
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 *DpimM* 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 *DpimM* 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 (Martín *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; Martín, 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 tetraene macrolide antifungal antibiotic produced by *Streptomyces natalensis*. As a polyene, 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. *Escherichia 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 wC31-derived phage (Rodicio *et al.*, 1985), was used for gene replacement experiments. *Streptomyces lividans* JII 1326 (Chater *et al.*, 1981) served as a host for phage propagation and transfection. Infection with w40D9 (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 SEQMAN. DNA and protein sequences were analysed with the NCBI World Wide Web BLAST server.

Construction of a *pimM* mutant. A 4 083 bp *AccI* fragment encompassing the entire *pimM* gene, and part of the *pimR* gene (Fig. 1), was cloned into an *AccI*-cut pUC19 vector to yield pMVM500. 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 pMVM500 as a template, a 1.4 kb DNA fragment containing the whole of *pimM* was amplified by PCR with primers DWM (59-AAAGCGGCAGATCTTCGGCAAGGATTC-39) and UPM (59-AGCAGAGATCTGGACGCGCAGCGGCACTC-39) (underlined sequences indicate *Bgl*II restriction sites). The PCR product was digested with *Bgl*II and ligated into a *Bam*HI-cut pUC19 vector to yield pMVM600.

The *pimM* gene was disrupted by KC515 phage-mediated gene replacement as follows. Plasmid pMVM600 was digested with *Apa*I and religated to yield pMVM620. This treatment eliminates a 387 bp *Apa*I 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 *Apa*I site. A 1 kb *Sac*I–*Pst*I 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, w40D9, 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 YEME 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 (59-TCCTGGATCCGCCCTGTGCCCGCTCACTTCACGAAG-TCG-39) and PMR (59-GGTTGGATCCTTCGGTTCGGTGGTGC-GGGCATTACGG-39) (the underlined sequences indicate *Bam*HI restriction sites). The PCR product was digested with *Bam*HI and ligated into *Bam*HI-cut pSET152 (Am^R, pUC18 replicon, wC31 *attP*; Bierman *et al.*, 1992) to yield pSETpimM. This plasmid was then transferred by conjugation from *E. coli* ET12567(pUZ8002) to the *S. natalensis* DPimM mutant as described by Enríquez *et al.* (2006).

Isolation of total RNA. *S. natalensis* ATCC 27448 and *S. natalensis* DPimM were grown for 48 h in YEME 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. RNeasy 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

