The two-component *phoR-phoP* system of *Streptomyces natalensis*: Inactivation or deletion of *phoP* reduces the negative phosphate regulation of pimaricin biosynthesis

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Abstract

The biosynthesis of the antifungal pimaricin in *Streptomyces natalensis* is very sensitive to phosphate regulation. Concentrations of inorganic phosphate above 1 mM drastically reduced pimaricin production. At 10 mM phosphate, expression of all the pimaricin biosynthesis (*pim*) genes including the pathway-specific positive regulator *pimR* is fully repressed. The *phoU-phoR-phoP* cluster of *S. natalensis* encoding two-component Pho system was cloned and sequenced. Binding of the response regulator PhoP to the consensus PHO boxes in the *phoU-phoRP* intergenic promoter region was observed. A *phoP*-disrupted mutant and a *phoR-phoP* deletion mutant were obtained. Production of pimaricin in these two mutants increased up to 80% in complex yeast extract–malt extract (YEME) or NBG media and showed reduced sensitivity to phosphate control. Four of the *pim* genes, *pimS1*, *pimS4*, *pimC* and *pimG* showed increased expression in the *phoP*-disrupted mutant. However, no consensus PHO boxes were found in the promoter regions of any of the *pim* genes, suggesting that phosphate control of these genes is mediated indirectly by PhoR-PhoP involving modification of pathway-specific regulators.

Keywords: Pimaricin; Polyene; Polyketide synthase; Phosphate regulation; PHO boxes; PhoP; DNA binding

1. Introduction

Streptomyces and many other soil-dwelling or aquatic actinomycetes synthesize an impressive array of secondary metabolites, many of which show interesting biological activities (von Döhren and Grafe, 1997; Martín et al., 2000). A significant part of the genome of *Streptomyces coelicolor* (Bentley et al., 2002) and *Streptomyces avermitilis* (Omura et al., 2001; Ikeda et al., 2003) is dedicated to genetic information encoding enzymes for the biosynthesis of secondary metabolites.

Streptomyces natalensis, a strain isolated from South Africa (Natal) soil (Struyk et al., 1958), produces the antifungal polyene macrolide pimaricin (commercial name, natamycin) (Aparicio et al., 1999; Farid et al., 2000; El-Enshasy et al., 2000). Pimaricin represents a prototype molecule of glycosylated polyenes (Martín, 1977; Aparicio et al., 2004) used as an antifungal agent. Pimaricin is widely used in the food industry to prevent mold contamination of cheese and other non-sterile foods. It has also increased interest in medicine for the treatment of mycosis in immunodeficient patients (Aparicio et al., 2004). Initial studies showed that this 26-membered tetraene macrolide is synthesized in *S. natalensis* by a complex polyketide synthase (PKS) (Aparicio et al., 1999). The sequenced region of 85 kb responsible for the biosynthesis of pimaricin encodes 13 PKS modules within five multi-functional enzymes, and 12 additional proteins that

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catalyze post-PKS modifications of the polyketide skeleton (tailoring enzymes), secretion, and regulation of gene expression (Aparicio et al., 2000; Antoñ et al., 2004; Mendes et al., 2001, 2005).

The biosynthesis of antibiotics and other secondary metabolites in batch cultures occurs in a growth-dependent manner and most of these metabolites are synthesized when the growth rate decreases below a threshold level (Martıń and Demain, 1980; Champness and Chater, 1994; Bibb, 2005). Onset of secondary metabolite biosynthesis occurs usually in response to phosphate depletion or carbon or nitrogen source limitation (Martıń and Demain, 1980; Doull and Vining, 1990; McDowall et al., 1999). Macrolide biosynthesis is particularly sensitive to phosphate concentration in the culture broth (Masuma et al., 1986; Liras et al., 1990; Farid et al., 2000). It is known that the wide-domain negative phosphate regulation is exerted in some cases at the transcription level (Asturias et al., 1990; Martıń et al., 1994; McDowall et al., 1999).

We reported previously that phosphate control of actinorhodin and undecylprodigiosin biosynthesis in *Streptomyces lividans* is mediated by the two-component *phoR-phoP* system (Sola-Landa et al., 2003). However, it is unclear if a similar control mechanism occurs in different species of *Streptomyces* producing distinct classes of antibiotics, e.g., macrolides, aminoglycosides, b-lactams, tetracyclines, etc., all of which are regulated by phosphate, but to a different extent (Martıń, 2004).

Two-component regulatory systems are very abundant in *Streptomyces* species and serve as sensors and transdu-cers of a variety of nutritional and environmental signals (Hutchings et al., 2004). In order to understand if there is a molecular mechanism of phosphate control common to all actinomycetes, it was of great interest to study the transcriptional control of the pimaricin genes (*pim*) of *S. natalensis* by phosphate and the role of the PhoR-PhoP system on its biosynthesis. In this work we have cloned and characterized the PhoR-PhoP system of *S. natalensis*. Disruption of *phoP* and deletion of *phoR-phoP* showed that, indeed, phosphate control of pimaricin biosynthesis is mediated by the PhoR-PhoP system.

2. Materials and methods

2.1. Bacterial strains, cloning vectors and culture conditions

S. natalensis ATCC 27448 was routinely grown in yeast extract–malt extract (YEME) medium (Kieser et al., 2000) without sucrose. Sporulation was achieved in TBO medium (Aparicio et al., 1999). For pimaricin production, the strain was routinely grown in YEME without sucrose. These media were supplemented with thiostrepton (50 mg/ml in solid medium and 10 mg/ml in liquid medium) when used for growth of *S. natalensis* mutants carrying the thiostrepton resistance marker. *Candida utilis* (syn. *Pichia jadinii*) CECT 1061 was used for antifungal bioassay experiments. Phage KC515 (c+ attP::tsr::vph), a FC31-derived vector (Rodicio et al., 1985) was used for gene disruption. *S. lividans* JII1326 (Chater et al., 1981) was used as a host for phage propagation and transfection. Infection with FP22 (the KC515 recombinant derivative used for phoP disruption) was carried out on R5 medium (Kieser et al., 2000). Standard conditions for cultivation of *Streptomyces* species and isolation of phages were as described by Kieser et al. (2000).

