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Research Paper

Engineered biosynthesis of novel polyenes: a pimaricin derivative produced by targeted gene disruption in *Streptomyces natalensis*

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Abstract

Background: The post-polyketide synthase biosynthetic tailoring of polyene macrolides usually involves oxidations catalysed by cytochrome P450 monooxygenases (P450s). Although members from this class of enzymes are common in macrolide biosynthetic gene clusters, their specificities vary considerably toward the substrates utilised and the positions of the hydroxyl functions introduced. In addition, some of them may yield epoxide groups. Therefore, the identification of novel macrolide monooxygenases with activities toward alternative substrates, particularly epoxidases, is a fundamental aspect of the growing field of combinatorial biosynthesis. The specific alteration of these activities should constitute a further source of novel analogues. We investigated this possibility by directed inactivation of one of the P450s belonging to the biosynthetic gene cluster of an archetype polyene, pimaricin.

Results: A recombinant mutant of the pimaricin-producing actinomycete *Streptomyces natalensis* produced a novel pimaricin derivative, 4,5-deepoxypimaricin, as a major product. This biologically active product resulted from the phage-mediated targeted disruption of the gene *pimD*, which encodes the cytochrome P450 epoxidase that converts deepoxypimaricin into pimaricin. The 4,5-deepoxypimaricin has been identified by mass spectrometry and nuclear magnetic resonance following high-performance liquid chromatography purification.

Conclusions: We have demonstrated that PimD is the epoxidase responsible for the conversion of 4,5-deepoxypimaricin to pimaricin in *S. natalensis*. The metabolite accumulated by the recombinant mutant, in which the epoxidase has been knocked out, constitutes the first designer polyene obtained by targeted manipulation of a polyene biosynthetic gene cluster. This novel epoxidase could prove to be valuable for the introduction of epoxy substituents into designer macrolides.

Keywords: Cytochrome P450 monooxygenase; Epoxidase; Pimaricin; Polyene; Polyketide synthase

1. Introduction

Pimaricin is a 26-member polyene macrolide antifungal antibiotic [1] produced by *Streptomyces natalensis* ATCC 27448, which is widely used in the food industry in order to prevent mould contamination of cheese and other non-sterile foods (i.e. sausages, ham, etc.). Polyenes are planar macrolide ring structures that interact with sterols with high affinity [1], and are therefore active against fungi, but not bacteria. Their antifungal activity lies in their interaction with membrane sterols, thus causing the alteration of membrane structure and leading to the leakage of cellular materials. Structurally, they are characterised by a hydroxylated macrocycle containing one (amino)sugar, but their distinct characteristic is the presence of a chromophore formed by a system of three to seven conjugated double bonds in the macrolactone ring. As other macrocyclic polyketides, pimaricin is synthesised by the action of so-called type I modular polyketide synthases (PKSs) [2,3] in a process resembling fatty acid biosynthesis [4–7], except that the β-keto function introduced at each elongation step may undergo all, part or none of a reductive cycle comprising β-ketoreduction and dehydration (other macrolide synthase products also suffer enoyl reduction (for an example see [8]). These enzymes usually consist

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of several extremely large polypeptides in which different modules (sets) of enzymatic activities catalyse each successive round of elongation [9,10], and so does pimaricin synthase. Recently, we have characterised the organisation of the pimaricin biosynthetic gene cluster and found 13 modules of a PKS distributed within five giant multienzyme proteins [3].

The structural variety in the polyene class of products arises, as in other macrocyclic polyketides, from the choice of monomers, the extent of β-ketoreduction and dehydration, and the stereochemistry of each chiral centre. Yet further diversity is produced by functionalisation of the polyketide chain by the action of glycosylases, methyltransferases and oxidative enzymes.

Such oxidative enzymes are usually cytochrome P450 monooxygenases which catalyse the site-specific oxidation of the precursors to many macrolide antibiotics. Examples include erythromycin [11–13], tylosin [14], oleandomycin [15], mycinamycin [16], narbomycin [17], epothilone [18,19], methymycin [20], and rapamycin [21] among others. However, although cytochrome P450s are common in macrolide gene clusters, there are considerable variations in the substrates utilised, and the positions of the resulting hydroxyl and/or epoxide substituents to such compounds introduced by these enzymes. It is therefore of great importance to identify and characterise additional macrolide monooxygenases in order to target the increasing number of novel polyketides obtained by combinatorial techniques [22–24]. Furthermore, the modification of the genes encoding such oxidative enzymes could itself lead to the generation of new polyketides.

Recently, the sequencing of the pimaricin gene cluster revealed the presence of the *pimD* gene (Fig. 1), whose deduced gene product bears striking sequence similarity to authentic cytochrome P450 monooxygenases [3]. Here we report the functional analysis of the *pimD* gene product by phage-mediated gene disruption, and the ability of the mutant thus obtained to synthesise a new pimaricin derivative, 4,5-deepoxypimaricin, as the sole fermentation product.

