Transcriptional Studies and Regulatory Interactions between the \( \text{phoR-phoP} \) Operon and the \( \text{phoU, mtpA, and ppk} \) Genes of \( \text{Streptomyces lividans} \ TK24 \)

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The PhoR/PhoP two-component system of \( \text{Streptomyces lividans} \) was previously shown to allow the growth of the bacteria at low \( \text{P}_i \) concentrations and to negatively control antibiotic production. The present study focuses on the transcriptional analysis of \( \text{phoR} \) and \( \text{phoP} \), along with the \( \text{phoU} \) and \( \text{mtpA} \) genes that are transcribed divergently from the \( \text{phoRP} \) operon in \( \text{S. lividans} \). The effect of \( \text{phoR}, \text{phoP}, \text{phoU}, \text{and ppk} \) mutations on transcription of these genes was examined under phosphate-replete and phosphate-limited conditions. We demonstrated that \( \text{phoR} \) and \( \text{phoP} \) were cotranscribed as a leaderless bicistronic transcript cleaved at discrete sites toward the 3′ end of \( \text{phoR} \). In addition, \( \text{phoP} \) could also be transcribed alone from a promoter located at the 3′ end of \( \text{phoR} \). The \( \text{phoU} \) and \( \text{mtpA} \) genes, predicted to encode metal binding proteins, were shown to be transcribed as monocistronic transcripts. The expression of \( \text{phoR-phoP}, \text{phoP}, \text{and phoU} \) was found to be induced under conditions of \( \text{P}_i \) limitation in \( \text{S. lividans} \ TK24 \). This induction, requiring both \( \text{PhoR} \) and \( \text{PhoP} \), was significantly weaker in the \( \text{phoU} \) mutant but much stronger in the \( \text{ppk} \) mutant than in the parental strain. The expression of \( \text{mtpA} \) was also shown to be up-regulated when \( \text{P}_i \) was limiting but independently of \( \text{PhoR}/\text{PhoP} \). The induction of \( \text{mtpA} \) expression was much stronger in the \( \text{phoU} \) mutant strain than in the other strains. This study revealed interesting regulatory interactions between the different genes and allowed us to propose putative roles for \( \text{PhoU} \) and \( \text{MtpA} \) in the adaptation to phosphate scarcity.

Phosphate (\( \text{P}_i \)) is a crucial component of all living organisms, as most of the essential cellular constituents, including ATP, contain \( \text{P}_i \). Considering the vital importance of \( \text{P}_i \) and its scarcity in the natural world, bacteria have evolved manifold strategies to cope with limited availability of \( \text{P}_i \). The bacteria first sense \( \text{P}_i \) limitation in the growth medium and then trigger strategies aimed at scavenging and transporting trace amounts of \( \text{P}_i \) that is usually present in the growth medium as metal phosphates (4, 35) or at recycling the phosphate present in some phosphate-rich cellular constituents (2, 10). These strategies allow the growth of the bacteria at low \( \text{P}_i \) concentrations. In \( \text{Escherichia coli} \) or \( \text{Bacillus subtilis} \), the two-component systems (\( \text{PhoB/PhoR} \) and \( \text{PhoP/PhoR} \), respectively) were shown to be involved in sensing \( \text{P}_i \) limitation (20, 28). An as-yet-unknown signal, indicating a \( \text{P}_i \) limitation, triggers autophosphorylation of the sensory kinase on a histidine residue. This phosphoryl group is then transferred to a conserved aspartate \( \text{D} \) of the \( \text{pho} \) box, usually present in the promoter regions of genes of the \( \text{pho} \) regulon (5, 33).

Recently, the genes encoding the two-component system of \( \text{Streptomyces lividans} \) 1326, \( \text{phoR} \) (encoding a 426-amino-acid [aa]-long sensor kinase) and \( \text{phoP} \) (encoding a 223-aa-long response regulator), related to analogous systems of \( \text{B. subtilis} \) and \( \text{E. coli} \), were cloned and interrupted (32). The \( \Delta \text{phoR} \) and \( \Delta \text{phoRP} \) mutants are unable to grow under conditions of extreme \( \text{P}_i \) scarcity (10 \( \mu \text{M} \)), as their ability to transport \( \text{P}_i \) and to excrete phosphatases is greatly reduced (32). These strains are thus prematurely starved of free \( \text{P}_i \), and their ability to produce antibiotics (actinorhodin and undecylprodigiosin) is enhanced compared to that of \( \text{S. lividans} \) TK24 (32). This observation is consistent with previous findings that \( \text{P}_i \) limitation is a major trigger for antibiotic production in \( \text{Streptomyces} \) species (3, 7, 21). The interruption of another gene of \( \text{S. lividans} \) TK24 (\( \text{ppk} \)), with a function related to \( \text{P}_i \) metabolism, was also previously reported to lead to a large increase in antibiotic biosynthesis (8). This gene encodes a protein catalyzing the reversible polymerization of the gamma phosphate of ATP into polyphosphate, a phosphate and energy storage polymer (8). However, its role in the control of antibiotic biosynthesis remains unclear.

