PimM, a PAS domain positive regulator of pimaricin biosynthesis in *Streptomyces natalensis*

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Sequencing of the DNA region on the left fringe of the pimaricin gene cluster revealed the presence of a 579 bp gene, *pimM*, whose deduced product (192 aa) was found to have amino acid sequence homology with bacterial regulatory proteins. Database comparisons revealed that PimM combines an N-terminal PAS domain with a C-terminal helix-turn-helix (HTH) motif of the LuxR type. Gene replacement of *pimM* from the *Streptomyces natalensis* chromosome with a mutant version lacking the HTH DNA-binding domain resulted in complete loss of pimaricin production, suggesting that PimM is a positive regulator of pimaricin biosynthesis. Complementation of the ΔpimM mutant with a single copy of *pimM* integrated into the chromosome restored pimaricin production. The insertion of a single copy of *pimM*, with its own promoter, into the *S. natalensis* wild-type strain boosted pimaricin production. Gene expression analyses in *S. natalensis* wild-type and ΔpimM by reverse transcriptase PCR (RT-PCR) of the pimaricin gene cluster revealed the targets for the PimM regulatory protein. According to these analyses, the genes responsible for initiation and first elongation cycles of polyketide chain extension are among the major targets for regulation. Other *pim* genes are differentially affected. Interestingly, our results indicate that PimM plays its regulatory role independently of PimR, the first pathway-specific regulator of pimaricin biosynthesis.

INTRODUCTION

Bacteria belonging to the genus *Streptomyces* have attracted great interest due to their well known ability to produce a variety of antibiotics and other secondary metabolites. Production of these compounds is regulated in response to nutritional status alteration and a variety of environmental conditions, and hence occurs in a growth-phase-dependent manner and is usually accompanied by morphological differentiation (Martin et al., 2000; Bibb, 2005).

Control of secondary metabolite production is a complex process involving multiple levels of regulation. The highest levels include genes that exert a pleiotropic control over one or more aspects of secondary metabolism, such as antibiotic production or morphological differentiation (for reviews see Champness & Chater, 1994; Martin, 2004). The lowest level, however, is composed of regulatory genes that only affect a single antibiotic biosynthetic pathway. These pathway-specific regulatory genes are usually found within the respective antibiotic biosynthesis gene cluster, a feature that has greatly facilitated their study. Recently, this scenario has been complicated by the finding of pathway-specific regulators that can alter the expression of other pathways, and also modulate the effects of regulators that act more globally (Huang et al., 2005).

Regulators often sense chemicals in the environment and respond to changes in their concentration. The most well known example is constituted by the two-component systems involved in phosphorelay signal transduction (Hoch, 2000; West & Stock, 2001; Foussard et al., 2001). In these systems, a sensory protein phosphorylates a response-regulator in response to an extracellular stimulus (such proteins are usually membrane proteins), and the latter then binds to specific promoter sequences in the DNA, activating or repressing transcription. Two-component regulatory systems are very abundant in *Streptomyces* species and serve as sensors and transducers of a variety of nutritional and environmental signals (Sola-Landa et al., 2003, 2005; Hutchings et al., 2004; Mendes et al., 2007a).

The PAS domain is a small regulatory module represented in proteins of all kingdoms of life. It is a signal module that monitors changes in light, redox potential, oxygen, overall

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Abbreviation: HTH, helix-turn-helix.

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energy level of a cell, and small ligands (Taylor & Zhulin, 1999). PAS domains were first found in eukaryotes, and were named after homology to the *Drosophila* period protein (Per), the aryl hydrocarbon receptor nuclear translocator protein (ARNT) and the *Drosophila* single-minded protein (Sim). Unlike most other sensors, proteins containing PAS domains are located in the cytosol, and therefore they detect internal signals, but they can also sense environmental factors that cross the cell membrane. The majority of prokaryotic PAS domains function as sensor modules of sensor kinases of two-component systems (Taylor & Zhulin, 1999).

Pimaricin is a tetratera macrolide antifungal antibiotic produced by *Streptomyces natalensis*. As a polycyclic, its antifungal activity lies in its interaction with membrane sterols, thus causing the alteration of membrane structure and leading to the leakage of cellular materials (Aparicio et al., 2004). As for other macrocyclic polyketides, pimaricin is synthesized by the action of so-called type I modular polyketide synthases (Aparicio et al., 2003), and its biosynthetic gene cluster has been characterized (Aparicio et al., 1999, 2000; Mendes et al., 2001, 2005, 2007b; Antón et al., 2004).

