Glycerol, ethylene glycol and propanediol elicit pimarin biosynthesis in the PI-factor-defective strain *Streptomyces natalensis npi287* and increase polyene production in several wild-type actinomycetes

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Production of pimarin by *Streptomyces natalensis* ATCC 27448 is elicited by the PI-factor, an autoinducer secreted by the producer strain during the rapid growth phase. Exogenous PI-factor restored pimarin production in a mutant strain *npi287* defective in PI-factor biosynthesis. During purification of the PI-factor, a second pimarin-inducing fraction different from PI-factor was isolated from the culture broth of wild-type *S. natalensis* ATCC 27448. After purification by HPLC and analysis by MS and NMR, this active fraction was shown to contain glycerol and lactic acid. Pure glycerol restored pimarin production in liquid cultures of the autoinducer-defective *npi287* mutant. A similar effect was exerted by ethylene glycol, 1,2-propanediol and 1,3-propanediol but not by higher polyalcohols or by glycerol acetate or glycerol lactate esters. Glycerol stimulated (30–270 %) the production of six different polyene macrolide antibiotics by their respective producer strains. Addition of glycerol to the inducer-defective *npi287* strain restored pimarin production but did not result in extracellular or intracellular accumulation of PI-factor. Exogenously added PI-factor was internalized by the cells in the presence of glycerol, and a mixture of both PI-factor and glycerol produced a slightly higher inducing effect on pimarin production than PI-factor alone. In summary, glycerol, ethylene glycol and propanediol exert a bypass of the PI-factor inducing effect on pimarin biosynthesis.

Received 28 February 2006

Revised 30 May 2006

Accepted 7 July 2006

INTRODUCTION

Many micro-organisms produce extracellular low-molecular-mass secondary metabolites (Vining, 1992; Martín *et al.*, 2000). Some of them serve as quorum-sensing signals or as intercellular cross-talk messengers (Horinouchi & Beppu, 1992; Kaiser & Losick, 1993; Nodwell & Losick, 1998; Federle & Bassler, 2003; Klose, 2006). The antibiotic activity of many of these molecules is well known (Berdy, 2005). Many *Streptomyces* species and some rare actinomycetes have the ability to produce a class of antifungal compounds, the polyene macrolides (Martín, 1977), that belong to the polyketide family (Omura & Tanaka, 1984; Aparicio *et al.*, 2004). In recent years, clusters of genes encoding enzymes for the biosynthesis of a few polyene macrolides have been reported (Aparicio *et al.*, 1999, 2000, 2003; Brautaset *et al.*, 2000; Caffrey *et al.*, 2001).

Pimarin is a glycosylated polyene macrolide produced by *Streptomyces natalensis*. Due to its particularly low toxicity, pimarin is important for antifungal therapy in animals and it is widely used in the food industry to prevent mould contamination of cheese and other non-sterile foods. The synthesis of pimarin in *S. natalensis* requires a complex polyketide synthase (PKS) and additional modification enzymes (Aparicio *et al.*, 2000). The *pim* gene cluster (85 kb) encodes 13 PKS modules within five multifunctional enzymes, and 12 additional proteins that catalyse post-PKS modifications of the polyketide skeleton (tailoring enzymes), or are involved in export and regulation of gene expression (Mendes *et al.*, 2001, 2005; Antón *et al.*, 2004).

