PI factor, a novel type quorum-sensing inducer elicits pimaricin production in *Streptomyces natalensis*

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Runing title: A novel “quorum sensing” inducer

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SUMMARY

A chemically novel autoinducer (PI factor) has been purified from cultures of the pimaricin producer *Streptomyces natalensis* ATCC27448. The chemical structure of the PI molecule was identified as 2,3-diamino -2,3-bis (hydroxymethyl) -1,4-butanediol. Pimaricin biosynthesis in *S. natalensis npi287*, a mutant impaired in pimaricin production, was restored by supplementation with either A-factor from *Streptomyces griseus* IFO13350 or with PI factor. *S. natalensis* did not synthesize A-factor. The PI autoinducer was active at very low concentrations (50 to 350 nM). A threshold level of 50 nM was required to observe the induction effect. The dose-response curve was typical of a “quorum sensing” type mechanism. The biosynthesis of PI factor was associated with cell growth of *S. natalensis*, both in defined and complex media. Supplementation of the wild type *S. natalensis* with pure PI (300 nM) resulted in a stimulation of 33 % of the production of pimaricin. These results indicate that the endogenous synthesis of PI factor is limiting for pimaricin biosynthesis in the wild type strain. This water-soluble PI factor belongs to a novel class of autoinducers in *Streptomyces* species different from the classical butyrolactone autoinducers. Since restoration of pimaricin production in the *npi287* mutants is conferred by both A-factor and PI factor, *S. natalensis* appears to be able to integrate different quorum signals from actinomycetes.

INTRODUCTION

Actinomycetes produce a wide variety of secondary metabolites [1-4], and among them polyene macrolides, which are antifungal compounds synthesized by more than one hundred different species of actinomycetes [5, 6]. From the biosynthetic point of view these compounds are a subclass of the widely distributed polyketides [7, 8, 9].
Pimaricin (Fig. 1) represents a prototype molecule of glycosylated polyenes [5] important for antifungal therapy and promising for its antiviral activity, stimulation of the immune response, and action in synergy with other antifungal drugs or antitumor compounds [10]. Pimaricin is produced by *Streptomyces natalensis* and it is widely used in the food industry to prevent mold contamination of cheese and other non-sterile foods (i.e. cured meats). Initial studies showed that the synthesis of this 26-membered macrolide tetraene in *S. natalensis* requires a complex polyketide synthase (PKS) [11]. The complete sequence and analysis of the PKS genes responsible for the biosynthesis of pimaricin has been reported. The sequenced region of 85 kb encodes 13 PKS modules within five multifunctional enzymes, and 12 additional proteins that presumably catalyze post-PKS modification of the polyketide skeleton (tailoring enzymes), export and regulation of gene expression [12, 13, 14].

Secondary metabolism and cell differentiation in actinomycetes are controlled by diffusible low molecular weight chemical substances called autoregulators [15, 16]. There are different types of autoregulatory factors [17]. They belong to at least five chemical classes: 1) the butyrolactone class (e.g. the A-factor of *Streptomyces griseus*), the virginia butanolide (VB) factors of *Streptomyces virginiae* [18] and similar compounds isolated from *Streptomyces coelicolor* [19, 20] and other actinomycetes [21]; 2) the homoserine lactones of Gram-negative bacteria [16, 22]; 3) the nucleotide-like B-factor of rifamycin-producing *Amycolatopsis* (*Nocardia*) *mediterranei* [23, 24]; 4) furanosyl borate diester, a novel quorum sensing inducer in Gram-negative bacteria [25]; and 5) the modified peptide pheromones of Gram-negative and Gram-positive bacteria [26, 27].

In an effort to understand the regulation of the commercially important antifungal pimaricin, it was of great interest to isolate and characterize the autoregulatory molecule of pimaricin biosynthesis in *S. natalensis*. 
We describe in this article the isolation of a class of mutants that is defective in pimaricin production because it lacked a pimaricin inducer. Complementation tests of this mutant and purification of the inducer molecule led to the identification of a novel hydrophilic pimaricin inducer (PI) molecule.

EXPERIMENTAL PROCEDURES

Strains

The wild type *Streptomyces natalensis* ATCC 27448 was used as the parental strain to isolate different *npi* mutants. *S. natalensis* cultures were maintained on solid TBO sporulation medium (containing per liter tomato paste 20 g; oat meal 25 g; agar 25 g) as described previously [12]. *Candida utilis* (syn. *Pichia jadinii*) CECT 1061 was used as test strain in the bioassay of the antifungal activity of pimaricin.

