Nitrogen source governs the patterns of growth and pristinamycin production in '*Streptomyces pristinaespiralis*'

François Voelker and Stéphane Altaba

Author for correspondence: François Voelker. Tel: +33 1 55 71 31 06. Fax: +33 1 55 71 33 52.
e-mail: francois.voelker@aventis.com

Aventis Pharma, Process Development
Biotechnology, 13 quai
Jules Guesde, 94400 Vitry
sur Seine, France

Phosphate-limited synthetic culture media were designed to investigate the growth and the pristinamycin production of '*Streptomyces pristinaespiralis*' using different nitrogen sources. During balanced growth, either mineral or organic nitrogen sources were readily utilized. However, glutamate and alanine were used as both nitrogen and carbon source, sparing the utilization of the primary carbon source, glucose. Valine was utilized only for its nitrogen and consequently 2-ketoisovalerate was excreted in the medium. Ammonium prevented the utilization of nitrate. Upon phosphate limitation, glycerol, originating from the breakdown of teichoic acids, was released, allowing the recovery of phosphate from the cell wall and the continuation of growth. Under such conditions, ammonium was excreted following the consumption of glutamate and alanine and was later reassimilated after exhaustion of the primary nitrogen source. The mode of utilization of valine prevented the production of pristinamycins due to excretion of 2-ketoisovalerate, one of their direct precursors. For other nitrogen sources, pristinamycin production was controlled by nitrogen catabolic regulation linked to the residual level of ammonium. In the case of nitrate, the negative regulation was alleviated by the absence of ammonium and production then occurred precociously. In the case of amino acids and ammonium, production was delayed until after exhaustion of amino acids and depletion of ammonium.

Keywords: '*Streptomyces pristinaespiralis*', streptogramin, nitrogen regulation, ammonium

INTRODUCTION

Type I pristinamycins (PI) and type II pristinamycins (PII) are two different families of components of the complex antibiotic pristinamycin produced by '*Streptomyces pristinaespiralis*', a filamentous, Gram-positive bacterium. PI and PII, and especially their main components, PIA, PIIA and PIIB (Fig. 1) are produced simultaneously during the culture of '*S. pristinaespiralis*'. PI are cyclic hexadepsipeptides belonging to the streptogramin B group. The PIA macrocycle (Fig. 1) consists of seven amino acid residues, two common and five modified, condensed by multifunctional peptide synthetases (Thibaut *et al.*, 1997). PII are poly-

unsaturated cyclic peptolides of the streptogramin A group. The PIIB macrocycle (Fig. 1) originates from seven molecules of acetate, four amino acids (valine, glycine, serine and proline) and a methyl group arising from methionine (Kingston *et al.*, 1983). PIIB is assumed to be synthesized by the coordinated action of polyketide synthetases and peptide synthetases. PIIA derives from PIIB by an oxidation catalysed by PIIA synthase (Thibaut *et al.*, 1995).

Both PI and PII contain nitrogen from two nitrogen donors, glutamate and glutamine (Fig. 2). Glutamate transfers its amino group and glutamine donates its amido group by the action of aminotransferases and amidotransferases, respectively. Glutamate is the nitrogen donor for the biosynthesis of 85% of the nitrogenous compounds of the cell while glutamine can transfer its nitrogen to 15% of the nitrogenous molecules. These two nitrogen donors can be synthesized from ammonium by glutamate dehydro-

Abbreviations: ADH, alanine dehydrogenase; AT, alanine transaminase; BCDH, branched-chain keto-acid dehydrogenase; GDH, glutamate dehydrogenase; GOGAT, glutamate synthase; GS, glutamine synthetase; PI, type I pristinamycin; PII, type II pristinamycin.

Fig. 1. Structures of the major pristina-mycins produced by '*S. pristinaespiralis*'. (a) PIA, (b) PIIA and (c) PIIB.

Fig. 2. Interconversion of nitrogenous molecules in the central nitrogen metabolism of *Streptomyces*. GDH, glutamate dehydrogenase; GS, glutamine synthetase; GOGAT, glutamate synthase; ADH, alanine dehydrogenase; AT, alanine transaminase.

genase (GDH) and glutamine synthetase (GS), making ammonium the third central molecule of nitrogen metabolism after glutamate and glutamine. Another reaction catalysed by glutamate synthase (GOGAT) is able to convert glutamine into glutamate (Schreier, 1993; Reitzer, 1996). Central nitrogen metabolism in *Streptomyces* is not well characterized and its regulation is still under investigation (Shapiro *et al.*, 1985; Brana *et al.*, 1986). The general view is that ammonium assimilation proceeds through GDH at high concentrations.

However, this enzyme is not present in all *Streptomyces* species (Brana *et al.*, 1986) and an alternative pathway involving alanine dehydrogenase (ADH) has been proposed in the same high ammonium conditions (Novak *et al.*, 1992). In this case, the synthesis of glutamate would require the function of an alanine transaminase (AT) and would yield the same global reaction.

When the level of ammonium is low, the GS-GOGAT pathway is used for the synthesis of glutamate.

