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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 Gräfe, 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; Antón 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 (Martín 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 (Martín 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; Martín 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, β-lactams, tetracyclines, etc., all of which are regulated by phosphate, but to a different extent (Martín, 2004).

Two-component regulatory systems are very abundant in *Streptomyces* species and serve as sensors and transducers 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 μg/ml in solid medium and 10 μg/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 ΦC31-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 ΦP22 (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 *Streptomyces* species and isolation of *Streptomyces* total and phage DNA were performed as previously described (Kieser et al., 2000; Mendes et al., 2001; Antón 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 performed 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 Δ*phoR-phoP* (abbreviated *ΔphoRP*) 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% β-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.