Chemistry & Biology
Article

# Cholesterol Oxidases Act as Signaling Proteins for the Biosynthesis of the Polyene Macrolide Pimaricin

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DOI 10.1016/j.chembiol.2007.01.010

## SUMMARY

**The gene cluster responsible for pimaricin biosynthesis in *Streptomyces natalensis* contains a cholesterol oxidase-encoding gene (*pimE*) surrounded by genes involved in pimaricin production. Gene-inactivation and -complementation experiments revealed that *pimE* encodes a functional cholesterol oxidase and, surprisingly, that it is also involved in pimaricin biosynthesis. This extracellular enzyme was purified from *S. natalensis* culture broths to homogeneity, and it was shown to restore pimaricin production when added to the mutant culture broths. Other cholesterol oxidases also triggered pimaricin production, suggesting that these enzymes could act as signaling proteins for polyene biosynthesis. This finding constitutes the description of a cholesterol oxidase gene with an involvement in antibiotic biosynthesis, and it broadens the scope of the biological functions for this type of oxidase.**

## INTRODUCTION

*Streptomyces* species produce a large variety of extracellular enzymes, some of them clinically important, and, in particular, cholesterol oxidases [1–4]. Cholesterol oxidase (EC 1.1.3.6) is a flavoprotein that catalyzes both the oxidation of cholesterol to 5-cholesten-3-one, with the reduction of molecular oxygen to hydrogen peroxide, and the isomerization of the $\Delta^5$ bond [5], via a mechanism analogous to that of the $\Delta^5$-3-ketosteroid isomerase from *Pseudomonas testosterini* [6], to yield 4-cholesten-3-one as the final product (Figure 1). This bifunctional enzyme is routinely used for the enzymatic transformation of cholesterol [7] and for determining levels of total serum cholesterol in clinical and food specimens in a coupled system with cholesterol esterase and peroxidase [8, 9] (for a review, see [10]). Additionally, due to its ability to convert cholesterol into 4-cholesten-3-one, the enzyme disrupts the midgut epithelial membrane when it is ingested by larvae of some Coleoptera (e.g., *Anthonomus grandis grandis* [boll weevil]) and Lepidoptera (e.g., *Heliothis virescens*, *Helicoverpa zea*, *Pectinophora gossypiella*), thus exhibiting potent insecticidal activity [11, 12].

Cholesterol oxidases have been isolated from several sources other than *Streptomyces*, including members of the genera *Brevibacterium* [13], *Schizophyllum* [14], *Burkholderia* [15], and *Rhodococcus* [16, 17], among others. The crystal structures of the enzymes from *Brevibacterium sterolicum* and *Streptomyces* sp. SA-COO have been determined at 1.8 Å and 1.5 Å resolutions, respectively [18–20]. Most of the cholesterol oxidases are monomeric and contain flavin-adenine dinucleotide (FAD) as a prosthetic group [13].

*Streptomyces natalensis* produces pimaricin, a 26-member tetraene, macrolide, antifungal antibiotic widely used in the food industry in order to prevent mold contamination of cheese and other nonsterile foods (i.e., cured meat, sausages, ham, etc.), as well as for the treatment of fungal keratitis [21, 22]. 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. As with other macrocyclic polyketides, pimaricin is synthesized by the action of so-called type I modular polyketide synthases [21].

We have previously sequenced the pimaricin biosynthetic gene cluster and demonstrated its involvement in pimaricin production by gene-disruption experiments [23, 24]. The gene cluster encodes 13 polyketide synthase modules within 5 multifunctional enzymes, as well as 12 additional proteins that presumably govern modification of the polyketide skeleton, export, and regulation of gene expression (Figure 2) [25–27]. One of these proteins, PimE, showing a convincing sequence identity with cholesterol oxidases, is encoded by a gene located in the center of the cluster. The location of such a gene was puzzling and prompted us to evaluate its functional role.

In this paper, we demonstrate that *pimE* encodes cholesterol oxidase by gene disruption. The mutant obtained by this technique lacks cholesterol oxidase and the ability

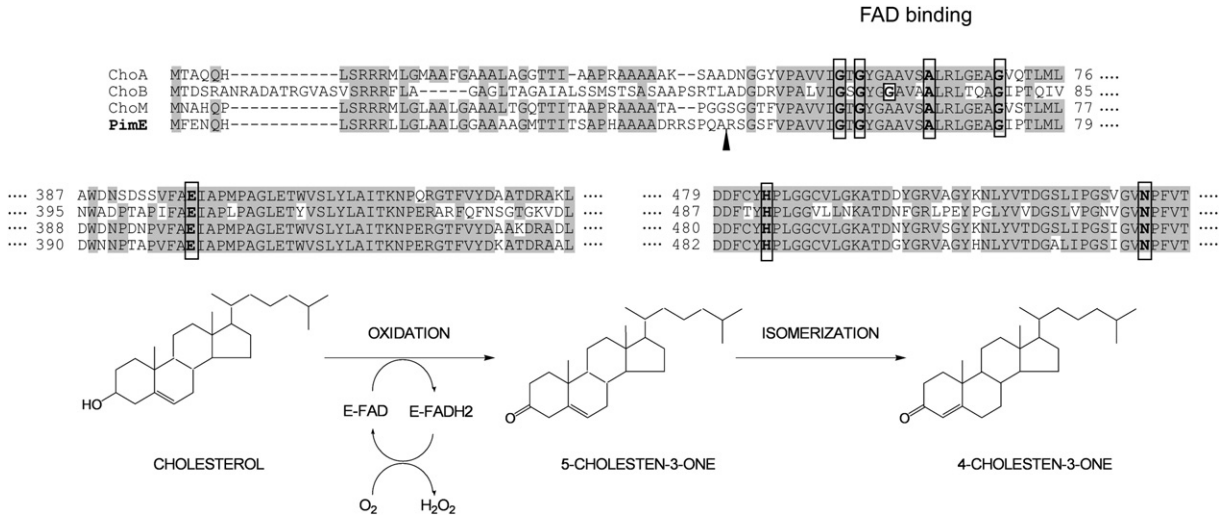


**Figure 1. Alignment of PimE with Other Cholesterol Oxidases**

ChoA, cholesterol oxidase from *Streptomyces* sp. SA-COO [2]; ChoB, enzyme from *Brevibacterium sterolicum* [28]; ChoM, cholesterol oxidase from *Streptomyces* sp. A19249 [12]. Numbers indicate amino acid residues from the N terminus of the protein (signal sequences included). The cleavage site of the PimE signal sequence is indicated by an arrowhead. The consensus amino acid residues for FAD binding are shown in bold and are boxed (upper alignment). The invariant Glu, His, and Asn residues (E356, H442, and N480 in mature PimE) that contribute to catalysis (see text) are also boxed (bottom alignments). The reaction catalyzed by the cholesterol oxidase is indicated at the bottom.

to produce pimaricin, suggesting that the cholesterol oxidase is a key enzyme on pimaricin biosynthesis. Moreover, complementation of the mutant with *pimE* restored both cholesterol oxidase activity and pimaricin biosynthesis. Also, addition of purified PimE or commercial cholesterol oxidases to the mutant broth triggered pimaricin production, suggesting that these enzymes could act as signaling proteins for polyene biosynthesis.

