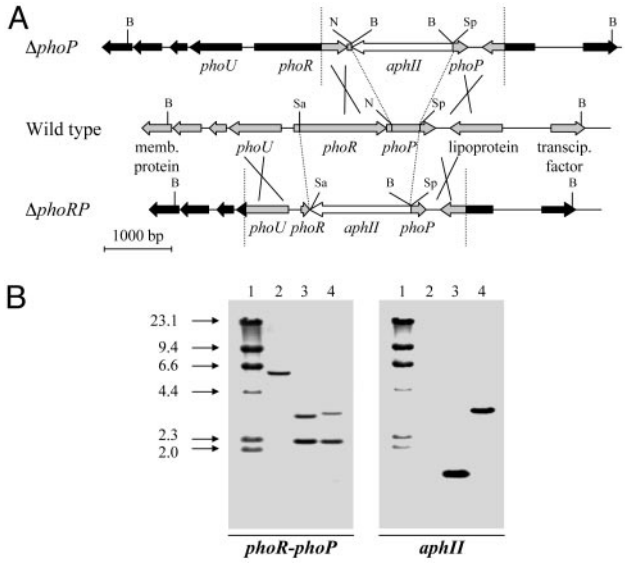

Fig. 3. (*A*) Strategies for obtaining the *S. lividans* deletion mutants Δ*phoP* and Δ*phoRP* by gene replacement with the kanamycin resistance gene (*aphII*) (white arrow). The organization of genes in the wild-type strain is shown in the center, and the crossing over resulting in Δ*phoP* and Δ*phoRP* mutants are in the upper and lower lines, respectively. DNA fragments originating from the integrating plasmid are shown in gray, and the remaining genes (in black) correspond to the wild-type chromosome. B, *Bam*HI; N, *Nru*I; Sa, *Sal*I; Sp, *Sph*I. (*B*) Hybridization patterns of *Bam*HI-digested DNA from *S. lividans* deletion mutants Δ*phoP* and Δ*phoRP* (lanes 3 and 4, respectively) as compared with that of the parental wild-type *S. lividans* strain (lane 2), using the *phoR-phoP* (*Left*) and the *aphII* (*Right*) probes. Note the lack of hybridization of the wild type with the *aphII* probe and the change in size of the hybridizing bands in the deletion mutants. DNA molecular size markers (lane 1; lambda DNA/*Hind*III digested) in kilobases are shown on the left.

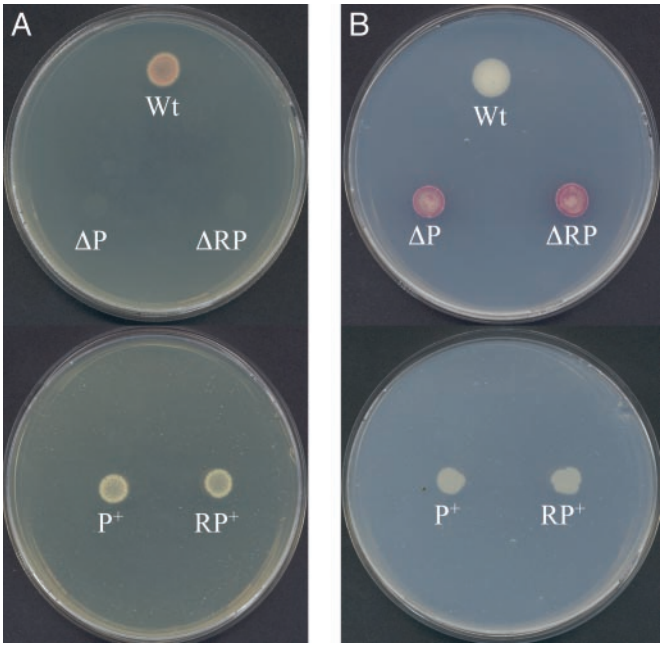

Fig. 4. (*A*) Lack of growth of the *S. lividans* Δ*phoP* and Δ*phoRP* mutants in asparagine-minimal medium at low (10 μM) phosphate concentration. (*B*) Growth in the same medium at 5 mM inorganic phosphate. Note that transformants *phoP*$^+$ and *phoRP*$^+$ grow well at either low or high phosphate concentrations. The Δ*phoP* and Δ*phoRP* deletion mutants overproduce red pigment, whereas the *phoP*$^+$ and *phoRP*$^+$ complemented transformants do not. Wt, wild type; ΔP, Δ*phoP*; ΔRP, Δ*phoRP*; P$^+$, *phoP*$^+$; RP$^+$, *phoRP*$^+$. Strains *phoP*$^+$ and *phoRP*$^+$ were grown in the presence of thiostrepton.

phosphorylation, including site His-165, and all of the residues involved in the phosphorylation of PhoP.