Here, we describe the cloning, sequencing and detailed characterization of a novel class of PAS domain-containing regulator in *S. natalensis* which does not belong to a two-component system, and demonstrate its role as a transcriptional activator for pimaricin biosynthesis in this bacterium.

**METHODS**

**Bacterial strains, cloning vectors and cultivation.** *S. natalensis ATCC 27448* was routinely grown in YEME medium (Kieser et al., 2000) without sucrose. Sporulation was achieved in TBO medium (Aparicio et al., 1999). For pimaricin production, the strain was grown in YEME without sucrose. The same media were supplemented with thiostrepton when used for *S. natalensis* 40D9-1 growth and/or metabolite production. *E. coli* strain XL1-Blue MR (Stratagene) was used as a host for plasmid subcloning in plasmids pBluescript (Stratagene), pUC18 and pUC19. *E. coli* ET12567 (pUZ8002) was used as donor in intergeneric conjugations. *Candida utilis* (syn. *Pichia jadinii*) CECT 1061 was used for bioassay experiments. Phage KC515 (c*"* attP::tsr::vph), a 4C31-derived phage (Rodicio et al., 1985), was used for gene replacement experiments. *Streptomyces lividans* III 1326 (Chater et al., 1981) served as a host for phage propagation and transfection. Infection with 40D9 (the KC515 recombinant derivative used for gene replacement) was carried out on R5 medium (Kieser et al., 2000). Standard conditions for culture of *Streptomyces* species and isolation of phages were as described by Kieser et al. (2000).

**Genetic procedures.** Standard genetic techniques with *E. coli* and in *vitro* DNA manipulations were as described by Sambrook & Russell (2001). Recombinant DNA techniques in *Streptomyces* species and isolation of *Streptomyces* total and phage DNA were performed as described by Kieser et al. (2000). Southern hybridization was carried out with probes labelled with digoxigenin by using the DIG DNA labelling kit (Roche Biochemicals). Intergeneric conjugation between *E. coli* ET12567(pUZ8002) and *S. natalensis* was performed as described by Enríquez et al. (2006).

**DNA sequencing and analysis.** Sequencing templates were obtained by random subcloning of fragments generated by controlled partial *Hae*III digestions. 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. Each nucleotide was sequenced a minimum of three times on both strands. Alignment of sequence contigs was performed using the DNASTAR program SEQUAN. DNA and protein sequences were analysed with the NCBI World Wide Web BLAST server.

**Construction of a pimM mutant.** A 4083 bp Accl fragment encompassing the entire *pimM* gene, and part of the *pimR* gene (Fig. 1), was cloned into an Accl-cut pUC19 vector to yield pMV5M00. This plasmid was then used as a source of DNA for both sequencing of *pimM* and for obtaining the DNA fragment used for gene replacement.

Using plasmid pMV5M00 as a template, a 1.4 kb DNA fragment containing the whole of *pimM* was amplified by PCR with primers DWM (5'-AAACGGCGAATCTTGGCCAAAGGATTG-3') and UPM (5'-AGCCAGAGATCGGACCCGAGGACACCTG-3') (underlined sequences indicate BglII restriction sites). The PCR product was digested with BglII and ligated into a BamHI-cut pUC19 vector to yield pMV600.

The *pimM* gene was disrupted by KC515 phage-mediated gene replacement as follows. Plasmid pMV600 was digested with *ApaI* and religated to yield pMV6620. This treatment eliminates a 387 bp *ApaI* fragment that encodes the C-terminal end (123 aa) of PimM which contains the helix–turn–helix (HTH) domain for DNA binding (Stevens et al., 1994), and results in a mutant *pimM* gene truncated beyond the new *ApaI* site. A 1 kb *SacI*–*PstI* fragment containing the mutant *pimM* sequence was cloned into the same sites of KC515 (Kieser et al., 2000). Transfection of *Streptomyces lividans* protoplasts (Kieser et al., 2000) resulted in a number of phage plaques that were screened by Southern hybridization for the presence of *pimM*-derived sequences. One of the recombinants, 40D9, was selected and used to infect *S. natalensis*, thus allowing selection for lysogen formation. Lysogens were selected by thiostrepton resistance on R5 medium. Gene replacement was sought by repeated rounds of non-selective growth in liquid YE medium without sucrose, and the loss of the phage was confirmed by genomic Southern hybridization.