Different types of autoregulatory effectors are known to occur in micro-organisms (Yamada & Nihira, 1999; Klose,

2006). These effectors are synthesized and secreted by the producer strains and internalized by other cells in the population (Robson *et al.*, 1997). The presence of butyrolactones in different species of *Streptomyces* and their role in triggering secondary metabolite biosynthesis is well documented (Horinouchi & Beppu, 1992). Other autoinducers belong to at least six different structural groups (Yamada & Nihira, 1999; Recio *et al.*, 2004). The biosynthesis of pimaricin is induced by PI-factor, a novel autoinducer produced by the parental wild-type strain *S. natalensis* ATCC 27448. The structure of PI-factor was determined as 2,3-diamino-2,3-bis-(hydroxymethyl)-1,4-butanediol and is clearly different from that of butyrolactones. The *S. natalensis* *npi287* mutant is unable to synthesize PI-factor and this compound restores pimaricin production in the *npi287* mutant when added externally (Recio *et al.*, 2004). The positive response to exogenous PI-factor indicates that this mutant is still able to internalize the PI autoinducer. Very little is known about the secretion and internalization (uptake) of autoinducers. The AI-2 of *Salmonella enterica* is internalized by the Lsr ACDB complex, an ABC-type transporter, and requires a functional *glpD* (glycerol-3-phosphate dehydrogenase) gene (Taga *et al.*, 2001; Xavier & Bassler, 2005).

A receptor protein for butyrolactones has been reported recently in *S. natalensis* (Lee *et al.*, 2005), but it is unknown whether this receptor is specific for classical butyrolactones or whether it may also interact with PI-factor.

During purification of the PI-factor (Recio *et al.*, 2004), we identified a second fraction able to induce production of pimaricin in the PI-factor defective *npi287* mutant. This fraction did not contain a butyrolactone, but, as reported in this article, NMR studies showed that it consisted of a mixture of glycerol and lactic acid. It was, therefore, important to study the effect of these compounds and their analogues on the biosynthesis of pimaricin.

METHODS

Strains. The following producers of polyene macrolides were used: *Streptomyces natalensis* ATCC 27448 (pimaricin producer), *S. eurocidicus* CECT 3259 (eurocidin producer), *S. cinnamoneus* CECT 3258 (fungichromin producer), *S. rimosus* CECT 3144 (rimocidin producer), *S. filipinensis* ATCC 23905 (filipin producer), *S. albulus* subsp. *tetrafungini* CECT 3238 (tetrafungin producer), *S. noursei* CECT 3240 (nystatin producer) and *S. griseus* IMRU 3570 (candicidin producer). These strains were maintained on solid TBO sporulation medium [containing, per litre: tomato paste (Cidacos), 20 g; oatmeal, 20 g; agar, 25 g]. *S. natalensis* *npi287*, a PI-factor-defective non-producer mutant (Recio *et al.*, 2004) was used to determine the pimaricin-inducing activity of different diols. *Candida utilis* (syn. *Pichia jardinii*) CECT 1061 was used as test strain in the bioassay of the antifungal activity of the diverse polyenes.

Culture media and quantification of polyene production in liquid cultures. The production of different polyenes was studied in NBG [nutrient broth (Oxoid) 13 g l⁻¹, glucose 5 g l⁻¹] medium in liquid cultures. The production of the polyenes was routinely quantified by spectrophotometry. A 0.5 ml aliquot of the culture was

extracted with 5 ml methanol and further diluted with methanol (if necessary). The UV-visible absorption spectrum was first determined to establish the wavelength of the different absorption peaks of each polyene. The absorption was determined at the wavelength corresponding to the most intense peak for each polyene (319 nm for pimaricin). The absorbance was converted to the respective polyene concentrations using the following molar absorption values in a 1 cm cuvette: candicidin, $\epsilon_1^1 = 1150 \text{ l mol}^{-1}$; nystatin, $\epsilon_1^1 = 790 \text{ l mol}^{-1}$; filipin, $\epsilon_1^1 = 1360 \text{ l mol}^{-1}$; fungichromin, $\epsilon_1^1 = 1231 \text{ l mol}^{-1}$. Since no molar absorption values were known for the other polyenes a unit was defined as the amount of polyene giving an absorption of 0.1 at the peak wavelength of the respective polyene.