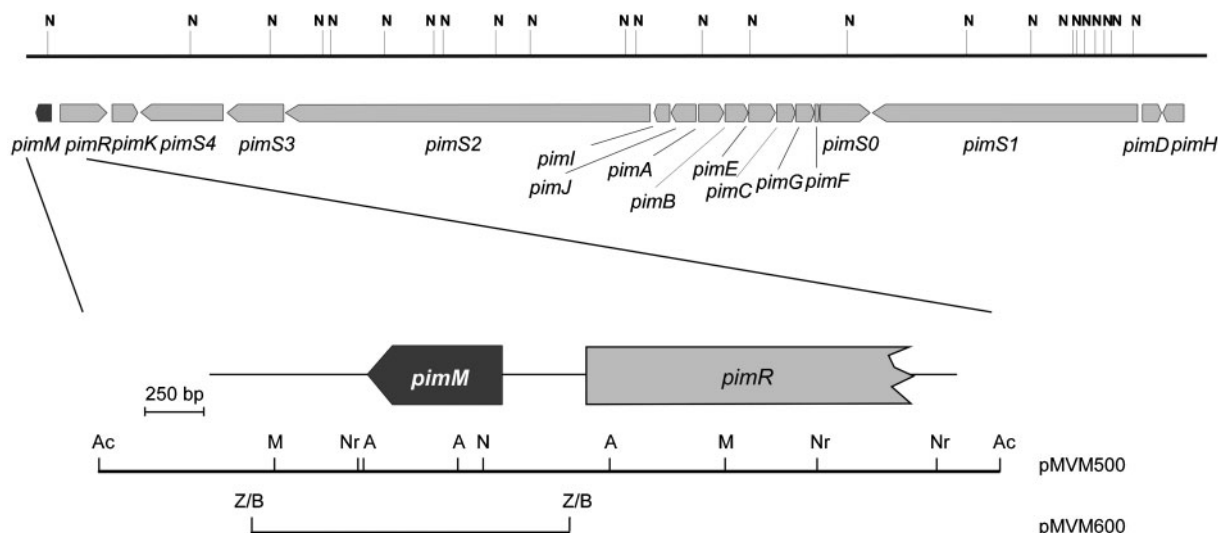


Fig. 1. Pimaricin biosynthetic gene cluster. The left fringe of the gene cluster is indicated in more detail, and includes *pimM* (in dark grey), and the 59 end of the transcriptional activator-encoding gene for pimaricin biosynthesis (*pimR*; Antón *et al.*, 2004). Arrowed boxes indicate the direction of transcription. A, *ApaI*; Ac, *Accl*; B, *BamHI*; M, *MluI*; N, *NotI*; Nr, *NruI*; Z, *BglII*. The inserts used for vector construction are indicated at the bottom.

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 uC for 40 min followed by heating at 94 uC for 2 min; amplification, 28 or 30 cycles of 98 uC for 15 s, 60–70 uC (depending on the set of primers used) for 30 s, and 72 uC 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 (59-TGGCGAGCCTTGATAGAACATTGAC-39) and PIMMAS (59-CCG-GTGGCGCAGGGTGAAC-39). The 59 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 *pimA* transcripts (PIMAAS) differs from that used by Antón *et al.* (2004), and its sequence is 59-GACCAGCAGCACCAGGGGGATGAC-39. The primers used for the analysis of *pimAB* mRNA stability were PIMABS (59-GCTGCTGCGCACCAGTACGACG-39) and PIMABAS (59-GCT-GAGGGTCTGCTGGAGGGTCTGCTGGAGGTTGTC-39). 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 59 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 20 945 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;

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 pimarin biosynthesis was tested by gene replacement. We used phage KC515, an *attP*-defective wC31 derivative (Rodicio *et al.*, 1985), to introduce DNA into *S. natalensis*. The recombinant phage used for *pimM* inactivation, w40D9, 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* DpimM. 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 DpimM digested with *MluI* were probed with a 431 bp