**Construction of plasmids for gene complementation and gene dosage increase.** In order to complement the *pimM* replacement mutant, a 1 kb DNA fragment containing the entire *pimM* gene, including its own promoter, was amplified by PCR with primers *PMD* (5'-TCTGTTATGGCGGCGGATCGCTATCTCATGGAGTCG3') and FMR (5'-GTTTATGTGGACTTGGTACCGTACGTGC-3') (the underlined sequences indicate *BamHI* restriction sites). The PCR product was digested with *BamHI* and ligated into *BamHI*-cut pSET152 (AmrR, pUC18 replicon, *ApaI* derived, *Bierman et al.*, 1992) to yield pSETpimM. This plasmid was then transferred by conjugation from *E. coli* ET12567(pUZ8002) to the *S. natalensis* APimM mutant as described by Enríquez et al. (2006).

**Isolation of total RNA.** *S. natalensis ATCC 27448* and *S. natalensis* *ApimM* were grown for 48 h in YE medium without sucrose (stationary phase). The cultures were then mixed with one volume 40 % (v/v) 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 % (v/v) β-mercaptoethanol. RNasey Mini Spin columns were used for RNA isolation according to manufacturer’s instructions. RNA preparations were treated with DNase I (Promega) to eliminate possible chromosomal DNA contamination.

**Gene expression analysis by reverse transcriptase PCR (RT-PCR).** Transcription was studied by using the SuperScript One-Step
RT-PCR system with Platinum Taq DNA polymerase (Invitrogen), using 5 ng total RNA as template. Conditions were as follows: first strand cDNA synthesis, 45 °C for 40 min followed by heating at 94 °C for 2 min; amplification, 28 or 30 cycles of 98 °C for 15 s, 60–70 °C (depending on the set of primers used) for 30 s, and 72 °C for 1 min. Primers (18- to 24-mers; Antón et al., 2004) were designed to generate PCR products of approximately 500 bp. The primers used for the detection of the pimM transcripts were PIMMS (5' - GCTGCTGCGCACCAGTACGACG - 3') and PIMMAS (5' - CCGGCGGAGGTTGTC-3'). The 5' end of PIMMAS is located 84 nt downstream from the pimM TGA stop codon. PCR products using these primers were 661 bp for the wild-type strain, and 274 bp for the mutant. The antisense primer used for the detection of the pimM transcripts (PIMAAS) differs from that used by Antón et al. (2004), and its sequence is 5'-GACCAGCAGCACCAGGGGGATGAC-3'. The primers used for the analysis of pimAB mRNA stability were PIMABS (5'-GCTGCTGCGCACCAGTACGACG-3') and PIMABAS (5'-GCTGCTGCTGCAAGGTCTGAGAGTGTC-3'). Negative controls were carried out with each set of primers and Platinum Taq DNA polymerase 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.

**Assay of pimaricin production.** Spectrophotometric determination of pimaricin was performed as described elsewhere (Recio et al., 2004). HPLC analysis of this antifungal was carried out as described by Recio et al. (2006). The fungicidal activity of pimaricin was tested by bioassay using C. utilis CECT 1061 as test organism.

**RESULTS**

**Cloning of pimM**

pimM was identified by genomic walking using an S. natalensis ATCC 27448 cosmid library (Aparicio et al., 1999) and DNA segments from pimR (which encodes a pathway-specific regulatory gene for pimaricin production, on the left end of the pimaricin gene cluster; Antón et al., 2004). The gene was sequenced from plasmid pMVM500 (see Methods) and turned out to be separated by 396 bp from the 5' end of pimR, orientated in a divergent fashion (Fig. 1). The initiating ATG codon of pimM is preceded by the sequence AGGGAG which could act as a ribosome-binding site. pimM is 579 bp long with an overall codon usage pattern in good agreement with that of typical Streptomyces genes; however, it contains a few codons that are rare in such a G+C-rich organism. The presence of one TTA codon could be of particular interest, since its involvement in the regulation of differentiation and secondary metabolism in Streptomyces has been proposed in other Actinomycetes (Leskiw et al., 1991).