Pimaricin was quantified by comparison with a sample of pure pimaricin (Sigma). The different *Streptomyces* species were grown in NBG medium, except for *S. noursei*, which was grown in SAO-23 medium (Brautaset *et al.*, 2000). The concentration of extracellular PI-factor was determined in liquid cultures in NBG, YED (yeast extract/dextrose [glucose]) and YEME (yeast extract/malt extract) (Recio *et al.*, 2004).

In addition to the spectrophotometric determination, pimaricin and filipin were also quantified by reverse-phase HPLC using a Waters 600 unit coupled to a PDA 996 detector equipped with a μ -Bondapak C₁₈ column (3.9 × 300 mm, particle size, 5 μ m). Pimaricin eluted at 14.6 min and filipin at 17.8 min using a mobile phase mixture consisting of a linear gradient of methanol/water (from 50:50, v/v, at time 0 to 90:10, v/v, at 30 min).

Purification of compounds from the second fraction of pimaricin-inducing activity. A second fraction different from PI-factor that had inducing activity on pimaricin production was isolated, as described previously (Recio *et al.*, 2004). Purification of this fraction was carried out by reverse-phase HPLC using a Waters system equipped with a Polarity C₁₈ column (3.9 × 150 mm, particle size 5 mm) (Waters). The active compound(s) eluted at 1.9 min using a mobile-phase mixture consisting of a linear gradient of acetonitrile/water (1:99, v/v, at time 0 to 70:30, v/v, at 15 min).

PI isolation and detection. The PI-inducing factor was isolated from *S. natalensis* ATCC 27448 cultures grown in YED medium for 24 h and purified as described previously (Recio *et al.*, 2004). The detection of the PI-factor inducing activity was routinely performed on SPG solid medium cultures of the PI-defective mutant *npi287*, as described before (Recio *et al.*, 2004). Solid SPG cultures of *S. natalensis* *npi287* produced pimaricin only when supplemented with PI-factor, in a dose-dependent response.

Extraction of intracellular PI-factor. *S. natalensis* *npi287* was grown in NBG medium with or without supplementation with 100 mM glycerol and the mycelium was collected by centrifugation at 5000 g. The collected mycelium was resuspended in 10 ml 50 mM Tris/HCl buffer pH 7.0 containing 2% Renex (Imperial Chemical Industries) and disrupted in a French press (Aminco). The disruption of the cells was confirmed by microscopic observation. The debris was removed by centrifugation at 14 000 g and the supernatant was concentrated 10-fold; the presence of PI-factor was tested by bioassay using the inducing test, as described above.

Determination of the pimaricin-inducing activity of different diols. The biological activity of glycerol and other polyhydroxylated compounds was determined by their ability to induce pimaricin production by the PI-factor-defective mutant *S. natalensis* *npi287*, in solid SPG medium and in liquid NBG medium. This activity was further tested in several *Streptomyces* strains, including wild-type *S. natalensis*, in liquid and solid medium. In liquid medium, glycerol (or other polyhydroxylated compounds) was added at different

concentrations and the production of polyene was compared with that of a control culture without additions. In solid medium tests the strains were grown as lawn cultures on TBO medium for 8 days; then agar plugs were taken from each plate and placed on NAG medium (NBG solidified with 20 g agar l⁻¹) in the vicinity of 0.7 cm diameter wells. The production of polyene was bioassayed after 48 h in the presence or absence of glycerol added to the neighbouring well, and the diameters of inhibition zones, indicative of the production of the antifungals, were measured.

NMR spectroscopy. The chemical structures of the compounds in the positive inducing samples were determined by NMR spectroscopy, using a combination of one-dimensional NMR (¹H-NMR and ¹³C-NMR) and two-dimensional shift-correlated NMR techniques (HMQC-HSQC and HMBC), for the complete ¹H and ¹³C signal assignments, and mass spectrometry.