2. Results

2.1. Disruption of the pimD gene

Since *S. natalensis* ATCC 27448 has so far proved absolutely resistant to transformation by conventional procedures, we took advantage of the ability of phage KC515, an *attP*-defective ∅C31 derivative [25], to infect *S. natalensis* in order to introduce DNA into this strain. The recombinant phage used for *pimD* disruption, ∅6D4, was constructed as described in Section 5, and used to infect *S. natalensis* in order to obtain lysogens. Because

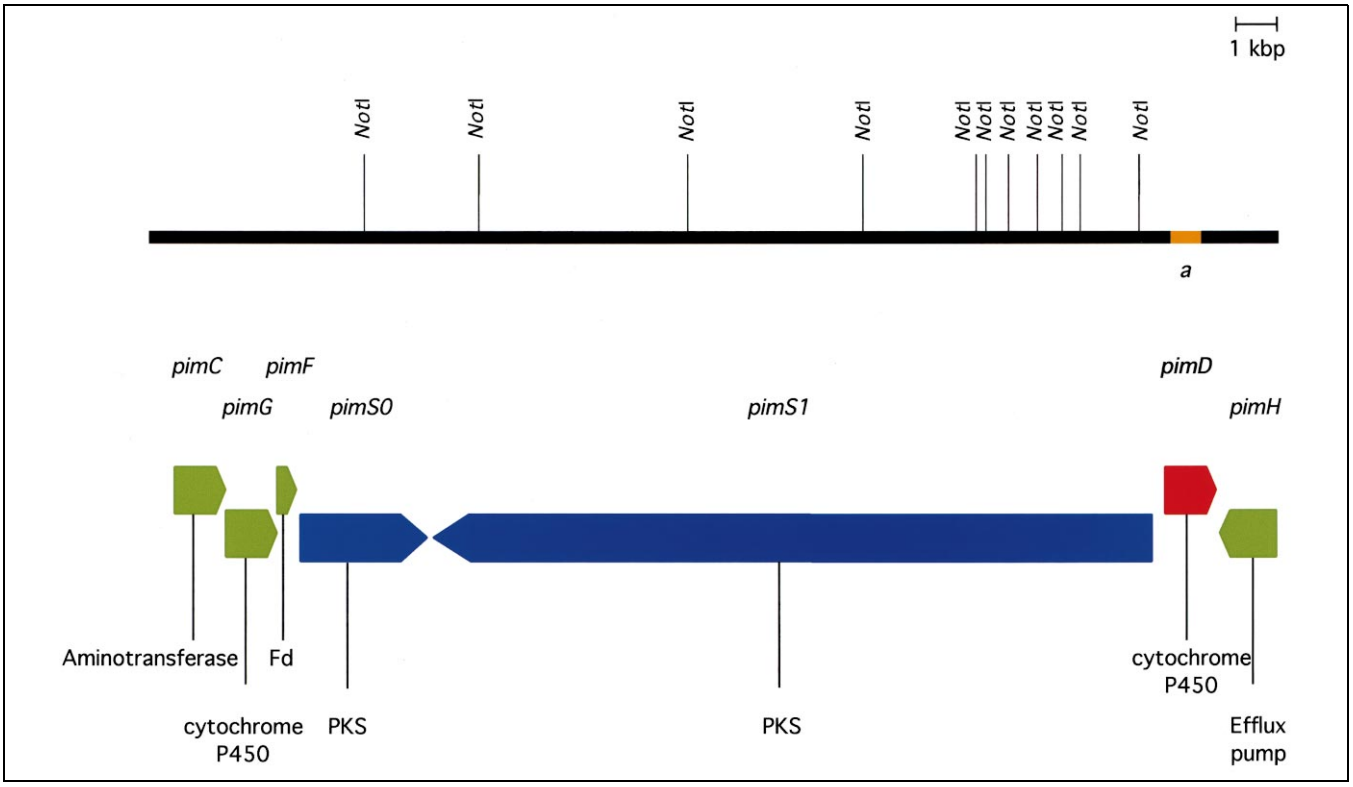

Fig. 1. A portion of the pimaricin biosynthetic gene cluster which contains some non-PKS genes, including *pimD* (in red) and the second cytochrome P450-encoding open reading frame (*pimG*) thought to be involved in pimaricin biosynthesis [3]. Pointed boxes indicate the direction of transcription. Fragment *a* was used for gene disruption (see Fig. 2). Only *Not*I sites are shown. Fd; ferredoxin.