**In silico analysis of the pimM gene product**

Computer-assisted analysis of the pimM gene product (192 aa with an estimated molecular mass of 20945 Da) showed a very high sequence identity (93.8%) with the whole of protein PTEF of Streptomyces avermitilis, a putative regulatory protein of 232 aa whose gene was found within the pte gene cluster and which is thought to be involved in the biosynthesis of the pentaene filipin ( Ikeda et al., 2003). Protein database comparisons revealed three additional counterparts, all encoded by regulatory genes of polyene biosynthetic clusters. These were AmphRIV (68% identity) (amphotericin; Carmody et al., 2004), NysRIV (66.8% identity) (Nystatin; Sekurova et al., 2004) and FscRI (67.5% identity) (FR008/Candididin; Chen et al., 2003). All of them have a PAS sensor-binding domain at the N terminus (Taylor & Zhulin, 1999; Hefti et al., 2004;

![Fig. 1. Pimaricin biosynthetic gene cluster. The left fringe of the gene cluster is indicated in more detail, and includes pimM (in dark grey), and the 5' end of the transcriptional activator-encoding gene for pimaricin biosynthesis (pimR; Antón et al., 2004). Arrowed boxes indicate the direction of transcription. A, Apal; Ac, Accd; B, BamHI; M, MluI; N, NotI; Nr, NruI; Z, BglII. The inserts used for vector construction are indicated at the bottom.](image-url)
SMART 00091) as well as an HTH motif of the LuxR type at the C terminus (SMART 00421) (Fig. 2). Interestingly, PimM is 17–49 aa shorter than its counterparts. When we analysed sequence identity at the DNA level, we found a strikingly high similarity between pimM and pteF (93.4 % identity along the whole length of pimM). This sequence identity is extremely high, and indicates that both regulatory genes probably have a common evolutionary origin. Curiously, the identity at the DNA level continued beyond the pimM ATG start codon, in the non-coding region upstream of ATG. A reassignment of the start codon according to the pteF coding sequence would yield a protein of identical size to PteF. However, the presence of a TGA triplet in the pimM-elongated sequence, 69 nt upstream from the pimM ATG start codon, removes such possibility.

**Gene replacement of pimM**

The involvement of pimM in pimaricin biosynthesis was tested by gene replacement. We used phage KC515, an attP-defective φC31 derivative (Rodicio et al., 1985), to introduce DNA into *S. natalensis*. The recombinant phage used for pimM inactivation, φ40D9, was constructed as described in Methods and used to infect *S. natalensis* to obtain lysogens. Because phage KC515 and its derivative lack attP, they can only form lysogens by homologous recombination into the chromosome (Fig. 3a).

Nine lysogens of *S. natalensis* were obtained by selection for thiostrepton resistance. One of these mutants was randomly selected and named *S. natalensis* 40D9-1. The identity of the mutant was confirmed by Southern hybridization (not shown). This mutant was then used to isolate thiostrepton-sensitive derivatives that had undergone a second recombination event deleting the integrated phage. These thiostrepton-sensitive isolates were obtained after 11 rounds of non-selective growth in YEME medium. Of the three colonies isolated, two were found to have reverted to the wild-type, while the other one harboured the desired change.

This mutant, where pimM had been replaced by a mutated version of it lacking the C-terminal HTH binding site, was named *S. natalensis* ΔpimM. The truncated protein is devoid of DNA-binding ability, and its function as a regulator should therefore be lost. Chromosomal DNAs isolated from *S. natalensis* ATCC 27448 and mutant ΔpimM digested with *Mlu*I were probed with a 431 bp