In this study, we focused on the transcriptional and regulatory analysis of the expression of \( \text{phoR}, \text{phoP}, \text{phoU} \) (corresponding to \( \text{SCO4228} \) and \( \text{SCO4227} \) of \( \text{Streptomyces coelicolor} \), respectively) in \( \text{S. lividans} \) TK24 and in \( \text{phoR}, \text{phoP}, \text{phoU} \) and \( \text{ppk} \) mutants, under conditions of \( \text{P}_i \) limitation or sufficiency. The gene corresponding to \( \text{SCO4228} \) of \( \text{S. coelicolor} \) was called \( \text{phoU} \) as it encodes a 229-aa-long protein possessing 28.1% identity in a 128-aa overlap with \( \text{PhoU} \) of \( \text{E. coli} \). In \( \text{E. coli} \), this gene is located downstream of \( \text{pstB} \) encoding the ATPase of the high-affinity phosphate ABC transporter, \( \text{PstSCAB} \) (34). \( \text{PhoU}-\text{like proteins} \) were recently shown to be metalloproteins containing two multinuclear iron clusters (18). The gene located downstream of \( \text{phoU}, \text{mtpA} \), encodes a 79-aa-long cysteine-rich protein be-
longing to the family of metallothionein proteins. These metal binding proteins are thought to be involved in the sensing of and/or resistance to oxidative stress in various organisms (9).

This study was aimed at elucidating the regulatory interactions existing between these different loci and their role in the adaptation to phosphate scarcity in S. lividans.

### MATERIALS AND METHODS

**Strains and media.** The Streptomyces strains used in this study are the original strain S. lividans TK24 (14) and the following derivatives: S. lividans TK24 phoP::Tn9, S. lividans TK24 phoR::Tn9, and S. lividans TK24 phoU::Tn9 (see below for the construction of the last three strains). Spores of the various S. lividans strains were spread on the surface of cellophane disks (from Cannings Packaging, Limited, United Kingdom) placed on the surface of the rich R2YE agar medium or on modified minimal medium, as described previously (13). After being autoclaved, the rich R2YE was shown to contain 1 mM P, as assayed with a phosphorus assay kit from Sigma Diagnostics. The usual concentration of K2HPO4 added to the R2YE is 0.37 mM (0×), bringing the final concentration of the free P in the medium to 1.37 mM. The addition of K2HPO4 was either omitted (-; final concentration, 1 mM) or added at a 10× level (+; final concentration, 4.7 mM) (see Fig. 5). In the modified medium, the K2HPO4 was added at either 0.44 mM (-; limitation) or 2.2 mM (+; sufficiency). The liquid growth media used were HT, TSB, and YEME (13).

**Construction of S. lividans TK24 phoP::Tn9, S. lividans TK24 phoR::Tn9, and S. lividans TK24 phoU::Tn9 mutant strains.** To disrupt the phoR and phoP genes, a 3.2-kb fragment encompassing these genes was amplified by PCR with the appropriate primers PhoRP5′ and PhoRP3′ (Table 1), derived from phoR (SCD8A.02; SC04229) and phoP (SCD8A.05; SC0230) of S. coelicolor. Total chromosomal DNA from S. lividans TK24 was used as template with the Turbo Pfu polymerase from Stratagene. The two PCR fragments were then cut first, then EcoRI followed by BamHI, for the upstream fragment and HindIII for the downstream fragment. They were cloned in a three-partner ligation into the thermosensitive vector pGM160, which was digested by BamHI, yielding, respectively, pSG113 and pSG114. These pGM160 derivatives, which carry the trp gene conferring resistance to thiostrepton or neomycin (Nos), were used to replace the wild-type phoP and phoR genes by their interrupted versions by the following procedure. The Nos′ colonies of S. lividans TK24 transformed with pSG113 and pSG114 were grown for 24 h at 30°C in TS medium containing Apra (20 μg·mL⁻¹). The mycelium was then harvested, homogenized, and used as a low-density inoculum to set up a culture in the same medium. These cultures were grown for 60 h at 41°C to eliminate the autonomously replicating plasmid. The mycelium was then harvested, homogenized, and fragmented by sonication to obtain small mycelial fragments that were plated on HT agar (29) containing apramycin (20 μg·mL⁻¹) and on HT agar containing neomycin (20 μg·mL⁻¹) to detect the Apra′ Nos′ colonies that had lost the replicative plasmid. The interruption of the gene was checked by Southern blotting and hybridization in comparison with the original strain (data not shown).