When utilized as source of nitrogen, amino acids can generate ammonium or glutamate (Fig. 2). Generally, the first step in the utilization of amino acids is the partition of the molecule into the amino group on one side and the carbon backbone on the other side. This can be done either by deamination of the amino acid, yielding free ammonium, or by transfer of the amino group to an acceptor, generally 2-oxoglutarate giving glutamate (Untrau *et al.*, 1994). In addition, the existence of two GSs in many *Streptomyces* species (Behrmann *et al.*, 1990; Kumada *et al.*, 1990) and the possible occurrence of two GDHs (NAD-dependent and NADP-dependent) that could function in either the assimilative or degradative direction (Vancurova *et al.*, 1989) has been shown. In '*S. pristinaespiralis*', the occurrence of enzymes of central nitrogen metabolism has not yet been investigated.

On an industrial scale, culture media for antibiotic

production generally contain complex nitrogen sources such as soybean meal. These sources have been selected for their ability to sustain high antibiotic titres and this property is supposed to be linked to the slow release of nitrogenous components during the course of the fermentation. More generally, several studies have shown that nitrogen assimilation is crucial for the regulation of antibiotic production but the mechanisms involved have not yet been unravelled. In addition, there is experimental evidence for repression of antibiotic production exerted by some nitrogen sources and especially ammonium (Aharonowitz, 1980; Brana & Demain, 1988).

In this study, we investigate the regulation of pristinamycin production by nitrogen using different nitrogen sources and synthetic culture media to control the supply and exhaustion of the major nutrient sources. The nitrogen sources were chosen from among inorganic (nitrate, ammonium) or organic (amino acids) compounds. They represent three main pathways of nitrogen assimilation, either by nitrate reduction, direct incorporation of ammonium into central nitrogen metabolism or catabolism of amino acids (Fig. 2). All of the growth experiments were carried out under controlled conditions in fermenters to prevent unwanted side effects such as oxygen limitation or pH effects.

We report the kinetics of growth and antibiotic production by '*S. pristinaespiralis*' when supplied with these different nitrogen sources, and characterize the different physiological phases occurring during the fermentations. Finally, the regulation of the production of pristinamycins by nitrogen in '*S. pristinaespiralis*' is discussed.

METHODS

Micro-organisms. '*S. pristinaespiralis*' Pr11 (Aventis Pharma) was used throughout this study. This strain was isolated after spontaneous mutation of '*S. pristinaespiralis*' ATCC 25486. Frozen mycelia from one single batch culture were maintained in 20% (v/v) glycerol in liquid nitrogen (-70°C) in 1 ml tubes and were used for all experiments reported here.

Media and culture conditions. Synthetic medium used as seed medium had the following composition (per litre of distilled water): 20 g sucrose, 0.3 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 5 g $(\text{NH}_4)_2\text{SO}_4$, 0.75 g K_2HPO_4 , 1 ml trace metal solution, 40 g MOPS. The pH was adjusted to 6.8 before autoclaving for 20 min at 110°C . Trace metal solution consisted of (per litre of distilled water): 45 g $\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$, 11 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 7 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 2 g $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 2 g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.4 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.4 g $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$. Synthetic medium used as production medium contained (per litre of distilled water): 40 g glucose, 0.3 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.2 g K_2HPO_4 , 1 ml trace metal solution. The nitrogen source was varied as described in the text. These media were sterilized for 20 min at 110°C . Cultures were initiated by inoculating 0.5 ml of frozen mycelia into a 300 ml baffled Erlenmeyer flask containing 40 ml seed medium. The culture was incubated for 26 h at 27°C on a rotary shaker at 325 r.p.m. Forty millilitres of the grown seed culture was then inoculated into a 2 l fermenter containing 1.2 l production medium. The pH and dissolved oxygen level were monitored using Ingold specific electrodes, and the pH

was maintained at 6.8 by the automatic addition of 3 M NaOH and 3 M HCl. Temperature was regulated at 27°C and an aeration rate of 1 vessel volume per minute (1 v.v.m.) was employed. The agitation rate was adjusted to keep the dissolved oxygen level above 30% saturation with a starting rate of 800 r.p.m.

Analytical procedures. For the estimation of biomass dry weight, 10 ml samples of culture were centrifuged (at 6000 g for 10 min) in preweighed tubes, the pellet was washed twice with distilled water and the tube plus pellet was dried at 110°C for 48 h. The first supernatant was kept for further analysis of the extracellular medium after filtration through 0.22 μm filters (Millipore).

Amino acid analyses of culture supernatants were performed with an amino acid analysis system from Applied Biosystems following the standard procedures. The system consisted of a model 420A derivatizer for pre-column derivatization of amino acids coupled to a model 130A separation system. Norleucine was used as internal standard.

The glucose, glycerol and 2-ketoisovalerate concentrations in culture supernatants were measured using an HPLC method. Separation was achieved at 56°C on an Aminex HPX-87H column. The column was eluted with a mobile phase of 0.005 M sulfuric acid at a flow rate of 0.8 ml min^{-1} . Column effluent was monitored using a differential refractometer (Shimadzu RID-6A).

Assays of residual phosphate and nitrate in culture supernatants were carried out by ion exchange chromatography using a Dionex DX 120 HPLC system and employing the standard operating procedures for assay of anions.