NMR spectra were recorded in CD₃OD at room temperature using a Bruker WM500 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 signals. The pulse programmes of the two-dimensional experiments were taken from the Bruker software library, and the parameters were as follows: 500/125 MHz gradient-selected HMQC spectra: relaxation delay $D_1 = 1.5$ s; 500/125 MHz gradient-selected HMBC spectra: relaxation delay $D_1 = 1.5$ s, evolution delay $D_2 = 3.33$ ms; delay for evolution of long-range coupling $D_6 = 60$ ms (Wilker *et al.*, 1993).

Mass spectra were recorded on an LC-TOF (Micromass). Glycerol mass was detected in the positive mode, using formic acid to facilitate ionization. Lactic acid measurement was carried out in the negative mode, using bicarbonate for ionization.

RESULTS

The second pimarin-inducing fraction consists of glycerol and lactic acid

During purification of the PI inducer using an induction test based on the restoration of pimarin production to the inducer-defective strain *npi287*, a second active fraction was isolated after fractionation of the lyophilized solvent extract of the culture broth (Recio *et al.*, 2004) through a Sephadex G10 column (Fig. 1). The compound(s) purified from the second active fraction (A2) had low molecular mass, lacked amino groups, since it was not derivatized with FMOC (fluorenylmethyl chloroformate), and eluted in reverse-phase HPLC with a retention time of 1.9 min (Fig. 1B).

Unexpectedly, the mass spectra (molecular masses of 93.2 and 89.3) and NMR spectra of the purified active fraction (Table 1) revealed that this fraction consisted of a mixture of glycerol and lactic acid. Exogenously added pure glycerol and lactic acid coeluted with the active fraction in HPLC.

The pimarin-inducing activity of the mixture of these two chemicals was confirmed using pure compounds. Addition of glycerol together with lactic acid to SPG solid medium cultures of the non-producer *npi287* strain triggered pimarin production. Such an effect was not observed when each compound was tested separately on solid medium cultures, but glycerol alone was sufficient to elicit pimarin production in liquid cultures (see below). A

stimulation of the production of pimarin in the wild-type *S. natalensis* by the mixture of both compounds was also observed in solid medium. Triacetylated glycerol, in contrast to pure glycerol, did not exert any inducing effect, although it was efficiently used as carbon source (when supplied instead of glucose). These results suggested that free alcohol groups are required to produce the inducing effect.

Glycerol elicits pimarin biosynthesis in liquid cultures of *S. natalensis npi 287* and stimulates production in the wild-type

The results observed in solid medium were compared with those in liquid medium. As shown in Fig. 2, glycerol alone or in combination with lactic acid triggered pimarin production in the *npi287* mutant. Lactic acid (D- or L-) when added alone did not produce any significant stimulating effect on pimarin production. Addition of lactic acid up to 5 mM had no significant effect on dry weight or pimarin production; lactic acid at 50 mM was inhibitory.

Glycerol alone triggered pimarin biosynthesis in liquid cultures of *npi287* in NBG medium at concentrations in the range 70–120 mM. At concentrations higher than 100 mM the stimulatory effect was smaller (Fig. 2). At these concentrations the glycerol had a stimulatory effect on biomass. The best supplementation for pimarin induction was a mixture of 100 mM glycerol and 100 μ M lactic acid. The levels of pimarin produced by the *npi287* mutant when supplemented with glycerol (23 μ g pimarin per mg dry weight or 400 μ g per ml) were of the same magnitude as those obtained with the specific inducer PI at 1 μ M concentration. However, whereas PI-factor was effective in the 100 nM to 1 μ M range, glycerol was only active at concentrations above 50 mM.