Cultures of *Streptomyces griseus* IFO 13350 (formerly described as *S. bikiniensis* IFO 13350; S. Horinouchi, personal communication) were used to produce A-factor in YMPG medium [28]. *S. griseus* HH1 (an A factor negative strain) was used to quantify the A-factor inducing activity.

Mutagenesis and isolation of non-producing clones

Spores of *S. natalensis* ATCC 27448 (about 10⁶ spores/ml) were suspended in 0.05 M Tris-Maleate buffer, pH 9.0 and mutated with N-methyl-N’-nitrosoguanidine (1 mg/ml) for 20 min at 30 ºC. Under those conditions the mortality after 20 min was about 50%.

The mutated spores were washed, diluted and plated in YED medium and incubated at 28ºC. When the colonies started to grow (24 h), agar plugs (7 mm diameter) containing individual colonies were cut out from the plates, incubated under high a_w (humidity) conditions for an additional 24 h and the pimaricin production by each clone was assayed on a
lawn of *C. utilis*. Mutants that did not produce inhibition zones were selected. The lack of production of pimaricin of the selected mutants was confirmed in liquid cultures in SPG medium [29]. Mutants that did not revert in liquid medium cultures were further analyzed by complementation tests.

**Culture media and quantification of pimaricin production in liquid cultures.**

Four different complex media were used for quantification of the PI factor concentration and its relationship to pimaricin production. These include: NBG medium [nutrient broth (Oxoid) supplemented with glucose (5 g/l)]; YEME medium (yeast extract 3 g/l; peptone 5 g/l; malt extract 3 g/l and glucose 10 g/l); TSB medium (Difco) and YED medium (yeast extract 10 g/l; glucose 10 g/l). In addition two defined media were also used to quantify the inducer production: *Streptomyces* MM [30] and Lechevalier defined medium [31].

The production of pimaricin in liquid cultures was routinely quantified by spectrophotometric determination at 319 nm. A 0.5 ml aliquot of the culture was extracted with 5 ml of methanol and diluted with distilled water; the concentration of pimaricin was determined as described previously [12] using a pure sample of pimaricin (Sigma Chem. Co) as standard.

**Complementation tests**

Complementation tests were performed between pairs of the 35 stable non-producer mutants using standard co-synthesis methods in solid YED medium. Each pair of *npi* mutants were grown as lawn cultures. Agar plugs were taken out from each of the growth zones and the production of pimaricin was bioassayed using *Candida utilis* as sensitive organism. Positive complementation was clearly detected by the production of pimaricin when the two
non-producer mutants were placed close to each other, whereas control plugs from each of the two non-producer mutants gave no inhibition zone when assayed separately (Fig. 1).

**Extraction and HPLC purification of the PI factor to homogeneity**

The culture broth (15 liters) from *S. natalensis* wild type strain grown for 24 h in YED medium in a Braun Biostat C fermentor was concentrated 10-fold in a vacuum evaporator. The concentrated broth was clarified by precipitating the proteins with HCl at pH 3.0 in the cold room. The clarified broth was adjusted to pH 7.0 with concentrated NaOH and extracted with ethyl acetate (see Experimental Procedures). The organic phase was then collected and concentrated to dryness under vacuum and the inducer was dissolved in 100 ml of 10% methanol (v/v) and applied to an active carbon column (30 x 3 cm) previously equilibrated with the same solvent (10% methanol, v/v). The retained compounds including the PI factor were fractionated by stepwise elution (flow 2 ml/min) with 50% methanol (v/v), 100% methanol, 10% ethylacetate in methanol (v/v), 50% ethylacetate in methanol (v/v) and pure ethyl acetate. Bioassays of the PI factor showed that it eluted in the second fraction (100% methanol). The inducing fraction was then concentrated, applied onto a Sephadex G10 column (2000 x 1 cm) and eluted with distilled water (flow 0.2 ml/min). This size exclusion chromatography yielded 40 ml of active fractions.