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**Fig. 2.** Domain structure and amino acid sequence alignments of parts of the PimM protein. (a) Predicted domain structure of PimM. PAS, PAS sensory domain (SMART 00091); LuxR HTH, DNA-binding domain of the LuxR type (SMART 00421). (b) Sequence comparison of the N-terminal PAS domains of PimM and other regulators of polyene biosynthetic gene clusters. AmphRIV, NysRIV, FscRI and PteF are transcriptional activators of amphotericin, nystatin, candicidin and filipin clusters, respectively. (c) Sequence alignment of the C-terminal LuxR-type HTH DNA-binding domains of PimM orthologues and GerE. GerE is the transcriptional regulator of spore formation in *Bacillus subtilis*. The horizontal bars at the top represent the location of the HTH motif in the proposed DNA-binding region. Numbers indicate amino acid residues from the N terminus of the protein. Identical residues are shown in bold. An asterisk (*) signifies a conservative substitution.
NotI–Ncol fragment covering most of pimM (Fig. 3a). A hybridizing band of 1.98 kb was found for the wild-type as expected (Fig. 3b), whereas in the mutant, the hybridizing band was 1.6 kb (Fig. 3b), indicating that a double crossover event had occurred. The observed hybridizing bands corresponded exactly to those expected, according to the integration process depicted in Fig. 3.

The new strain S. natalensis DpimM had growth and morphological characteristics identical to those of S. natalensis wild-type when grown on solid or liquid media, suggesting that PimM has no role in bacterial growth or differentiation. The spore counts of both strains were similar after growth for 9 days at 30°C on TBO plates. The spores of both strains were serially diluted and plated on minimal medium. Both strains grew well in minimal medium, showing an identical growth curve, which indicates that genes involved in amino acid biosynthesis were not affected. Also, no differences in pigment production were observed in the mutant, suggesting that the regulator might be specific for pimaricin biosynthesis.

**Inactivation of pimM blocked pimaricin biosynthesis and gene complementation restored antifungal production**

The fermentation broth produced by the mutant strain generated by phage-mediated gene replacement, S. natalensis ΔpimM, was extracted with butanol and analysed for the presence of pimaricin. Both the microbiological bioassay against C. utilis and HPLC indicated that no pimaricin was being produced by the mutant strain ΔpimM (Fig. 4). This result indicated that PimM was a positive regulator for pimaricin biosynthesis, which together with its high sequence similarity with transcriptional activators of polyene biosynthesis (see above) raised the question of which gene/genes were the potential target of PimM activity.

To confirm that the disruption of pimM was directly responsible for the abolition of pimaricin production, we complemented the pimM-disrupted mutant with pimM. A DNA fragment containing pimM plus its putative promoter region was inserted into the integrative vector pSET152 (Bierman et al., 1992), giving rise to pSETpimM (see Methods). The recent development of a method for intergeneric gene transfer from E. coli to S. natalensis by conjugation (Enríquez et al., 2006) enabled its use for the introduction of pSETpimM into the pimM-disrupted mutant. The plasmid was thus transferred from E. coli ET12567(pUZ8002) to S. natalensis ΔpimM. Introduction of pSETpimM restored pimaricin biosynthesis to wild-type levels. These results were fully consistent with those obtained upon replacement of the pimM gene, and confirmed the involvement of PimM in pimaricin biosynthesis.

**Duplication of gene dosage increases pimaricin production**

When we introduced pSETpimM into S. natalensis wild-type, whereas no significant change in the growth curve in flask cultures was observed, a substantial increase in the specific production of pimaricin was detected (Fig. 5), thus suggesting that PimM availability does constitute a bottleneck in the biosynthesis of the antifungal. The increase in

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**Fig. 3.** Gene replacement of pimM. (a) Predicted restriction enzyme polymorphism caused by gene replacement. The MluI restriction pattern before and after replacement is shown. The probe is indicated by thick lines. The fragment used for gene disruption is derived from pMVM620 (see text for details). A, Apal; M, MluI; N, NotI; Nc, NcoI; S, SacII; Sa; SacI. (b) Southern hybridization of MluI-digested chromosomal DNA of wild-type (lane 1) and ΔpimM (lane 2) strains.
pimaricin production ranged between 2.4-fold after 48 h of growth to 1.5-fold after 96 h, and taking into consideration the similar growth curves of the strain with an extra copy of \( \text{pimM} \) and the parental strain, one can conclude that it is mostly derived from a higher volumetric production in the strain with a double gene dosage, which reached 1.48 g l\(^{-1} \) at its maximum (72 h).