## RESULTS

### In Silico Analysis of the *pimE* Gene Product

The translated amino acid sequence of PimE (549 amino acids) showed a very high end-to-end sequence identity with cholesterol oxidases. These values ranged from 59% for ChoB, the cholesterol oxidase of *Brevibacterium sterolicum* [28], to 82% for ChoA, the enzyme from *Streptomyces* sp. SA-COO [2], or 91% for RimD, the enzyme from *S. diastaticus* [29]. All of them show the invariant Glu, His, and Asn residues (E356, H442, and N480 in mature PimE) that are involved in the enzyme activity [18, 30] (Figure 1).

PimE presents a twin-arginine translocation signal sequence (InterPro, IPR006311) between amino acid residues 7 and 38. By analogy with other *Streptomyces* extracellular enzymes, including other cholesterol oxidases, this potential signal sequence was predicted to be 45 amino acids long (see below); thus, the mature enzyme would contain 504 residues and have a molecular mass of 54,883 Da.

The consensus sequence for FAD-binding GxGxxGxxxAxxxxxxG [31] is located near the amino-terminal end of the mature protein at amino acids 12–28 (Figure 1). Interestingly, the third invariant Gly in the FAD-binding motif is substituted by Ala in PimE and in all of the previously published cholesterol oxidases from *Streptomyces*. Enzymatic analyses of *S. natalensis* culture broths (see Experimental Procedures) readily revealed cholesterol oxidase activity.

### Transcriptional Analysis of the *pimE* Gene

A previous gene-expression analysis by reverse transcriptase-polymerase chain reaction (RT-PCR) of the pimaricin gene cluster in *S. natalensis* ATCC 27448 revealed a different transcription profile for *pimE* when compared with those of its surrounding genes (Figure 2) [26]. Such observation suggested that *pimE* was actually transcribed from a monocistronic operon, in contrast with our initial assumption that *pimE* was a part of a large operon governing the expression of seven genes [24]. In order to confirm this finding, we carried out northern hybridization experiments. A single RNA transcript of about 1.8 kb (data not shown) hybridized with the probe used (see Experimental Procedures). Given that the gene is 1650 bp in size, this result indicates that *pimE* is transcribed as an independent transcriptional unit under the culture conditions used.

### Disruption of the *pimE* Gene

We used phage KC515, an *attP*-defective ØC31 derivative [32], in order to introduce DNA into this strain. The recombinant phage used for *pimE* disruption, Ø1D3, was constructed as described in Experimental Procedures, and it was used to infect *S. natalensis* in order to obtain lysogens. Because phage KC515 and its derivative lack

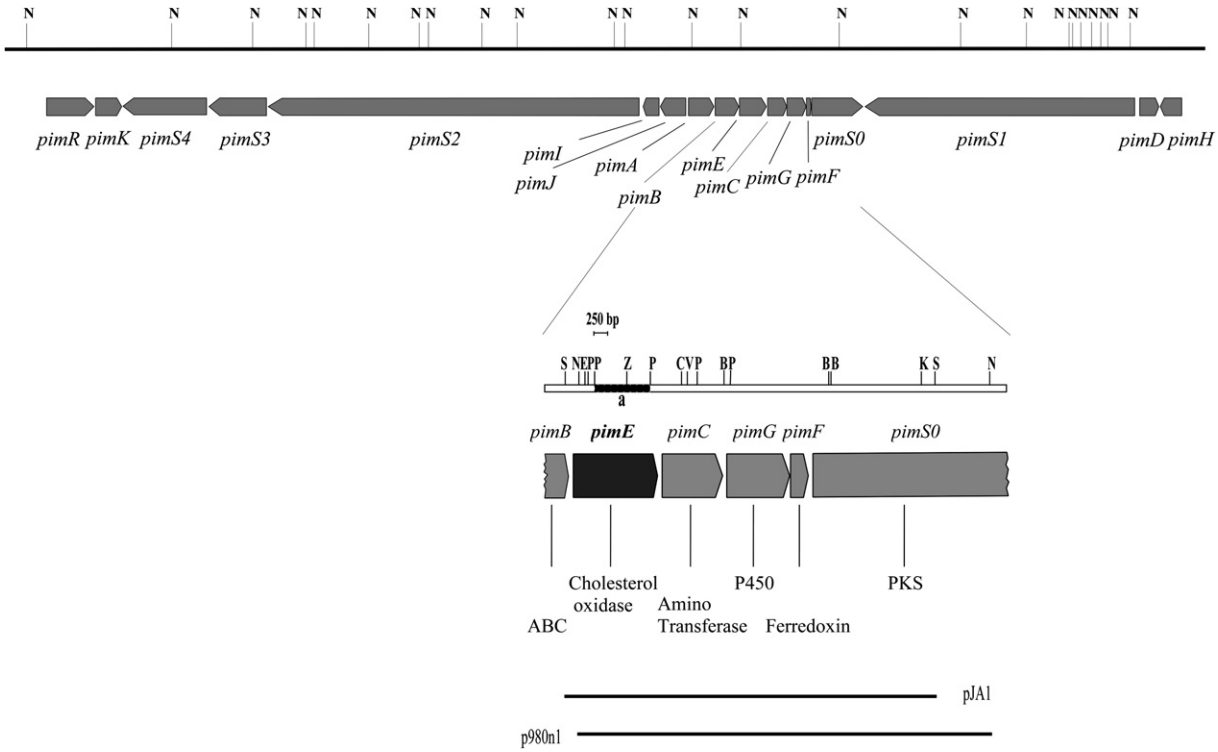


**Figure 2. Pimaricin Biosynthetic Gene Cluster**

The central section is indicated in more detail and includes a portion of an ABC transporter-encoding open reading frame (*pimB*), *pimE* (in black), an amino transferase-encoding gene (*pimC*) thought to be involved in mycosamine biosynthesis, a cytochrome P450 monooxygenase-encoding gene (*pimG*) and its partner ferredoxin (*pimF*) for electron transfer, and the 5′ end of the first polyketide synthase (PKS) gene (*pimS0*) involved in the initiation of pimaricinolide construction [24]. Pointed boxes indicate directions of transcription. Fragment *a* was used for gene disruption (see text). B, BamHI; C, ClaI; E, EcoRI; K, KpnI; N, NotI; P, PvuII; S, SphI; V, EcoRV; Z, BglII. The inserts used for vector construction are indicated at the bottom.

*attP*, they can only form lysogens by homologous recombination into the chromosome (Figure 3A).