Similar studies were performed with the wild-type strain in NBG medium supplemented with glycerol or with the glycerol/lactic acid mixture. Glycerol alone produced a significant stimulatory effect on pimarin production (up to 125 μ g per mg dry weight) similar to that exerted by the glycerol/lactic acid mixture. The stimulatory effect was higher when glycerol (with or without lactic acid) was added at inoculation time or at 14 h than when added at 24 h after inoculation. The effect of the addition at 24 h was small but still detectable (Fig. 3). The higher effect observed when glycerol is added early to the cultures, as compared to the lower stimulation when added at 24 h, is consistent with an indirect role of glycerol, rather than a nutrient (precursor) effect. Furthermore, when glucose in the NBG medium was replaced by glycerol as carbon source, the pimarin yield was lower (about 45–50 %), indicating that the glycerol stimulation is not merely a nutrient effect.

The lack of requirement of lactic acid for the glycerol stimulatory effect observed in liquid cultures suggests that the different physiological conditions in submerged cultures makes lactic acid unnecessary.

Fig. 1. Separation of pimarinic-inducing fractions from the culture broth of *S. natalensis* ATCC 27448. (A) Fractionation of the lyophilized solvent extract dissolved in water through a Sephadex G10 column. The two active fractions A1 and A2 are shaded. Size markers: sucrose (solid line), glucose (dotted line) and NaCl (dashed line). The initial peak (fractions 14–15) corresponds to the dextran blue marker. Note that the active fraction A2 eluted between glucose (M_r 180) and NaCl (M_r 58.5). The elution peak of NaCl was followed by precipitation with a silver nitrate solution. (B) HPLC analysis in a C_{18} column of the compounds in fraction A2 of the Sephadex G10 column. The pimarinic-inducing compound eluted with a retention time of 1.9 min and showed a relatively polar nature (see text). (C) Inducing effect of pimarinic production in the mutant strain *S. natalensis* *npi287* by fraction A2 and by PI-factor (purified from fraction A1). a, Negative fraction from the G10 column (100 μ l); b, Fraction 2 (100 μ l); c, pure PI (100 μ l of a 100 ng ml⁻¹ solution).

Table 1. ¹H- and ¹³C-NMR chemical shift assignments for glycerol (1) and lactic acid (2) (CD₃OD)

No.	1			2		
	δ ¹³ C (p.p.m.)	DEPT	HMQC (¹ H, p.p.m.) [J (Hz)*]	δ ¹³ C (p.p.m.)	DEPT	HMQC (¹ H, p.p.m.) [J (Hz)*]
1	64.79	CH ₂	3.54 dd (11.20; 6.10) 3.61 dd (11.20; 4.90)	183.17	C	
2	74.24	CH		70.28	CH	4.11 a (7.0)
3	64.79	CH ₂		21.76	CH ₃	1.28 d (6.8)

*J values in parentheses.

Fig. 2. Effect of glycerol or glycerol plus lactic acid on pimarin production by *S. natalensis npi287* in liquid cultures in NBG medium. Panels A1, B1 and C1, growth kinetics; panels A2, B2 and C2, pimarin production (specific yield). Panels A, glycerol plus lactic acid mixture; panels B, glycerol alone; panels C, lactic acid alone. Vertical bars indicate SEM values of three determinations from duplicate cultures.

Glycerol lactate esters do not induce pimarin production

The possible effect of lactate esters of glycerol was tested using glycerol monolactate, glycerol dilactate (not shown)

and glycerol trilactate on solid or liquid cultures of the *npi287* mutant. None of them induced pimarin biosynthesis (Fig. 4 and not shown). This result supports the lack of inducing activity observed with glycerol acetate esters described above.

Fig. 3. Effect of the addition of glycerol (100 mM) at different times (vertical arrows) on the growth kinetics (A) and specific pimaricin production (B) in cultures of *S. natalensis* ATCC 27448 in NBG medium. Vertical bars indicate SEM values of three determinations from duplicate cultures. ◆, Unsupplemented control; ■, glycerol added at 0 h; ▲, glycerol added at 14 h; ●, glycerol added at 24 h.