2.2. Genetic procedures

Standard genetic techniques with *E. coli* and in vitro DNA manipulations were as described by Sambrook and Russell (2001). Recombinant DNA techniques in *Strepto-myces* species and isolation of *Streptomyces* total and phage DNA were performed as previously described (Kieser et al., 2000; Mendes et al., 2001; Antoñ et al., 2004). Southern hybridization was carried out with probes labeled with digoxigenin by using the DIG DNA labeling kit (Roche Biochemicals).

A *S. natalensis* ATCC 27448 library (Aparicio et al., 1999) was screened using probes derived from the *S. coelicolor* A3(2) *pho* genes (Sola-Landa et al., 2003). For *phoR* the probe used was a 1011 bp *Pvu*I internal fragment and for *phoP* the 435 bp *Not*I-*Kpn*I internal fragment.

2.3. DNA sequencing and analysis

DNA sequencing was accomplished by the dideoxynucleotide chain-termination method using the Perkin–Elmer Amplitaq Gold Big Dye-terminator sequencing system on double-stranded DNA templates with an Applied Biosystems 310 sequencer (Foster City, CA, USA). Each nucleotide was sequenced a minimum of three times on both strands. Alignment of sequence contigs was per-formed using the DNA Star program Seqman (Madison, WI). DNA and protein sequences were analyzed with the EBI BLAST server.

2.4. Isolation of total RNA

S. natalensis ATCC 27448 and *S. natalensis* P22-8 *phoP* or D*phoR-phoP* (abbreviated D*phoRP*) mutants were grown for 48 h in YEME medium without sucrose; the cultures were then mixed with the same volume of 40% glycerol, and mycelia were harvested by centrifugation and immediately frozen by immersion in liquid nitrogen. Frozen mycelium was then broken by shearing in a mortar, and the frozen lysate was added to buffer RLT (Qiagen) in the presence of 1.5% b-mercaptoethanol. RNeasy Mini Spin columns were used for RNA isolation. RNA preparations were treated with DNase I (Promega) in order to eliminate possible chromosomal DNA contamination.

2.5. Gene expression analysis by RT-PCR

Transcription was studied by using the SuperScript™ One-Step reverse transcriptase-polymerase chain reaction (RT-PCR) system with Platinum® Taq DNA polymerase (Invitrogen), using 5 ng of total RNA as template. Conditions were as follows: first-strand cDNA synthesis, 45 1C for 40 min followed by heating at 94 1C for 2 min; amplification, 28 or 26 cycles of 98 1C for 15 s, 60–70 1C (depending of the set of primers used) for 30 s, and 72 1C for 1 min). Primers (18–24 mers; Antoñ et al., 2004) were designed to generate PCR products of approximately 500 bp. Negative controls were carried out with each set of primers and Platinum® Taq DNA polymerase in order to confirm the absence of contaminating DNA in the RNA preparations. The identity of each amplified product was corroborated by direct sequencing of the PCR product.

2.6. Construction of phoP-disrupted mutants

The *phoP* gene was disrupted by a phage-mediated technique using the KC515 phage, an *attP*-defective FC31 derivative (Rodicio et al., 1985). Due to the absence of the *attP* site, lysogens can only be formed by homologous recombination. A 330 bp *Pvu*II *phoP* internal fragment was subcloned into the pBluecript KS+ *Sma*I site originating the pMVMPho3 plasmid. Then, the *Bam*HI-*Pst*I fragment containing the *phoP* internal segment was cloned into the *Bam*HI-*Pst*I sites of KC515. Transfection of *S. lividans* protoplasts (Kieser et al., 2000) resulted in a number of phage plaques that were screened by Southern hybridization for the presence of *phoP*-derived sequences. One of the recombinants, phage FP22, was randomly selected and used to infect *S. natalensis* thus allowing the selection for lysogen formation. Lysogens were selected by thiostrepton resistance on R5 medium and confirmed by Southern hybridization.

2.7. Isolation of phoR-phoP deletion mutants

Deletion of *phoR* and *phoP* genes of *S. natalensis* was made by replacing the wild-type genes with a cassette containing an apramycin-selective marker using a PCR-based system (Gust et al., 2003). The plasmid pIJ773 containing the apramycin resistance gene (*aac*(3)IV) and the *oriT* replication origin was used as a template. The mutant was constructed using the oligonucleotides 5⁰-tgggggagtgtccgacctgccgcatacgctggagtcatgATTCCGGGGA TCCGTCGACC-3⁰ and 5⁰-*tgctgtggccggccaccggccctggccgc ggccggccta*TGTAGGCTGGAGCTGCTTC-3⁰ as the forward and reverse primers, respectively (the sequence identical to *phoR* is underlined and in lower case and the sequence identical to *phoP* is in lower case italics). These two long PCR primers (59 and 58 nt) were designed to produce a deletion of *phoR* just after its start codon and a deletion of all *phoP* leaving only its stop codon behind. The 3⁰ sequence of each primer matches the right or left end of the disruption cassette (the sequence is shown uppercase in both primers). The extended resistance cassette was amplified by PCR and *E. coli* BW25113/pIJ790 bearing FA1 cosmid was electro-transformed with this cassette. Isolated mutant cosmid was introduced into non-methylat-ing *E. coli* ET12567 containing the RP4 derivative pUZ8002. Then the mutant cosmid was transferred to *S. natalensis* by intergeneric conjugation (Enrıquez et al., 2006). Double cross-over exconjugants were screened for their kanamycin sensitivity and apramycin resistance.

2.8. PhoP-binding and electrophoretic mobility shift assays

Interaction between DNA-binding domain of the response regulator PhoP ($PhoP^{DBD}$) and the PhoP-binding sequences (PHO boxes) was evidenced by electrophoretic mobility shift assays (EMSA) performed as reported previously using pure $PhoP^{DBD}$-GST (Sola-Landa et al., 2005).