The concentration of ammonium in culture supernatants was determined with an Orion ammonium electrode model 95-12 with ammonium chloride as standard in conjunction with a digital ion analyser model from Kent.

Extraction, separation and quantitative analysis of pristinamycins were done as follows. One vol. culture broth was mixed with 2 vols HPLC mobile phase (see below) and shaken at 325 r.p.m. for 20 min. The mixture was filtered through a paper filter and the filtrate was analysed by HPLC. PIA, PIIA and PIIB were separated at 23°C on a Nucleosil C8 column eluted at a flow rate of 1 ml min^{-1} with a mobile phase consisting of 37% (v/v) 0.1 M potassium phosphate buffer pH 3.0 and 63% (v/v) acetonitrile. Pristinamycins were detected and quantitated by their absorbance at 260 nm.

RESULTS AND DISCUSSION

Utilization of carbon and nitrogen sources for growth

Glucose was supplied as the carbon source in all of the media investigated. A range of nitrogen sources, inorganic (ammonium and nitrate) and organic (different amino acids) were tested. In Fig. 3, the consumption of the carbon source, the consumption of the nitrogen sources and the formation of biomass are shown during the growth phase. In these synthetic media, nitrogen and carbon sources were simultaneously consumed. In the medium with ammonium nitrate (Fig. 3f) where two nitrogen sources were supplied together, ammonium was consumed first and nitrate was only utilized after exhaustion of ammonium in the medium. All of the nitrogen sources investigated can be considered as good

Fig. 3. Kinetics of utilization of carbon and nitrogen sources for growth in different media. Concentrations of glucose (▲) and nitrogen sources (● in a-e; ●, ammonium and ▼, nitrate in f), and biomass dry weight (■) are shown. Nitrogen sources supplied in the different media were: (a) ammonium chloride, 5 g l⁻¹; (b) sodium nitrate, 7 g l⁻¹; (c) alanine, 10 g l⁻¹; (d) glutamate, 15 g l⁻¹; (e) valine, 15 g l⁻¹; and (f) ammonium nitrate, 4 g l⁻¹.

nitrogen sources for the growth of '*S. pristinaespiralis*' since the levels of biomass obtained within 35 h of cultivation were high (between 5.9 and 7.9 g l⁻¹; Table 1). Lower amounts of biomass were obtained with mineral nitrogen sources such as ammonium and nitrate even though the glucose consumption was generally higher than in the other media, except in the medium

with valine (Table 1). The medium using glutamate as sole nitrogen source allowed earlier growth without a lag phase. For this reason, glutamate seemed to be the most available nitrogen source among the mineral and organic sources studied. Glutamate has been shown to be an excellent source of nitrogen for the growth of *Streptomyces* (Williams & Katz, 1977) and is even

Table 1. Main characteristics of growth in relation to the nitrogen source

Nitrogen source	Maximum biomass, X (g l ⁻¹)	Glucose consumed, G (g l ⁻¹)	Yield, YX/G (g g ⁻¹)
NH ₄ Cl (5 g l ⁻¹)	5.9	14.5	0.41
NaNO ₃ (7 g l ⁻¹)	6.0	18.4	0.33
Alanine (10 g l ⁻¹)	7.9	10.5	0.75
Glutamate (13 g l ⁻¹)	8.5	8.4	1.01
Valine (15 g l ⁻¹)	7.1	17.4	0.41

Fig. 4. Carbon and nitrogen balances for utilization of valine during growth. (a) Carbon balance. Carbon of valine remaining is shown by open bars and carbon of 2-ketoisovalerate produced by filled bars. (b) Nitrogen balance. Nitrogen of valine remaining is shown by open bars and nitrogen of biomass produced by filled bars.

preferred to ammonium in *Streptomyces griseofuscus* (Zhang *et al.*, 1996). In *Streptomyces*, ammonium has been reported to be an inhibitory substrate for growth, especially when supplied in high concentrations (Ives & Bushell, 1997). This could explain the lag phase observed in the medium with ammonium. Nitrate has been shown to be widely used as a nitrogen source by *Streptomyces* (Dekleva *et al.*, 1985), but with some exceptions (e.g. *Streptomyces clavuligerus*; see Aharonowitz & Demain, 1978). However, its assimilation is inducible and can be repressed by a better nitrogen source such as ammonium

(Shapiro & Vining, 1984). Our strain of '*S. pristinae-spiralis*' was able to assimilate nitrate (Fig. 3b) and the results demonstrated that repression of nitrate assimilation by ammonium is also active. In a medium with ammonium and nitrate as nitrogen sources (Fig. 3f), ammonium was assimilated first and nitrate was taken up only after ammonium exhaustion. Therefore, the lag phase in the case of nitrate-containing medium can be explained by the carryover of ammonium from the inoculum stage.

Only one important by-product was detected during growth for all of the media. Upon consumption of valine as the sole nitrogen source, 2-ketoisovalerate, the organic acid derived from valine by removal of the amino group, was produced and accumulated in the medium up to a concentration of 7.6 g l⁻¹. Carbon and nitrogen balances for valine utilization in the medium with valine as sole nitrogen source showed that the nitrogen part of valine was totally assimilated into biomass, whereas its carbon backbone was not assimilated but totally excreted in the medium as 2-ketoisovalerate (Fig. 4). Other branched-chain amino acids, i.e. leucine and isoleucine, followed the same pattern of assimilation when used as nitrogen source. Indeed, the organic acids 2-ketoisocaproate and 2-keto-3-methylvalerate have been shown to accumulate in the medium in flask studies with leucine or isoleucine, respectively, as sole nitrogen sources (data not shown). The consequences of excretion of such a by-product are discussed below.