1,2- or 1,3-propanediol and ethylene glycol but not other polyalcohols trigger pimaricin production

A series of glycerol analogues including ethylene glycol, 1,2- and 1,3-propanediol, treitol, erythritol, arabitol, ribitol and xylitol, and organic acids related to lactic acid including β -hydroxypropionic acid, pyruvic acid, malic acid, tartaric acid, α -hydroxybutyric acid, succinic acid, β -hydroxyvaleric acid, glutaric acid, α -hydroxyglutaric acid and the cyclic compounds propiolactone and γ -butyrolactone were tested for their inducing effect.

Of these compounds, only ethylene glycol and 1,2- or 1,3-propanediol, alone or in combination with lactic acid, showed an inducing effect on pimaricin production (Fig. 4). The positive effect was observed both on solid medium (ethylene glycol or glycerol and lactic acid) (Fig. 4A) and in liquid cultures (ethylene glycol, glycerol or propanediol with or without lactic acid) (Fig. 4B) using cultures of either the *np1287* mutant or the parental *S. natalensis* wild-type strain. At the same concentration (100–200 mM), ethylene glycol or propanediol showed a smaller induction effect than glycerol.

Glycerol increases the production of six different polyene macrolides by their producer organisms

To study if the glycerol stimulation of polyene macrolide production was a general phenomenon for other *Streptomyces* species, we tested the effect of these compounds on the production of nystatin by *S. noursei*, rimocidin by *S. rimosus*, candicidin by *S. griseus* IMRU 3570, filipin by *S. filipinensis*, tetrafungin by *S. albulus*, eurocidin by *S. eurocidicus* and fungichromin by *S. cinnamoneus*. As an additional control, the effect of glycerol on the production of streptomycin by *S. griseus* IFO 13350 was also tested. These studies were done on solid medium and in liquid cultures in NBG medium. In all cases the results in liquid cultures agreed with those observed on solid medium.

The production of six out of seven different polyene macrolides tested was stimulated by glycerol (100 mM); the degree of stimulation depended on the producer strain (Fig. 5). A high increase in polyene macrolide yields was observed in the cultures producing nystatin (90 % increase

Fig. 4. Comparative inducing effect of ethylene glycol, 1,3- and 1,2-propanediol and glycerol on pimaricin production in cultures of the *np1287* mutant. (A) Assays in solid SPG medium: a, negative control (water, 100 μl); b, ethylene glycol + lactic acid (50 μl each); c, glycerol + lactic acid (50 μl each); d, PI-factor (100 μl of 100 ng ml⁻¹). (B) Effect of 100 mM glycerol (●), 150 mM ethylene glycol (×), 140 mM 1,2-propanediol (□), 140 mM 1,3-propanediol (△), 150 mM glycerol monolactate (■) and 150 mM glycerol trilactate (▲) on pimaricin production in liquid cultures in NBG medium of the *np1287* mutant. ◆, Unsupplemented control.

Fig. 5. Effect of glycerol (100 mM) on the production (specific yields) of seven different polyene macrolide antifungals by their respective producer strains grown on the media indicated in Methods. ◆, Unsupplemented control; ■, +100 mM glycerol. The experiments were done in triplicate flasks. Vertical bars indicate SEM values of three determinations.

at 96 h), candicidin (270 % increase at 96 h), tetrafungin (60 % at 72 h), filipin (90 % at 72 h) and fungichromin (200 % at 96 h). Moderate increases were obtained in the

production of rimocidin (30 % at 72 h) and no increment was observed in eurocidin production by *S. eurocidicus* at the glycerol concentrations tested. The wild-type isolates of

S. albulus and *S. rimosus* (producers of tetrafungin and rimocidin, respectively) yielded very low levels of the respective polyenes under the conditions used (not optimized) but the stimulatory effect of glycerol was observed in both cases.

Glycerol addition had no effect at all on the production of streptomycin by *S. griseus* IFO 13350. The effect of glycerol on the A-factor concentration in *S. griseus* cultures was also tested. No differences were found in A-factor concentration in culture broths of *S. griseus* IFO 13350 with or without supplementation with glycerol (not shown).