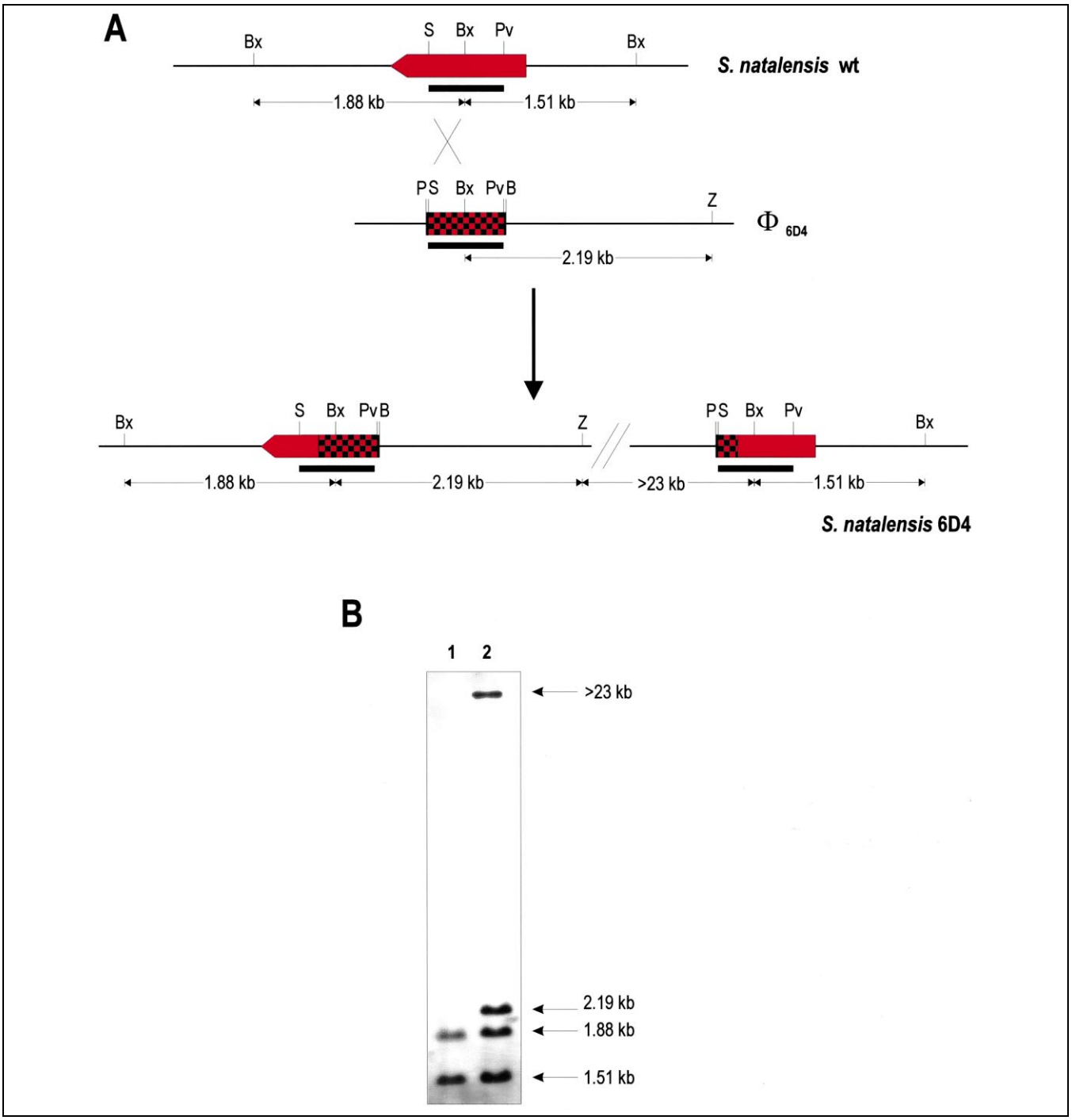

Fig. 2. Disruption of *pimD*. (A) Predicted restriction enzyme polymorphism caused by gene disruption. The *Bst*XI–*Bgl*II restriction pattern before and after disruption is shown. The probe is indicated by thick lines. The fragment used for gene disruption derives from fragment *a* in Fig. 1 (see text for details). B, *Bam*HI; Bx, *Bst*XI; P, *Pst*I; Pv, *Pvu*II; S, *Sma*I; Z, *Bgl*II. (B) Southern hybridisation of the *Bst*XI–*Bgl*II-digested chromosomal DNA of the wild type (lane 1) and the mutant (lane 2).

phage KC515, and its derivative, lack *attP*, they can only form lysogens by homologous recombination into the chromosome (Fig. 2A).

Six lysogens of *S. natalensis* were obtained by selection for thiostrepton resistance and tested for the lack of pimaricin production. One of these disrupted mutants was randomly selected, and named *S. natalensis* 6D4. The identity of the mutant was confirmed by Southern hybridisation (Fig. 2B). Chromosomal DNAs isolated from *S. natalensis* ATCC 27448 and mutant 6D4 and digested with both *Bst*XI and *Bgl*II were probed with the 667-bp *Pvu*II–*Sma*I fragment used to construct the KC515 derivative utilised for gene disruption (see Section 5). Hybridising bands of 1.9 kb and 1.5 kb were found for the wild