To disrupt the phoU gene, two 1.0-kb DNA fragments corresponding to the regions located upstream and downstream of phoU were amplified by PCR, using the appropriate pairs of primers PhoU3′/PhoU5′ (Table 1), derived from phoU (SCD8A.07; SC05372) and phoU (SCD8A.06; SC0689). Total chromosomal DNA from S. lividans TK24 was used as template with the Turbo Pfu polymerase from Stratagene. The two PCR fragments were then cut first, then EcoRI followed by XbaI for the upstream fragment and HindIII for the downstream fragment. They were cloned in a three-partner ligation into the thermosensitive vector pGM160 (25) and cut by XbaI and HindIII, yielding pSG115. This plasmid was then cut by EcoRI; the 1,777-bp EcoRI DNA fragment carrying the cassette phoU (6) conferring resistance to apramycin (Apra) was cloned into pSG110 cut by NoI or by NcoI, yielding pSG111 and pSG112, respectively. The BglII fragments carrying the inserts of pSG111 and pSG112, respectively, were then cloned into the thermosensitive vector pGM160 (25) and digested by BamHI, yielding, respectively, pSG113 and pSG114. These pGM160 derivatives, which carry the trp gene conferring resistance to thiostrepton or neomycin (Nos), were used to replace the wild-type phoP and phoR genes by their interrupted versions by the following procedure. The Nos′ colonies of S. lividans TK24 transformed with pSG113 and pSG114 were grown for 24 h at 30°C in TS medium containing Apra (20 μg·mL⁻¹). The mycelium was then harvested, homogenized, and used as a low-density inoculum to set up a culture in the same medium. These cultures were grown for 60 h at 41°C to eliminate the autonomously replicating plasmid. The mycelium was then harvested, homogenized, and fragmented by sonication to obtain small mycelial fragments that were plated on HT agar (29) containing apramycin (20 μg·mL⁻¹) and on HT agar containing neomycin (20 μg·mL⁻¹) to detect the Apra′ Nos′ colonies that had lost the replicative plasmid. The interruption of the gene was checked by Southern blotting and hybridization in comparison with the original strain (data not shown).

**TABLE 1. List and sequence of primers used in this study and their utilization**

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<tr>
<th>Primer</th>
<th>Primer sequence</th>
<th>Purpose</th>
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<tbody>
<tr>
<td>PhoRP5′</td>
<td>5′-CTGCGGATCCGGcgccggggtgaggggctagcgc 3′</td>
<td>3.2-kb DNA fragment carrying phoR and phoP</td>
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<td>PhoRP3′</td>
<td>5′-CTCGGACCTCGGcattgctggcggggtgaggggctagcgc 3′</td>
<td>1-kb DNA fragment located upstream of phoU</td>
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<tr>
<td>PhoU5′ EcoRI</td>
<td>5′-TACGAAATTGCGATCAGGATTTTCTCCTGTAAGGTGGGCTAGCgc 3′</td>
<td>1-kb DNA fragment located downstream of phoU</td>
</tr>
<tr>
<td>PhoU3′ XbaI</td>
<td>5′-AGTTCGAGGACGGCGAggtcagcggcgtagg 3′</td>
<td>Northern blot</td>
</tr>
<tr>
<td>PhoU5′ HindIII</td>
<td>5′-ACGAGCTTGAAGGGCCGGAgaaggtcagcggcgtagg 3′</td>
<td>Probe P1</td>
</tr>
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<td>PhoU3′ EcoRI</td>
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<td>Probe P4</td>
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<td>PhoR5′</td>
<td>5′-ACGGATCGGCGGGGAggtcagcggcgtagg 3′</td>
<td>High-resolution S1 mapping</td>
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<td>PhoR3′</td>
<td>5′-ACGTTTGCGGACGGCGGAGGCGGAGGctagcgc 3′</td>
<td>PCR fragment P5 (5′ regions of phoR and phoU)</td>
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<tr>
<td>PhoU5′</td>
<td>5′-ATGCGGAGGACGGCGAggtcagcggcgtagg 3′</td>
<td>PCR fragment P6 (5′ region of phoP)</td>
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<td>PhoU3′</td>
<td>5′-GATGGCGCGTGCATCAACGGGcgtagcgc 3′</td>
<td>Promoter probing</td>
</tr>
<tr>
<td>MtpA5′</td>
<td>5′-ACTGGCAGGACGGTTGGCCGAGTCGAGGcgtagcgc 3′</td>
<td>PCR fragment I (proximal 5′ region of phoP)</td>
</tr>
<tr>
<td>MtpA3′</td>
<td>5′-GGACCACGACGACCGGAGGCGGAGGcgtagcgc 3′</td>
<td>PCR fragment II (distal 5′ region of phoP)</td>
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Cloning for gene disruption