The stimulatory effect of glycerol does not involve intracellular or extracellular PI-factor accumulation in strain *npi* 287

As described previously, the PI-factor induces pimarcin production both in the *npi*287 mutant and in the wild-type strain (Recio *et al.*, 2004). To elucidate if the glycerol effect was mediated through the PI signal-transduction cascade, experiments were performed in which the PI-induction system was fully activated (saturated) with high levels of PI (2 μ M). Parallel cultures were supplemented with glycerol alone (100 mM) or with glycerol (100 mM) and PI-factor (2 μ M). The results (Fig. 6) showed that glycerol produced a higher increase of pimarcin than PI alone and the induction pattern was different. The PI-factor exerted an earlier stimulatory effect (48 h) than glycerol (maximum effect at 72 h) when added separately. Glycerol was still able to increase slightly the production of pimarcin in the PI-saturated system when both PI and glycerol were added simultaneously (Fig. 6).

Fig. 6. Combined effect of glycerol (100 mM) and PI-factor (2 μ M) on the production of pimarcin by cultures of *S. natalensis* ATCC 27448. ◆, Unsupplemented control; ■, +100 mM glycerol; ●, +2 μ M PI; ▲, +100 mM glycerol and 2 μ M PI. Note that the mixture of both compounds exerted a slightly higher stimulatory effect but was not additive. Results are the mean of three experiments using duplicate flasks in NBG medium. Vertical bars indicate SEM values of three determinations from triplicate cultures.

Strain *npi*287 lacked PI; therefore, to test the possibility that glycerol might be converted into the PI-factor, the intracellular and extracellular levels of the PI-factor were measured, as described in Methods, in strain *npi*287 grown with increasing concentrations of glycerol (70–120 mM). When supplemented with glycerol, strain *npi*287 produced pimarcin, but no detectable levels of intracellular or extracellular PI-factor were found in this mutant, either without or with glycerol supplementation (not shown). This result indicates that the effect of glycerol is indeed exerted through a mechanism different from that involving PI-factor biosynthesis.

The extracellular levels of PI-factor were also determined in cultures of the wild-type strain ATCC 27448 in three different media (NBG, YED and YEME) with or without 100 and 200 mM glycerol. The levels of PI-factor were different in the three media, being about fourfold higher in YED medium than in YEME or NBG, but in all three cases there were only minor differences in cultures with or without glycerol, indicating that glycerol is not a direct precursor of PI-factor.

DISCUSSION

Intercellular communication between different bacteria or between bacteria and higher organisms is becoming more evident (Kaiser & Losick, 1993; Fray, 2002; Federle & Bassler, 2003). Secondary metabolism and cell differentiation in actinomycetes are controlled by diffusible low-molecular-mass compounds (Robson *et al.*, 1997; Horinouchi & Beppu, 1992). These belong to at least six different chemical classes (Recio *et al.*, 2004) and there are probably other unknown types of cross-talk signals.

The production of pimarcin by *S. natalensis* is probably induced by PI-factor through a quorum-sensing-type mechanism. The PI-factor-defective mutant *S. natalensis npi*287 regained pimarcin biosynthesis ability when supplemented with small amounts of PI-factor (Recio *et al.*, 2004). A gene encoding a butyrolactone-receptor protein has been reported recently in *S. natalensis* (Lee *et al.*, 2005). However, we have never found a true butyrolactone in *S. natalensis*. It would be desirable to test if the receptor protein is specific for butyrolactones or whether it may interact with the PI-factor.

In this work a second pimarcin-inducing fraction purified from culture broths of the parental strain was shown by NMR studies to contain glycerol and lactic acid. Indeed, pure commercial glycerol and lactic acid restored pimarcin production to strain *npi*287 in solid medium. When tested in liquid medium, glycerol alone was sufficient to trigger pimarcin biosynthesis. The different requirement for lactic acid in solid and liquid medium is probably related to the distinct physiological conditions in solid and submerged cultures.