Southern analysis of both Δ*phoP* and Δ*phoRP* mutants revealed the presence of a single copy of the *aphII* marker integrated at the expected location in the *phoR-phoP* locus (Fig. 3).

The *S. lividans* Mutants Δ*phoP* and Δ*phoRP* Do Not Grow at Low Phosphate Concentrations. In *E. coli*, several inorganic- and organic-phosphate transport proteins (e.g., the *pst* system) are known to be regulated by PhoR-PhoP. As shown in Fig. 4, both the Δ*phoP* and Δ*phoRP* mutants were unable to grow in *Streptomyces* asparagine-minimal medium containing 10 μM inorganic phosphate, whereas the parental *S. lividans* strain grew well at this low phosphate concentration. At high phosphate concentration (5 mM) the deletion mutants were able to grow, showing more intense red pigmentation than the parental strain.

Growth of both *S. lividans* Δ*phoP* and Δ*phoRP* mutants was restored by complementation with plasmid pIJ*phoRP* containing both *phoR-phoP* genes and its complete promoter region. Transformation of Δ*phoP* and Δ*phoRP* with this plasmid gave rise to *S. lividans* strains *phoP*$^+$ and *phoRP*$^+$ that were perfectly able to grow on asparagine-minimal medium. The complemented *S. lividans phoP*$^+$ and *phoRP*$^+$ transformants did not show the red color of the Δ*phoP* or Δ*phoRP* mutants in asparagine-minimal medium with 5 mM phosphate (Fig. 4*B*).

The Δ*phoRP* Mutant Is Partially Defective in Inorganic Phosphate Uptake. To confirm that the lack of growth in asparagine-minimal medium with reduced phosphate concentration was due to a defect in phosphate uptake, the cellular incorporation of ^{32}P-labeled phosphate during 2.5 or 7.5 min was determined. The parental strain takes inorganic phosphate rapidly and reaches an equilibrium level at 7.5 min, whereas the Δ*phoRP* mutant showed a drastic reduction in the uptake of inorganic phosphate (Fig. 5).

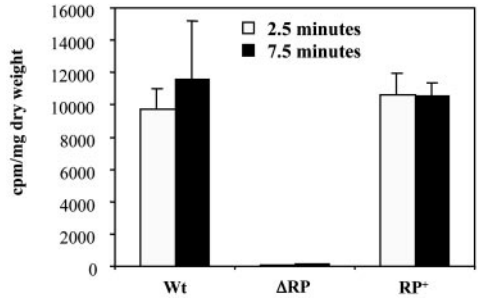

Fig. 5. Uptake of ^{32}P-labeled phosphate after 2.5 min (open vertical bars) or 7.5 min (black bars) in the wild-type *S. lividans* (Wt), the Δ*phoRP* deletion mutant (ΔRP), and the complemented transformant *phoRP*$^+$ (RP$^+$). Thin vertical bars indicate SD of the mean.

The complemented mutant *phoRP*$^+$ showed the same behavior as the wild-type strain, although the uptake of phosphate at short times (e.g., 2.5 min) was higher, probably because of the positive effect on transport of the oversynthesis of the phosphate regulatory proteins in the transformant containing the *phoR-phoP* genes in a multicopy plasmid.

The PhoR-PhoP System Regulates AP. When the parental *S. lividans* and the deletion mutants Δ*phoP* and Δ*phoRP* were compared on R5 liquid medium that supports a good AP formation, the total amount of extracellular protein was similar until 48 h of cultivation, but it was reduced in cultures at 60 and 72 h when extracellular enzymes are known to accumulate (Fig. 6*A*). The extracellular AP was drastically reduced in the *S. lividans* Δ*phoP* and Δ*phoRP* mutants when compared with the parental strain (Fig. 6*B*). Complementation of each deletion mutant with the *phoR-phoP* cluster restored the formation of AP.