PCR fragment P5 (5′ regions of phoR and phoU)

Northern blot

Probe P1

Probe P2

Probe P3

Promoter probing

Northern blot

Probe P4

Northern blot

PCR fragment I (proximal 5′ region of phoP)

PCR fragment II (distal 5′ region of phoP)
ping experiments, were determined with a Beckman spectrophotometer at 260 nm. The concentration and integrity of RNA were verified by electrophoresis with an agarose gel stained with ethidium bromide. Northern blot analysis of the phoP (P1), phoR (P2), phoU (P3), and mtpA (P4) transcripts in S. lividans TK24, S. lividans TK24 phoU::\( \text{H}9024 \), S. lividans TK24 phoR::\( \text{H}9024 \), and the S. lividans TK24 phoU::\( \text{H}9024 \) strains were carried out. For this purpose, 20 \( \mu \)g of RNA was denatured with a glyoxal-dimethyl sulfoxide mixture (34a); fractionated on a 1% agarose gel; transferred to an Hybond-XL membrane; hybridized at 65°C with the PCR DNA probes P1, P2, P3, and P4; and labeled with \( \text{[\( ^{32} \text{P} \)]ATP} \) with the T7 quick prime kit (Pharmacia). Molecular weight standards from BRL were treated in the same way as the RNA samples and labeled as the DNA probes. The pairs of synthetic oligonucleotides used to amplify the probe P5, encompassing the 5’ region of the equivalent genes in the S. coelicolor genome.

High-resolution S1 nuclease mapping to determine the 5’ ends of the phoP, phoR, and phoU transcripts was performed as described previously (16). A total of 40 \( \mu \)g of RNA was hybridized to approximately 0.02 pmol of a DNA probe amplified by PCR with primers labeled at their 5’ ends with \( \text{[\( ^{32} \text{P} \)]ATP} \) and T4 PolyNucleotide kinase from Biolabs. The synthetic oligonucleotides used to amplify the probe P5, encompassing the 5’ regions of phoU and phoR, and the probe P6, encompassing the 5’ region of phoP (Table 1), were derived from the sequence of the equivalent genes in the S. coelicolor genome.

Promoter probe assays. To test the activity of the phoR and phoU promoters, transcriptional fusions were made between a Sall restriction DNA fragment encompassing the 5’ regions of these two divergent genes in both orientations and the promoterless reporter gene \( \text{aph} \) carried by the Streptomyces multicopy plasmid, pIJ487 (38), yielding pIJ487/\( \text{phoP} \) and pIJ487/\( \text{phoU} \), respectively. In these transcriptional fusions, the promoter fragments direct the expression of the promoterless aminoglycoside phosphotransferase gene \( \text{aph} \) conferring resistance to kanamycin to S. lividans (38). To test for promoter activity in region I (from 165 bp upstream to 90 bp downstream of the PhoP GTG start codon) or region II (from 335 bp upstream to 127 bp upstream of the PhoR GTG start codon) located at the 5’ end of phoP, PCR fragments I and II were amplified using the appropriate pairs of primers (Table 1) and cloned into pIJ487 (38), yielding pIJ487/\( \text{phoP1} \) and pIJ487/\( \text{phoP2} \), respectively. The PCR fragments were first cloned into pIJ2925 and transformed into E. coli (15). They were then cloned in the correct orientation as EcoRI-HindIII fragments into pIJ487. Promoter strength was estimated by plating convenient dilutions of spores of S. lividans TK24, S. lividans ppk::\( \text{H}9024 \), and S. lividans TK24 phoU::\( \text{H}9024 \) carrying the pIJ487 derivatives on plates of minimal medium containing 2.2 mM or 0.44 mM K2HPO4 in the presence or absence of 20 \( \mu \)g/ml kanamycin. The number of colonies was estimated in each case, and the percentage of survival was calculated.

Test of sensitivity to \( \text{H}_2\text{O}_2 \). Spores (105) of S. lividans TK24 and of its phoU, phoP, and ppk mutants were plated on the rich medium R2YE (13) in which no K2HPO4 (1 mM final concentration) or 3.7 mM K2HPO4 (4.7 mM final concentration) was added. A 5-mm-diameter cellulose disk, wetted with 20 \( \mu \)l of 100 mM H2O2, was deposited in the center of the plate just after spreading. Plates in triplicate were incubated 60 h at 30°C. Diameters of the zones of growth inhibition were measured, giving an estimate of the sensitivity and/or resistance of the strain to an externally applied oxidative stress. Results obtained were highly reproducible.