Several lysogens of *S. natalensis* were obtained by selection for thiostrepton resistance and were tested for the lack of cholesterol oxidase production. One of these disrupted mutants was randomly selected and was named *S. natalensis* D3. The identity of the mutant was confirmed by Southern hybridization (Figure 3B). Chromosomal DNAs isolated from *S. natalensis* ATCC 27448 and mutant D3 and digested with BglII and NcoI were probed with the 1111 bp PvuII fragment used to construct the KC515 derivative utilized for gene disruption (see Experimental Procedures). Hybridizing bands of 2.35 kb and 1.4 kb were found for the wild-type as expected (Figure 3B). However, in the disrupted mutant, three bands of 1.4 kb, 2.2 kb, and 11.4 kb were also detected (Figure 3B), indicating that a single crossover event had occurred with the phage derivative. The observed hybridizing bands corresponded exactly to those expected from to the integration shown in Figure 3.

The mutant strain *S. natalensis* D3 showed growth and morphological characteristics identical to those of *S. natalensis* wild-type when grown on solid or liquid media, indicating that PimE has no role on growth or differentiation of the producer strain.

## Cholesterol Oxidase Production Is Blocked in the *pimE*-Disrupted Mutant

The fermentation broth of the mutant strain generated by phage-mediated gene disruption, *S. natalensis* D3, was tested for cholesterol oxidase production. No traces of cholesterol oxidase activity could be detected by the extremely sensitive spectrophotometric assay (see Experimental Procedures), even when using 10-fold concentrated broths (data not shown), suggesting that PimE is actually a functional cholesterol oxidase, and that it is the only enzyme with such an activity produced by *S. natalensis* under the experimental conditions tested.

## Functional Inactivation of *pimE* also Blocks Pimaricin Production

Further analysis of the disrupted mutant for pimaricin production in complex medium (see Experimental Procedures) showed that it was also blocked in the production of the antifungal macrolide. The microbiological bioassay against *Candida utilis* showed no traces of fungicidal activity (Figure 4), as compared with that of the wild-type strain. Furthermore, the high-performance liquid chromatography (HPLC) analysis of butanol extracts from culture supernatants indicated that no pimaricin was being produced by the mutant *S. natalensis* D3 (Figure 4).

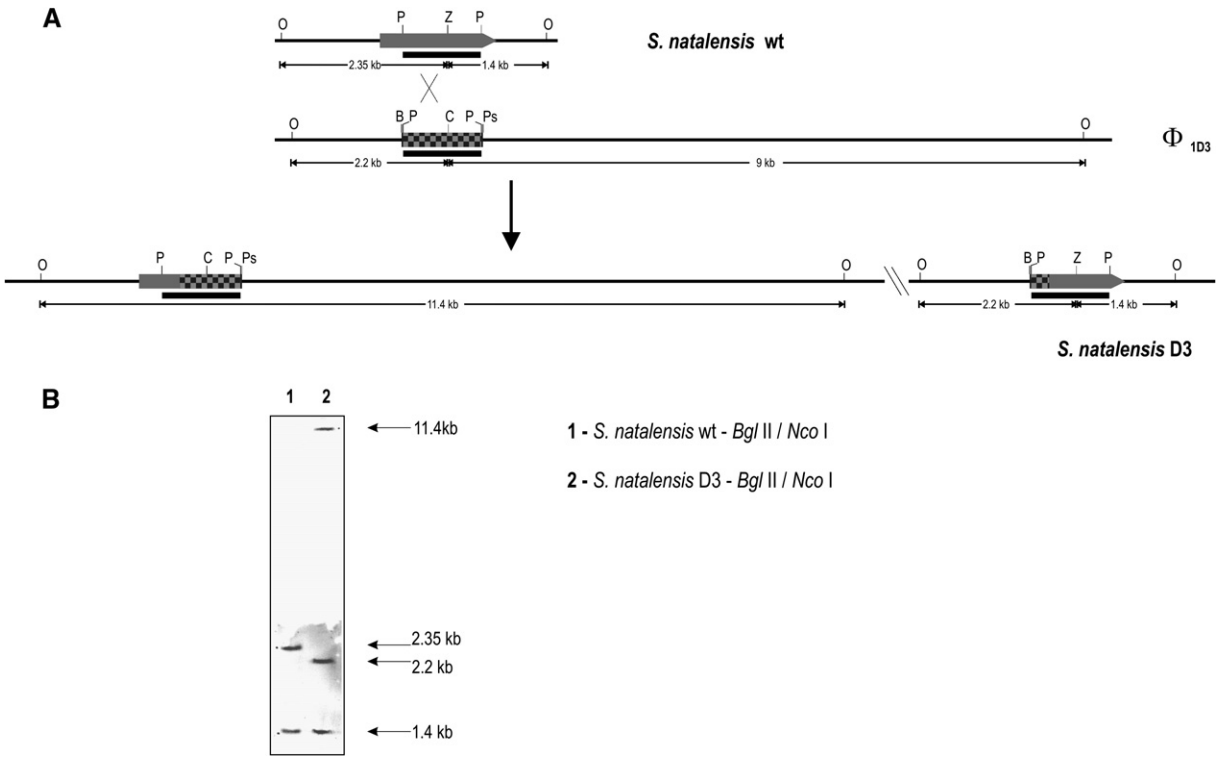


**Figure 3. Gene Disruption of *pimE***

(A) Predicted restriction enzyme polymorphism caused by gene disruption. The BglII-NcoI restriction pattern before and after disruption is shown. The probe is indicated by thick lines. The fragment used for gene disruption was fragment *a* from Figure 2 (see text for details). B, BamHI; C, ClaI; O, NcoI; P, PvuII; Ps, PstI; Z, BglII.

(B) Southern hybridization of the BglII-NcoI-digested chromosomal DNA of the wild-type (lane 1) and the mutant (lane 2) strains.

In order to discard any possible polar effects of gene disruption on the transcription of other genes of the cluster, RT-PCR was carried out for all of the *pim* genes. Primers for RT-PCR were specific to sequences within *pim* genes and were designed to produce cDNAs of ~500 bp [26]. A primer pair designed to amplify a cDNA of the *lysA* gene (encoding diaminopimelate decarboxylase) [26] was used as an internal standard of primary metabolism genes not involved in pimaricin biosynthesis. All genes showed a transcriptional pattern

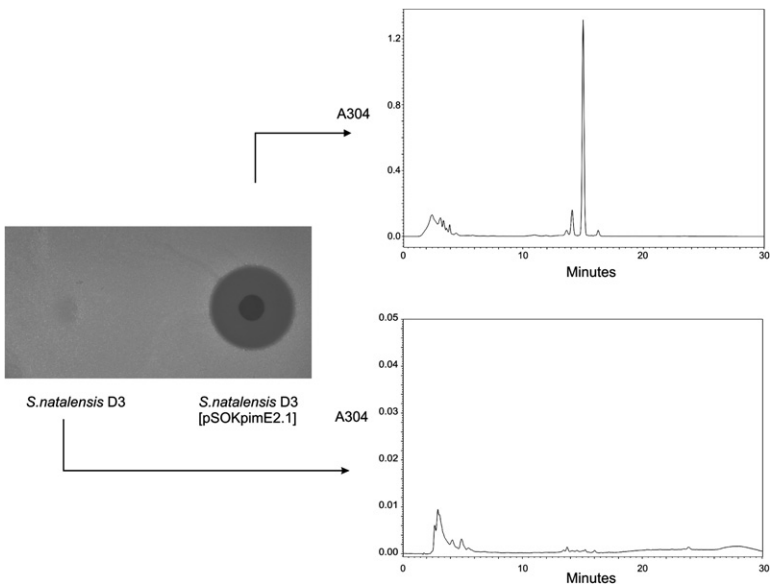


**Figure 4. Disruption of PimE Blocks Pimaricin Production, and Gene Complementation Restores Antifungal Biosynthesis**

Comparison of HPLC analyses of butanol-extracted broths from *S. natalensis* D3 (bottom) and *S. natalensis* D3 transformed with pSOK-pimE2.1 (top). Detection was carried out at $A_{304}$. Cells were grown for 48 hr at 300 rpm and 30°C in YEME medium. Bioassays for pimaricin production, using *C. utilis* as test organism, are included at the left.