2.9. Assay of pimaricin production

To assay pimaricin in culture broths, 0.5 ml of culture was extracted with 4 ml of butanol, and the organic phase was diluted in water-saturated butanol to bring the OD at 319 nm in the range of 0.1–0.4 absorbance units. Control solutions of pure pimaricin (DSM, Delft, The Netherlands) were used as standard. To confirm the identity of pimaricin, an UV–visible absorption spectrum (absorption peaks at 319, 304, 291 and 281 nm) was routinely made in a Hitachi U-2001 spectrophotometer. Quantitative determi-nation of pimaricin was performed after separation in a Waters 600 HPLC with a diode array ultraviolet detector set at 304 nm, fitted with a m-Bondapack RP-C18 column (10 mm; 3.9 × 300 mm). Elution was performed with a gradient (1 ml/min) of methanol according to the following program (methanol concentration: 50% 0–3 min, up to 90% 3–12 min, 90% 12–20 min, down to 50% 20–25 min, 50% 25–30 min). The retention time for pimaricin was 14.5 min.

3. Results

3.1. Phosphate decreases the production of pimaricin in both complex and defined media

Increasing concentrations of inorganic phosphate (2–10 mM) added at inoculation time reduced drastically the specific production (pimaricin per unit of dry weight) of this macrolide in three different complex media (YED, NBG, YEME) (Fig. 1) and at the same time stimulated the growth rate of the culture. The strong effect of phosphate was in agreement with previous information for other phosphate-controlled polyene macrolides such as candicidin (Asturias et al., 1990). Similar results were obtained when exogenous phosphate was added at inoculation time to cultures in defined Lechevalier

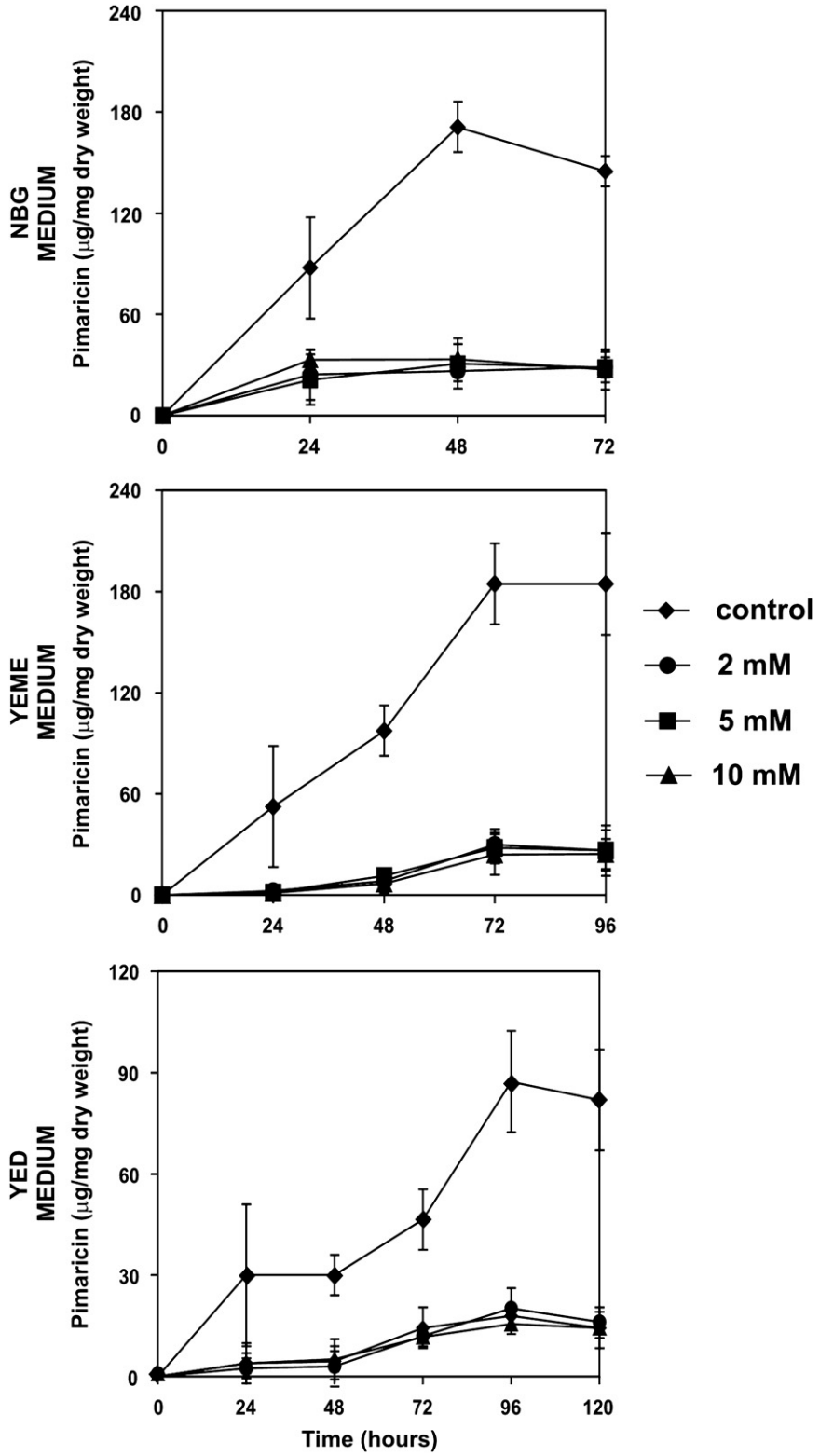

Fig. 1. Effect of increasing inorganic phosphate concentrations (2, 5 and 10 mM) on the specific production of pimaricin (expressed as mg of pimaricin per mg of dry weight) by the wild-type *S. natalensis* ATCC 27448 in three different culture media (YEME, NBG and YED). Data are the average of three duplicate flasks. Vertical bars indicate standard deviation of the mean values.

medium (not shown), although the pimaricin levels obtained in this medium were only about 5% of those obtained in complex YEME or NBG media. Because of the drastic effect exerted by 2–10 mM phosphate, the range of phosphate tested in the subsequent experiments was reduced to 1–5 mM.