**Transcriptional control of pimaricin production**

Total RNA was prepared from *S. natalensis* wild-type and mutant \( \Delta \text{pimM} \) after growth for 48 h (when pimaricin is actively produced; Mendes et al., 2001) and used as template for gene expression analysis by RT-PCR. Primers for RT-PCR were specific to sequences within \( \text{pim} \) genes (see Methods) and were designed to produce cDNAs of approximately 500 bp. A primer pair designed to amplify a cDNA of the \( \text{lysA} \) gene (encoding diaminopimelate decarboxylase) was used as an internal control. Transcripts were analysed from the 18 genes of the \( \text{pim} \) cluster, including \( \text{pimM} \), after 28 PCR cycles. In the case of \( \text{pimM} \), transcripts were analysed using a sense primer located before the deletion (PIMMS), and an antisense primer located after the stop codon (PIMMAS) (see Methods). Whenever 28 cycles did not yield a product, analysis was repeated at 30 cycles. These analyses were carried out at least three times for each primer pair.

All 18 genes were transcribed at 48 h in *S. natalensis* wild-type; however, when we analysed the transcription pattern in *S. natalensis \( \Delta \text{pimM} \), we found no transcripts for the genes \( \text{pimD, pimS1, pimS0, pimF, pimG, pimC and pimB} \) (Fig. 6a) even after increasing the number of cycles to 30 (Fig. 6a), indicating that the promoters controlling the expression of these genes are likely targets, directly or indirectly, for PimM regulatory control. Similarly, no transcripts were found for the genes \( \text{pimI and pimL} \) (Fig. 6a), but in these cases increasing the number of cycles allowed the detection of a very small amount of their transcripts (Fig. 6a), thus suggesting that the mutant retains some transcription of these \( \text{pim} \) genes albeit at very low levels. Transcription was also substantially reduced for \( \text{pimK and pimA} \), and for the polyketide synthase-encoding genes \( \text{pimS2, pimS3 and pimS4} \), although in these cases, the transcripts were clearly detected after 28 cycles of amplification (Fig. 6a). Interestingly, no difference in the transcription pattern was observed for the genes \( \text{pimR, pimE and pimH} \) (Fig. 6a), thus suggesting that \( \text{pimM} \) and the first regulatory gene for pimaricin biosynthesis \( \text{pimR} \) (Antón et al., 2004) function independently of each other (see Discussion). A similar transcription pattern was also observed for \( \text{pimM} \), although with different size RT-PCR amplification products for the wild-type and the mutant; given that primers are located at both sides of the deletion, this indicates that transcription proceeded unabated across the site of the in-frame deletion in \( \text{pimM} \). This result indicates that the gene replacement does not have a polar effect on the transcription of genes located downstream from \( \text{pimM} \), and also that PimM is not auto-regulated. The transcription pattern of \( \text{lysA} \) (a primary metabolism gene) was comparable in *S. natalensis \( \Delta \text{pimM} \) and in the parental strain (Fig. 6a), thus validating the results described above.

Analysis of the transcription pattern of the \( \text{pimM} \) gene in the *S. natalensis \( \Delta \text{pimR} \) mutant previously obtained (Antón et al., 2004) revealed that \( \text{pimM} \) is not regulated by PimR (Fig. 6b).

The strict control of PimM on the transcription of \( \text{pimS0} \), which encodes the polyketide synthase responsible for starting polyketide chain construction (Aparicio et al.,
is sufficient to explain the lack of pimaricin production in the mutant. *pimS0* most probably forms a multicistronic operon together with *pimC, pimG* and *pimF* (Antón et al., 2004), and hence all of them are controlled coordinately. The genes *pimD* and *pimS1* are transcribed as monocistronic units as can be deduced from their chromosomal arrangement in a divergent fashion (Fig. 6a). The lack of *pimS1* transcription in the mutant strain could also account for the lack of pimaricin production in the mutant.

The differential control of PimM on the transcription of *pimJ* and *pimI* when compared with that of *pimS2, pimS3* and *pimS4* was somehow unexpected because, due to the absence of apparent transcriptional terminators in the short intergenic regions between them, all these genes were thought to form an operon resulting in a transcript of more than 42 kb (Aparicio et al., 2000). These results now could indicate that the above-indicated genes are actually transcribed from at least two different transcriptional units, namely *pimJI* and *pimS2S3S4*. However, in the absence of evidence indicating that all the transcripts are equally stable it is also possible that the multicistronic transcript could be processed and subject to different rates of RNA degradation.