**Table 1. Purification of PimE Cholesterol Oxidase**

| Pool | Protein (mg) | Activity (U) | Specific Activity (U/mg Protein) | Yield (%) | Purification (Fold) |
|---|---|---|---|---|---|
| Broth | 188.6 | 141.45 | 0.75 | 100 | 1 |
| Ammonium sulfate | 32.4 | 66.19 | 2.04 | 46.8 | 2.7 |
| DEAE-FF | 12.8 | 41.01 | 3.2 | 29 | 4.2 |
| Superdex 75 | 3.1 | 37.66 | 12.15 | 26.6 | 16.2 |

comparable to that of the parental strain (data not shown), indicating that gene disruption did not have any polar effect on their transcription.

Taken together, all of these results suggest the involvement of the *pimE* gene product in the biosynthesis of the pimaricin molecule and constitute the first, to our knowledge, indication of the involvement of cholesterol oxidases in polyene macrolide biosynthesis.

## Complementation of *pimE*-Disrupted Mutants Restores Cholesterol Oxidase Activity and Pimaricin Production

To confirm that the disruption of *pimE* was directly responsible for the observed loss of cholesterol oxidase activity and also pimaricin production, we complemented the *pimE*-disrupted mutant with *pimE*. A DNA fragment containing *pimE* plus its putative promoter region was inserted into the self-replicating vector pSOK201 [33], giving rise to pSOKpimE2.1 (see Experimental Procedures). The recent development of a method for intergeneric gene transfer from *E. coli* to *S. natalensis* by conjugation [34] enabled its use for the introduction of pSOKpimE2.1 into the *pimE*-disrupted mutant. The plasmid was thus transferred from *E. coli* ET12567 [pUZ8002] to *S. natalensis* D3. Introduction of pSOKpimE2.1 restored cholesterol oxidase activity in the mutant, as well as pimaricin biosynthesis (Figure 4). These results were fully consistent with those obtained upon disruption of the *pimE* gene, and they confirm the involvement of PimE cholesterol oxidase in pimaricin biosynthesis.

## Purification of PimE Reveals It as an Authentic Cholesterol Oxidase

The enzyme was purified from *S. natalensis* culture broths after growth for 48 hr in YEME medium. A summary of the specific activity and recovery of the enzyme during the purification procedure is given in Table 1. Each purification step was also analyzed by SDS-PAGE, followed by Coomassie staining (Figure 5). The purified enzyme showed a homogenous protein band on SDS-PAGE. The enzyme was purified 16.2-fold with a yield of 26.6% from the culture broth.

The N-terminal sequence of the mature PimE protein obtained from the gel filtration column was Arg-Ser-Gly-Ser-Phe-Val-Pro-Ala-Val, which fully agrees with the prediction based on sequence alignments with the sequence deduced from the *pimE* gene (see above). The molecular mass of the mature enzyme was estimated to be ~55 kDa by SDS-PAGE (Figure 5), which is slightly higher than the predicted value deduced from the sequence (54.9 kDa).

Enzymatic assays with the purified enzyme showed the production of 4-cholesten-3-one when using cholesterol as substrate (Figure 5), thus demonstrating that PimE is an authentic cholesterol oxidase. The enzyme was also able to oxidize ergosterol, albeit at a low level; 22% of the activity observed when using cholesterol as substrate was reached.

## Exogenous Cholesterol Oxidase Restores Pimaricin Production in *pimE*-Disrupted Mutants

The availability of purified PimE protein allowed its addition to culture broths of the mutant strain in order to check its role as a putative signal protein for pimaricin biosynthesis. Cells were grown in YEME medium containing increasing concentrations of PimE, and pimaricin production was assessed after 48 hr of growth. Surprisingly,

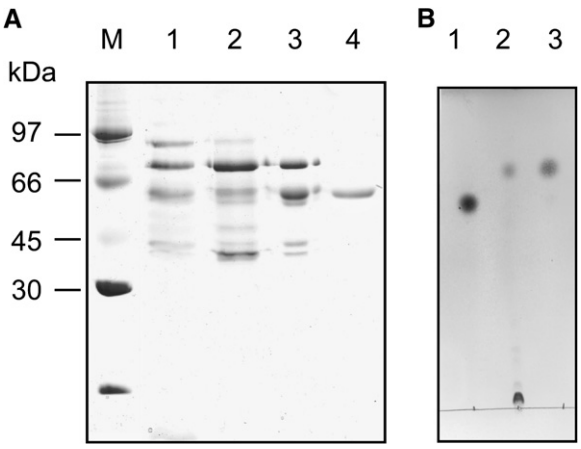


**Figure 5. Purification of PimE and Determination of Its Enzymatic Product**

(A) SDS-PAGE (10% polyacrylamide) of the broth and partially purified fractions. The gel was stained with Coomassie brilliant blue. Lanes: M, molecular weight markers: phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa); 1, broth; 2, active fractions after ammonium sulfate precipitation; 3, active fractions after anion-exchange chromatography; 4, active fractions after Superdex 75 gel filtration chromatography.

(B) Oxidation of cholesterol by purified PimE. The TLC migrations of cholesterol (lane 1) and 4-cholesten-3-one (lane 3) standards are shown. Lane 2 shows the formation of 4-cholesten-3-one after incubation of PimE with cholesterol.