The PI factor was purified by reverse phase HPLC using a Waters 600 unit coupled to a PDA 996 detector equipped with a Polarity C18 column (3.9 x 150 mm; particle size, 5 μm). The PI factor elutes at a retention time of 2.5 min using a mobile phase mixture consisting of a linear gradient of acetonitrile-water (from 1:99 (v/v) at time 0 to 70:30 (v/v) at 15 min). The pure PI factor was derivatized with FMOC (fluorenylmethyl chloroformate) as described by Sim and Perry [32].
Determination of PI factor biological activity

The biological activity of PI factor was determined by its ability to induce pimaricin production by mutant strain *S. natalensis npi287* in solid SPG medium. After allowing growth of *S. natalensis npi287* for 2 days at 30 °C, samples of culture broths (100 µl) or of different fractions from the PI factor purification process were added to wells (7 mm diameter) in the agar layer. The plate was then overlayed with a culture of *C. utilis* in soft agar and incubated for 24 h at 28 °C. The diameter of the *C. utilis* inhibition zone after induction of pimaricin in strain *npi287* was proportional to the amount of PI factor in the sample.

Acetylation of PI factor

Acetylation of PI factor (0.1 mg) was made with a mixture of acetic acid and acetic anhydride (4 ml each) for 24 h at room temperature in the dark. The resulting product was lyophilized and the acetylated PI was dissolved in dichloromethane. For the deacetylation process acetyl-PI was hydrolyzed by drop-wise addition of concentrated NaOH (5 g) in methanol (5 ml) and the conversion of acetyl-PI to PI was followed by TLC.

NMR Spectroscopy

The structure elucidation of PI factor was established by NMR spectroscopy, using a combination of 1D NMR methods (1H-NMR and 13C-NMR) and 2D shift-correlated NMR techniques (HMQC – HSQC and HMBC), for the complete 1H and 13C signal assignments.

NMR spectra were recorded in D2O at room temperature using a Bruker WM 500 spectrometer [500 MHz (1H NMR) and 125 MHz (13C NMR)]. Chemical shifts are given on the δ-scale and were referenced to the solvent and to dioxane as internal signal. The pulse programs of the 2D experiments were taken from the Bruker software library and the parameters were as follows: 500 / 125 MHz gradient-selected HMQC spectra [33], relaxation...
delay $D_1=1.5$ s; evolution delay $D_2=3.33$ ms; delay for evolution of long range coupling (HMBC) $D_6=60$ ms.

The ES+ mass spectrum was recorded on a HP 1100-MSD using CF$_3$COOH 0.1% as the source of ionization.

RESULTS

Classes of $S. natalensis$ mutants impaired in pimaricin biosynthesis and cross-feeding studies

A total of 384 non-producer mutants impaired in pimaricin ($npi$) biosynthesis ($npi$-1 to $npi$-384) were isolated after NTG mutagenesis as described in “Experimental Procedures”. Some of them reverted or were unstable. After several rounds of selection 35 stable $npi$ mutants were selected (Table 1) and assayed in pair-wise complementation tests on solid YED medium (Fig. 1). Based on the results of the complementation tests, the mutants were divided into 11 classes (A to K in Table 1).

A group of seven mutants ($npi$-6, $npi$-12, $npi$-54, $npi$-64, $npi$-86, $npi$-98 and $npi$-137) (class H in Table 1) were unable to complement any other mutant class or vice versa, probably because these mutants were blocked in one of the pimaricinolide synthases [11, 12] and the large intermediates that these enzymes produce could not diffuse out of the cells.

Non-producer mutants of classes A, B, C, F, and J were all able to complement $npi$287 (class G) (Table 1). The results of these complementation tests indicated that $npi$ mutants of classes A, B, C, F and J were able to produce a substance that complements pimaricin production in $npi$287 and that these mutants were blocked later in the biosynthetic pathway. Mutant $npi$287 responded clearly to the spent culture broth of the wild type $S. natalensis$ ATCC27448 (see below) and was therefore used as test strain (converter) for the presence of the putative inducer. Mutant classes D, E, I and K failed to complement the
inducer-requiring class G (see below) and, therefore, may also contain mutations related to the inducer biosynthesis.

**Mutant npi287 recovers normal pimaricin production when supplemented with**

*S. natalensis* **wild type culture broth or with A-factor from S. griseus**

Since initial studies indicated that mutant *npi287* recovered pimaricin production in co-synthesis experiments with different *S. natalensis* mutant strains we tested its complementation with spent culture broths of the parental strain *S. natalensis* ATCC 27448. Results showed that mutant *npi287* recovered full pimaricin production levels when supplemented with culture broths of the *S. natalensis* wild type strain grown for 24 h in either YED, NB or YEME media suggesting that the inducer was secreted by the wild type strain.