These results were confirmed by Western analysis using anti-AP antibodies. The immunoblot analysis clearly indicated that the extracellular AP is absent in the Δ*phoP* and Δ*phoRP* mutants (Fig. 6*C*) and is restored by complementation with the

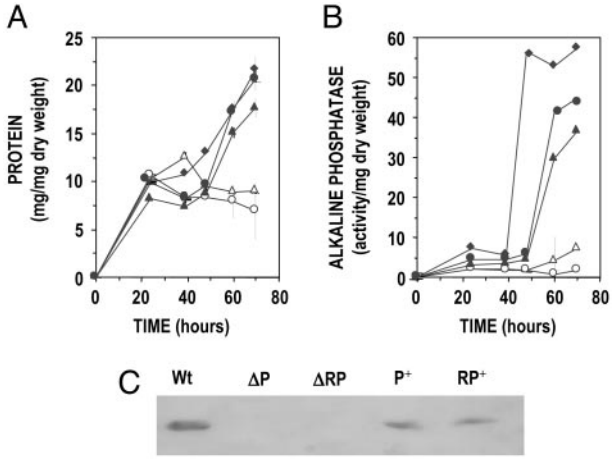

Fig. 6. Total extracellular protein (*A*) and AP activity (*B*) in R5 liquid cultures of wild-type *S. lividans* (filled diamonds), the Δ*phoP* (open triangles), and Δ*phoRP* (open circles) mutants and the complemented strains *phoP*$^+$ (filled triangles) and *phoRP*$^+$ (filled circles). Experiments were done in triplicate. Thin vertical bars indicate SD of the mean. *phoP*$^+$ and *phoRP*$^+$ strains were grown in the presence of thiostrepton. (*C*) Western blot analysis using anti-AP antibodies of extracellular proteins of *S. lividans* wild-type (Wt), Δ*phoP* (ΔP), Δ*phoRP* (ΔPR), *phoP*$^+$ (P$^+$), and *phoRP*$^+$ (RP$^+$) strains grown in R5 liquid medium. Note that the AP is restored by complementation of the deletion mutants with the *phoR-phoP* cluster (transformants P$^+$ and RP$^+$).

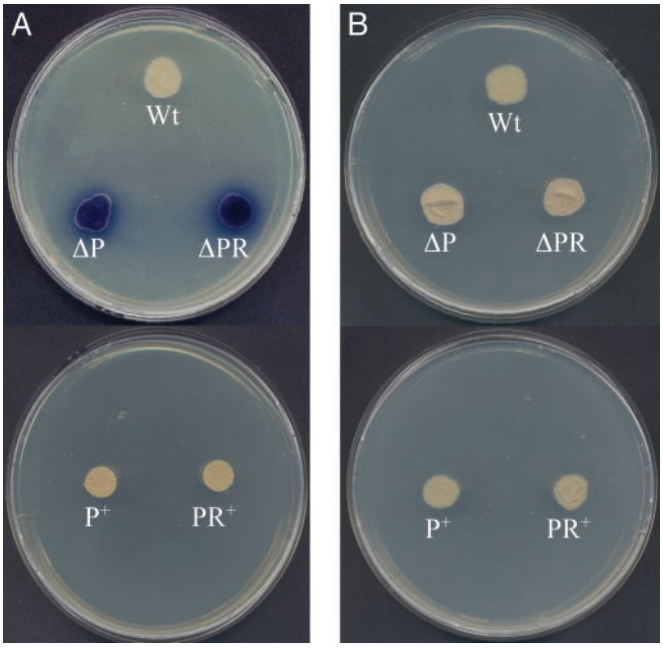

Fig. 7. Growth and actinorhodin production on R5 solid medium containing low (0.37 mM; *A*) or high (1.85 mM; *B*) phosphate concentration of the wild-type *S. lividans*, the Δ*phoP* and Δ*phoRP* deletion mutants, and the complemented transformants *S. lividans phoP*$^+$ and *phoRP*$^+$. Note that there is a very high actinorhodin (blue pigment) production in the Δ*phoP* and Δ*phoRP* deletion mutants (*A*) growing under a low phosphate concentration but not in the plate with a high phosphate level. The complemented transformants *phoP*$^+$ and *phoRP*$^+$ did not overproduce actinorhodin, even under low phosphate concentrations. Abbreviations in the photograph are as in Fig. 4. *phoP*$^+$ and *phoRP*$^+$ strains were grown in the presence of thiostrepton.