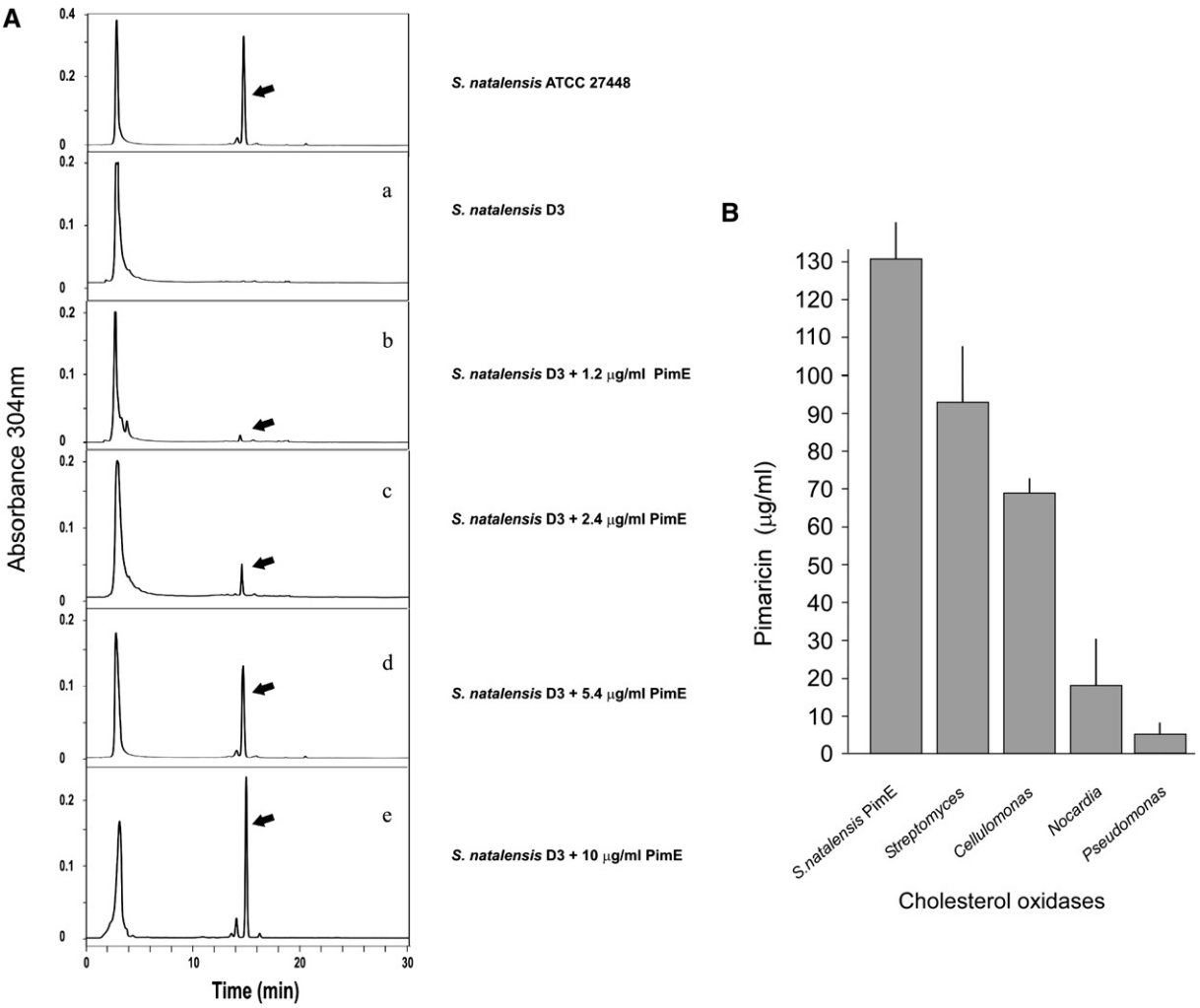


**Figure 6. Addition of Cholesterol Oxidase to *S. natalensis* D3 Culture Broths Restores Pimaricin Biosynthesis**

(A) *S. natalensis* D3 cells were grown in YEME medium, and pimaricin production was assessed after 48 hr of growth. The results of analytical HPLC after addition of purified PimE to the culture media are shown as follows: (a) no enzyme; (b) 1.2 μg/ml; (c) 2.4 μg/ml; (d) 5.4 μg/ml; (e) 10 μg/ml PimE. Chromatographic peaks corresponding to pimaricin are indicated by arrows. *S. natalensis* wild-type is included for comparative purposes. Note that broths of the mutant strain have been concentrated 5-fold.

(B) Effect of different cholesterol oxidases on pimaricin production by *S. natalensis* D3. Enzymes used are: PimE, *Streptomyces* sp. enzyme, *Cellulomonas* sp. enzyme, enzyme from *Nocardia erythropolis*, and *Psesudomonas fluorescens* enzyme. All enzymes were added at 10 μg/ml concentration. Pimaricin production was assessed after 48 hr of growth. Note that pimaricin production of *S. natalensis* ATCC 27448 under identical conditions was 760 μg/ml. Error bars indicate the standard deviation values of the mean of three determinations.

enzyme concentrations as low as 1 μg/ml in the culture media were enough to trigger pimaricin production. Figure 6A shows the production of pimaricin after addition of growing concentrations of PimE enzyme to the cultures as measured by HPLC. The identity of the pimaricin peak was assessed by LC/MS. This unexpected result prompted us to investigate whether other cholesterol oxidase enzymes could also produce the same effect. We tested four cholesterol oxidases (10 μg/ml) from different microorganisms: namely, *Streptomyces* sp., *Cellulomonas* sp., *Pseudomonas fluorescens*, and *Nocardia erythropolis*. Interestingly, all of them triggered pimaricin production to some extent. Figure 6B shows the production of pimaricin upon addition of the different cholesterol oxidases to the culture broth. The cholesterol oxidase from *Streptomyces* sp. turned out to be the most effective, reaching levels of 72% of the pimaricin obtained with PimE. Cholesterol oxidase from *Cellulomonas* sp. also resulted in high levels of pimaricin production (53% of the positive control). Less effective was the addition of the enzyme from *Nocardia* (14%) and that from *Pseudomonas* (4%). These results provide strong evidence for a signaling role for PimE in pimaricin biosynthesis, and they prove that such signal can be substituted by proteins with a similar protein structure and equivalent enzymatic activity.

In order to test whether the effect observed was attributable to the enzymatic activity of the protein on cholesterol, we also assayed pimaricin production after addition

of increasing concentrations of cholesterol oxidase canonical reaction products such as $H_2O_2$ or cholestenone (10 pM to 10 nM) to the mutant's culture broth. However, we could not detect any pimaricin being produced under the conditions used (data not shown). This suggests that the enzyme might be using a substrate other than cholesterol during the signaling process.

We thus decided to repeat the assay with the addition of PimE cholesterol oxidase preincubated with cholesterol or ergosterol. PimE cholesterol oxidase (10 μg/ml; 20 μM) was treated with 20 μM cholesterol or ergosterol for 10 min at 30°C, and it was then added to the cells. Pimaricin production was assessed after 72 hr. Interestingly, pimaricin biosynthesis was not observed in the samples in which the enzyme had been preincubated with ergosterol. Although samples in which the enzyme had been preincubated with cholesterol showed pimaricin production, the production level was only 13% of that observed in the control experiment in which PimE was maintained for 10 min at 30°C in the absence of added sterols, thus indicating that an occupied active site is not needed for the effect, and that ergosterol and cholesterol are unlikely to constitute the actual substrates for the enzyme in the signaling process.

To discard the possibility that PimE cholesterol oxidase acts on a medium component, the equivalent assay was performed with resting cells. Cells were grown for 36 hr in YEME medium, and they were then extensively washed with 50 mM MOPS buffer (pH 7.2) and suspended in the same buffer. PimE cholesterol oxidase was then added (2.4 μg/ml), and cells were kept under agitation for an additional 48 hr. Pimaricin production was then assessed. Interestingly, pimaricin production was triggered in the mutant, reaching 9.6 μg pimaricin/ml (data not shown), thus suggesting that PimE either acts directly on its cellular target (a receptor) or on a strain-specific product.