In Fig. 5, the glucose consumed during the fermentations and the biomass produced are given in terms of carbon mass. In the media containing mineral nitrogen sources, glucose was the only carbon source available and its consumption resulted in the production of biomass and energy. Consequently, the difference between the carbon consumed and the carbon assimilated into biomass was assumed to be converted into carbon dioxide to generate energy for biosynthesis (Fig. 5a, b). The pattern was the same with valine because, as shown previously, valine was only used as a nitrogen source (Fig. 5d). However, for alanine, consumption of glucose alone cannot account for the biomass produced and we have to consider that glucose and alanine are both used as carbon and energy sources (Fig. 5c). The same phenomenon is observed in the medium with glutamate, where glucose was not the only carbon source (high yield of

Fig. 5. Assimilation of carbon into biomass in different media. Consumption of carbon from glucose (●), assimilation of carbon into biomass (■) and consumption of carbon from both alanine and glucose (▲, panel c only) are shown. Nitrogen sources supplied were: (a) ammonium chloride, 5 g l⁻¹; (b) sodium nitrate, 7 g l⁻¹; (c) alanine, 10 g l⁻¹; and (d) valine, 15 g l⁻¹. Hatched areas represent the amount of carbon necessary for the build-up of biomass.

biomass per unit of glucose in Table 1). It is then clear that the consumption of glucose during growth is more important in media with mineral nitrogen sources or valine than in media with amino acids, used both as nitrogen and carbon sources, explaining the yields reported in Table 1. This has already been noticed by several other authors (Williams & Katz, 1977; Shapiro & Vining, 1983). It appears that certain keto-acids, especially those with long carbon chains (arising from the catabolism of branched-chain amino acids) are less suitable as a carbon source than products of the catabolism of glucose because they are excreted in the extracellular medium. To our knowledge, this is the first time that the excretion of 2-ketoisovalerate has been observed upon consumption of valine. Excretion and further reassimilation of isobutyrate, the next product in the catabolism of valine, was reported by Lounes *et al.* (1995) in fermentations with glycerol as carbon source. The availability of the carbon skeleton of the amino acids is reflected by its ability to support the growth of *Streptomyces* when supplied as sole carbon and nitrogen source. Romano & Nickerson (1958) reported that for

Streptomyces fradiae, glutamate and alanine supported good growth whereas all the branched-chain amino acids (valine, leucine and isoleucine) did not permit growth. This is in agreement with our observations that glutamate and alanine were readily utilized as carbon sources for the growth of '*S. pristinaespiralis*' whereas valine was not.

The nitrogen sources investigated here can be classified into three groups according to their mode of utilization by '*S. pristinaespiralis*'. The mineral nitrogen sources, ammonium and nitrate, were utilized only as nitrogen source. Branched-chain amino acids (valine, leucine and isoleucine) were consumed only for their nitrogen content, the carbon backbone being excreted into the medium as organic acid. Other amino acids, such as glutamate and alanine, were utilized as both nitrogen and carbon sources. However, the consumption of the amino acids must be driven primarily by the need for nitrogen because they represented the sole source of nitrogen for the cell. Once those needs have been satisfied, the carbon skeleton (the keto-acid) can be

Fig. 6. Response to phosphate limitation in media with different nitrogen sources. Concentrations of phosphate ($\square$), nitrogen sources ($\blacktriangledown$), ammonium excreted ($\blacktriangle$) and glycerol ($\circ$) are shown. Nitrogen sources supplied in the different media were: (a) ammonium chloride, 5 g l⁻¹; (b) sodium nitrate, 7 g l⁻¹; (c) alanine, 10 g l⁻¹; and (d) valine, 15 g l⁻¹. The time of phosphate exhaustion is indicated by a vertical line.

utilized concurrently with glucose present in the medium. In the case of amino acids whose corresponding keto-acids enter directly into central metabolism (e.g. glutamate and alanine), these keto-acids appeared to be utilized preferentially and glucose was used only as a complementary carbon source. In contrast, for keto-acids derived from branched-chain amino acids, glucose was preferred as a carbon source and consequently, the keto-acids were excreted into the medium.

Metabolic transitions linked to nutrient exhaustions

In this study, synthetic culture media were designed in order to prevent the exhaustion of glucose as the carbon and energy source during the course of fermentation, ensuring at the same time enough carbon for antibiotic synthesis. Phosphate was provided so as to be the limiting nutrient, as confirmed in Fig. 6. Phosphate was always the first major nutrient (out of carbon, nitrogen and phosphorus sources) to be exhausted in the different

media, the time of exhaustion corresponding to the maximum respiration rate and to the minimum dissolved oxygen value (Fig. 6). However, biomass accretion and uptake of carbon and nitrogen sources persisted after phosphate depletion. Increase in biomass after phosphate exhaustion was recorded in all of the media (up to 40% in the medium with glutamate), except in the one with valine. Owing to reserves of phosphate, growth can continue but at a slower rate after exhaustion of phosphate from the medium (Wanner & Egli, 1990). Phosphate storage materials can include polyphosphates, RNA or teichoic acids in Gram-positive organisms (Mundry & Kuhn, 1991). The occurrence of teichoic acids serving as cell wall polymers has been demonstrated in *Streptomyces* by Naumova *et al.* (1980). It is only after the exhaustion of these reserves of phosphate that the growth of the cell really stopped.