Our first working hypothesis was that the pimaricin inducer might belong to the A-factor butyrolactone family since such class of inducing compounds are common among *Streptomyces* species [15, 34]. To test this hypothesis mutant *npi287* was supplemented with increasing concentrations of HPLC-purified A-factor from *S. griseus* IFO 13350. As shown in Fig. 2, the production of pimaricin by *npi287* was restored by addition of A-factor.

To discern if the pimaricin inducer was a butyrolactone or a different molecule we followed initially the purification procedure described for the virginia butanolides from *Streptomyces virginiae* [18]. The procedure is based on extraction of the butyrolactones with ethyl acetate under acidic pH conditions (pH 2.0) followed by concentration of the organic phase, and application of the concentrate through an active carbon column followed by stepwise elution with 50% methanol in water (v/v) or 100% methanol. As a control, A-factor was purified from cultures of *Streptomyces griseus* IFO 13350. As expected, A-factor was purified using this protocol and eluted in the 100% methanol fraction.
Surprisingly, in contrast to what occurs with butyrolactones, a significant proportion of the pimaricin inducing compound remained in the aqueous phase after ethylacetate extraction at either acidic, neutral or slightly basic (pH 7.5) pH values, suggesting that the inducer was a hydrophilic molecule.

**The purified PI factor is different from A-factor**

The PI factor was purified as indicated in Experimental Procedures. After elution from the Sephadex G10 column the biologically active fractions were further purified by reverse phase HPLC chromatography. Aliquots were derivatized with FMOC for an easy detection (Fig. 3). The pure PI compound was used for mass spectrometry and NMR analyses.

The *S. griseus* IFO13350 A-factor was purified by following its biological activity on the test strain *S. griseus* HH1, a mutant lacking streptomycin production due to its deficiency in A-factor biosynthesis. As indicated above, the purified *S. griseus* A-factor containing fraction elicited pimaricin production in the *S. natalensis npi287* mutant (Fig. 2) as also did the crude culture broth of the streptomycin-producing parental strain of *S. griseus* IFO13350. These results indicated that pimaricin production by our *npi287* strain responded to A-factor.

Interestingly, the opposite tests were negative. Neither the wild type *S. natalensis* culture broth nor the HPLC-pure PI factor restored streptomycin production to the *S. griseus* HH1 mutant, further indicating that the PI factor was different from A-factor and specific for *S. natalensis*. In contrast to the well-known stimulation of sporulation of *S. griseus* HH1 exerted by A-factor, the pure PI factor did not stimulate sporulation of the wild type *S. natalensis* or the *npi287* mutant.

These results, together with the capability of the PI factor (but not of A-factor) to react with FMOC, clearly supported the proposal that the nature of both compounds is different. The ability of the PI factor to react with FMOC suggested the presence of amino groups in its
structure, while the lack of reaction of A-factor under the same conditions confirmed the absence of such groups in the A-factor structure.

To study whether any traces of PI factor might be produced by the S. griseus strain, comparative HPLC analysis of the pure PI and the A-factor containing fraction were performed. Results (Fig. 3) showed that the active PI factor derivatized with FMOC eluted at 11.0 min (Fig 3A and C), whereas no peak could be observed at 11.0 min in the A-factor chromatogram after FMOC derivatization (Fig. 3B).

**Chemical structure of the PI factor**

The $^1$H NMR spectrum of PI Factor (Fig. 4) showed only a signal at $\delta$ 3.71 ppm as a singlet whereas its $^{13}$C NMR spectrum contained two signals, which were assigned to one methylene group ($\text{CH}_2$, $\delta$ 58.76 ppm) and one quaternary carbon atom at $\delta$ 60.87 ppm. This was confirmed by means of a DEPT study. Assignment of the carbon atom of the methylene group was carried out by using a HMQC spectrum, which showed a correlation peak via $^1$J$_{\text{H,C}}$ with the methylene protons at $\delta$ 3.71 ppm. In the HMBC spectrum one key correlation peak via $^3$J$_{\text{C,H}}$ was obtained between the methylene protons and the quaternary carbon atom at $\delta$ 60.87 ppm.