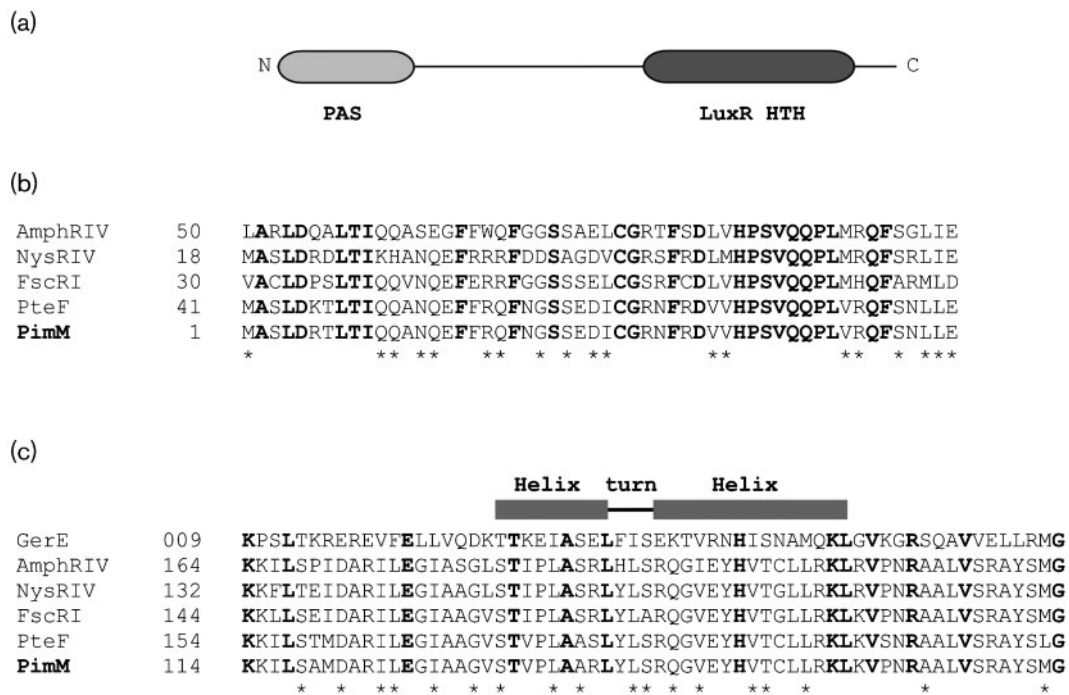


Fig. 2. Domain structure and amino acid sequence alignments of parts of the PimM protein. (a) Predicted domain structure of PimM. PAS, PAS sensory domain (SMART 00091); LuxR HTH, DNA-binding domain of the LuxR type (SMART 00421). (b) Sequence comparison of the N-terminal PAS domains of PimM and other 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.

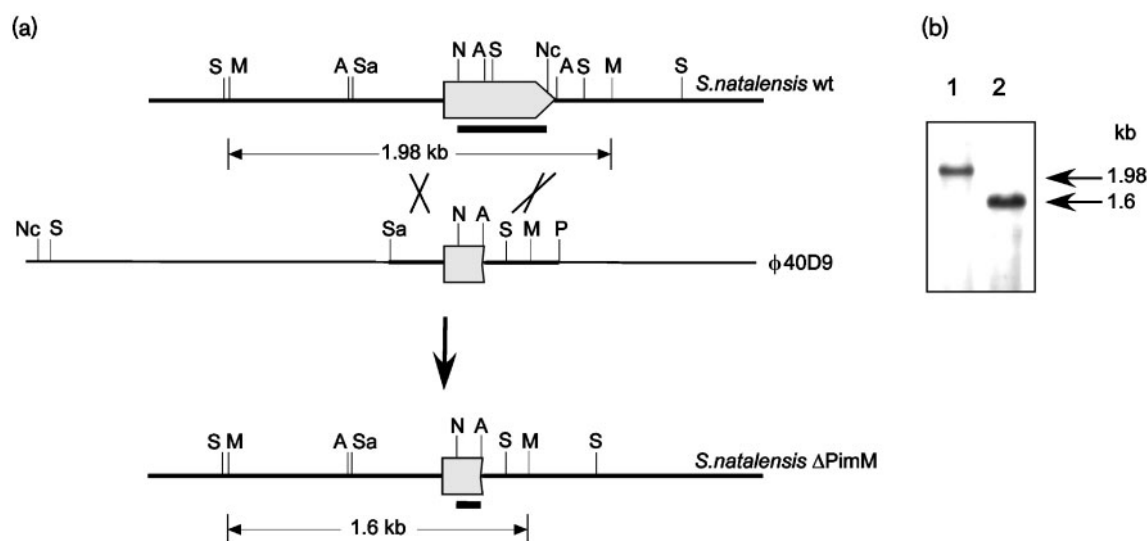


Fig. 3. Gene replacement of *pimM*. (a) Predicted restriction enzyme polymorphism caused by gene replacement. The *MluI* restriction pattern before and after replacement is shown. The probe is indicated by thick lines. The fragment used for gene disruption is derived from pMVM620 (see text for details). 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 *DpimM* (lane 2) strains.

NotI–*NcoI* 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 DpimM*, 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 *DpimM* (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 (Enr iquez *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 DpimM*. 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