Although results with washed resting cells corroborated the signaling role of PimE in pimaricin biosynthesis, it could be argued that the washing conditions used were not sufficient to extract all sterols from the cell membrane that may have accumulated from the medium. In order to eliminate such a possibility, we repeated the assay by growing cells in a minimal medium (PMM) without yeast extract and malt extract (see Experimental Procedures). Cells were grown for 96 hr in PMM in the presence of 10 μg/ml PimE cholesterol oxidase, and pimaricin production was assessed every 24 hr. Although pimaricin production by the wild-type strain in this medium is greatly reduced when compared with that in YEME medium (only 67 μg/ml after 72 hr of incubation), we could detect pimaricin being produced by mutant cultures 48 hr after addition of PimE; the amount of pimaricin produced reached a maximum of 1.8 μg pimaricin/ml at 72 hr (data not shown). This result excludes the possibility of PimE interacting with sterols, and it corroborates our previous conclusion.

The involvement of the enzymatic activity in the signaling process was demonstrated by using cholesterol oxidase inhibitors in the resting cell signaling assay. Cells were grown for 36 hr in YEME medium, and they were then extensively washed with 50 mM MOPS buffer (pH 7.2) and suspended in the same buffer containing PimE (14 μg/ml) that had been previously treated with 2 mM $HgCl_2$ or $AgNO_3$ for 30 min at 24°C. Both salts inhibit PimE in vitro cholesterol oxidase activity when cholesterol is used as substrate (70% and 95%, respectively) (unpublished data). Cells were then kept under agitation, and pimaricin production was assessed every 24 hr. Interestingly, we could not detect any pimaricin being produced in the samples that had been preincubated with the salt, even after 144 hr of incubation, whereas in the control experiment in which PimE was maintained for 30 min at 24°C in the absence of salt, we readily detected pimaricin after 48 hr of incubation; the amount of pimaricin produced reached a maximum of 22 μg pimaricin/ml at 96 hr, thus indicating that the observed effect is dependent on the enzymatic activity of the protein.

Taken together, these results support the involvement of PimE enzymatic activity in the signaling process, but they do not support the possibility of an interaction between PimE and a medium component, or with putative sterols, suggesting a direct interaction with the cells or with a strain-specific product. The actual target for the signal protein remains to be established.

### PimE Does Not Cooperate with Pimaricin on Antifungal Activity

In higher eukaryotes, cholesterol oxidases interact with the membrane lipid bilayer in order to bind its steroid substrate [19], and this action yields cholest-4-en-3-one, which significantly alters the structural integrity of the membrane [35, 36]. This production of cholestenone from cellular cholesterol is the basis for the insecticidal activity of cholesterol oxidases against Coleoptera [11].

Recently, it has been proposed that the tight packing interaction of cholesterol and similar sterols with saturated membrane lipids (lipid rafts) can be attributed to the properties of their small polar 3-OH group, among other factors [36]. The small polar group could help position the sterol at the proper depth in the bilayer to allow tight packing with lipids, and to prevent exposure of sterol hydrocarbon to water. The elimination of the polar OH group in the product of cholesterol oxidase activity would therefore lead to a more porous and less stable membrane. If this were also true for fungal membranes in which ergosterol promotes raft formation more strongly than cholesterol [37], then PimE cholesterol oxidase could be directly involved in weakening the structured ergosterol-lipid interactions and thus collaborating with the antifungal action of pimaricin or even showing its own antifungal activity.

In order to test this hypothesis, putative antifungal activity of purified cholesterol oxidase was tested by bioassay against *C. utilis*. No antifungal activity could be detected regardless of the amount of cholesterol oxidase used in the assays (data not shown). Similarly, we could not detect any cooperative effect with the antifungal action of pimaricin.

## DISCUSSION

Cholesterol oxidases (EC 1.1.3.6) are enzymes produced by a variety of microorganisms [10], including life-threatening pathogens such as *Rhodococcus equi*, *Mycobacterium tuberculosis*, and *M. leprae* [17]. Although cholesterol oxidases have proven to be very useful for human applications that require detection or disruption of cholesterol-containing membranes, their biological role in the cell remains partly unknown. Originally, these enzymes were found to participate in the first step of a metabolic pathway for utilizing cholesterol as a carbon and energy source [38]. Recently, other cellular roles have been described; gene-disruption studies of the cholesterol oxidase gene *choE* in *Rhodoccocus equi* have indicated that cholesterol oxidase is a major membrane-damaging factor, thus contributing to pathogenicity and leading to the characteristic lesions found in infected animals and humans [17]. In the present study, we have used gene-disruption and -complementation experiments to demonstrate that *pimE* encodes a functional cholesterol oxidase in *S. natalensis*, but, more interestingly, that the encoded protein PimE is a key enzyme in the biosynthesis of the polyene macrolide pimaricin. To our knowledge, this finding constitutes the first description of a cholesterol oxidase gene with an involvement in antibiotic biosynthesis, and it broadens the scope of the biological functions for this type of oxidase.

In a previous work, we found that *pimE* was the primary cellular target of PimR, the pathway-specific regulator of pimaricin biosynthesis in *S. natalensis* [26]. PimR is the archetype of a new, to our knowledge, class of regulators that combines an N-terminal domain corresponding to the SARP family of transcriptional activators, a central domain with similarity to the nucleotide triphosphate-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 it also reduced the transcription of all of the key enzyme-encoding genes for pimaricinolide construction to very low levels, thus blocking pimaricin production completely and suggesting that PimE was somehow involved in pimaricin biosynthesis [26]. The lack of pimaricin production upon gene disruption of *pimE* and the recovery of antibiotic biosynthesis upon gene complementation further support our previous observations, and they clearly demonstrate the involvement of PimE in the biosynthesis of this antifungal macrolide.

Given that PimE is a functional cholesterol oxidase of extracellular nature, and that transcription of the remaining *pim* genes is not affected in *S. natalensis* D3 mutant, no obvious function could be predicted for it in pimaricin biosynthesis. We thus decided to purify it in order to assay its effect when added to the mutant cultures. Surprisingly, addition of PimE triggered pimaricin production, and the effect observed was dependent on the amount of the enzyme added. This result provides strong evidence for the participation of PimE in a signal transduction pathway that leads to the final production of the antibiotic. Furthermore, PimE could be substituted by other cholesterol oxidases, including the enzymes from *Streptomyces* sp., *Cellulomonas* sp., *Nocardia erythropolis*, and the Gram-negative bacterium *Pseudomonas fluorescens*, thus indicating that cholesterol oxidase enzymes may fulfill a signaling role in pimaricin biosynthesis. The target of these proteins remains to be established; however, our results discard the possibility that PimE interacts with a medium component, and with putative sterols, suggesting a direct interaction with the cells or with a strain-specific product. Also, our results indicate that the signaling effect is dependent on the enzymatic activity of the protein, but also that cholesterol and ergosterol are unlikely to constitute the actual substrates for the enzyme in the signaling process. It is noteworthy that the *Pseudomonas* enzyme is able to function as signal, although weakly, for pimaricin biosynthesis in our assay system. Unfortunately, this enzyme's sequence is not available at the databases, but, belonging to *Pseudomonas*, it is likely to have little sequence similarity with *Streptomyces* enzymes. This would explain the weak effect of this enzyme under our assay system. These results now raise the question of what is the target for the signal protein. Further experimental analyses will provide the answer to this question.