The production of glycerol was observed in all of the media investigated following phosphate depletion as can

Fig. 7. Nutrient concentrations and pristinamycin production in media with initial glutamate concentrations of 15 g l⁻¹ (a) and 13 g l⁻¹ (b). Concentrations of phosphate (■), glutamate (●), glycerol (○), ammonium excreted (□) and pristinamycins (▲) are shown. The first vertical line (from the left) indicates the time of phosphate exhaustion, the second the time of glutamate exhaustion and the third the time of onset of pristinamycin production.

be seen in Figs 6 and 7. The magnitude of this production varied according to the nitrogen source in the medium. Higher production levels were obtained in the alanine- and glutamate-containing media with concentrations up to 1 g l⁻¹ (Figs 6c, 7). In the other media (with ammonium, nitrate and valine) the production of glycerol was lower with maximum amounts of 100–200 mg l⁻¹ (Fig. 6a, b, d). This production of glycerol seemed to be specific to phosphate exhaustion because it was not observed when nitrogen exhaustion occurred first (data not shown). The glycerol can be reassimilated in the late part of the fermentation, especially in alanine- and glutamate-containing media (Figs 6, 7). No production of glycerol by *Streptomyces* has been previously reported in the literature upon phosphate limitation. However, the replacement of teichoic acids by teichuronic acids in order to recover phosphate has been described in *Bacillus* (Grant, 1979). In this case, glycerol should be concomitantly released. The discrepancy in the amounts of glycerol produced in the different media could be explained either by immediate utilization, or by release of the glycerol in some cases. In media containing mineral nitrogen sources or valine, one part of the glycerol was directly recycled for cellular needs and used together with glucose. Under such circumstances, glucose was almost certainly diverted to the synthesis of teichuronic acids, replacing teichoic acids in the cell wall. As for certain organic acids (pyruvate or 2-oxoglutarate), their carbon-rich part was utilized preferentially and glycerol was totally released. The production of ammonium occurred only in alanine- and glutamate-containing media (amino acids used as nitrogen and carbon sources) after phosphate exhaustion (Figs 6c, 7). In these cases, ammonium originated

from the deamination of the amino acid and reached high levels in the extracellular medium (more than 200 mg l⁻¹, Figs 6c, 7). Rapid consumption of the nitrogen source still continued after depletion of phosphate, leading to ammonium accumulation. Carbon metabolism rather than nitrogen metabolism was then controlling the consumption of the amino acid. In the case of nitrogen sources only used for their nitrogen content, the control over consumption was more stringent because the consumption of the nitrogen source slowed down after phosphate depletion (Fig. 6a, b). In the case of nitrate assimilation, it has been shown that uptake into the cell is tightly controlled in order not to generate excess ammonium (Shapiro & Vining, 1984). As in the case of valine, the consumption of the nitrogen source almost stopped at the time of phosphate depletion in the medium (Fig. 6d). Owing to the large quantity of ketoisovalerate excreted, we postulate a feedback inhibition of this product on its formation, blocking any further catabolism of valine and consequently any excretion of ammonium.

With glutamate as the nitrogen source, a thorough look at the concentration of nutrients in the extracellular medium after phosphate depletion allowed the reconstruction of the different metabolic phases (Fig. 7). The first phase, immediately after phosphate exhaustion, was characterized by a large release of both ammonium and glycerol (Fig. 7). Ammonium originated from the catabolism of glutamate (the sole nitrogen source) that was still consumed at a high rate after phosphate exhaustion. However, at this time, glutamate seemed to be consumed primarily as a carbon source because of the excretion of ammonium. This would mean that 2-oxoglutarate, the product of deamination of glutamate,

Table 2. Characteristics of pristinamycin production in relation to the nitrogen source

Nitrogen source	Biomass at production peak, X (g l ⁻¹)	Pristinamycin production peak, P (mg l ⁻¹)	Production phase	Yield, YP/X (mg g ⁻¹)
NH ₄ Cl (5 g l ⁻¹)	5.7	57	Idiophase	10.0
NaNO ₃ (7 g l ⁻¹)	1.5	12	Trophophase	8.0
Alanine (10 g l ⁻¹)	7.9	75	Idiophase	9.5
Glutamate (13 g l ⁻¹)	5.5	61	Idiophase	11.1

