A link between primary and secondary metabolism: malonyl-CoA formation in *Streptomyces ambofaciens* growing on ammonium ions or valine

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Growth of *Streptomyces ambofaciens* in the presence of valine led to a greater production of spiramycin and excretion of short-chain fatty acids compared with growth on ammonium ions as nitrogen source. We determined the activities of enzymes that lead to the formation of malonyl-CoA, a key intermediate in the biosynthesis of spiramycin. In valine-grown cultures, the activities of acetyl-CoA carboxylase and pyruvate dehydrogenase were increased during the antibiotic production phase. During this idiophase, oxaloacetate dehydrogenase activity was much higher in cultures growing on ammonium compared with valine. Two pathways are proposed concerning the mode of malonyl-CoA formation: a route via oxaloacetate dehydrogenase following growth on ammonium, and a route via acetyl-CoA carboxylase after growth on valine.

Keywords: *Streptomyces ambofaciens*, spiramycin, malonyl-CoA, nitrogen source

INTRODUCTION

The branched lactonic ring of spiramycin requires acetate, propionate and butyrate as precursors for its biosynthesis (Omura *et al.*, 1977). These latter compounds are final products of some pathways of primary metabolism. They are synthesized from glycolysis, the tricarboxylic acid cycle and the catabolism of fatty acids and/or amino acids; thus they occupy a key position between primary and secondary metabolism. The polymerization of activated acyl units to form the macrolide lactonic ring is catalysed by a polyketide synthetase enzyme system, similar to the events leading to the synthesis of long-chain fatty acids (Neusil & Hostalek, 1986).

The incorporation of precursors into the macrolide occurs in the form of activated substrates, so the enzymic activities ensuring the formation of the latter appear essential. In general, two kinds of key enzymes are

implicated in these initial steps: acylkinases coupled with acylphosphotransferases, and carboxylases which lead from acetyl-, propionyl- or butyryl-CoA to malonyl-, methylmalonyl- or ethylmalonyl-CoA respectively.

In *Streptomyces fradiae*, a tylosin producer, the production of fatty acids (Vancura *et al.*, 1987) and methylmalonyl-CoA from propionyl-CoA (Omura & Tanaka, 1986) is correlated with tylosin synthesis and is repressed by ammonium ions (Vu-Trong & Gray, 1987), phosphate (Vu-Trong *et al.*, 1981) or glucose (Vu-Trong *et al.*, 1980). In *Streptomyces ambofaciens*, fatty acids are activated by acylkinases and acylphosphotransferases (Khaoua *et al.*, 1992). Therefore, a compound such as malonyl-CoA must play a significant part in macrolide antibiotic biosynthesis as well as in fatty acid biosynthesis.

However, carboxylation of acetyl-CoA is not the sole pathway leading to malonyl-CoA, which may also be synthesized from oxaloacetate through oxaloacetate dehydrogenase action (see Fig. 6). In *Streptomyces aureofaciens*, malonyl-CoA synthesis for tetracycline formation occurs via acetyl-CoA carboxylase, phosphoenolpyruvate carboxylase then oxaloacetate dehydrogenase (Behal *et al.*, 1977). On the other hand, the availability of short-chain fatty acids seems to be a determining factor for spiramycin biosynthesis in *S. ambofaciens*; such fatty acids increase the

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Abbreviations: DCW, dry cell weight; ACC, acetyl-CoA carboxylase; AK, acetate kinase; OADC, oxaloacetate decarboxylase; OADH, oxaloacetate dehydrogenase; PDC, pyruvate decarboxylase; PDH, pyruvate dehydrogenase; TCA, trichloroacetic acid.

Fig. 1

Fig. 2

Fig. 1. Growth as dry cell weight (■), ammonium consumption (●) and spiramycin production (□) (a) and acetate (▲), butyrate (○) and propionate (◆) production in the presence of 50 mM ammonium chloride (b).

Fig. 2. Growth as dry cell weight (■), and spiramycin (□), acetate (▲) and isobutyrate (○) production in a culture with 50 mM valine.

activity of the activation enzymes and spiramycin production, and an excess of ammonium lowers acylkinase activity (Khaoua *et al.*, 1990).

The present work was performed as part of studies of the relationship between primary and secondary metabolism, and the regulation of spiramycin biosynthesis in *Streptomyces ambofaciens*. Our objective was to gain insight into the potential use of different pathways in malonyl-CoA biosynthesis, when spiramycin was produced by this organism in the presence of the two nitrogen sources, ammonium ions and valine.

METHODS

Micro-organisms. *Streptomyces ambofaciens* RP 181110 (Rhône-Poulenc) was the spiramycin-producing strain. *Bacillus subtilis* ATCC 6633 was used for the determination of the antibiotic.