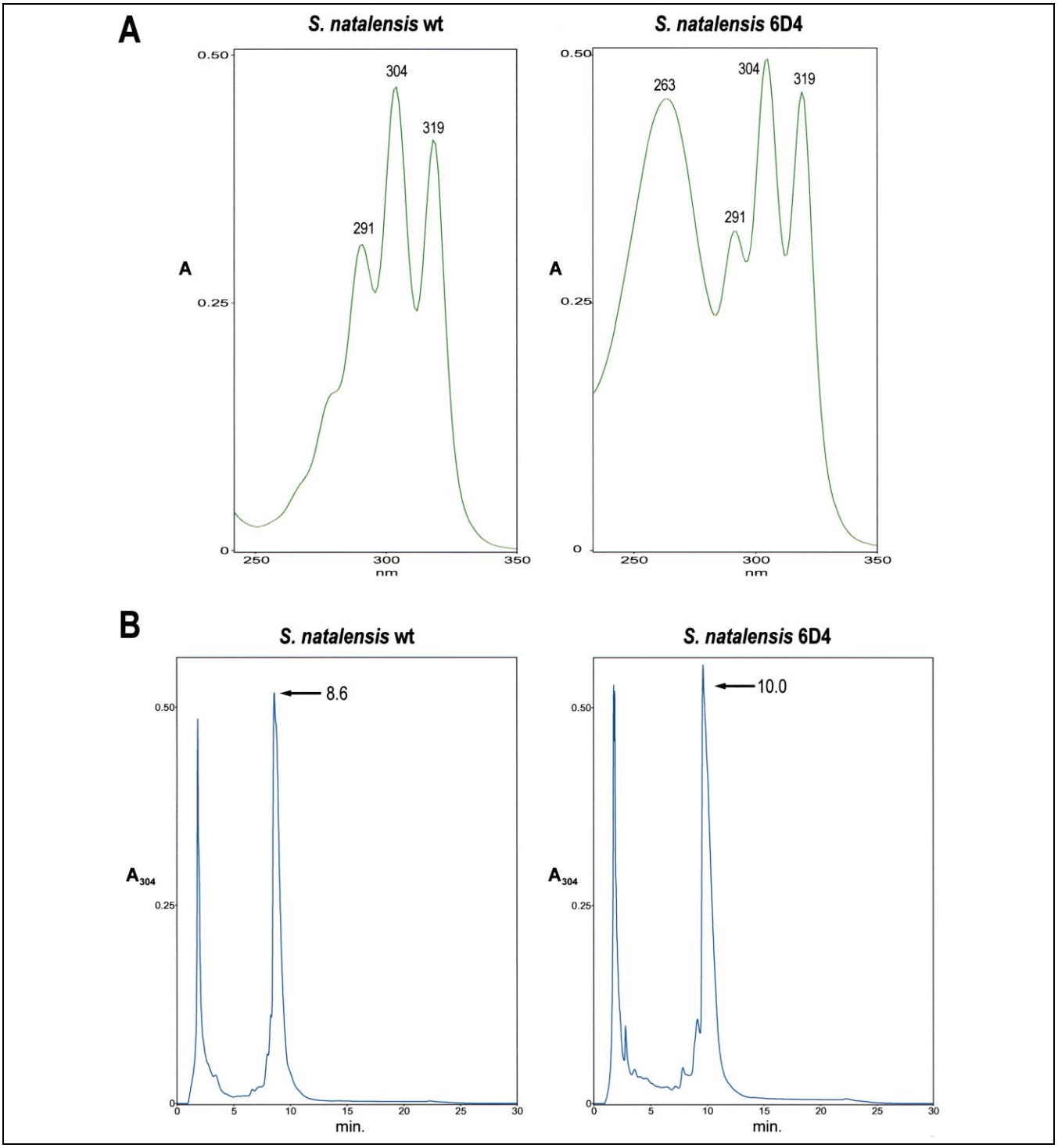

Fig. 3. Comparison of spectral (A), and HPLC (B) analyses of butanol-extracted broths from *S. natalensis* wild type and 6D4 strains. (A) The peaks of maximum absorption are indicated in both cases. Note that the typical chromophore absorption spectrum of a tetraene is present in the mutant strain. (B) Arrows indicate the position of pimaricin or deepoxypimaricin, and the elution times of both compounds under the separation conditions used (see text). Detection was carried out at $O.D._{304\,nm}$. The UV–visible absorption spectra of both peaks are identical to the spectra shown in (A).

type as expected (Fig. 2B). However, in the disrupted mutant, two new bands of 2.2 kb and more than 23 kb were also detected (Fig. 2B), indicating that a single crossover event had occurred with the phage derivative. The observed hybridising bands corresponded exactly to those expected according to the integration shown in Fig. 2.

2.2. *A novel tetraene macrolide is formed in the pimD-disrupted mutant*

The broth produced by the mutant strain generated by phage-mediated gene disruption was readily distinguishable from pimaricin synthesised by the wild type by spec-

trophotometric analysis of butanol-extracts of the culture broths (see Section 5). Comparison of the UV–visible absorption spectrum of both *S. natalensis* ATCC 27448 and 6D4, revealed the typical absorption spectrum of the polyene chromophore in both cases, but also a new absorption peak in the mutant broth at 263 nm (Fig. 3A), suggesting that a new compound was being produced by the recombinant strain.

To rule out the possibility of *S. natalensis* 6D4 producing a mixture of pimaricin and the new product, the extracted broth was concentrated to dryness and subjected to high-performance liquid chromatography (HPLC) and thin layer chromatography (TLC). HPLC revealed a major new peak with a slightly longer retention time (1.4 min under the separation conditions assayed, see Section 5) than the pimaricin present in the *S. natalensis* wild type broth (Fig. 3B), whereas these two compounds were not well resolved by TLC (not shown). Further spectral analysis of the compound present in the new HPLC peak corroborated our previous observation, yielding an identical UV–visible absorption spectrum to the one in Fig. 3A. The presence of the typical chromophore absorption spectrum of a tetraene with peaks at 319, 304, 291 and 281 nm, together with the apparent absence of pimaricin in *S. natalensis* 6D4 broths strongly suggested that the new product was a pimaricin-like molecule, and possibly a precursor in its biosynthesis.

The existing correlation between the deduced domain structure of the pimaricin synthase proteins [3] and the pimaricinolide product indicates that in the final pimaricin molecule there are two functions which do not derive from the PKS, and could result from post-PKS oxidation catalysed by P450 monooxygenases [3]. These include the epoxy group at C4–C5, which should originate from a double bond, and the exocyclic carboxyl function at C12 that should derive from a methyl group (Fig. 4).