The concentration of glycerol required for optimal effect on pimarcin biosynthesis (50–100 mM) might suggest that the triggering effect could be related to a nutritional effect. This

does not seem to be the case, since the *npi287* mutant does not produce pimarin when supplemented with other carbon sources (glycerol esters, glucose, acetate or sucrose) that are excellent carbon sources for growth of *S. natalensis* and for pimarin production. Furthermore, glycerol, when used instead of glucose as carbon source, decreased the yield of pimarin, i.e. the inducing effect of glycerol appears to be additional to the effect of biosynthetic precursors provided by glucose.

The high concentration of glycerol required suggests that it may play an indirect role, rather than having a direct effect on pimarin biosynthesis, e.g. a role in membrane permeability or signal transduction across the membrane. Glycerol is well known to serve as a signal that elicits aggregation and cell differentiation, resulting in fruiting body formation in *Myxococcus xanthus* (Dworkin & Gibson, 1964; Killeen & Nelson, 1988). A $\Delta glpT$ *M. xanthus* mutant defective in the glycerol permease aggregates even in rich medium. Since swarming and aggregation was delayed by glycerol 3-phosphate (G3P), it seems that cell binding and/or catabolism of glycerol is an important step in glycerol regulation of differentiation (Moraleta-Muñoz *et al.*, 2001). Recently glycerol metabolism has been shown to be also involved in regulation of the uptake of the quorum-sensing autoinducer AI-2 in *Escherichia coli*, an autoinducer that is structurally related to PI-factor (Xavier & Bassler, 2005). A mutation in the *glpD* gene encoding G3P-dehydrogenase causes a defect in AI-2 internalization.

The effect of glycerol on pimarin production in both the PI-factor-defective *npi287* mutant and the wild-type *S. natalensis* was also exerted by ethylene glycol and 1,2- or 1,3-propanediol but not by threitol or higher polyalcohols. Neither acetate nor lactate esters of glycerol exerted any effect. These results indicate that the effect of the active compounds is due to the signalling effect of 2-carbon or 3-carbon hydroxylated compounds. Ethylene glycol is known to interact with the cell membranes and it is not catabolized by the glycerol pathway; therefore, a purely nutritional effect of these compounds seems very unlikely.

Addition of glycerol to cultures of strain *npi287* did not restore detectable levels of intracellular or extracellular PI-factor in this strain, thus excluding a direct precursor role of glycerol for PI biosynthesis. In addition, the stimulatory effect of glycerol on pimarin biosynthesis in the wild-type is not accompanied by increased extracellular PI levels in this strain. In summary, glycerol, ethylene glycol and propanediol exert a bypass of the PI inducer in strain *npi287*.

Glycerol has a stimulatory effect on the biosynthesis of six different polyene macrolides by the respective wild-type producer strains, but no effect on streptomycin production by *S. griseus* IFO13350 was observed. Glycerol has been reported to stimulate the production of nystatin (Jonsbu *et al.*, 2002) and it is known to be a good carbon source for other *Streptomyces* species (Kaiser *et al.*, 1994; Borodina *et al.*, 2005). Our results suggest that the effect of glycerol

might be specific for polyketides, particularly for polyene macrolides, although this phenomenon should be studied on other antibiotic-producing *Streptomyces* species.

ACKNOWLEDGEMENTS

This work was supported by grants of the CICYT (Madrid, Spain) to J. F. M. (BIO2003-01489) and to J. F. A. (BIO2004-00066) and by a Special Genomics Action (GEN2003-20245-C09-01 and GEN2003-20245-C09-08). E. R. received a fellowship of the Diputación de León, León, Spain. We acknowledge the excellent technical assistance of B. Martín, J. Merino and M. Álvarez.

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