Given that the antifungal activity of polyene antibiotics is based on their interaction with membrane sterols, causing the alteration of membrane structure and leading to the leakage of cellular materials [22], it is conceivable that PimE, besides its signaling role in antibiotic biosynthesis, could facilitate the antifungal action of pimaricin, or even show its own antifungal activity. The product of cholesterol oxidase is cholestenone, which alters the integrity of the membrane [35]. However, our results discard this hypothesis; therefore, the results observed can only be attributed to the involvement of PimE in a signal transduction cascade.

Computer-assisted analysis of the *pimE* gene product showed a very high (more than 80%) end-to-end sequence identity with several cholesterol oxidases, including ChoA and ChoM from the *Streptomyces* sp. strains SA-COO and A19249, respectively [2, 12], and, interestingly, with PteG, a putative cholesterol oxidase whose encoding gene was found within the *pte* gene cluster of *S. avermitilis* [39]. The *pte* gene cluster encodes the pentaene filipin, a 28-membered polyene macrolide very similar in size to pimaricin. Recently, another cholesterol oxidase gene (*rimD*) had been found to be encoded by the gene cluster responsible for the biosynthesis of the 28-membered tetraenes rimocidin and CE-108 in *S. diastaticus* var. 108 [29]. Although the availability of sequenced polyene gene clusters is limited, it seems that the presence of a cholesterol oxidase-encoding gene could constitute a genetic marker for the gene clusters of small-size polyenes (26–28 members). Bearing in mind the role of PimE in pimaricin biosynthesis, it is conceivable that the enzymes encoded by these genes could also fulfill a similar role in the biosynthesis of their respective polyene macrolides. It is therefore plausible that the genes that encode cholesterol oxidases might have been acquired in the process of evolution by the polyene antibiotic

 

gene clusters in order to provide a prompt response to the presence of fungi (whose cell envelopes contain ergosterol) in the environment via expression of the polyene antibiotic biosynthesis genes.

Thus far, the presence of common regulatory circuits controlled by cholesterol oxidase enzymes cannot be extended to polyenes of large aglycones since no cholesterol oxidase-encoding genes have been found in the gene clusters for the recently characterized nystatin [40], amphotericin [41], or candicidin [42, 43] polyene macrolides.

## SIGNIFICANCE

**Polyenes represent a major class of antifungal agents characterized by the presence of a series of conjugated double bonds in their planar, hydroxylated macrolide ring structure. Despite their general interest, very little is known about the factors that modulate their biosynthesis. Thus far, only transcriptional regulatory genes and low-molecular weight inducers have been found to be involved in macrolide regulation. This report describes an authentic cholesterol oxidase (encoded by *pimE* of the pimaricin cluster), an extracellular enzyme traditionally considered to have a nutritional role in bacteria, as an essential element for the biosynthesis of the tetraene pimaricin. The discovery of the involvement of cholesterol oxidases in a signal transduction cascade for the production of polyenes is totally unexpected, and it broadens the scope of the biological functions for this type of oxidase. Additional experiments that examine the precise mechanism by which cholesterol oxidase modulates polyene biosynthesis will aid in the understanding of the molecular mechanism of signal transduction.**

## EXPERIMENTAL PROCEDURES

### Materials

Sepharose Q XL, Superdex 75 columns, and Mr standards for SDS-PAGE were obtained from Amersham Pharmacia Biotech; ProBlott polyvinylidene difluoride membranes and sequencing reagents were obtained from Applied Biosystems, Inc. Commercial cholesterol oxidases were obtained from *Pseudomonas fluorescens*, *Streptomyces* sp., *Cellulomonas* sp. (Sigma), and *Nocardia erythropolis* (Fluka). All other reagents were obtained from commercial sources and were of analytical grade.

### Bacterial Strains, Cloning Vectors, and Cultivation

*S. natalensis* ATCC 27448 was routinely grown in YEME medium [44] without sucrose. Sporulation was achieved in TBO medium [24]. For pimaricin production, the strain was grown in YEME medium without sucrose, as described elsewhere [25]. The minimal medium used for pimaricin production was PMM ($MgSO_4 \cdot 7H_2O$, 0.2 g/l; $FeSO_4 \cdot 7H_2O$, 0.01 g/l; $CaCl_2$, 1 g/l; MOPS, 2 g/l [pH 7.0]; L-asparagine, 2 g/l; glucose, 20 g/l; glycerol, 5 g/l; $KH_2PO_4$+$K_2HPO_4$, 1 mM; $CuSO_4 \cdot 5H_2O$, 0.7 μM; $H_3BO_3$, 0.4 μM; $MnSO_4 \cdot H_2O$, 0.12 μM; $ZnSO_4 \cdot 7H_2O$, 14 μM; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.013 μM). The same media were supplemented with thiostrepton when used for *S. natalensis* D3 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* CECT 1061 was used for bioassay experiments. Phage KC515 ($c^+$ attP::tsr::vph), a ØC31-derived phage [32], was used for gene-disruption experiments. *Streptomyces lividans* JII 1326 [45] served as a host for phage propagation and transfection. Infection with Ø1D3 (the KC515 recombinant derivative used for gene disruption) was carried out in R5 medium [44]. Standard conditions for culture of *Streptomyces* species and isolation of phages were as described by Kieser et al. [44].

### Genetic Procedures

Standard genetic techniques with *E. coli* and in vitro DNA manipulations were as described by Sambrook and Russell [46]. Recombinant DNA techniques in *Streptomyces* species and isolation of *Streptomyces* total and phage DNA were performed as previously described [44]. Southern hybridizations were carried out with probes labeled with digoxigenin by using the DIG DNA labeling kit (Roche). Intergeneric conjugation between *E. coli* ET12567 [pUZ8002] and *S. natalensis* was performed as described [34].

### Isolation of Total RNA and Northern Analysis

*S. natalensis* ATCC 27448 was grown for 48 hr in YEME medium without sucrose (stationary phase of growth), the cultures were then mixed with one volume 40% glycerol, and mycelia were harvested by centrifugation and were immediately frozen by immersion in liquid nitrogen. Frozen mycelia were then broken by shearing in a mortar, and the frozen lysate was added to buffer RLT (QIAGEN) in the presence of 1.5% β-mercaptoethanol. RNeasy Mini Spin columns were used for RNA isolation according to the manufacturer's instructions. RNA preparations were treated with DNase I (Promega) in order to eliminate possible chromosomal DNA contamination. After separation in denaturing agarose gels, the RNA was transferred to nylon membranes. Northern hybridization was performed by standard methods [46] with a 1,111 bp PvuII probe internal to *pimE*.