**RESULTS**

**Phenotypes of the phoR, phoP, and phoU mutant strains of S. lividans TK24 on solid minimal medium.** Mutants of the phoR, phoP, and phoU genes of S. lividans TK24 were constructed as described in Materials and Methods. On minimal medium containing 0.44 mM K2HPO4 (P, limitation conditions), S. lividans TK24 and the phoU mutant strain were pale pink, whereas the phoR and phoP mutant strains were reddish, indicating the production of a red pigment (likely undecylprodigiosin). The colonies of the phoR mutant strain were larger and not as bright red as those of the phoP mutant strain, indicating that the growth defect and the ability to produce the red pigment were more pronounced in the phoP mutant strain than in the phoR mutant strain (Fig. 1). These phenotypes are consistent with those described previously (32).
Transcriptional and regulatory analysis of the expression of phoR-phoP operon. Northern blot analysis shown in Fig. 2B using the probe P2, representing almost the whole kinase down to 22 bp upstream of the TGA stop codon, demonstrated that the phoR and phoP genes were cotranscribed as an ~2-kb bicistronic mRNA. However, in addition to the ~2-kb transcript, another smaller band of ~1 kb was also visible but present mainly in the ppk mutant strain. High-resolution S1 mapping experiments showed that the transcriptional start site and the putative translational start site of phoR proposed by the Sanger Centre coincided (Fig. 3). The rather canonical sequence TAACCT is proposed as the 10 promoter sequence, whereas the sequence GGGCCG, located 16 bp upstream of it, was in position for a σ70 promoter sequence (Fig. 4A). The promoter of phoR was tested for functionality by cloning a 520-bp SalI fragment in the proper orientation in the promoter probe plasmid pIJ487 (38). The results shown in Fig. 2B and 3 indicate that the expression of phoR and thus that of the phoR-phoP operon was weakly inducible when P_i was limiting in S. lividans TK24. This induction was even weaker in the phoU mutant strain, undetectable in both the phoR and phoP mutant strains, and much stronger in the ppk mutant strain than in the parental strain (Fig. 2 and 3; Table 2).

Transcriptional and regulatory analysis of phoP expression. Northern blot analysis shown in Fig. 2B, using the probe P1 corresponding to a 650-bp DNA fragment internal to phoP (Fig. 2A), confirmed that the phoR and phoP genes were cotranscribed as an ~2-kb bicistronic mRNA. In addition to the ~2-kb bicistronic phoR-phoP transcript, at least three other smaller, discrete transcripts of approximately 1, 0.9, and 0.7 kb were detected. The latter followed a regulatory pattern similar to that of the bicistronic transcript. Nonetheless, the smallest 0.7-kb transcript was much more intense than the other two in the ppk mutant strain when grown under P_i limitation and was not detected with the probe P2 (phoR), suggesting that it may correspond to the transcription of phoP alone.

To test this hypothesis, the 5' ends of the putative phoP
transcript were determined by high-resolution S1 nuclease mapping (Fig. 3). This analysis revealed the presence of four 5’ ends mapping toward the 3’ end of phoR that were mainly detectable in the ppk mutant strain under conditions of P_i limitation. Two strong discrete and regulated bands, whose intensity varied with P_i availability, mapped 222 bp and 285 bp upstream of the putative phoP GTG start codon (region II) (Fig. 4B). Furthermore, the other two more diffuse bands...
TABLE 2. Estimation of the promoter strength of DNA fragments carrying the promoter regions of the genes phoR, phoP, and phoU, cloned into the promoter-probe plasmid pIJ487 in the appropriate orientation and transformed into *S. lividans* TK24 (wt), *S. lividans* TK24 ppp::Δ*phoP* (ppk), and *S. lividans* TK24 phoP::Δ*tec* (phoP)*a*.

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<tbody>
<tr>
<td>wt</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>2</td>
<td>60</td>
</tr>
<tr>
<td>ppp mutant</td>
<td>0.33</td>
<td>1</td>
<td>10</td>
<td>100</td>
</tr>
<tr>
<td>phoP mutant</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>1.3</td>
<td>2.5</td>
</tr>
</tbody>
</table>

*a* *S. lividans* TK24 containing pIJ487 was used as a kanamycin-sensitive control strain. Appropriate dilutions of spores of different strains were spread on plates of modified minimal medium containing either 2.2 mM K₂HPO₄ (Pᵢ sufficiency), 0.44 mM K₂HPO₄ (Pi limitation, +) or 0.44 mM KH₂PO₄ (Pᵢ limitation, −) in the presence or absence of 20 µg of kanamycin/ml. The number of colonies was estimated in each situation, and the percentage of survival was calculated.