3.2. Phosphate controls negative expression of the pimaricin biosynthesis genes

The degree of expression of different *pim* genes in control (unsupplemented) and phosphate-supplemented cultures was studied by RT-PCR. Total RNA was prepared from *S. natalensis* wild type after growth for 48 h in YEME medium in the absence or presence of added phosphate (10 mM), and used as template for gene expression analysis by RT-PCR. Primers for RT-PCR were specific to sequences within the 17 *pim* genes (Antoñ et al., 2004) and were designed to produce cDNAs of approximately 500 bp. A primer pair designed to amplify a cDNA of the *lysA* gene (encoding diaminopimelate decarboxylase) (Antoñ et al., 2004) was used to amplify the *lysA* mRNA as an internal standard of primary metabolism genes not involved in pimaricin biosynthesis.

All 17 *pim* genes were transcribed at 48 h in *S. natalensis* in the absence of phosphate. However, when we analyzed the transcription pattern in the presence of 10 mM phosphate, we found no transcripts for any of the genes of the cluster (Fig. 2). It is interesting that the transcription pattern of the *lysA* (control) gene in phosphate-supplemented cultures was comparable to that in its absence (Fig. 2, panel B). This result indicates that *lysA* (a primary metabolism gene) is not repressed by phosphate, thus validating the rep-ression described above for the 17 genes of pimaricin biosynthesis.

In the presence of lower concentrations of phosphate (1, 2.5 or 5 mM) amplification of the transcripts of all the *pim* genes was still observed, indicating that the degree of transcription of the *pim* genes at low phosphate concentrations is sufficient to be detected by the RT-PCR reaction.

3.3. Cloning of the phoR-phoP cluster of S. natalensis

The *phoR-phoP* cluster of *S. natalensis* was identified by screening the *S. natalensis* ATCC 27448 cosmid library (Aparicio et al., 1999) with two probes internal to the homologous *S. coelicolor* genes: (i) a 1011 bp *Pvu*I fragment for the *phoR* gene; and (ii) a 435 bp *Not*I-*Kpn*I fragment for the *phoP* gene. A cosmid, FA1, was found to hybridize with both probes, suggesting that both *phoR* and *phoP* were linked. A 4.0 kb *Pst*I fragment hybridizing with both probes, was identified in the FA1 cosmid, cloned into *Pst*I-digested pBluescript KS+ vector (named pMVMPho1) and finally sequenced.

The *Pst*I fragment was 3940 bp long. Bioinformatic analysis of this nucleotide sequence showed three complete and one incomplete open reading frames (Fig. S1, Supplementary material 1) with the characteristic codon bias (high GC content in the third position of triplets) of *Streptomyces* species. Sequence analysis of the four deduced proteins showed that three of these genes corresponded to the *phoU-phoR-phoP* cluster in *S. nata-lensis*. They have a similar genetic organization with the *phoU-phoR-phoP* clusters found in *S. coelicolor*, *S. avermitilis* and *S. lividans* chromosome (Fig. S1). Also, the nucleotide sequence and the deduced amino acid sequences proved to be very similar. The first gene, named *phoU*, is 678 bp long and encodes a 225-aa protein (a putative phosphate transduction signal modulator) (Ghorbel et al., 2006); it showed high similarity with the SAV3974 protein from *S. avermitilis* (85% identical amino acids), the SCO4228 protein from *S. coelicolor* (85% identity) and the PhoU protein from *S. lividans* (82% identity), all of which are homologous to the *E. coli* PhoU protein (Surin et al., 1986).

Upstream from *phoU* and divergently transcribed, we found a second gene, named *phoR*, encoding a 427-aa

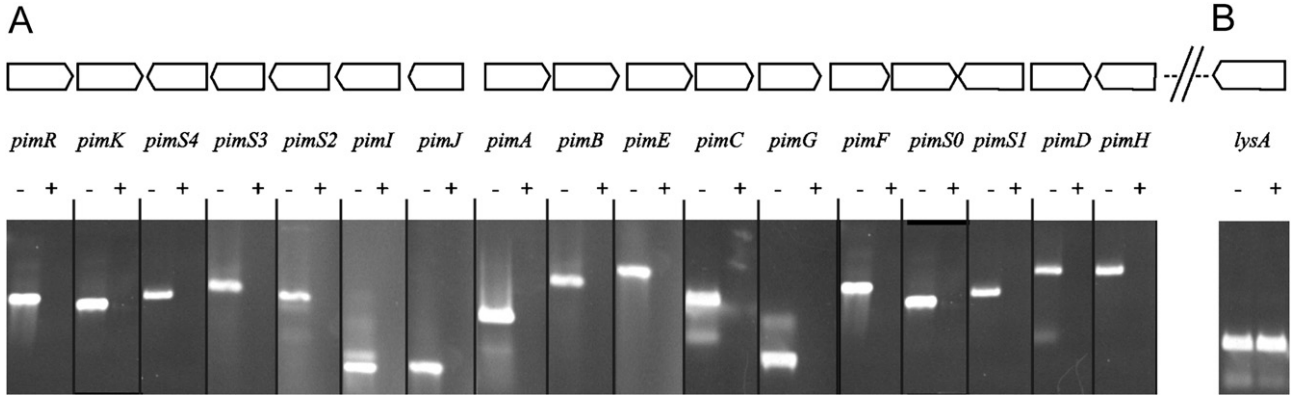

Fig. 2. Repression by phosphate of the expression of the 17 different *pim* genes in *S. natalensis* as shown by RT-PCR after 28 PCR cycles. (A) The arrangement of the genes in the *pim* cluster is indicated by open arrows in the map above the gels (the length of the arrows is not proportional to the size of the genes). RT-PCR analyses were performed on *S. natalensis* wild type in the absence (—) or presence (+) of 10 mM added phosphate as indicated in Materials and Methods. The identity of each amplified PCR product was corroborated by direct sequencing. The absence of contaminating DNA in the RNA samples was confirmed by PCR reactions without reverse transcriptase. Due to its small size, transcription of *pimF* was assessed by using a reverse primer located within the coding sequence of the gene located downstream from it (i.e., *pimS0*) (Antoñ et al., 2004). (B) Expression of the *lysA* gene (meso-diaminopimelate decarboxylase) located outside the *pim* cluster, was determined, as a control. This analysis was carried out three times for each primer pair.