The low-field nature of the chemical shifts ($\delta_\text{H}$ and $\delta_\text{C}$) of the methylene group suggested the presence of an oxygenated group (-CH$_2$OH). This was easily confirmed by comparison with the NMR spectra of the acetylated derivative PIa (Fig. 4). The $^1$H NMR spectrum of the acetylated derivative (recorded in CDCl$_3$) showed two singlets at $\delta$ 1.25 and 4.43 ppm, which were attributed to a methyl group ($\text{CH}_3$COO-) and a methylene group ($\text{CH}_3$$\text{COOCH}_2$), respectively; and the $^{13}$C NMR spectrum showed four signals at $\delta$ 20.70 ppm ($\text{CH}_3$), $\delta$ 58.10 ppm (C), $\delta$ 62.71 ppm ($\text{CH}_2$) and $\delta$ 170.60 ppm (CO).
The downfield shift of the quaternary carbon atom and the variation of the chemical shifts by the pH change (see Table 2), suggested the presence of an amine group. This was confirmed by means of the mass spectrum. The PI compound gave an ion at m/z 91 [M+2H]+/2 on the positive electrospray (ES+) indicating a double-charged species. On the basis of all available data the structure of compound PI is proposed to be 2,3-diamino-2,3-bis(hydroxymethyl)-1,4-butanediol (Fig. 4).

Dose response: The PI factor works at low concentrations

The availability of the pure PI compound allowed us to quantify its inducing effect using the standard inducer assay with the npi287 strain (Fig. 5). The npi287 strain clearly responded to PI concentrations of 100 nM and the pimaricin production showed a linear response up to 350 nM. The inducer increased the diameter of the pimaricin inhibition zone up to a concentration of 350 nM and the assay was saturated at concentrations above 400 nM. A threshold level of PI of about 50 nM was always required to detect the induction of pimaricin production. These results are consistent with a cooperative effect typical of a “quorum-sensing” type mechanism.

Following addition of PI factor (200 ng/ml) to npi287 mutant cultures, most of the PI factor was taken up by the cells and only 10 ng/ml remained in the culture broth 24 hours after addition. The PI level in the broth increased to 22 and 15 ng/ml at 48 h and 72, respectively, suggesting that a residual level is released after initial binding to the cells.

The production of pimaricin by the wild-type strain S. natalensis ATCC 27448 is stimulated by exogenous PI factor
In quorum sensing systems the autoinducer signal is secreted by some cells in the culture population and the inducer is incorporated by other cells to trigger differentiation or other biochemical switches.

The production of pimaricin by the parental strain *S. natalensis* ATCC 27448 in NBG, TSB and YEME media was stimulated by the addition of 300 nM PI factor in the three culture media. Since the wild type strain synthesizes endogenous PI factor, the stimulatory effect of exogenous inducer indicates that its biosynthesis is limiting for pimaricin production in the wild type. Furthermore the results indicate that PI factor is taken up by *S. natalensis* or at least it triggers at the membrane level a signal cascade leading to overproduction of pimaricin.

**Kinetics of formation of PI factor in cultures of *S. natalensis* in defined and complex media**

The time of synthesis of the PI factor is relevant to trigger the onset of pimaricin biosynthesis. Furthermore, the level of inducer formed may be limiting for total pimaricin accumulation. To analyze the time-course of PI factor biosynthesis, *S. natalensis* ATCC 27448 was grown in two defined media, namely MM for *Streptomyces* [30] and Lechevalier medium [31], and in four complex media TSB, NBG, YED and YEME (see Experimental Procedures) that are known to support high pimaricin production.

The two defined medium supported low yields of pimaricin (not shown). In both *Streptomyces* MM and Lechevalier media, PI factor was synthesized during growth and a peak (8 to 10 ng/ml) was observed at the end of rapid growth phase of *Streptomyces natalensis* (equivalent to the exponential growth phase of unicellular bacteria). Biosynthesis of pimaricin in these two defined media was parallel to the growth of the culture with a delay of about 12 h with respect to the inducer formation. These results indicate that the synthesis of PI is related to the growth phase.
All complex media were found to support much higher levels of pimaricin production ranging from 620 µg/ml in TSB medium to 920 µg/ml in YME medium (Fig. 6). In the four complex media the PI factor was formed earlier than pimaricin coinciding with the end of the rapid growth phase and reached levels of PI between 25 ng/ml in YME and NBG and 45 ng/ml in TSB media that were clearly higher (about three- to four-fold) than the level of inducer in defined media. A special mention deserves the YED medium in which PI factor accumulated to levels of 140 ng/ml. Production of pimaricin in this medium did not match the high level of PI factor observed. These results indicate that biosynthetic steps other than the level of PI factor are limiting for pimaricin production above PI saturation levels in this medium.