mapped within the 50 bp preceding the putative phoP GTG start codon (region I) (Fig. 4B). To assess the presence of a promoter activity in these two regions, the appropriate PCR regions (I and II) (Fig. 4; Table 1) were cloned into the promoter probe plasmid pIJ487. The PCR region I showed promoter activity (pIJ487:phoPp) (Table 2), whereas the PCR region II did not (data not shown). Consequently, the 5′ end detected 285 bp and 222 bp upstream of the putative phoP GTG start codon (Fig. 3) are likely to constitute processing sites ps1 and ps2, respectively, yielding the approximately 520-bp and 0.9-kb bands detected with probe P1 (phoP) shown in Fig. 2B. These products would be expected to encompass the whole phoP and the last 285 nt (ps1) or the last 222 nt (ps2) of phoR, respectively. The mapping of these putative processing sites (Fig. 3) revealed that they were located in close proximity to AT, TA, or AA dinucleotides (Fig. 4B) that are known to be present in the cleavage sites of the RNase E endoribonuclease of *E. coli* (11, 23). Considering the promoter probe activity of the PCR fragment I (pIJ487:phoPp [Table 2]), we propose that the DNA-RNA hybrid mapping at the T/C, 38 nt upstream of the GTG start proposed by the Sanger Centre, is the real transcriptional start site of phoP. This is supported by the fact that the near-canonical −10 (TACAGC) and −35 (TCCCTCA) promoter sequences, with a spacing of 17 bp, could be found 6 bp upstream of the start site. The two bands, located approximately 10 nt upstream of this putative T/C transcriptional start, map in a short AT-rich stretch that might correspond to a third processing site (ps3). The band located 4 nt downstream of the putative T/C transcriptional start map at a G/C within the loop of a small stem-loop structure that might be a substrate for the S1 nuclease. The specific degradation of the phoR part of the phoR-phoP cotranscript, as well as the transcription of phoP alone, is consistent with the necessity to produce larger amounts of the regulator PhoP than of the sensor kinase PhoR.

The results shown in Fig. 2B and 3 clearly indicate that the expression of phoP alone was weakly inducible in conditions of Pᵢ limitation in *S. lividans* TK24. This induction was even weaker in the phoU mutant strain, undetectable in both the phoR and phoP mutant strains, and much stronger in the ppp mutant strain. Furthermore, the results shown in Table 2 indicate that in all strains and conditions tested, the activity of phoPp is 30- to 100-fold higher than that of phoRp and phoUp.

Transcriptional and regulatory analysis of phoU expression. The Northern blot analysis shown in Fig. 2B that used the probe P3 and corresponding to phoU (Fig. 2A), allowed the detection of an ~0.8-kb transcript that was slightly longer than expected for phoU (~0.7 kb). The hybridization of the same Northern blot with a DNA probe corresponding to the 239-bp open reading frame (mtpA), located downstream of phoU, gave an independent ~0.3-kb band (Fig. 2B), confirming that phoU was transcribed alone. The 5′ end of the phoU transcript was mapped by high-resolution nuclease S1 mapping. The results shown in Fig. 3 revealed the presence of a unique 5′ end at the A/A, located 80 nt upstream of the putative ATG start codon, yielding an 80-nt-long untranslated mRNA leader. The proposed −10 and −35 sequences for this promoter were TAC AGG and TGGGGGT, respectively, with a spacing of 16 bp. To test the functionality of the putative phoU promoter, a 520-bp SalI fragment was cloned in the proper orientation in the promoter probe pIJ487 and indeed showed promoter activity (Table 2), whereas a PCR fragment starting 20 bp downstream of the putative transcriptional start and ending at the SalI site within phoU had no activity (data not shown). The results shown in Table 2 and Fig. 2B and 3 clearly indicate that the expression of phoU is weakly inducible in *S. lividans* TK24 when Pᵢ is limiting. This induction was undetectable in the phoR and phoP mutant strains and much stronger in the ppp mutant strain than in *S. lividans* TK24.

Transcriptional and regulatory analysis of mtpA expression. Northern blot analysis using the probe P4 corresponding to mtpA (Fig. 2A) allowed the detection of an ~0.3-kb-long monocistronic transcript. The expression of this gene was detected in the five strains tested when Pᵢ was limiting but was much stronger in the phoU mutant strain than in the other strains. Under conditions of Pᵢ sufficiency, the expression of this gene was undetectable in the parental strain and in the ppp and in the phoU mutants but was clearly detectable in the phoR and phoP mutants. As mtpA is overexpressed in a phoU mutant and encodes a small metallothionein-like protein thought to be involved in sensing and/or resistance to oxidative stress (9), we tested the ability of *S. lividans* TK24 and of the phoU, phoP, and ppp mutants to cope with an...
adaptation to Pi scarcity remains to be elucidated. As expected, phoR mutants to H2O2. phoR was clear in the parental strain but undetectable in the