was utilized preferentially as the other carbon sources, probably to replenish the tricarboxylic acid cycle in a time of energy deprivation. Consequently, glycerol accumulated in the medium as a by-product. The second phase was initiated by the exhaustion of the amino acid, and the exhaustion of the keto-acid as carbon source must have occurred more or less simultaneously (Fig. 7). This time corresponded to the peak in the concentration of ammonium. The ammonium was then reutilized by the strain to satisfy its nitrogen needs until its exhaustion in the medium. The residual carbon sources were then glycerol and glucose, and both were used simultaneously. Interestingly, we did not observe any repression of glycerol consumption by glucose as has been observed in other *Streptomyces* spp. (Hodgson, 1982). This kinetic profile was observed in two fermentations with different initial amounts of glutamate as the sole nitrogen source (15 vs 13 g l⁻¹) in Fig. 7. For the fermentation with a higher initial amount of glutamate, more residual glutamate (5 vs 2 g l⁻¹) was present at the time of phosphate depletion and consequently ammonium was excreted in larger amounts (280 vs 160 mg l⁻¹) before exhaustion of glutamate. That meant that more keto-acid was obtained, sparing the utilization of glycerol. Consequently, the level of glycerol released was higher (1230 vs 715 mg l⁻¹). Similar excretion and consumption profiles were observed with ammonium and glycerol in the medium with alanine (Fig. 6c).

In conclusion, on the basis of the events occurring after growth limitation by phosphate, and especially on the basis of the products excreted, it is possible to distinguish three groups of media. These groups are the same as those already identified according to the utilization of nitrogen sources during growth. The first group, where the production of glycerol was low and where no production of ammonium was observed, corresponded to media with mineral nitrogen sources, and the second group, with a higher level of glycerol production and excretion of ammonium, corresponded to media where amino acids were used as nitrogen sources. The medium with valine was an exceptional case because of the excretion of 2-ketoisovalerate during growth.

Characteristics of the production of pristinamycin

The typical kinetics of production of pristinamycins in synthetic media are shown in Fig. 7. The production phase lasted for less than 10 h and the maximum titres

were always below 100 mg l⁻¹. Type II pristinamycins (A and B) represented the major fraction of the total pristinamycins (above 80 %), the remainder being pristinamycin IA (see Fig. 1). Type II pristinamycins (but not type I pristinamycins) are subject to degradation in the fermentation broth (unpublished results). Therefore, one peak of production and then a slow decrease in the pristinamycin titre were observed (Fig. 7). In Table 2, maximum production levels are given together with the biomass at the time of maximum production. Their ratio gives a tentative yield of pristinamycins per unit of biomass. Results for media containing valine have been omitted because of the absence of production throughout the course of the fermentation. In the medium with nitrate as nitrogen source, production occurred very early during growth with a resulting low amount of biomass (1.5 g l⁻¹). For media with ammonium, alanine or glutamate, production took place during the stationary phase, after phosphate exhaustion. The yield of pristinamycins per unit of biomass was very similar for all of the media investigated. Regardless of nitrogen source, high biomass production was a necessary condition to obtain a good level of pristinamycin production. This could reflect a limited amount of precursors of pristinamycin per unit of biomass in these media. Apparently, the limitation cannot be alleviated by changing the nitrogen source.

It is interesting to correlate the onset of pristinamycin production with the metabolic events occurring during the course of fermentation, and especially with the exhaustion of nutrients. The carbon and energy source, glucose, was always in excess during the course of fermentation. The presence of phosphate did not prevent the onset of production because its level remained high during the production phase in medium with nitrate (Table 3). Moreover, in media with an initial growth limitation linked with nitrogen exhaustion where phosphate was still present, the production of pristinamycin was also observed (data not shown). Similarly, the presence of glycerol was unimportant. Glycerol was not present in the fermentation broth in the nitrate-containing medium at the time of production whereas its concentration reached about 900 mg l⁻¹ in the glutamate-containing medium (Table 3). Amino acids were not present in any media at the time of production. Interestingly, the initial amount of amino acid appeared to play a role in the onset of production. In media with glutamate, the more amino acid present, the more the

Table 3. Levels of various nutrients at onset of pristinamycin production in relation to the nitrogen source

Nitrogen source	Phosphate (mg l ⁻¹)	Ammonium (mg l ⁻¹)	Amino acids (g l ⁻¹)	Glycerol (mg l ⁻¹)
NH ₄ Cl (5 g l ⁻¹)	Exhausted	<280	–	60
NaNO ₃ (7 g l ⁻¹)	>485	ND	–	ND
Alanine (10 g l ⁻¹)	Exhausted	>170	Exhausted	>550
Glutamate (13 g l ⁻¹)	Exhausted	71	Exhausted	695
Glutamate (15 g l ⁻¹)	Exhausted	120	Exhausted	880

ND, Not detected.

onset of production was delayed (Fig. 7). The influence of ammonium on production was not straightforward. In the case of media with ammonium as nitrogen source, production was delayed until low levels of residual ammonium were reached (Table 3). In addition, the same delay in production that was observed with high levels of glutamate was also seen when the medium contained a higher concentration of ammonium. In media containing amino acids, which lead to accumulation of ammonium after phosphate exhaustion (e.g. Fig. 7), ammonium was present at moderate concentrations (100–200 mg l⁻¹) and did not prevent production. The production of pristinamycin began during the period when ammonium was being consumed as a secondary nitrogen source after exhaustion of the amino acid used as primary nitrogen source.