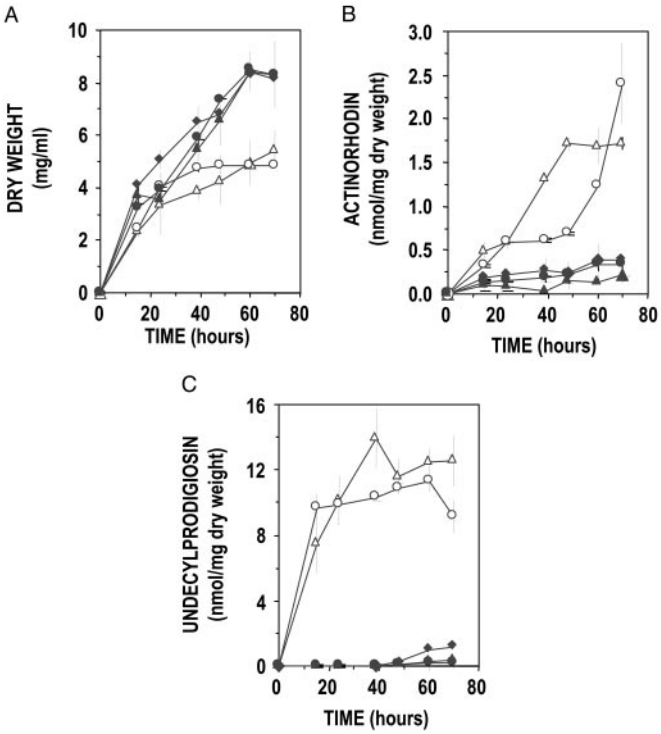

Fig. 8. Growth (*A*), actinorhodin (*B*), and undecylprodigiosin (*C*) production in R5 liquid cultures of *S. lividans* wild type (filled diamonds), Δ*phoP* (open triangles), Δ*phoRP* (open circles), and the complemented transformants *phoP*$^+$ (filled triangles) and *phoRP*$^+$ (filled circles). Note that high levels of actinorhodin and undecylprodigiosin are synthesized in the Δ*phoP* and Δ*phoRP* deletion mutants but not in the complemented *phoP*$^+$ and *phoRP*$^+$ strains. Experiments were done in triplicate. Thin vertical bars indicate SD of the mean. *phoP*$^+$ and *phoRP*$^+$ strains were grown in the presence of thiostrepton.

phoR-phoP cluster, i.e., formation of AP depends on the PhoR-PhoP regulatory system.

The Production of Actinorhodin and Undecylprodigiosin Is Strongly Regulated by the PhoR-PhoP System. *S. lividans* contains the actinorhodin gene cluster but, unlike *S. coelicolor*, it does not synthesize actinorhodin efficiently. When the *S. lividans* Δ*phoP* and Δ*phoRP* mutants were compared with the parental *S. lividans* strain, it was observed that both mutants were actinorhodin hyperproducers in solid R5 medium with low phosphate (0.37 mM; Fig. 7*A*). The overexpression of the actinorhodin genes was suppressed in both deletion mutants by complementation with the *phoR-phoP* cluster (strains P$^+$ and RP$^+$ in Fig. 7). When excess phosphate was added to the R5 medium the production of actinorhodin was still controlled by phosphate in the Δ*phoP* and Δ*phoRP* mutants, as shown by the reduction in blue pigment production (Fig. 7*B*), indicating that the regulatory effect of PhoR-PhoP is bypassed at high phosphate concentrations.

The biosynthesis of actinorhodin and undecylprodigiosin was quantified in R5 liquid cultures. As shown in Fig. 8, both the *S. lividans* Δ*phoP* and Δ*phoRP* overproduced actinorhodin and undecylprodigiosin, reaching levels up to 2.5 nmol (1.59 μg) of actinorhodin and 14 nmol (5.49 μg) of undecylprodigiosin per milligram of dry weight. The oversynthesis of both antibiotics in the deletion mutants in liquid R5 medium was again suppressed by complementation with the *phoR-phoP* regulatory genes (Fig. 8).

Discussion

Many secondary metabolites, including antibiotics, pigments, immunomodulators, and siderophores, produced by actinomycetes and other Gram-positive and -negative bacteria are strongly repressed by high inorganic phosphate concentrations in the culture medium (2). Production of these compounds has to be performed under phosphate-limiting conditions (4).