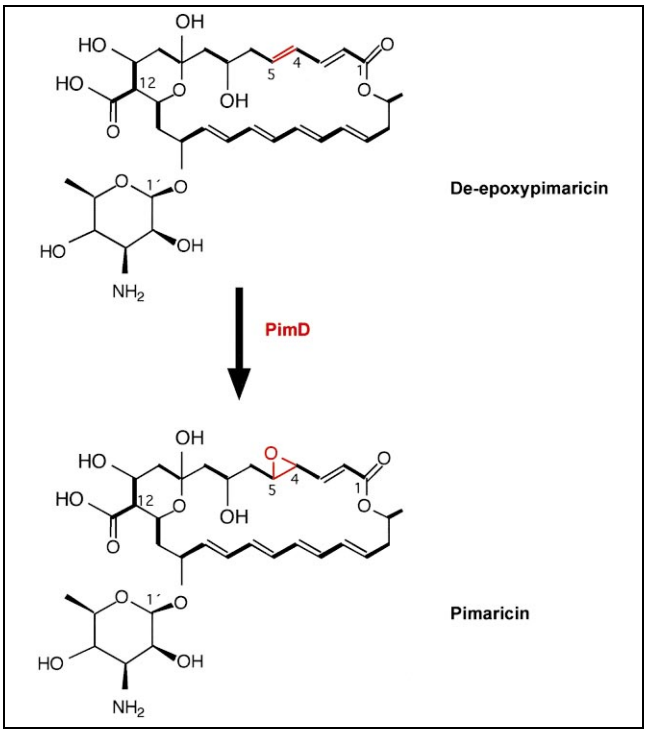

Fig. 4. Proposed conversion of 4,5-deepoxypimaricin into the pimaricin molecule by PimD.

2.3. *The novel compound corresponds to 4,5-deepoxypimaricin*

To distinguish between the two latter possibilities, the compound present in the new peak was analysed by liquid chromatography-mass spectroscopy (LC-MS) and LC-MS-MS using atmospheric-pressure chemical-ionisation (APCI) techniques, and compared with authentic pimaricin. The molecular mass of the new compound was thus found to be 649, 16 masses lower than pimaricin (Fig. 5; see Section 5 for the fragmentation pattern of pimaricin), which was in agreement with the loss of one oxygen atom. Furthermore, both pimaricin and the new component showed a fragment loss of 163 (corresponding to the aminosugar mycosamine), thus suggesting that no changes had been introduced in the mycosamine moiety. Moreover, the masses of the principal peaks in the spectrum of the new compound (Fig. 5) were in close agreement with the values expected (i) for the molecular ion of protonated deepoxypimaricin ($MH^+ = 649.9$), (ii) for a dehydrated deepoxypimaricin species ($MH^+ - 18 = 631.8$), (iii) for a mycosamine-minus species ($MH^+ - \text{sugar} = 487.0$), (iv) for the dehydrated mycosamine-minus deepoxypimaricin ($MH^+ - \text{sugar} - 18 = 469.0$), (v) for an identical species further dehydrated ($MH^+ - \text{sugar} - 2H_2O = 451.1$), and (vi) for a dehydrated carboxyl-minus mycosamine-minus deepoxypimaricin ($MH^+ - \text{sugar} - 18 - COOH = 425.1$). However, a fragment loss of 44 from the unknown compound suggested that it could still lose a carboxylic acid group.

In order to corroborate that the new component retained the exocyclic carboxyl group of pimaricin, it was dissolved in deuterium-labelled methanol. Such treatment allows the replacement of all exchangeable H-atoms for D-atoms. LC-MS-MS analysis of the new compound measured in a constant infusion mode yielded a *m/z* 659 (not shown) that corresponds with a molecular mass of 657, which indicated that the new compound still had eight exchangeable H-atoms (like pimaricin), and therefore retained the carboxyl group.

The spectrum of the novel compound was compared with that of pimaricin using 2D nuclear magnetic resonance (NMR) spectroscopic methods (COSY, TOCSY, C–H correlation). The NMR data of the pimaricin derivative (Table 1) indicated that the signals of C4/H4 and C5/H5 of the pimaricin molecule (epoxide function) were absent, although owing to overlap of double bonds were not observed directly. Nevertheless, the signal of C6/H6 showed coupling to a double bond while the signals of C7/H7 and C8/H8 remained as in the spectrum of pimar-