On the basis of the characteristics of the production of pristinamycin, we can distinguish four groups of nitrogen sources. The medium with valine was once again an exceptional case, with no pristinamycin production. When nitrate was utilized as nitrogen source, production took place during the trophophase with plenty of nutrients (glucose, nitrate, phosphate) still remaining in the medium. This nitrogen source resulted in a poor production of pristinamycins, possibly linked with the low level of biomass. In media with ammonium as nitrogen source, production was delayed until after the exhaustion of extracellular phosphate and was apparently linked with the residual level of the nitrogen source in the medium. With alanine and glutamate, production started after depletion of both extracellular phosphate and amino acids but with some ammonium still present in the medium.

Possible regulation of production of pristinamycin

The production of antibiotics can take place only if three conditions are fulfilled. First, it is necessary to have the cell factories to synthesize the molecule, i.e. enough biomass; secondly, building blocks, i.e. precursors, have to be present; and thirdly, the workers, i.e. the enzymes to process these building blocks into antibiotics, should be present and active. Specific regulatory factors of antibiotic production have also been reported in the literature. A-factor and virginiae butanolides, compounds with a gamma-butyrolactone structure, have

been shown to be involved in the regulatory cascade leading to streptomycin and virginiamycin production respectively (Hara & Beppu, 1982; Yamada *et al.*, 1987). Although the occurrence of similar compounds is known in '*S. pristinaespiralis*' cultures (Paquet *et al.*, 1992), their possible role in the onset of pristinamycin production is not discussed in this paper. In our experiments, the amount of biomass seems to be important only for the quantity of pristinamycin produced and had no influence on the onset of production. Consequently, the absence of production or the delay observed with some nitrogen sources could result either from a shortage of precursors or from a negative regulation of the enzymes involved in pristinamycin biosynthesis.

The absence of production of pristinamycin throughout the fermentation with valine as the sole nitrogen source can be explained by the specificity of the catabolism of valine in this strain. The first steps of branched-chain amino acid catabolism are well established in *Streptomyces* (Zhang *et al.*, 1999; Fig. 8). In the case of '*S. pristinaespiralis*', the branched-chain keto-acid dehydrogenase (BCDH) enzyme is negatively regulated in a medium with valine, as demonstrated by the excretion of 2-ketoisovalerate. Hafner *et al.* (1991) demonstrated that a functional BCDH is mandatory for the synthesis of avermectins because the starters originate exclusively from the degradation of either isoleucine or valine (Fig. 8). A BCDH⁻ mutant is unable to produce avermectin unless supplied with isobutyrate or methylbutyrate. The same characteristics have been demonstrated for type II pristinamycin synthesis, which shares the same starters, 2-methylbutyryl-CoA or isobutyryl-CoA, with avermectin biosynthesis. The inhibition or repression of BCDH could be explained in two ways. Nitrogen regulation mediated by excess ammonium generated by the degradation of valine can be proposed. It has been reported by Lounes *et al.* (1995) that ammonium repressed BCDH in *Streptomyces ambofaciens*. However, in our work, ammonium was not likely to accumulate because valine was consumed at the same rate as biomass was formed and ammonium was never excreted in the medium throughout the course of the fermentation (Fig. 6d). The hypothesis of carbon catabolic regulation mediated by glucose is more appealing. According to our results, glucose was utilized as a preferred carbon source compared to the carbon

Fig. 8. Pathways of valine and isoleucine catabolism in *Streptomyces* (Zhang *et al.*, 1999). The amino acid is first converted to its corresponding keto-acid (2-ketoisovalerate for valine) by either a deaminase or a transaminase. The second step is a decarboxylation by a BCDH to give acyl-CoA (isobutyryl-CoA for valine).

backbone of valine by '*S. pristinaespiralis*'. The target of this possible catabolic regulation obviously cannot be the first step of valine catabolism which provides nitrogen to the cell, but would be the second step catalysed by BCDH, preventing any further catabolism of the carbon backbone of valine.

For other nitrogen sources, a specific shortage in precursors of pristinamycin was not obvious. Nutrients or their catabolites could be responsible for a mechanism of catabolic regulation where pristinamycin production is first prevented by the presence of the regulator and its disappearance triggers the production. Phosphate would be the first candidate for promoting this regulation because its exhaustion initiated the transition to the stationary phase. In addition, its involvement in the regulation of antibiotic production is well documented (Martin, 1977). However, phosphate did not seem to play a role in catabolic repression of pristinamycin production because in the case of medium with nitrate, production started with a still high residual level in the medium. Moreover, in several media where production took place during stationary phase, the onset was not brought on immediately by the exhaustion of phosphate in the extracellular medium. The occurrence of carbon catabolic regulation mediated by glucose was not likely to take place in the fermentations studied here with the exception of the case of valine discussed above. A high level of residual glucose throughout the fermentation did not prevent the synthesis of pristinamycin in any case. Carbon catabolic regulation in *Streptomyces* has been shown to be brought on by catabolism of glucose through glucose kinase and to prevent the utilization of poorer carbon sources, especially glycerol (Hodgson, 1982). In our case, glucose was the initial carbon source, although not the preferred one. Other carbon metabolites, keto-acids or glycerol, can be utilized together with glucose. The necessity for amino acids to be totally cleared from the medium before production can take place could be an indication of the influence of certain keto-acids. It has been shown that amino acids, and more precisely their carbon backbone, were the pre-

ferred carbon source after exhaustion of phosphate in the medium. A type of catabolic regulation of the assimilation of carbon sources then took place. This catabolic regulation, obviously promoted by keto-acids or their metabolites, could also act on the production of pristinamycin. This would explain the late production observed when more glutamate (and consequently more 2-oxoglutarate) was supplied at the beginning of the fermentation (Fig. 7).