Regulation of phoR-phoP, phoP, and phoU expression in the ppk mutant strain. The expression of phoR-phoP, phoP, and phoU was shown to be much higher in the ppk mutant strain than in the parental strain S. lividans TK24 under conditions of P limitation, suggesting that an unknown signal of P limitation is somehow amplified in the ppk mutant strain. As expected, the greater intensity of this signal correlates with an enhancement of antibiotic biosynthesis (8). It was previously stated that a situation of P limitation correlates with a low intracellular adenylate charge (19, 22), suggesting that a low energetic charge might be the real trigger for antibiotic biosynthesis in Streptomyces and thus the real signal for P limitation. If this hypothesis is correct, the ppk mutant strain could be predicted to have a lower intracellular adenylate charge than the wild-type strain in conditions of P scarcity, as it overproduces antibiotic. This prediction, which has to be confirmed experimentally in vivo, is consistent with the proven in vitro nucleoside diphosphate kinase activity of Ppk, which regenerates ATP from ADP and polyphosphate when the ATP/ADP ratio in the reaction mixture is low (8).

Regulation of mtpA expression in S. lividans TK24 and in the ppk, phoR, and phoP mutants of this strain. MtpA belongs to the family of metallothionein proteins known to be involved in the sensing of and/or the resistance to oxidative stress in various organisms (9). The oxyradical-scavenging capacity of proteins of this family is thought to be linked to their sulfhydryl nucleophilicity that neutralizes hydroxyl radicals, as well as to their ability to sequester transition metal (iron and copper), responsible for oxidative stress, since they display Fenton reactivity (36). The expression of these proteins is usually induced by an oxidative stress (1). The expression of mtpA was detectable in S. lividans TK24 and in the ppk mutant when P was limiting. Conditions of P limitation are known to generate an internal oxidative stress in E. coli (24) or Sinorhizobium meliloti (17), and our results suggest that this is also the case in S. lividans (Fig. 5). The expression of mtpA, like that of other metallothionein proteins (1), might thus be induced by the internal oxidative stress linked to P limitation. The phoR and phoP mutant strains were previously shown to be impaired in P scavenging and transport (32), becoming P limited much earlier than the parental strain, even when grown in a P-sufficient medium. This early limitation is likely to be responsible for the early induction of mtpA expression detected in these strains, even under P-sufficient conditions. The expression of mtpA, unlike that of phoR-phoP, phoP, phoU (this study), and ppk (unpublished data), is not under the positive control of PhoR/PhoP but might be indirectly negatively controlled by PhoR/PhoP via PhoU (see below).

Regulation of phoR, phoP, and mtpA expression in the phoU mutant. The induction of the expression of mtpA is greatly

externally applied oxidative stress under conditions of P sufficiency or scarcity.

Sensitivity of S. lividans TK24 and of phoU, phoP, and ppk mutants to H2O2. In this test, the diameters of the zones of growth inhibition were comparable (±2 mm) in three independent experiments and gave an estimate of the resistance and/or sensitivity of the strains to an externally applied oxidative stress. The results in Fig. 5 indicate that the diameters of the zones of growth inhibition were significantly larger for all strains when Pi was limiting. It is also noteworthy that the phoP and ppk mutants were both more sensitive to H2O2 than S. lividans TK24, whereas the phoU mutant strain was much more resistant.

DISCUSSION

The aim of this study was to establish the transcriptional activity of phoR and phoP and that of the divergent genes phoU and mtpA, together with the study of the regulation of expression of these genes in the parental strain S. lividans TK24 and in the isogenic mutants phoR, phoP, phoU, and ppk under conditions of phosphate scarcity or sufficiency.

Regulation of phoR-phoP, phoP, and phoU expression in S. lividans TK24 and in phoR and phoP mutants. The induction of the expression of phoR-phoP, phoP, and phoU under P limitation was clear in the parental strain but undetectable in the phoR and phoP mutant strains (Fig. 2B and 3), indicating that both PhoR and PhoP are necessary for their own transcription and that of phoU. The iron binding protein PhoU thus belongs to the regulon pho of S. lividans TK24, but its role in the adaptation to Pi scarcity remains to be elucidated. As expected from other systems, the autoregulation of the response regulator PhoP requires the sensor kinase PhoR (28, 30, 31). The more drastic phenotype of the phoP mutant compared to the phoR mutant (Fig. 1) is consistent with the observation that phoP could be transcribed alone and suggests that PhoP might be weakly active, conceivably phosphorylated by an alternative phosphate donor (12, 37), in the absence of its cognate kinase PhoR. Furthermore, phoP might also be negatively regulated by a repressor, primarily present in a Pi-sufficient medium. This repressor is expected to be titrated out in the multicopy situation, as the activity of phoPp is severalfold higher than that of phoRp and phoUp in all the strains and under the conditions tested (Table 2). The fact that the very low level expression of phoPp in a phoP mutant strain is still twofold higher under conditions of P limitation (Table 2) is consistent with this hypothesis.