To confirm that the PI inducer is produced during growth of S. natalensis cultures and is not present in the complex media used in these experiments all media were tested by HPLC before inoculation and after 48 h of incubation with S. natalensis. Results are shown in Fig. 7 for the NBG medium. There was no PI inducer in the complex medium before inoculation and it clearly accumulated after 48 h of incubation in this medium. To confirm the absence of PI, the NBG medium was primed with pure PI inducer. As shown in Fig. 7B, the HPLC elution profile of the PI-supplemented culture medium confirmed the lack of this molecule in the starting culture medium.

DISCUSSION

Intercellular communication has been reported in a variety of Gram-negative bacteria including Vibrio fischeri, Myxococcus xantus [35] Erwinia caratovora [22] and Gram-positive bacteria such as Bacillus subtilis and Streptomyces species [36, 20] among others.

Strain specificity of autoinducers is common among Streptomyces species due to the formation of either different inducer molecules [18] or to differences in their stereochemistry.
Several of these molecules including A-factor, the virginia butanolides and related compounds belong to the butyrolactone family [15, 18, 20]. The butyrolactones appear to occur in many but not all *Streptomyces* strains [34]. However other types of autoinducers occur in some actinomycetes [23, 24]. A recent report described a novel boron-containing autoinducer [25] and it seems likely that novel types of “quorum sensing” effectors will be discovered.

As reported in this article a novel type of autoinducer has been found in the pimaricin producer *S. natalensis*. This autoinducer complements the non producer *S. natalensis* class G mutant. The molecule 2,3-diamino-2,3-bis(hydroxymethyl)-1,4-butanediol (PI factor) is symmetrical and has not been reported before in the microbial world. The tetraacetylated derivative of PI showed no inducing ability indicating that the hydroxyl groups of this molecule are strictly required for interaction with its receptor protein. The PI inducer has an entirely novel chemical structure, that is only distantly related to the homoserine lactone and the furanosyl diester [25] inducer families.

It is interesting that class G mutant *npi*287 also recovers pimaricin production when complemented with A-factor, suggesting that there is a mechanism of signal integration that switches gene expression in response to either PI factor or A-factor. However we have shown that *S. natalensis* does not produce A-factor. It will be interesting to know if the PI factor induction is mediated by a butyrolactone-type receptor [38, 39, 40] regulating induction of antibiotic biosynthesis in an A–factor-like fashion. Cross-talk between populations co-existing in the soil [36] may be explained by the use of specific or wide-domain inducers. Some classes of inducers (e.g. butyrolactones) may affect a variety of *Streptomyces* species whereas others (e.g. PI factor) might have a narrow specificity.

The time course of biosynthesis of the PI inducer in complex media shows that formation of PI factor is parallel to growth, in six different media tested both defined and
complex. It is important to note that in mycelial organisms such as *Streptomycetes*, there is not a clear-cut separation of the growth phase and the production phase, in contrast to what occurs in unicellular bacteria. Therefore, onset of pimaricin biosynthesis by old cells occurs while young cells in the hyphal tips are still growing.

The dose-response curve of pimaricin to increasing concentrations of PI factor shows a sigmoidal curve with a minimal threshold level. When PI factor was added to cultures of the *npi287* mutant unable to produce it, PI was initially taken up by the cells and a small amount was then released into the culture broth. These results clearly indicate that the PI inducer shows a standard “quorum sensing” type of kinetics: the PI molecule is, therefore, secreted and signals to other cells that they must start to produce pimaricin in response to biomass accumulation, nutrient limitation or other environmental conditions. This “quorum sensing” type of response requires low concentrations of inducer [41], usually in the nanomolar range, as occurs with the PI factor.

An important question is whether higher levels of inducer increase further pimaricin biosynthesis. As shown in this article addition of 300 nM PI factor to cultures of the pimaricin producer wild type *S. natalensis* strain increased pimaricin production between 20 and 40% in different media. These results suggest that under standard culture conditions, the concentration of extracellular PI factor in complex media is still limiting for pimaricin production. Therefore metabolic engineering of the PI factor biosynthesis is a subject of great interest for improving pimaricin production.