Interestingly, *pimA* and *pimB* showed a different transcription pattern. While transcription of *pimA* was reduced in *S. natalensis* ApimM when compared to the parental strain, no transcripts could be detected for *pimB* in the mutant (Fig. 6a), even after increasing the number of cycles to 30 (Fig. 6a). Since both genes are thought be translationally coupled, given that their coding sequences overlap (*pimB* start codon is located 23 bp upstream from the *pimA* stop codon; Aparicio et al., 2000), the most likely explanation for this result is the fast degradation of *pimB* transcripts. To check this possibility we designed primers to give an amplification band covering the 3′ end (last 142 nt) of *pimA* and the 5′ end (first 465 nt) of *pimB* (Fig. 7). The result of the RT-PCR analysis showed a band of similar intensity to that obtained with *pimA* primers, thus indicating that the 3′ end of the *pimAB* mRNA was particularly prone to degradation in the mutant.

**DISCUSSION**

Two distinct regulators of pimaricin biosynthesis are encoded by genes located in the *pim* cluster, *pimR* and *pimM*. PimR is the archetype of a novel class of regulators that combines an N-terminal domain corresponding to the SARP family of transcriptional activators, a central domain with similarity to the NTP-binding motif of the LuxR family of DNA-binding proteins, and a C-terminal domain that resembles guanylate cyclases. Gene disruption of *pimR* totally abrogated *pimE* transcription, and also reduced the transcription of all the key enzyme-encoding genes for pimaricinolide construction to very low levels, thus blocking pimaricin production completely (Antón et al., 2004).

Sequencing of the left-hand side of the pimaricin gene cluster revealed the presence of a gene, *pimM*, which could play a role as a regulator for pimaricin biosynthesis in *S. natalensis*. Computer-assisted analysis of PimM revealed that it has an N-terminal region strikingly similar to PAS sensory domains (Taylor & Zhulin, 1999; Hefti et al., 2004) and a C-terminal region with a LuxR-type HTH motif for DNA binding. The presence of a PAS-like domain within PimM suggests that this protein could respond to the energy levels in the cell (Ponting & Aravind, 1997), while the HTH motif suggests the ability of PimM to bind DNA...
(Stevens et al., 1994) and thus regulate the expression of pimaricin genes. The absence of pimaricin production upon disruption of the gene by removal of the HTH domain clearly indicates that PimM is an activator of pimaricin biosynthesis. It constitutes the second activator of pimaricin biosynthesis, after PimR (Antón et al., 2004).

Our results indicate that the control of pimaricin biosynthesis exerted by PimM takes place through the specific transcriptional activation of some key enzyme-encoding genes for pimaricinolide construction. These include pimS0 and pimS1, the genes that encode the polyketide synthases responsible for polyketide chain initiation and the first four elongation cycles of polyketide chain construction (Aparicio et al., 2000). The lack of expression of any of them could explain the lack of pimaricin production in the mutant. PimM also controls the expression of other genes such as pimC (encoding transaminase, putatively involved in the biosynthesis of the mycosamine moiety of pimaricin), pimG (cytochrome P450 mono-oxygenase candidate for the formation of the exocyclic carboxyl group) and pimF (ferredoxin) (Mendes et al., 2005). This is not surprising, since they are thought to form a multicistronic operon together with pimS0 (Antón et al., 2004). The epoxidase-encoding gene pimD is also controlled by PimM. pimD is transcribed as a monocistronic unit whose expression is controlled most probably by a bidirectional promoter, given the divergent orientation of pimD and pimS1.

Besides these major targets for PimM control, other genes display reduced transcription upon gene disruption of pimM. Among these, the most strongly affected by the lack of PimM are pimJ (GDP-mannose dehydratase) and pimI (discrete thioesterase putatively involved in the removal of non-productive decarboxylated extender acyl groups from the polyketide synthase; Kim et al., 2002). Others, like pimS2, pimS3 and pimS4, the remaining three polyketide...
target for the NysRIV regulator (Sekurova et al., 2004), as occurs with pimS0 (functionally equivalent to nysA) for PimM. Similarly, NysRIV also controls expression from the nysH promoter, which drives the expression of the ABC transporter for nystatin secretion, as seen with the operon pimAB in the case of PimM. All these similarities suggest that both regulators might follow a similar regulatory pattern for the expression of their respective polyenes, and that this pattern could be shared by the homologous regulatory genes found in other polyene biosynthetic gene clusters.

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Regulation of pimaricin biosynthesis


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