Medium and culture conditions. Medium and culture conditions were the same as described by Lebrihi *et al.* (1992).

Preparation of the cell extracts. Cell-free extracts were prepared as described in Khaoua *et al.* (1992). Protein concentrations in the extracts were determined using the Lowry method.

Enzyme activities

These were determined from at least three cultures in each growth condition. For each culture three time points were taken to establish the rate of enzymic reaction. The mean values of all these assays were calculated. The variation was no more than 10%, due mainly to the variability between cultures.

Pyruvate decarboxylase (PDC) and oxaloacetate decarboxylase (OADC). These activities were determined colorimetrically by measuring the amount of ferrocyanide liberated during the oxidative decarboxylation of pyruvate or oxaloacetate using ferricyanide as electron acceptor (Reed & Williams, 1966). The activities are expressed as $\mu\text{mol product formed min}^{-1} (\text{mg protein})^{-1}$.

Pyruvate dehydrogenase (PDH) and oxaloacetate dehydrogenase (OADH). The reaction mixture contained, in a final volume of 0.75 ml: phosphate buffer, pH 7.0, 100 mM; MgCl_2 , 1.33 mM;

NAD⁺, 8 mM; cysteine hydrochloride, 4 mM; thiamin pyrophosphate, 0.8 mM; HS-CoA, 0.24 mM; oxaloacetate or pyruvate, 4 mM. The reaction was started by adding enzyme extract, allowed to continue for 10 min at 25 °C, and stopped by addition of trichloroacetic acid (TCA; 10%, w/v). After centrifugation the acetyl-CoA and malonyl-CoA produced was measured in the supernatant fluid by HPLC. A control assay was performed under the same conditions by adding TCA before the enzyme. Enzyme activities are expressed as nmol product formed min⁻¹ (mg protein)⁻¹.

Acetyl-CoA carboxylase (ACC). The reaction mixture contained, in a final volume of 0.24 ml: Tris/HCl buffer, pH 8.0, 62.5 mM; acetyl-CoA, 0.2 mM; KHCO₃, 20.4 mM; ATP, 6.25 mM; MgCl₂, 25.0 mM. The reaction was started by adding the enzyme extract, allowed to continue for 10 min at 37 °C, and stopped by addition of TCA (10%). After centrifugation the malonyl-CoA produced was measured by HPLC. A control was performed by adding TCA before the enzyme. The activity is expressed as μmol malonyl-CoA formed min⁻¹ (mg protein)⁻¹.

Acetate kinase (AK). This activity was measured according to the method described by Röse (1955), and is expressed as μmol product formed min⁻¹ (mg protein)⁻¹.

High-performance liquid-chromatography (HPLC) analysis. Coenzyme A, acetyl-CoA and malonyl-CoA in the assay mixtures were detected by HPLC, measuring UV absorption at 254 nm. The equipment comprised a multisolvent delivery system (Spectraphysics SP8800 model), U6K injector, variable wavelength detector (Spectrafocus system), personal computer (IBM) and C18 column (Colosil, 6 mm $\times$ 15 cm), 5 μm particle diameter. The sample volume was 25 μl . Separation was achieved with a linear gradient from 0 to 30% methanol in phosphate buffer, 50 mM, pH 5.3, for 25 min, at a flow rate of 1 ml min⁻¹.

Short-chain fatty acid measurements. Short-chain fatty acids in the bacterial culture were quantified by gas chromatography (IGC 121 EL model, Intersmat) using a Propak Q80, 100 mesh column (2 m $\times$ 2 mm) and a flame ionization detector. The column temperature was 180 °C. Known amounts of standard short-chain fatty acids were used for calibration procedures (external standard). A sample (8 μl) of external standard or sample supernatant was mixed with 2 μl of internal standard (prepared with 4 ml of methanol + 6 ml of 6 M HCl in a final volume of 100 ml with distilled water). A sample (2 μl) of the resulting mixture was injected for analysis.

Measurement of growth. The dry cell weight (DCW) was determined according to the method described by Lebrihi *et al.* (1987). Under these conditions, 1 g DCW l⁻¹ corresponded to an OD of 2 at 660 nm.

Determination of spiramycin. This was done by the conventional disc diffusion method using *Bacillus subtilis* as the sensitive strain. Triplicate assays were done; the variation was less than 10%.

Chemical products. Spiramycin was supplied by Rhône-Poulenc Santé; the other reagents were supplied by Sigma.