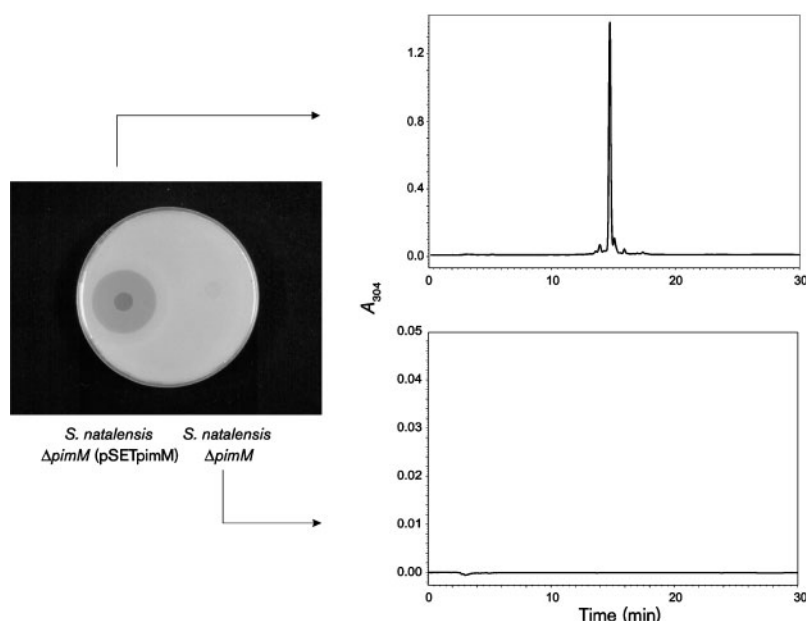


Fig. 4. Replacement of PimM blocks pimarin production, and gene complementation restores antifungal biosynthesis. Comparison of HPLC analyses of butanol-extracted broths from *S. natalensis* Δ pimM (bottom) and *S. natalensis* Δ pimM transformed with pSETpimM (top). Detection was carried out at A_{304} . Cells were grown for 48 h with shaking at 300 r.p.m. and at 30 °C in YEME medium. Bioassays for pimarin production are included on the left using *C. utilis* as test organism.

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 *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 pimarin production

Total RNA was prepared from *S. natalensis* wild-type and mutant *DpimM* after growth for 48 h (when pimarin 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 *pim* genes (see Methods) 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) was used as an internal control. Transcripts were analysed from the 18 genes of the *pim* cluster, including *pimM*, after 28 PCR cycles. In the case of *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* *DpimM*, we found no transcripts for the genes *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 *pimJ* and *pimI* (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 *pim* genes albeit at very low levels. Transcription was also substantially reduced for *pimK* and *pimA*, and for the polyketide synthase-encoding genes *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 *pimR*, *pimE* and *pimH* (Fig. 6a), thus suggesting that *pimM* and the first regulatory gene for pimarin biosynthesis *pimR* (Antón *et al.*, 2004) function independently of each other (see Discussion). A similar transcription pattern was also observed for *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 *pimM*. This result indicates that the gene replacement does not have a polar effect on the transcription of genes located downstream from *pimM*, and also that PimM is not auto-regulated. The transcription pattern of *lysA* (a primary metabolism gene) was comparable in *S. natalensis* *DpimM* and in the parental strain (Fig. 6a), thus validating the results described above.

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

The strict control of PimM on the transcription of *pimS0*, which encodes the polyketide synthase responsible for starting polyketide chain construction (Aparicio *et al.*,

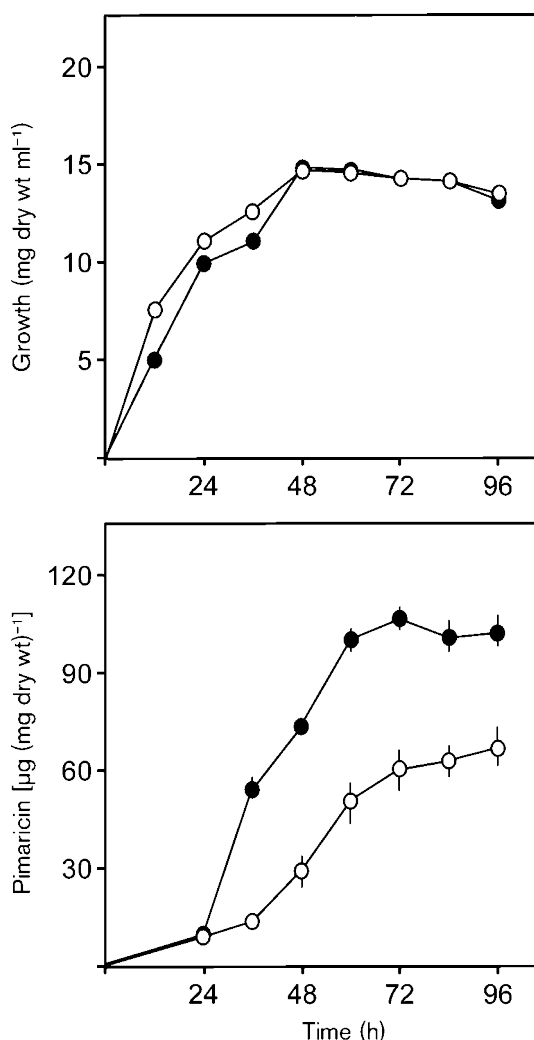


Fig. 5. Increase in *pimM* gene dosage increases pimaricin production. Specific production of pimaricin [expressed as mg pimaricin (mg dry wt)⁻¹] in YEME medium without sucrose (lower panel). Open circles, production by the wild-type strain; solid circles, production observed in the strain with an extra copy of *pimM*. Data are the means of three duplicate flasks. Vertical bars indicate the SD values. Growth curves are shown in the upper panel.