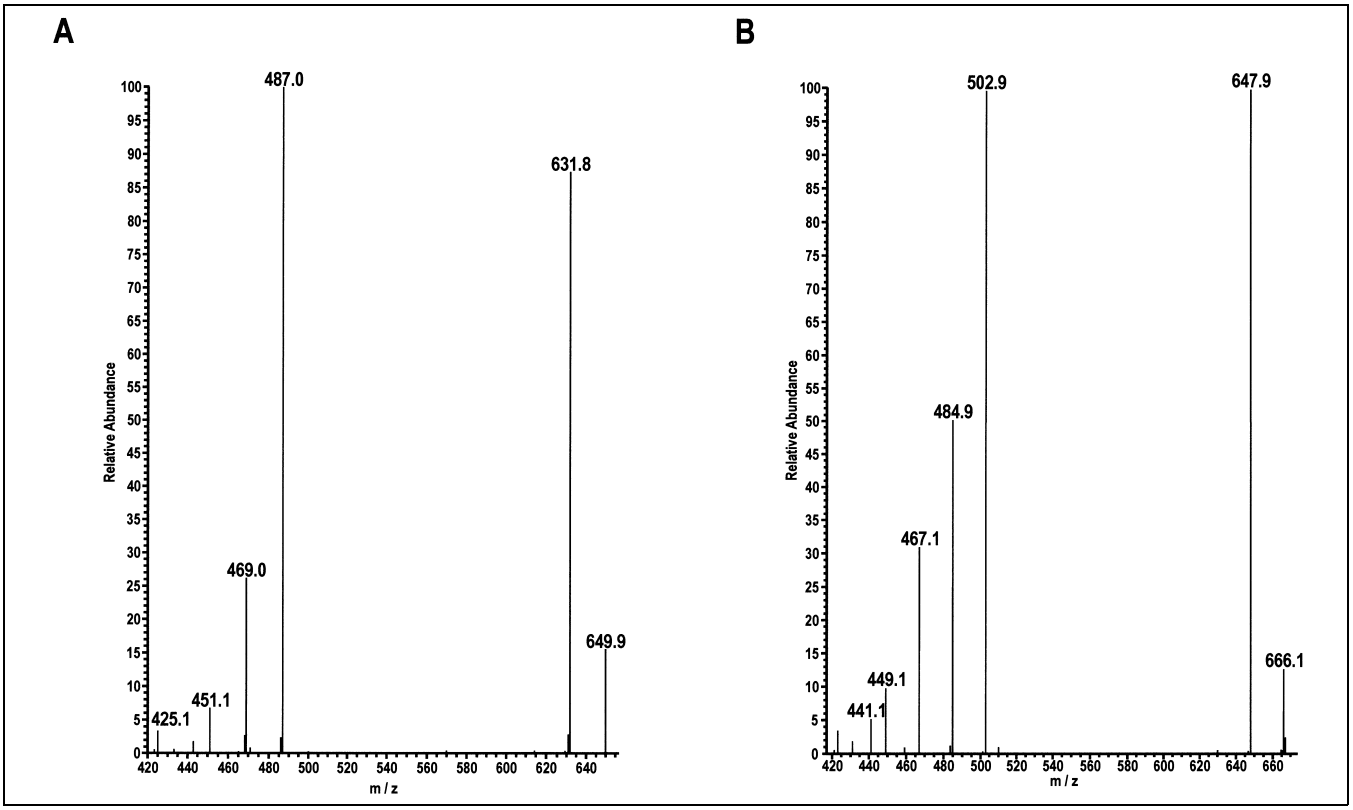

Fig. 5. MS identification of deepoxypimaricin produced by *S. natalensis* 6D4. (A) Spectrum showing the molecular ion MH^+ (649.9) of this new compound and dehydration and mycosamine-minus species (see text for a detailed description of the signals). (B) Spectrum of pimaricin, showing the molecular ion MH^+ (666.1) of the complete molecule, and its fragmentation pattern.

icin. Therefore, the double bond of the new molecule should be on C4–C5. Besides, the signal of H3 shifted downfield due to the conjugation of C2 = C3 with C4 = C5 double bonds.

Taken together, all the data identified the material present in the peak as 4,5-deepoxypimaricin as a sole product (Fig. 4), indicating that the gene product of *pimD* is responsible for the epoxidation of the pimaricinolide ring at C4–C5, and that our previous deduction, from the sequence analysis of *pimS3*, suggesting the introduction of a double bond by PimS3 (a single-module enzyme of the pimaricin PKS corresponding to module 11) was correct [3].

Table 1
Selection of ^1H and ^{13}C NMR data for 4,5-deepoxypimaricin and pimaricin[a]

Position[b]	^1H δ (ppm)	^{13}C δ (ppm)
2	5.58 (6.29)	121.9 (125.0)
3	6.92 (6.55)	146.3 (144.9)
4	6.07[c] (3.32)	132.2 (54.5)
5	6.07[c] (2.89)	134.9 (59.0)
6	2.16 (2.10)	43.7 (40.1)
7	4.03 (4.26)	69.4 (66.6)
1′	4.47 (4.63)	99.7 (98.1)

Only the chemical shifts of the most relevant signals are listed. The signal of C1′ is included to show that the sugar moiety is still present in the mutant.

[a]Data in parentheses are chemical shifts for equivalent positions in pimaricin.

[b]Positions are labelled according to their number in the polyketide backbone (Fig. 4).

[c]Overlapping signals.

2.4. Biological activity and yield of 4,5-deepoxypimaricin

HPLC and LC-MS provided evidence for the accumulation of 4,5-deepoxypimaricin as the sole PKS product in *S. natalensis* 6D4 culture broths. In order to characterise the biological activity of the new product; we initially compared bioassays performed with butanol-extracted culture broths from early stationary phase-grown *S. natalensis* 6D4 cells with those of the parent strain. In every case analysed we found a consistent halo of growth inhibition of the *Candida utilis* CECT 1061 cells used as test organism (not shown), indicating that 4,5-deepoxypimaricin retains antibiotic activity.

Under the growth conditions used (see Section 5) the yields of pimaricin and 4,5-deepoxypimaricin in YEME medium without sucrose were very similar, in the range 220–310 μg of product per mg of dry cells (about 0.7 g/l). After purification of the product by HPLC, the antibiotic activity of deepoxypimaricin was found to be substantially lower than that of pimaricin, showing a minimal inhibitory concentration of 70 μg/ml, 10 times higher than the one for pimaricin.