The characterization of the PI inducer opens the possibility of genetic modification of its biosynthesis to understand the molecular mechanism of signal transduction.

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LEGENDS TO THE FIGURES

FIG. 1. **Pair-wise complementation test for classification of npi mutants.** Chemical structure of pimaricin (panel A) and cosynthesis method used for the complementation of npi mutants of *S. natalensis*. Two *npi* (non-producer) mutants A and B are unable to produce pimaricin when assayed separately as agar-plug cultures on a lawn of *Candida utilis* but one of them (A, the converter) regains pimaricin production when grown close together to the “donor” strain B.

FIG. 2. **Induction of pimaricin production.** Pimaricin inducing activity of PI factor (A), A-factor (B) and acetylated PI factor (C) on *S. natalensis* npi287. Note that PI factor has a strong inducing activity whereas its acetylated derivative has no inducing effect. The *S. griseus* A-factor has also some inducing activity on *S. natalensis* npi287. One hundred µl of each compound (100 ng/ml) were applied to each well.

FIG. 3. **HPLC detection of derivatized inducing factors.** HPLC-analysis of FMOC-derivatized (A) pure PI factor, (B) A-factor (90% purity) and (C) A-factor (90% purity) mixed with PI factor. PI factor-FMOC elutes at 11.8 min (arrow). A-factor does not react with FMOC. Note that the *S. griseus* A-factor preparation has no traces of PI factor.

FIG. 4. **Structure of the PI factor.** The chemical structure determined for the PI factor corresponds to 2,3-diamino-2,3-bis(hydroxymethyl)-1,4-butanediol. Its tetraacetylated PI derivative (PIa) is also shown.
FIG. 5. **Dose-Response of pimaricin production by *S. natalensis npi287* to increasing concentration of PI factor (100 to 500 nM).** Panel A. Bioassay: 100 nM (A); 150 nM (B); 200 nM (C); 350 nM (D). Panel B. Dose-response curve in solid medium. Panel C, Dose-response in liquid NBG medium.

FIG. 6. **Time course of PI factor and pimaricin formation in cultures of wild type *S. natalensis.*** Four complex media TSB, NBG, YEME and YED were used; (■) dry weight (upper panels); (□) PI factor (dotted line); (●) pimaricin (continuous line, lower panels).

FIG. 7. **PI factor is synthesized *de novo* by *S. natalensis.*** HPLC analysis showing the absence of PI factor in complex NBG medium at inoculation time (A); NBG medium supplemented with PI factor (0.2 µg/ml) (B) and NBG culture broth 48 h after inoculation with *S. natalensis.* (C) The PI-FMOC peak is shaded. The expected position of PI-FMOC in panel A is indicated by an inclined arrow. Similar results were obtained in TSB, YEME and YED media.
**TABLE 1. Complementation classes of npi (non-producer mutants) of *S. natalensis***

| Classes | Complemented classes |
|---------|----------------------|
| A. npi380 | B, C, D, E, G, J, K |
| B. npi16, npi235 | A, C, E, G |
| C. npi275 | A, B, D, G, I, J |
| D. npi31 | A, C |
| E. npi38 | A, B |
| F. npi30, npi71, npi83, npi85, npi116, npi140, npi148, npi178, npi226, npi238, npi249, npi276 | G |
| G. npi287 | A, B, C, F, J |
| H. npi6, npi12, npi54, npi64, npi86, npi98, npi137 | None |
| I. npi22, npi255 | C |
| J. npi79, npi88, npi169, npi218, npi384 | A, C, G |
| K. npi39, npi271 | A |
**TABLE 2.** $^1$H NMR and $^{13}$C NMR spectral data of PI (in D$_2$O; chemical shifts in $\delta$ ppm)

|       | pH = 5.20  |        | pH = 8.05  |        |
|-------|------------|--------|------------|--------|
| Group | $^1$H      | $^{13}$C | $^1$H      | $^{13}$C |
| CH$_2$| 3.71 s     | 58.76  | 3.66 s     | 59.44  |
| C     |            | 60.87  |            | 59.93  |
Fig. 1
Fig. 4
Fig. 7

Absorbance (arbitrary units)

Elution time (min.)
PI factor, a novel type quorum-sensing inducer elicits pimaricin production in Streptomyces natalensis
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