Two-component systems (22) provide a conserved mechanism for the coordinate control of gene expression in response to different environmental factors (19, 23). Three putative *phoR-phoP*-like systems, were found by computer search in the genome of *S. coelicolor* (24). We have found that the *phoR-phoP* system showing the best match to the homologous *E. coli* system is linked to a putative *phoU* gene (encoding a modulator of phosphate transduction; ref. 12) and that the three genes are expressed from a bidirectional promoter region. The *phoU-phoR-phoP* organization is very similar in *S. lividans* and *S. coelicolor*. The best match of the *S. lividans* and *S. coelicolor* PhoR and PhoP proteins were, respectively, with the *Mycobacterium bovis* two-component SenX3 (41.6% identical amino acids) and the RegX3 (72.7% identity) system of unknown function (25).

PhoR of *S. lividans* corresponds to the sensor protein of two-component systems. Sensor proteins autophosphorylate in a conserved histidine (corresponding to His-165 in the *S. lividans* protein; ref. 25). In addition to His-165, PhoR also contains all of the consensus motifs of the kinase domains (26).

PhoP is closely related to the RO$_{II}$ subfamily of response regulators (26). It contains a helix-turn-helix motif in its C-terminal region similar to that identified in the OmpR protein (27, 28) that is also present in the RegX3 protein of *M. bovis* (25). In the two-component systems the mechanisms of signal transduction involve, first, autophosphorylation of the sensor protein (PhoR), followed by transfer of the phosphate group onto a

conserved aspartate residue of the response regulator (Asp-49 in the *S. lividans* PhoP; Figs. 1 and 2).

The AP encoded by *phoA* is a prototype member of the *pho* regulon and is known to be repressed by phosphate in *Streptomyces* species (15). As shown in this article, PhoA is not formed in the *S. lividans* Δ*phoP* and Δ*phoRP* deletion mutants and its synthesis is restored by complementation of the deletion mutants with the *phoR-phoP* cluster. These results indicate that formation of PhoA is under positive control of the PhoR-PhoP system as occurs in *E. coli* (12, 29) and *B. subtilis* (30, 31). The drastic reduction in phosphate uptake in the *S. lividans* Δ*phoRP* deletion mutant suggests that some components of a high-affinity phosphate transport system also depend on the PhoR-PhoP system. However, growth of the deletion mutants occurs at high phosphate concentration, indicating that alternative phosphate transport is still possible in the absence of the PhoR-PhoP system at high phosphate concentrations.

An interesting finding of this work was the observation that production of both actinorhodin and undecylprodigiosin is greatly increased in the Δ*phoP* or Δ*phoRP* mutants either in solid or liquid medium. These results clearly indicate that the biosynthesis of secondary metabolites is under the control of the PhoR-PhoP system. The biosynthesis of actinorhodin is known to be regulated negatively by phosphate (5). The wild-type *S. lividans*, unlike *S. coelicolor*, produces very low levels of actinorhodin and undecylprodigiosin. The large increase in the production of those secondary metabolites in the Δ*phoP* and Δ*phoRP* mutants indicates that these secondary metabolites are repressed by a form of PhoP (i.e., a negative regulation) that accumulates in response to high phosphate concentration in the culture medium. Alternatively, the PhoR-PhoP system may activate the formation of a specific repressor protein for phosphate-controlled promoters by a cascade mechanism. In either case, the Δ*phoP* and Δ*phoRP* deletion mutants are largely derepressed in secondary metabolite biosynthesis because they lack a functional PhoP protein.

These regulatory proteins may serve to integrate different input signals (32) that affect secondary metabolite biosynthesis, e.g., the negative role exerted by polyphosphate through autophosphorylation of the polyphosphate glucokinase (7).

The kinetics of expression of different regulatory proteins in *S. coelicolor* has been reported recently (11). One of the genes studied SCD8A.03 corresponds to *phoP* in *S. lividans*. The pattern of expression of this gene coincides with that of SCD8A.01c that corresponds to *phoU* in our cloned fragment. Both genes are induced (derepressed) at the middle of the growth phase coinciding with the depletion of phosphate in the growth medium.

In summary, all available evidence suggest that inorganic phosphate concentrations control the pattern of expression of a series of genes involved in the biosynthesis of different types of antibiotics in *S. lividans* and probably in *S. coelicolor* and other *Streptomyces* species. The PhoR-PhoP system may serve as a general transduction system for the expression of genes involved in secondary metabolism.

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