3. Discussion

The widespread clustering of the biosynthetic genes needed to make a particular secondary metabolite [26] has been a tremendous aid in the isolation of genes involved in polyketide biosynthesis [7], and also a good indication for the assignment of functions to genes whose role in the biosynthesis would not be otherwise obvious. Such reasoning was used to propose a function for the *pimD* gene product on the tailoring of the pimaricinolide aglycone [3]; however, the sole presence of a gene within a biosynthetic gene cluster does not justify its involvement in biosynthesis. The existence of genes within a secondary metabolite biosynthetic gene cluster, which are not essential for its biosynthesis, has been reported. Thus, in the mithramycin gene cluster it has been shown that *mtmOI* and *mtmOIII* (genes encoding oxygenases) are not essential for mithramycin biosynthesis [27], and in the erythromycin gene cluster it has been demonstrated that *eryBI* (encoding a β-glucosidase) is also not essential for erythromycin biosynthesis [28]. In this paper we describe the first polyene derivative obtained by targeted manipulation of a polyene biosynthetic gene cluster. We have used phage-mediated gene disruption to produce a *S. natalensis* mutant inactivated in the *pimD* gene. The strain so generated produced a pimaricin derivative as a shunt metabolite, thus establishing the link of *pimD* with pimaricin biosynthesis.

Sequencing analysis of the pimaricin PKS genes prompted us to predict, based on the general colinearity and processivity of type I PKSs [9,10,29,30], that the epoxide of pimaricin, at C4–C5, could originate from a double bond introduced by PimS3 during polyketide chain elongation by the pimaricin PKS. PimS3 contains a single module for chain extension (module 11) and has β-ketoacyl-ACP synthase, acyltransferase, β-ketoacyl-ACP reductase, β-hydroxyacyl-thioester dehydratase and acyl carrier protein functional domains. Therefore, such a module would generate a *trans* double bond between carbons 4 and 5 of the pimaricinolide product of the PKS (Fig. 4) [3]. Post-PKS epoxidation was hence expected to occur on such double bond to yield the epoxy functionality of pimaricin. On the basis of sequence comparison with authentic cytochrome P450 monooxygenases, PimD was proposed as a possible candidate to catalyse such epoxidation. However there is a second hydroxylase (PimG) in the pimaricin biosynthetic cluster that could also fulfil such role [3].

As *pimD* is transcribed on a monocistronic RNA (Fig. 1), expression of the remaining genes of the pimaricin biosynthetic gene cluster should not be affected by any polar effect of its disruption. The production of 4,5-deepoxypimaricin by *S. natalensis* 6D4 (Fig. 4), as verified by LC-MS and NMR, in which *pimD* has been disrupted, corroborated our previous assumptions about *pimD* transcription and polyketide chain assembly, and demonstrated that PimD is in fact the enzyme responsible for the formation of the epoxide functionality in the final pimaricin molecule.

A preliminary characterisation of the biological activity of the new product, 4,5-deepoxypimaricin, indicates lower antibiotic activity than pimaricin. However, other parameters important for the applicability of this new product such as host-range, toxicity and acid or alkali stability will establish the future economical importance of the finding presented here.

The accumulation of 4,5-deepoxypimaricin in the mutant as a major product also indicates that the reaction catalysed by PimD does not affect the modifications introduced by other tailoring enzymes on their pimaricinolide substrate. Therefore, either the epoxidation reaction is the last step in the biosynthetic pathway as has been suggested in oleandomycin biosynthesis [31], or the modifying enzymes for the latter steps of the pathway do not discriminate against substrates with variations at C4–C5. This lack of substrate discrimination in polyketide tailoring enzymes is not unprecedented. Inactivation of the C6 hydroxylase in the erythromycin A biosynthetic pathway by plasmid-mediated gene disruption of *eryF* results in the production of 6-deoxyerythromycin A [12], thus implying that the remaining tailoring enzymes of the erythromycin pathway can utilise 6-deoxyerythronolide B as substrate. Further characterisation of the pimaricin biosynthetic route will permit the validation of one of these hypotheses.

With the development of combinatorial biosynthesis [32], a growing number of novel macrolide analogues have already been produced by genetic engineering macrolide gene clusters [10,12,33–45]. In many cases, however, the modified compounds lack post-PKS hydroxylations, possibly owing to the apparent strict substrate specificities of these oxidising enzymes. The identification of novel macrolide monooxygenases with activities toward alternative substrates is, therefore, a crucial aspect for the oxidative tailoring of novel polyketides, and should facilitate the search for new bioactive macrolide compounds. This novel epoxidase could thus prove valuable for the introduction of epoxy substituents into designer macrolides in the future.

4. Significance

Polyenes represent a major class of antifungal agents characterised by the presence of a series of conjugated double bonds in their planar hydroxylated macrolide ring structure. Despite their general interest, very little was known about their biosynthetic routes, and only the recent description of the biosynthetic gene clusters for pimaricin and nystatin has provided some clues for the rational design of polyene derivatives with improved properties. This report addresses the functional disruption of the

gene *pimD*, which encodes an authentic cytochrome P450 monooxygenase, as a mean to generate a derivative of the polyene pimaricin. The 4,5-deepoxypimaricin accumulated by the recombinant mutant of the pimaricin-producing actinomycete *S. natalensis* shows that PimD is the enzyme responsible for the introduction of the peculiar epoxy moiety in the pimaricin macrolactone structure. This novel epoxidase could prove valuable for the introduction of epoxy substituents into designer macrolides in the future. The work represents the first description of a polyene analogue obtained by targeted manipulation of a polyene biosynthetic gene cluster.