protein. Comparison of the PhoR protein with the protein sequence database revealed 82% identity with the SAV3973 protein from *S. avermitilis* and 79% identity with the SCO4229 protein from *S. coelicolor* and the PhoR protein from *S. lividans* (Sola-Landa et al., 2003). PhoR displays all of the characteristics of the sensor proteins of the PhoR family of two-component systems, including the H, N, D/F and G motifs (Fig. S1, panel B). The putative phosphorylation site was located at His-165 (in the H motif) by homology with the well-known sensor kinases of *E. coli*.

Downstream from *phoR*, and only five nucleotides apart, we located the third complete gene named *phoP*. It encodes a 223-aa protein showing high similarity with proteins SAV3972 from *S. avermitilis* and SCO4230 from *S. coelicolor* (85% identical amino acids in both cases) and the PhoP protein from *S. lividans* (81% identity). It encodes the putative response regulator of the two-component system and it consists of two domains: (i) an amino-terminal response regulator receiver domain (amino acid 1–113), which receives the signal from the sensor protein by means of phosphorylation of the Asp-49 residue, and (ii) a carboxyl-terminal HTH DNA binding domain (amino acid 190–201) similar to that of the OmpR family (Fig. S1, panel C). All three proteins constitute a putative regulatory/modulation protein complex involved in the phosphate control system (Sola-Landa et al., 2003; Martín, 2004). A fourth incomplete open reading frame (named *lpp*) encodes a putative lipoprotein.

3.4. Four PHO boxes are present in the phoRP-phoU bidirectional promoter region

The nucleotide sequences protected by the PhoP response regulator in several promoters of phosphate-regulated genes in *S. coelicolor* (so-called PHO boxes) have been identified recently (Sola-Landa et al., 2005). Two boxes, each consisting of two 11-nucleotide repeated units (DRU-U1 to DRU-U4) were found in the *S. coelicolor phoU* direction. The footprinting also revealed a 46-nucleotide coincident region protected in the complemen-tary strand (*phoR* sense) (Sola-Landa et al., 2005).

A comparative study of the sequence of the bidirectional promoter *phoRP-phoU* of *S. natalensis* with that of *S. coelicolor* revealed the presence of eight 11-nucleotide direct repeats (i.e., four full PHO boxes, Fig. 3). The conservation of the PHO boxes with respect to the standard $^{1}G^{G/T}TCAYYYR^{G/C}G^{11}$ PHO box sequence was even better in the *S. natalensis phoU-phoR* promoter region than in *S. coelicolor*.

3.5. PhoP binds with high affinity to the phoU-phoR promoter region of S. natalensis

Binding of purified PhoP^{DBD}-GST to the PHO boxes in the *phoU-phoR* region of *S. natalensis* was studied using a 490 bp *Pvu*I fragment of this region obtained from plasmid pMVMPho1 and compared to the binding to the same region of *S. coelicolor*. As shown in Fig. 3, PhoP^{DBD}-GST binds strongly to the *S. natalensis phoU-phoR* region resulting in a total retardation of the digoxigenin-labeled fragment that did not occur with the same affinity in *S. coelicolor* in which part of the labeled DNA fragment remained unretarded at the same protein concentration (compare panels 4B and C). Two retardation DNA–protein bands were observed in *S. coelicolor*, whereas three bands (arrows) were observed in the *S. natalensis* DNA fragment and most of the complex corresponds to the highest-molecular-weight band indicating that probably four PhoP dimers are bound to the region of *S. natalensis*.

3.6. A PhoP defective mutant shows increased pimaricin yield and is less sensitive to phosphate control

Initially, *S. natalensis* could not be transformed by the classical protoplast transformation procedure. In order to disrupt the *phoP* gene, we took advantage of the ability of phage KC515, an *attP*-defective FC31 derivative to infect *S. natalensis*. A recombinant phage was constructed containing a 330 bp *Pvu*II internal fragment of *phoP* inserted into the phage KC515 (Fig. S2, Supplementary material). *S. natalensis* was infected in order to obtain lysogens which had integrated the recombinant phage by homologous recombination (Fig. S2, panel A).

Ten lysogens were obtained by selection for thioestrepton resistance. One of these mutants was randomly selected and named *S. natalensis* P22-8. The mutant identity was confirmed by Southern hybridization (Fig. S2, panel B). Chromosomal DNAs isolated from the parental strain *S. natalensis* ATCC 27448 and mutant P22-8 were digested with both *Pvu*I and *Pst*I or with both *Bgl*II and *Pst*I and probed with the 330 bp *Pvu*II fragment of *phoP* used to construct phage FP22. Hybridization bands of 1.1 and 1.3 kb for the *Pvu*I-*Pst*I digestion, and 1.9 and 2.9 kb for the *Bgl*II-*Pst*I digestion were obtained for the mutant (Fig. S2, panel B). These hybridization patterns indicate that a single crossover event had occurred with the phage derivative and correspond exactly to the patterns expected according to the integration shown in Fig. S2, resulting in a *phoP*-defective mutant.