RESULTS

Growth and spiramycin production in the presence of ammonium or valine

Growth was very similar with the two nitrogen sources (Figs 1 and 2), both of which were totally consumed at the end of growth (not shown). We measured the production

of spiramycin during growth of *S. ambofaciens* in a basal medium containing 50 mM NH₄Cl (Fig. 1) or valine (Fig. 2). Antibiotic production occurred during the stationary phase of growth, and was higher in the presence of valine. The specific production, at the time when production was maximal, was 4.5 and 18.5 mg (g DCW)⁻¹ with NH₄Cl and valine respectively. Analysis of the culture medium, with NH₄Cl as nitrogen source, showed the presence of various fatty acids (Fig. 1): acetate made up the greatest proportion of these acids (0.17 g l⁻¹ at a maximum); propionate and butyrate concentrations were significant whilst isobutyrate was less so. With valine as nitrogen source, more acetate and isobutyrate were excreted than in the case of growth with ammonium (5 g l⁻¹ and 2.8 g l⁻¹ respectively) (Fig. 2); excretion of the latter preceded spiramycin appearance in the medium by 24 h (Fig. 2).

Fig. 3. Growth as dry cell weight (■), and acetate kinase (□) and acetyl-CoA carboxylase (▲) specific activity in cultures with 50 mM ammonium (a) or valine (b).

Fig. 4

Fig. 5

Fig. 4. Growth as dry cell weight (■), and activities of pyruvate dehydrogenase (●) and oxaloacetate dehydrogenase (▲) (a) and pyruvate decarboxylase (●) and oxaloacetate decarboxylase (▲) (b) in culture with 50 mM ammonium.

Fig. 5. Growth as dry cell weight (■), and activities of pyruvate dehydrogenase (●) and oxaloacetate dehydrogenase (▲) (a) and pyruvate decarboxylase (●) and oxaloacetate decarboxylase (▲) (b) in culture with 50 mM valine.

Acetate kinase and acetyl-CoA carboxylase

During the exponential growth phase AK activity seemed to be slightly greater in cells grown on valine: the maximum specific activities were 0.134 and 0.09 $\mu\text{mol min}^{-1} \text{mg}^{-1}$ for cells cultivated with valine (Fig. 3b) or NH_4Cl (Fig. 3a) respectively. This AK activity decreased during the exponential phase then showed a peak during the stationary phase; this peak was of the same order of magnitude with each of the two nitrogen sources ($0.1 \mu\text{mol min}^{-1} \text{mg}^{-1}$).

Using identical culture conditions ACC activities were determined in cell extracts (Fig. 3a, b). In the presence of ammonium the specific activity of this enzyme was $1 \mu\text{mol min}^{-1} \text{mg}^{-1}$ in the early exponential growth phase; it then fell rapidly and disappeared although a very slight peak of activity ($0.05 \mu\text{mol min}^{-1} \text{mg}^{-1}$) was noted

during the stationary phase at the time of spiramycin production. This peak of ACC during the latter period was significantly higher in the medium with valine: the maximum reached $1 \mu\text{mol min}^{-1} \text{mg}^{-1}$.

Pyruvate decarboxylase and pyruvate dehydrogenase

We measured the decarboxylation activity of the pyruvate dehydrogenase complex. Though this decarboxylation reaction occurs with dehydrogenation, we chose to name the activity pyruvate decarboxylase to distinguish it from pyruvate dehydrogenase, which transforms pyruvate to acetyl-CoA.

The use of this assay for measurement of the PDH activity (see Methods) led to a particular profile in the HPLC analysis after reaction: in all cases the amount of acetyl-

Fig. 6. Pathways of malonyl-CoA formation in presence of ammonium or valine. ACC, acetyl-CoA carboxylase; AK, acetate kinase; APT, acetylphosphotransferase; PDH, pyruvate dehydrogenase; PDC, pyruvate decarboxylase; OADH, oxaloacetate dehydrogenase; OADC, oxaloacetate decarboxylase.

CoA was low, whereas a proportionally higher level of malonyl-CoA was observed. We assumed that acetyl-CoA formed in the PDH reaction might be rapidly transformed in malonyl-CoA through the ACC reaction. We thus actually measured the formation of acetyl-CoA and malonyl-CoA from pyruvate as the result of PDH activity.

PDC and PDH activities were measured during growth on NH_4Cl (Fig. 4a, b) or valine (Fig. 5a, b). In both cases the specific activity of PDC, after a slight decrease in the initial growth phase, rose during the stationary phase to attain levels of 0.1 and 0.08 $\mu\text{mol min}^{-1} \text{mg}^{-1}$ respectively. The profile of PDH activity differed. In cultures grown on NH_4Cl it reached 8 $\text{nmol min}^{-1} \text{mg}^{-1}$ during the exponential phase then rapidly diminished and disappeared totally during the stationary phase. In cultures grown on valine this PDH activity reached 16 $\text{nmol min}^{-1} \text{mg}^{-1}$ at the beginning of the exponential phase, decreased at the end of this phase, then showed a peak (9 $\text{nmol min}^{-1} \text{mg}^{-1}$) during the stationary phase; this was accompanied by an increase in spiramycin excretion.