Regulation of phoR-phoP, phoP, and phoU expression in the ppk mutant strain. The expression of phoR-phoP, phoP, and phoU was shown to be much higher in the ppk mutant strain than in the parental strain S. lividans TK24 under conditions of P limitation, suggesting that an unknown signal of P limitation is somehow amplified in the ppk mutant strain. As expected, the greater intensity of this signal correlates with an enhancement of antibiotic biosynthesis (8). It was previously stated that a situation of P limitation correlates with a low intracellular adenylate charge (19, 22), suggesting that a low energetic charge might be the real trigger for antibiotic biosynthesis in Streptomyces and thus the real signal for P limitation. If this hypothesis is correct, the ppk mutant strain could be predicted to have a lower intracellular adenylate charge than the wild-type strain in conditions of P scarcity, as it overproduces antibiotic. This prediction, which has to be confirmed experimentally in vivo, is consistent with the proven in vitro nucleoside diphosphate kinase activity of Ppk, which regenerates ATP from ADP and polyphosphate when the ATP/ADP ratio in the reaction mixture is low (8).
enhanced in a phoU mutant of *S. lividans* TK24 when Pi is limiting, whereas the expression of phoR-phoP and phoP is reproducibly lower than in the parental strain (Fig. 2B). We do not know whether PhoU directly represses mtpA expression or if mtpA expression is triggered by the physiological changes resulting from the interruption of phoU. In addition, it is not clear whether the overexpression of mtpA is or is not responsible for the down-regulation of phoR-phoP and phoP expression, observed with the phoU mutant strain, and for the enhanced resistance of the phoU mutant to oxidative stress. MtpA has structural (CXXC motifs) and functional similarities with Spx from *B. subtilis* (26). Spx is a small protein acting as an activator of the expression of genes encoding functions involved in maintaining thiol homeostasis. An spx null mutant is more sensitive to oxidative stress than a wild-type strain (27, 39). Spx interacts with the carboxy-terminal part of the alpha subunit of RNA polymerase, preventing the interaction of the latter with some transcriptional activators (27). By analogy with Spx, one can speculate that MtpA interferes with the PhoP-stimulated transcription of phoR-phoP and phoP and might be at least partly responsible for the very low expression of the latter in the phoU mutant strain.

**Link between phosphate limitation and oxidative stress.** A limitation in Pi obviously triggers the production of antibiotics, as well as an enhanced sensitivity to an external oxidative stress (Fig. 5). The synthesis of antibiotics requires the accumulation of ad hoc precursors stemming from primary metabolism that should be stimulated in these conditions. This stimulation is obviously more intense in the ppk mutant strain that over-produces antibiotics than in the original strain. In the ppk mutant strain, the strong induction of the expression of phoR-phoP and thus of genes of the pho regulon (Fig. 2 and 3) is expected to lead to a stimulation of P, uptake that is necessary to fulfill the needs of an active metabolism. A stimulation of central metabolic pathways leads to the generation of reduced cofactors that should be reoxidized by the respiratory chain. However, if the production of reduced cofactors exceeds the oxidative capability of the respiratory chain, reactive oxygen species (including H2O2, O2•−, and OH•) accumulate within the cell, generating an oxidative stress. The internal accumulation of these free reactive radical species could explain the increased sensitivity of the ppk mutant (and of the phoP mutant) to an externally applied oxidative stress (Fig. 5). The role of PhoU in this regulation remains unclear. If the overexpression of mtpA is triggered by the physiological changes resulting from the interruption of phoU, we can propose that PhoU might be involved in the resistance to mild oxidative stress, whereas MtpA might deal with harsher stress. In the absence of PhoU, the increase in the intracellular oxidative stress might trigger the expression of more efficient mechanisms for reduction of this stress, involving MtpA. These mechanisms, via the down-regulation of the expression of genes of the pho regulon, would lead to a reduction of the P, supply. This reduction will lead to a “slowing down” of metabolism and thus to a reduction of the internal oxidative stress. Alternatively, if PhoU directly represses mtpA expression, it could be seen as a moderator of the response to oxidative stress, aimed at limiting the “slowing down” of the metabolism.

**Conclusions.** This study clearly demonstrated interesting regulatory interactions between the genes phoR, phoP, and ppk, related to the metabolism of P, and the putative iron binding proteins encoded by phoU and mtpA. We propose that a P, limitation that is thought to correlate with a low adenylate charge triggers the activation of the central metabolic pathways, generating an internal oxidative stress. An oxidative stress being potentially deleterious for the cell, the bacteria has to precisely regulate the delicate balance between the necessary stimulation of its central metabolism to produce indispensable ATP and the potentially deleterious effects of oxidative stress resulting from this stimulation. The metalloproteins PhoU and MtpA are thought to be involved in this fine tuning.

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