5. Materials and methods

5.1. Bacterial strains, cloning vectors and cultivation

S. natalensis ATCC 27448 was routinely grown in YEME medium [46] without sucrose. Sporulation was achieved in TBO medium (2% (w/v) tomato paste, 2% (w/v) oats flakes, 2.5% (w/v) agar) at 30°C. For pimaricin production, the strain was grown in YEME without sucrose. The same media were supplemented with thiostrepton when used for *S. natalensis* 6D4 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. *C. utilis* (syn. *Pichia jadinii*) CECT 1061 was used for bioassay experiments. Phage KC515 (*c*$^+$ *attP::tsr::vph*), a ∅C31-derived phage [25], was used for gene disruption experiments. *Streptomyces lividans* JII 1326 [47] served as a host for phage propagation and transfection. Infection with ∅6D4 (the KC515 recombinant derivative used for disruption) was carried out on R5 medium [46]. Standard conditions for culture of *Streptomyces* species and isolation of phages were as described by Kieser et al. [46].

5.2. Genetic procedures

Standard genetic techniques with *E. coli* and in vitro DNA manipulations were as described by Sambrook et al. [48]. Recombinant DNA techniques in *Streptomyces* species and isolation of *Streptomyces* total and phage DNA were performed as previously described [46]. Southern hybridisation was carried out with probes labelled with digoxigenin by using the DIG DNA labelling kit (Roche Molecular Biochemicals).

5.3. Construction of a pimD mutant

The *pimD* gene was disrupted by KC515 phage-mediated single crossover integration as follows. A 667-bp *Pvu*II–*Sma*I fragment internal to the *pimD* gene was cloned into a *Hin*cII-cut pUC18 vector to yield pMVM1. This plasmid was then used as a source of DNA for subcloning into KC515 [25]. A 690-bp *Bam*HI–*Pst*I fragment (both sites belong to the polylinker of pUC) was ligated into the *Bam*HI–*Pst*I sites of KC515. Transfection of *S. lividans* protoplasts [46] resulted in a number of phage plaques that were screened by Southern hybridisation for the presence of *pimD*-derived sequences. One of the recombinants, ∅6D4, was 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 genomic Southern hybridisation.

5.4. Spectral analysis, bioassay, and metabolite concentration determination

To assay pimaricin in culture broths, 0.5 ml of culture was extracted twice with 4 ml of butanol, and the organic phase was diluted in water-saturated butanol to bring the O.D. at 319 nm in the range of 0.1 to 0.4 absorbance units. Control solutions of pure pimaricin (DSM, Delft, The Netherlands) were used as control. To confirm the identity of pimaricin, an UV–visible absorption spectrum (absorption peaks at 319, 304, 291 and 281 nm) was routinely determined in a Hitachi U-2001 spectrophotometer. The fungicidal activity of pimaricin or 4,5-deepoxypimaricin was tested by bioassay using *C. utilis* CECT 1061 as test organism. Quantitative determination of pimaricin, or its derivative, was performed with a Shimadzu VP HPLC with a diode array ultraviolet detector set at 304 nm, fitted with a μ-Bondapack RP-C18 column (10 μm; 3.9×300 mm). Elution was with a gradient (1.5 ml/min) of 100% methanol (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). Retention time for pimaricin was 8.6 min. TLC was performed on silica 60 F254 plates (Merck), and elution was carried out with water:butanol:acetic acid (4:5:1).

5.5. Structural elucidation of 4,5-deepoxypimaricin

Fermentation broths were harvested by centrifugation after 2 days of growth at 300 rpm and 30°C, and the supernatant extracted with eight volumes of butanol. This was repeated once, and the solvent was removed from the combined extracts in vacuo to yield a dry powder. Two gram of the residue thus obtained was dissolved in methanol (10 ml) and centrifuged to remove particulate matter before the supernatant (50 μl) was analysed using LC-MS and LC-MS-MS.

The fragmentation pattern for authentic pimaricin (Fig. 5) was as follows: $(M)H^+$ $m/z = 666.1$; $(M-H_20)H^+$ $m/z = 647.9$; (M−mycosamine ring)H^+ $m/z = 502.9$; (M−mycosamine ring-$H_2O)H^+$ $m/z = 484.9$; (M−mycosamine ring−$2H_2O)H^+$ $m/z = 467.1$; (M−mycosamine ring−$3H_2O)H^+$ $m/z = 449.1$; (M−mycosamine ring−H_2O−COOH)H^+ $m/z = 441.1$.

To replace all exchangeable H-atoms of the pimaricin derivative for D-atoms, the dry pellet (see above) was washed twice with deuterium-labelled methanol, and redissolved in the same solvent (2 ml). This solution was then centrifuged, and the clear supernatant (50 μl) used for analysis.

All LC-MS and LC-MS-MS experiments were carried out using a LCQ apparatus (ThermoQuest, Breda, The Netherlands). A pimaricin solution (5 mg/ml methanol) was used for tuning. Analyses were run using an APCI interface and positive ionisation

mode. Chromatography and column characteristics were as indicated above. NMR spectra were recorded using a Bruker DRX-600 spectrometer equipped with a 5-mm inverse triple resonance probe with self-shielded gradients. Samples were dissolved in CD_3OD before injection. Assignment was achieved by means of standard 2D techniques (COSY, TOCSY, and HSQC), and by comparison with the spectrum of pimaricin.

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