2000), 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 *pimJ* 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* D*pimM* 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 to 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 39 end (last 142 nt) of *pimA* and the 59 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 39 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

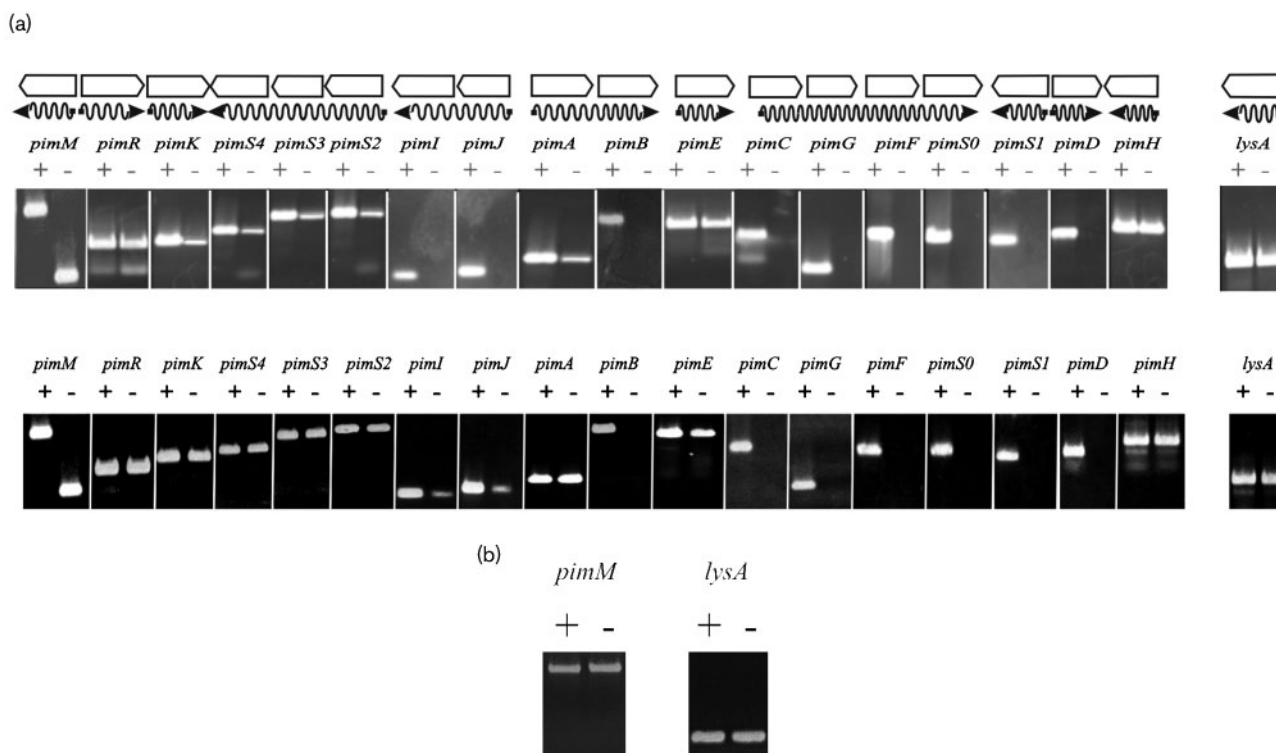


Fig. 6. Gene expression analysis of the pimarinic gene cluster by RT-PCR. (a) Analysis was carried out on *S. natalensis* wild-type (+) and *DpimM* (–) strains as indicated in Methods after 28 (top panel) and 30 (bottom panel) PCR amplification cycles. The identity of each amplified product was corroborated by direct sequencing. The absence of contaminating DNA in the RNA samples was assessed by PCR. 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*). Transcription of *pimM* or its mutated version was assessed by using a reverse primer located after the deletion, downstream from the stop codon (note the different size of the amplification products). Transcription of the *lysA* gene (encoding diaminopimelate decarboxylase) located outside the *pim* cluster was also assessed as an internal control. A diagram with the organization of the genes within the pimarinic cluster and their putative transcripts is also included (the length of the arrows is not proportional to the size of the genes). (b) Comparative expression of the *pimM* gene in the wild-type *S. natalensis* (+) and in a previously obtained *DpimR* mutant (–) (Antón *et al.*, 2004) after 28 PCR amplification cycles.