Results of cultures of the *phoP*-disrupted P22-8 mutant in complex YEME medium showed that this mutant behaves as a pimaricin overproducer in the absence of added phosphate, reaching a 110–120% of the pimaricin produced by the wild-type strain (Fig. S3, supplementary material). Moreover, the mutant still produced high pimaricin levels in phosphate (1 mM)-supplemented YEME medium (similar to the production in non-supplemented cultures), in contrast to what occurred in the parental wild-type strain where biosynthesis of pimaricin was severely reduced by 1 mM and suppressed by higher concentrations of phosphate (Fig. 1). At 2.5 mM phosphate, the *phoP* mutant still produced about 50% of the pimaricin level in the unsupplemented cultures in

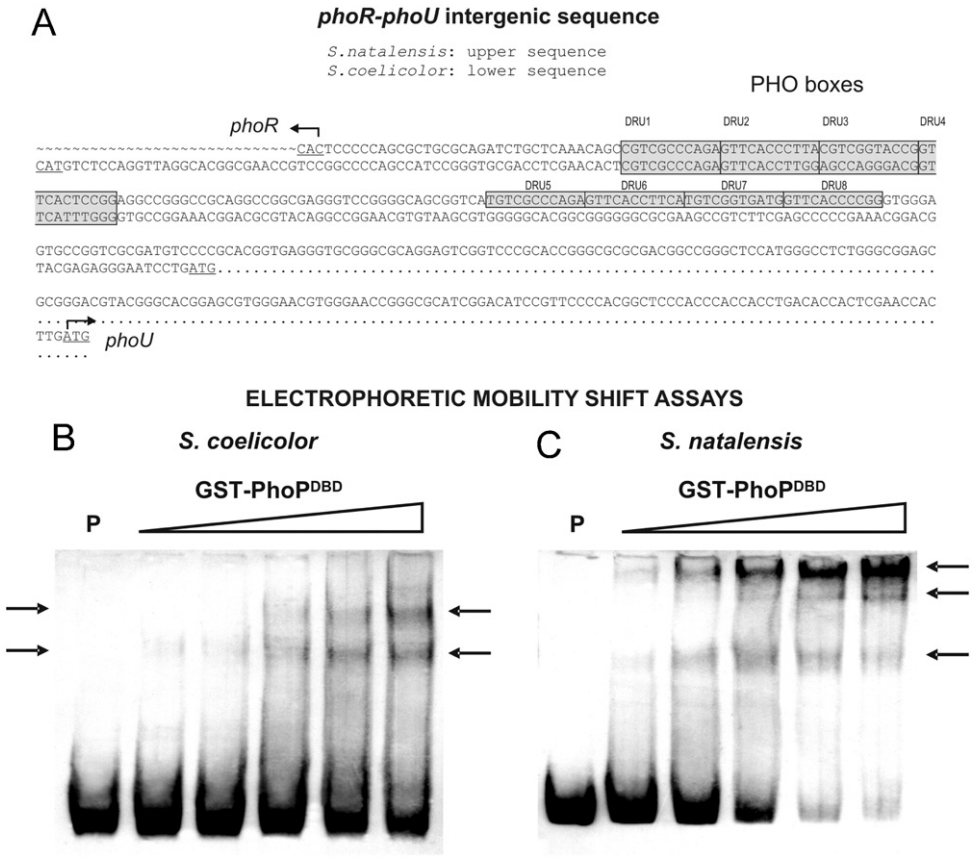

Fig. 3. (A) PHO boxes (PhoP-binding sequences) in the *phoU-phoRP* intergenic region of *S. natalensis* and *S. coelicolor*. Note the presence in *S. natalensis* of eight direct repeats (DRU1–DRU4 and DRU5–DRU8) forming four complete PHO boxes. The ATG and GTG triplets corresponding to the amino terminal end of PhoU and PhoR, respectively, are underlined. Note that the intergenic region is larger in *S. natalensis* than in *S. coelicolor*. (B and C) Binding of pure PhoP^{DBD}-GST to the intergenic *phoU-phoR* region of *S. coelicolor* (B) and *S. natalensis* (C), as shown by electrophoretic mobility shift assays. The DNA fragments were labeled with digoxigenin and mixed with increasing concentrations of PhoP^{DBD}-GST (0.2, 0.4, 0.8, 1.6 and 3.2 pMoles of PhoP^{DBD}-GST protein). Lane P, probe without protein, as indicated in the figure. Note the formation of two DNA–protein bands in *S. coelicolor* and at least three in *S. natalensis* (arrows); one of them corresponds to a high-molecular-weight (slow-moving) complex.

YEME medium, whereas the parental strain was unable to synthesize significant amounts of pimaricin at this phosphate concentration. The regulation by high phosphate concentrations observed in the *phoP* P22-8 mutant suggests that alternative phosphate control mechanisms, other than that mediated by PhoP occur in *S. natalensis* (see Discussion).

The disrupted mutant P22-8 cultures in YEME medium accumulated significant amounts of an extracellular polymeric material (see below) that may affect the yield of pimaricin production by this mutant.

The behavior of the *S. natalensis* P22-8 *phoP* mutant was studied in more detail in defined Lechevalier medium. Growth of the *phoP* mutant required higher phosphate concentration than the wild-type strain. Normal growth of the P22-8 *phoP* mutant in defined Lechevalier medium was strictly dependent upon the phosphate concentration. Even at 2.5 mM phosphate, the growth rate was still lower than that of the parental strain (not shown). Growth limitation was not significant in the complex YEME or NBG media, suggesting that organic phosphate or other components in the complex media compensate the deficiency in inorganic phosphate transport in the *phoP*-disrupted mutant.

3.7. Four pim genes showed differential phosphate regulation in the phoP-disrupted mutant

In order to understand the changes in *pim* gene expression in the *phoP*-disrupted mutant, total RNA was prepared from *S. natalensis* wild-type and mutant P22-8 after growth for 48 h in YEME without added phosphate and used as template for gene expression analysis by RT-PCR as indicated above. Transcripts from the 17 genes of the *pim* cluster were analyzed after 28 PCR cycles, since differences in transcription patterns could be observed using this number of cycles. Whenever 28 PCR cycles revealed clear different transcription intensities between the parental strain and the disrupted mutant, the analysis was repeated at 26 cycles. Four genes showed clear differences in their expression levels in both strains after 26 cycles: two of the pimaricinolide synthase genes, *pimS1*, and *pimS4* (Aparicio et al., 2000); *pimC* encoding an aminotransferase needed for mycosamine biosynthesis; and *pimG* which