### Construction of a *pimE* Mutant

The *pimE* gene was disrupted by KC515 phage-mediated single-crossover integration as follows: A 7.6 kb NotI fragment encompassing most of the *pimE* gene (lacking 107 bp from the 5′ end of the gene), *pimC*, *pimG*, *pimF*, and part of the *pimS0* gene (Figure 2) was cloned into a NotI-cut pBluescript vector to yield p980n1. This plasmid was then used as a source of DNA for gene disruption. A 1,111 bp PvuII fragment (fragment "a" in Figure 2) was ligated into a SmaI-cut pBluescript, which was subsequently cut with BamHI and PstI (both sites belong to the polylinker), and the resulting fragment was then cloned into the BamHI-PstI sites of KC515. Due to the absence of SacI sites within the insert, the recombinant phages were selected by digestion of the ligation mixture with SacI (KC515 is susceptible to digestion, while KC515 derivatives are resistant). Transfection of *S. lividans* protoplasts resulted in a number of phage plaques that were screened by Southern hybridization for the presence of *pimE*-derived sequences. One of the recombinants, Ø1D3, was selected and used to infect *S. natalensis*, thus allowing the selection for lysogen formation. Lysogens were selected by thiostrepton resistance on R5 medium [44] and were confirmed by genomic Southern hybridization.

### Complementation of *pimE*

In order to complement *pimE* deletion mutants, a 6.9 kb SphI fragment containing the entire *pimE* gene, including its own promoter, *pimC*, *pimG*, *pimF*, and part of the *pimS0* gene (Figure 2), was cloned into a SphI-cut pUC vector to yield pJA1. This plasmid was then digested with BamHI (one of the sites belonging to the polylinker), and the resulting 2,992 bp fragment was ligated into a BamHI-cut pSOK201 ($Neo^R$, $Am^R$, ColE1 + pSG5 replicons [33]) to yield pSOKpimE2.1. This plasmid was then transferred by conjugation from *E. coli* ET12567 [pUZ8002] to the *S. natalensis pimE*-disrupted mutant (D3) as previously described [34].

### Cholesterol Oxidase Assay

Cholesterol oxidase activity was assessed as described previously [9]. The assay mixture contained 50 mM potasium phosphate (pH 7.0), 5 mM sodium cholate, 1 mM cholesterol, 7 mM phenol, 1.4 mM 4-aminoantipyrine, 0.33% Triton X-100, and 7 U horseradish peroxidase. For determination of cholesterol oxidase activity, the appearance of quinoneimine dye, which is formed by the reaction with 4-aminoantipyrine, phenol, and peroxidase, was measured espectrophotometrically at 500 nm. One unit of activity was defined as the formation of 1 μmol hydrogen peroxide (0.5 μmol of the red dye) per min at pH 7.0 and 37°C.

### Assay of Pimaricin Production

Culture broths were extracted with butanol and processed as previously described [25]. To confirm the identity of pimaricin, a 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 was tested by bioassay by using *C. utilis* CECT 1061 as the test organism. Quantitative determination of pimaricin was performed with a Waters 600 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 performed with a gradient (1 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 14.5 min. HPLC-MS assays were carried out on an LC-TOF (Micromass) by using electrospray as the source and the positive ionization mode.

### Gene Expression Analysis by RT-PCR

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

### Cholesterol Oxidase Purification

All purification procedures were carried out at 4°C. *S. natalensis* ATCC 27448 was grown in YEME medium and the broth collected after 48 hr. Cells were removed by filtration under vacuum, and the broth (1320 ml) was used as the source of enzyme.

#### *Step 1: Ammonium Sulfate Fractionation*

Ammonium sulfate was added in order to bring the concentrated broth to 70% saturation, and the mixture was stirred overnight at 4°C. After centrifugation, the pellet was dissolved in buffer A (50 mM Tris-HCl [pH 7.0], 1 mM EDTA, 1 mM 1,4-dithiothreitol).

#### *Step 2: Anion-Exchange Chromatography*

The enzyme solution (32.4 mg protein and 141.45 U of enzyme in 440 ml) was dialyzed against buffer A. Then, it was divided into two aliquots, and each aliquot was applied to a DEAE-FF column (1 cm × 5 cm, 5 ml) equilibrated with buffer A (flow rate of 1 ml/min). The column was washed with 50 ml of this buffer. The cholesterol oxidase enzyme did not bind to the column. This solution was then concentrated in Amicon Ultra 15 concentrators. The final volume of pooled active fractions was 120 ml (12.8 mg of protein, 41.01 U).

#### *Step 3: Superdex 75 Chromatography*

The former solution was used as the source of material for size-exclusion chromatography. For each chromatography, 2 ml of the former enzymatic preparation was applied (flow rate of 0.1 ml/min) to a Hi Load 16/60 Superdex 75 column (1.6 cm × 60 cm; 120 ml) equilibrated with buffer A containing 150 mM NaCl and 5 mM sodium deoxycholate. In the absence of sodium deoxycholate, the oxidase eluted over a wide range of fractions and was not separated from impurities. Fractions were collected and assessed for the presence of cholesterol oxidase by SDS-PAGE. The final volume of the pooled cholesterol oxidase-pure fractions was 54 ml (3 mg protein, 37.66 U).

### Analytical Methods

Protein concentrations were estimated by using the Bradford method [47] or by measuring the absorbance of column eluates at 280 nm. SDS-PAGE was performed on 10%–12% polyacrylamide gels by following standard procedures. After electrophoresis, gels were stained either with Coomassie brilliant blue R-250 or with silver. Alternatively, gels were transferred to ProBlott polyvinylidene difluoride membranes according to Matsudaira [48] and were subjected to Edman degradation on an Applied Biosystems 477A pulsed-liquid protein sequencer. TLC plates (Silica Gel G-60, Merck) were developed in a mobile phase of toluene/ethyl acetate/formic acid (80:20:5 vol/vol/vol), and compounds were stained with 20% phosphomolibdic acid in ethanol.

## ACKNOWLEDGMENTS

This work was supported by Spanish Ministry of Education and Science grants to J.F.A. (BIO2001-0040, BIO2004-00066, and GEN2003-20245-C09-08/NAC) and J.F.M. (BIO2003-01489), and by the Agencia de Desarrollo Económico from Castilla y León (Proyecto Genérico 2005). M.V.M. received a fellowship of the Fundação para a Ciência e a Tecnologia (PRAXIS XXI/BD/15850/98). N.A. and J.S.-A. were the recipients of Formación de Profesorado Universitario fellowships from the Ministerio de Educación, y Ciencia (AP2002-1446 and AP2005-3644, respectively). S.M.G. received a fellowship from the University of León. We thank J. Merino, B. Martín, M. Álvarez, and A. Casenave for their excellent technical assistance.

Received: September 7, 2006
Revised: January 11, 2007
Accepted: January 17, 2007
Published: March 23, 2007

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