(Stevens *et al.*, 1994) and thus regulate the expression of pimarinic genes. The absence of pimarinic production upon disruption of the gene by removal of the HTH domain clearly indicates that PimM is an activator of pimarinic biosynthesis. It constitutes the second activator of pimarinic biosynthesis, after PimR (Antón *et al.*, 2004).

Our results indicate that the control of pimarinic biosynthesis exerted by PimM takes place through the specific transcriptional activation of some key enzyme-encoding genes for pimarinolide 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 pimarinic 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 pimarinic), *pimG* (cytochrome P450 mono-oxygenase candidate for the formation of the exocyclic carboxyl group) and *pimF* (ferredoxin) (Aparicio *et al.*, 2003). 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* (Mendes *et al.*, 2005) 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

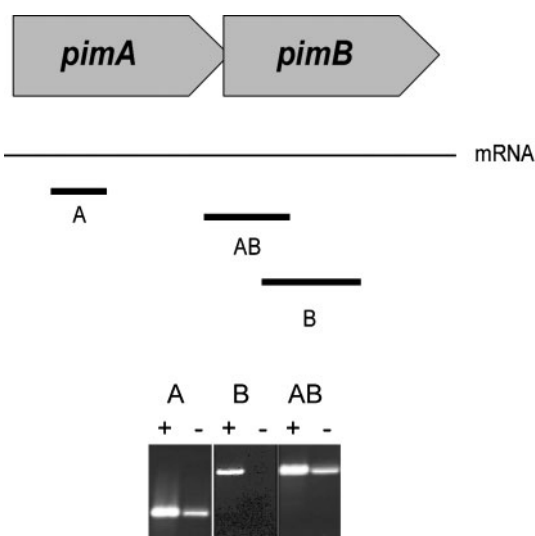


Fig. 7. Gene expression analysis of the *pimAB* operon by RT-PCR. Comparative expression in the wild-type *S. natalensis* (+) and in the *DpimM* mutant (–) was assessed using primers corresponding to different locations in the mRNA (see text). Expected amplification cDNA bands are indicated for the various primer pairs. The RT-PCR analysis was carried out after 28 PCR amplification cycles.

synthase genes responsible for the formation of the pimaricinolide (Aparicio *et al.*, 2000), *pimA* and *pimB*, which encode a putative heterodimer ABC transporter involved in pimaricin secretion (Aparicio *et al.*, 2003), and *pimK*, involved in the attachment of the mycosamine moiety during pimaricin biosynthesis (unpublished results), are also affected, although to a reduced extent.

PimM either controls multiple pimaricin biosynthetic promoters directly or activates another hierarchical regulator(s). This does not seem to be the case for *pimR*, the transcriptional activator of pimaricin biosynthesis first characterized by Antón *et al.* (2004). PimR, as well as PimM, is not auto-regulated (Antón *et al.*, 2004) and does not control *pimM* expression (Fig. 6b). Similarly, PimM does not control the expression of *pimR*, and neither that of *pimH* (efflux pump) nor *pimE* (cholesterol oxidase; Mendes *et al.*, 2007b). Since *pimE* seems to be the main target of PimR (Antón *et al.*, 2004), most probably both regulators belong to different regulatory circuits.

Only NysRIV amongst PimM orthologues has been characterized, in this case by gene disruption and *in vivo* complementation experiments (Sekurova *et al.*, 2004). According to these studies, *nysRIV* most probably directly controls the expression of nystatin biosynthetic genes, and that is also the case of *pimM* for the *pim* genes. Interestingly, the promoter that drives the expression of *nysA*, the gene responsible for the initiation of nystatin biosynthesis (Brautaset *et al.*, 2000), seems to be the major

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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