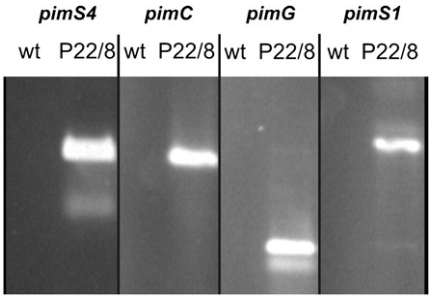

Fig. 4. Comparative expression of *pimS4*, *pimC*, *pimG* and *pimS1* in the wild-type *S. natalensis* and in the *phoP*-disrupted mutant P22-8, as shown by RT-PCR using primers corresponding to each gene. RNA was extracted from cultures in YEME medium without added phosphate. The RT-PCR analysis was carried out after 26 PCR amplification cycles. The remaining genes of the *pim* cluster showed a similar transcription pattern in both *S. natalensis* wild type and the mutant after 26 (no significant amplification) or 28 PCR cycles (amplification observed, see text). The *lysA* gene was used as control, as in Fig. 2 (not shown).

encodes a P450 monooxygenase putatively involved in the macrolide ring modification (Aparicio et al., 2003). In those cases, transcription was clearly observed in the P22-8 mutant after 26 PCR cycles, whereas it was absent in the parental strain (Fig. 4). Expression of the other *pim* genes is not observed after 26 PCR cycles in cultures of the P22-8 mutant in unsupplemented YEME medium, as occurs also with these genes in the parental strain.

These results suggest that the promoters of the above-mentioned four *pim* genes are regulated in the wild-type strain by PhoP either directly or indirectly, and hence pimaricin production is negatively regulated. In the P22-8 mutant, where PhoP is not present, transcription of these four genes is derepressed at higher phosphate concentrations than in the wild-type strain.

3.8. Deletion of phoR-phoP genes of S. natalensis

The P22-8 mutant might be unstable, losing the phage insert, unless supplemented with thiostrepton. Therefore, the REDIRECT gene replacement strategy was used for obtaining a deletion mutant lacking *phoR-phoP*, as indicated in Materials and Methods. One transformant resistant to apramycin and sensitive to kanamycin was randomly selected and the mutation was verified by PCR (Fig. S4, supplementary material) and Southern blot (Fig. S4, panel B) analysis. A 1462 bp PCR band corresponding to the extended apramycin resistance cassette (that replaced the *phoR-phoP* genes) was amplified only when using DNA of the mutant strain as template (Fig. S4, panel A). A hybridizing 3.9 kb *Pst*I band was observed for the wild type when probed with *phoP* (643 bp fragment), whereas no band was observed for the mutant; additionally, a 2 kb band was observed for the mutant when probed with the *aac*(3)IV (apramycin resistance) fragment (935 bp; Fig. S4, panel B), as expected. These hybridization results confirmed that the *phoR-phoP* genes have been deleted and replaced by the apramycin resistance marker in this mutant (hereafter named D*phoRP*).

3.9. Both the disrupted and the D*phoRP mutant showed a higher optimal phosphate concentration for pimaricin production*

The optimal phosphate concentration and the repressing phosphate levels for the wild type, the disrupted P22-8 and the D*phoRP* mutant were compared in YEME and NBG medium. Cultures in YEME medium, particularly those of the D*phoRP* mutant, were distorted by the extracellular accumulation of large amounts of a fibrillar material, probably a hyphal sheath component that may include pimaricin, that was not accumulated by the parental strain. This polymeric compound is not produced in NBG medium and, therefore, this medium was used for further studies.

Results of three different experiments showed that in all cases the best pimaricin production by cultures of the two mutants was obtained in NBG with 1 mM added phos-phate, whereas the production of the wild type was reduced by 50% by this phosphate concentration (Fig. 5).

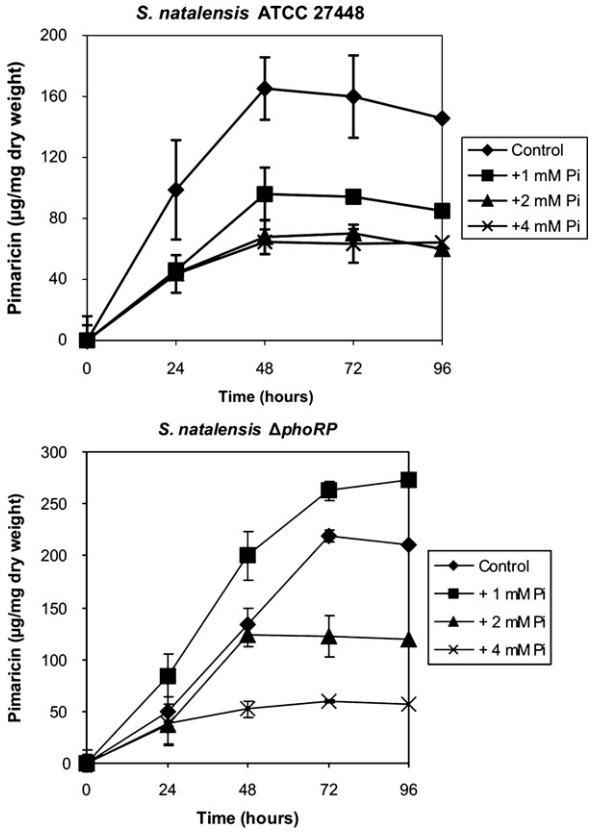

Fig. 5. Comparative effect of increasing phosphate concentrations on pimaricin production in NBG medium by the deletion D*phoRP* mutant (lower panel) and the parental strain (upper panel). Data are the average of four samples from duplicate experiments. Vertical bars indicate the standard deviation values. Note that the D*phoRP* mutant overproduces pimaricin both in phosphate unsupplemented and in medium supplemented with 1 mM phosphate.

The results were similar for both the P22-8-disrupted mutant and the D*phoRP* mutant and indicate that inactivation of *phoP* or deletion of *phoR-phoP* switches the optimal phosphate concentration to higher levels and allows the use of industrial complex media that are frequently rich in phosphate.