Oxaloacetate decarboxylase and oxaloacetate dehydrogenase

We assayed the enzyme complex OADH that leads to malonyl-CoA formation from oxaloacetate. This assay is analogous to the pyruvate dehydrogenase complex; thus,

under the same conditions, we followed the first step of the OADH reaction which results in decarboxylation of oxaloacetate (termed OADC).

When cultures were growing on NH_4Cl (Fig. 4a, b) or valine (Fig. 5a, b), the OADC specific activity followed the same pattern as the specific activity of PDC. At the end of culture this OADC activity was 0.13 and 0.09 $\mu\text{mol min}^{-1} \text{mg}^{-1}$ respectively. In the presence of NH_4Cl the specific activity of OADH showed two peaks, during the exponential growth phase (5 $\text{nmol min}^{-1} \text{mg}^{-1}$) and the stationary phase (4 $\text{nmol min}^{-1} \text{mg}^{-1}$). In the presence of valine this activity increased during the exponential phase (5 $\text{nmol min}^{-1} \text{mg}^{-1}$) then decreased during the stationary phase and totally disappeared after 6 d of cultivation.

DISCUSSION

We have followed the specific activities of different enzymes that lead to the formation of malonyl-CoA, in cellular extracts of *S. ambofaciens* grown in media containing NH_4Cl or valine as nitrogen source.

It was shown previously that the activation of short-chain fatty acids in *S. ambofaciens* is carried out by acylkinase (measured as acetate and propionate kinase) coupled with acylphosphotransferase during the antibiotic production phase (Khaoua *et al.*, 1992); the synthesis of

acylkinase and spiramycin production during this phase is reduced in the presence of excess ammonium. Our results showed that the activation of acetate by AK activity could occur in the presence of either of the two nitrogen sources.

Acetyl-CoA carboxylase is considered to be one of the key enzymes in the biosynthesis of fatty acids and macrolide antibiotics. In *S. ambofaciens* growing in the presence of NH_4Cl or valine this enzyme was active during the exponential growth phase, when primary biosynthetic functions such as fatty acid biosynthesis must occur. In the stationary phase this ACC activity was negligible when the bacteria were grown with NH_4Cl ; however, in medium containing valine the activity of ACC was greater. This difference between the activity levels of ACC parallels the greater production of spiramycin with valine compared with NH_4Cl .

In addition to the catabolism of fatty acids and certain amino acids, acetyl-CoA may also be synthesized from pyruvate by PDH, so synthesis of malonyl-CoA from pyruvate occurs by the successive actions of PDH and ACC. In *S. ambofaciens*, malonyl-CoA was formed from pyruvate via PDH during the exponential growth phase in both media used, but during the stationary phase solely in the medium with valine; PDC activity, as a part of PDH, was present in both cases. The observed spiramycin concentrations appeared to correlate with a corresponding PDH activity level.

In the presence of NH_4Cl , when the ACC and PDH activities were quite negligible, production of spiramycin nonetheless occurred. If malonyl-CoA is a direct intermediate in biosynthesis of the antibiotic, we could assume the existence of another pathway for malonyl-CoA synthesis under these conditions. The evidence for OADH activity suggested that transformation of pyruvate into malonyl-CoA could also occur from oxaloacetate via this activity. This hypothesis was supported by the OADC activity observed. Studies of *S. aureofaciens* have also indicated that the formation of malonyl-CoA is not catalysed solely by acetyl-CoA carboxylation (Behal *et al.*, 1977); these authors established a significant role for phosphoenolpyruvate carboxylase and OADH in the formation of malonyl-CoA required for tetracycline biosynthesis.

We argue that valine utilization by *S. ambofaciens* results in higher concentrations of short-chain fatty acids in the medium than in the case of NH_4Cl utilization. Maintenance of the biomass level after growth on valine might be related to assimilation of fatty acids excreted in the medium. The greater spiramycin production in medium with valine may reflect the levels of fatty acid precursors (acetate and isobutyrate, particularly) and acyl-CoA derivatives resulting from catabolism of the amino acid. It has been shown in *S. ambofaciens* that the negative effect exerted by NH_4Cl on the activation systems of short-chain

fatty acids and on spiramycin production was reduced by addition to the growth medium of fatty acids (Khaoua *et al.*, 1992) or catabolic intermediates of valine (Lebrihi *et al.*, 1992).

Based on our present understanding we propose a model of possible pathways of malonyl-CoA formation in *S. ambofaciens* from intermediates of primary metabolism (Fig. 6) with the different nitrogen sources.

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