The various kinetics of pristinamycin production observed in media differing only in the nitrogen source suggest a strong influence of nitrogen metabolism on the production of antibiotics, as already outlined by several authors (Gräfe, 1982; Shapiro, 1989). Occurrence of early production with nitrate as sole nitrogen source compared to late production with either ammonium or some amino acids (alanine and glutamate) could be the result of the absence of regulation by the nitrogen source in the first case and of a negative action in the other cases. This hypothesis is supported by the experiments where production was delayed when using higher initial amounts of ammonium or glutamate as nitrogen source. Nitrogen catabolic regulation has been documented for production of several antibiotics. A negative action of ammonium has already been observed, especially for macrolides (Tanaka *et al.*, 1986) and beta-lactams (Brana *et al.*, 1985; Castro *et al.*, 1985). The direct involvement of an amino acid has been shown in the mechanism of nitrogen catabolic repression of actinomycin D production by glutamate (Foster & Katz, 1981). The preferred utilization of ammonium over nitrate as nitrogen source is evidence for the occurrence of a mechanism of nitrogen catabolite regulation. Moreover, nitrate assimilation is tightly controlled in order not to generate excess ammonium. Therefore, absence of catabolic repression mediated by ammonium is likely to occur in this case, allowing early production of pristinamycin. The hypothesis of ammonium catabolic regulation is also sustainable in the media with ammonium as sole nitrogen source where the high initial concentrations will prevent pristinamycin production

and where the consumption of ammonium will determine the time of onset of production. However, extracellular ammonium was not always totally exhausted at that time. This could be an indication that there is a lower threshold level that triggers production. As for an organic nitrogen source, it is difficult to distinguish between regulation exerted either by the amino acid per se or by ammonium generated by the catabolism of this amino acid. During growth, amino acids were used in the first place as nitrogen sources and in this case no ammonium was excreted. High intracellular levels of ammonium were then not likely to be produced. After phosphate exhaustion, amino acids were used preferentially as carbon source, almost certainly by direct deamination because ammonium was excreted in the extracellular medium. In this case, the probability of excess intracellular ammonium was high. However, whereas pristinamycin production always started after total exhaustion of the amino acids, residual ammonium was still present in the medium at that time. This would once again sustain the hypothesis of a lower threshold level if ammonium regulation is involved. Nitrogen regulation could also be linked, not specifically to catabolic repression, but to the general nitrogen status of the cell. When intracellular ammonium is high, it is generally recognized that nitrogen assimilation in *Streptomyces* proceeds either by the GDH pathway or by the ADH pathway. However, these dehydrogenases exhibit high affinity constant values and cannot be responsible for assimilation of ammonium when the intracellular level falls down to a threshold value. In this case, the GS-GOGAT pathway takes precedence for assimilation of ammonium (Paress & Streicher, 1985; Fisher & Wray, 1989). It is striking that the conditions under which the GS-GOGAT pathway is known to operate are also the conditions of pristinamycin production in our experiments (nitrate-containing medium or medium with low ammonium). Therefore, the system responsible for the switch between the different assimilation pathways could also be triggering the production of antibiotics.

Concluding remarks

In this study, the conditions for controlled culture of '*S. pristinaespiralis*' in synthetic media with several single nitrogen sources and using phosphate as limiting nutrient were established. For each case, the main physiological phases of the culture were identified and their features characterized. This knowledge of the patterns of utilization of carbon and nitrogen sources was a prerequisite for the study of the influence of nitrogen metabolism on pristinamycin production. The growth of '*S. pristinaespiralis*' is well supported by various organic and inorganic nitrogen sources. Pristinamycin production is independent of the nitrogen source supplied in terms of specific production. However, the nature and the amount of the nitrogen source are both critical in determining the onset of antibiotic production. The results of this study suggest that nitrogen catabolic repression of pristinamycin production is taking place.

Moreover, ammonium, either directly supplied as a nitrogen source or originating from the breakdown of amino acids, may play a central role in this negative regulation. Difficulties were encountered during this study in using amino acids as nitrogen sources. Amino acids are sources of carbon as well as nitrogen catabolites, and both are able to exert a regulatory effect. Regulation of pristinamycin production by carbon rather than by nitrogen catabolites is therefore not excluded when using amino acids. In addition, some amino acids have to be considered as direct precursors for antibiotic synthesis and not only as nutrient sources. This study represents the first step toward an understanding of the determinants of pristinamycin production. It will be interesting to investigate the occurrence of nitrogen catabolic repression under different culture conditions, especially under nitrogen limitation. Moreover, to elucidate the mechanism of nitrogen regulation of pristinamycin production, enzymic studies are needed.

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