4. Discussion

As shown in this article, the biosynthesis of the polyene macrolide pimaricin in wild-type *S. natalensis* is very sensitive to repression by inorganic phosphate. Concentra-tions of phosphate as low as 2 mM when added to complex YEME medium suppress almost completely the production of pimaricin, in contrast to the lower sensitivity of other classes of secondary metabolites such as cephamycins (Aharonowitz and Demain, 1977; Romero et al., 1984) that are only inhibited by phosphate concentrations above 10–20 mM. In this respect, the high sensitivity of pimaricin biosynthesis to phosphate is very similar to that of candicidin, another polyene macrolide (Martín and Demain, 1976; Asturias et al., 1990), suggesting that expression of the macrolide synthase genes is probably more sensitive to phosphate than expression of genes encoding other types of antibiotics (e.g., peptide antibiotics formed by non-ribosomal peptide synthetases). The biosynthesis of tetracyclines (McDowall et al., 1999) and some other polyketides formed by type II PKSs (e.g., actinorhodin) (Doull and Vining, 1990; Sola-Landa et al., 2003) is also particularly sensitive to phosphate repression. In a given strain (e.g., *S. coelicolor*), the expression of genes encoding different secondary metabolites is affected differentially due to the involvement of distinct signal transduction cascades (Martín, 2004). The negative control of pimaricin biosynthesis by phosphate is exerted at the transcription level, as shown by RT-PCR studies of the *pim* genes. No transcripts were detected in the presence of 10 mM phosphate for any of the 17 *pim* genes analyzed, including the transcriptional activator *pimR*. Transcriptional control by phosphate of genes involved in antibiotic biosynthesis has been reported in a few other cases (Martín et al., 1994). It is clearly established that phosphate control of the biosynthesis of candicidin and oxytetracycline is exerted at the transcriptional level (Asturias et al., 1990; McDowall et al., 1999).

Advanced studies on phosphate control of secondary metabolite biosynthesis have been performed with the model actinomycetes *S. coelicolor* and *S. lividans* (Doull and Vining, 1990; Hobbs et al., 1992). Phosphate regula-tion of expression of the actinorhodin (*act*) and undecyl-prodigiosin (*red*) genes is mediated by the PhoR-PhoP two-component regulatory system (Sola-Landa et al., 2003).

In this work, we have characterized the *phoR-phoP* cluster of *S. natalensis*. The cloned genes encode a PhoR-PhoP two-component system of class IIIA (Fabret et al., 1999). The response regulator PhoP belongs to the OmpR type, as in most class IIIA two-component systems (Hutchings et al., 2004). The *phoU-phoR-phoP* arrangement is conserved in the four *Streptomyces* species studied so far (Fig. S1).

An important finding reported in this article is that expression of at least four *pim* genes and, therefore, pimaricin biosynthesis is controlled by the PhoR-PhoP system. Indeed, disruption of *phoP* (resulting in the loss of the response regulator PhoP) leads to derepression of *pimS1*, *pimS4* (involved in the formation of the polyketide aglycone), *pimC*, which encodes an aminotransferase needed for mycosamine biosynthesis, and *pimG*, encoding a P450 monooxygenase (Aparicio et al., 2000, 2003). This results in the increased production of pimaricin (in the range of 25–30%) by the *phoP* mutants in complex media such as YEME or NBG supplemented with 1 mM phosphate. Similar results were observed when either the phage-disrupted *phoP* mutant or the *phoR-phoP* deleted null mutant were tested in submerged cultures in fermen-tors. However, when more phosphate is added to the fermentation (2.5 mM or higher) biosynthesis of pimaricin by both *phoP* mutants is still partially sensitive to phosphate, mainly because high phosphate concentrations stimulate growth and there is a rapid termination of the production phase in the *phoP* mutants.

The *phoR-phoP* operon is autoregulated by PhoP. Four consensus PHO boxes (PhoP-binding sites in DNA protection experiments; Sola-Landa et al., 2005) were found in the intergenic *phoU-phoRP* region of *S. natalensis* containing the *phoU* and *phoRP* promoters, whereas only two PHO boxes occur in the same intergenic region of *S. coelicolor*. The degree of conservation of this sequence in the direct repeats of each PHO box appears to be related to the intensity of phosphate repression (Sola-Landa et al., 2005; A. García-Rodríguez and J.F. Martín, unpublished results). PhoP-binding experiments confirmed that there is a strong binding of PhoP to the four PHO boxes in the *phoU-phoR* intergenic region of *S. natalensis*; this finding indicates that expression of *phoU* and *phoRP* is strongly self-regulated by PhoP. Since the regulation is positive (A. Sola-Landa and J.F. Martín, unpublished data), the presence of four PHO boxes will lead to a burst of *phoR-phoP* expression following phosphate starvation (detected by the PhoP sensor) until a later decay of the encoded proteins; this may explain the strong phosphate regulation observed in this strain.

No PHO boxes were found in the promoter regions of *pimS1*, *pimS4*, *pimC* and *pimG* genes, thus excluding direct binding of PhoP to the promoters of these genes. There appears to be a signal transduction cascade involving sequential protein phosphorylation/dephosphorylation (i.e., a phosphorelay) (Umeyama et al., 2002) from PhoP to the pathway-specific regulators, rather than a direct activation by PhoP of the *pim* genes.

Some of the complex media used for industrial pimaricin production may contain enough phosphate to reduce antibiotic production. The inorganic phosphate content of unsupplemented YED, YEME and NBG media were

1.60, 0.68 and 0.80 mM, respectively, that at least in the case of YED is repressive for pimaricin biosynthesis, which correlates with the lower production obtained in this medium.

Phosphate-deregulated mutants were previously isolated by classical mutation and selection techniques in several *Streptomyces* species of industrial interest (Martín et al., 1979; Hanel et al., 1984), but directed disruption or deletion of *phoP*, as described in this article is a simpler procedure to obtain clean phosphate-deregulated strains.

Accession number—The sequences reported in this article have been deposited in the GenBank database under the accession number AM176576.

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