Organic acid excretion by Streptomyces lividans TK24 during growth on defined carbon and nitrogen sources

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Cultures of Streptomyces lividans TK24 grown in defined media containing certain rapidly used carbon and nitrogen sources excreted high levels of organic acids. These were identified by HPLC and enzymic assays as pyruvic acid and 2-oxoglutaric acid. Acidification occurred only with glucose as the principal carbon source, and depended on the nitrogen source used. With nitrate as the sole nitrogen source, high levels of pyruvate and small amounts of 2-oxoglutarate were produced. Carbon from D-[U-14C]glucose was converted into both organic acids. Combining glucose with a selection of amino acids as primary nitrogen/secondary carbon sources yielded less pyruvate and more 2-oxoglutarate. Carbon from both 14C-labelled glucose and amino acids was metabolized to both organic acids. Adding nitrate to this combination caused a reversion of the acid production pattern to that of the glucose-nitrate combination, as if the amino acids were absent. Addition of ammonium salts to any combination of carbon and nitrogen sources completely prevented organic acid formation.

Keywords: Streptomyces lividans TK24, metabolism, pyruvic acid, 2-oxoglutaric acid, HPLC

INTRODUCTION

The excretion of significant amounts of organic acid metabolites into the extracellular medium has been observed in cultures of many different Streptomyces spp. During a previous study (Wrigley-Jones, 1991; Wrigley-Jones et al., 1993), Streptomyces lividans TK24 was observed to produce copious amounts of organic acid during rapid growth in a complex medium containing glycerol as the primary carbon source. A rise in pH towards the end of fermentations indicated that the organism reassimilated the organic acid, possibly due to exhaustion of the glycerol. Unchecked acid production reduced the culture pH to as low as pH 2.0–3.0, which subsequently had a deleterious effect on the growth of the organism. It was suggested that, in a recombinant organism, the metabolic imbalance indicated by acid production might affect the synthesis of active secreted products. Analysis of the organic acids showed that they were not lactate, citrate, oxaloacetate, malate, succinate or glutamate (unpublished data), but their identity was not determined.

S. lividans 66 and derivatives such as TK24 are popular hosts for gene cloning and expression of recombinant proteins and antibiotics (Hopwood et al., 1983; Gusek & Kinsella, 1992). Production of organic acids in certain types of media may affect the yield of recombinant materials from S. lividans (Payne et al., 1990; DelaCruz et al., 1992), and may need to be prevented. An understanding of the central metabolism of this important organism would give the information needed to manipulate central carbon and nitrogen flux for increased antibiotic or recombinant protein production.

METHODS

Organism. Streptomyces lividans 66 strain TK24 (SLP2- SLP3- str-6) was used for all experiments and was obtained from Professor D. A. Hopwood, John Innes Centre, Norwich, UK (Hopwood et al., 1983).

Spore preparation. Sporulating cultures were grown at 28 °C for 7 d from a spore suspension spread on half-strength tryptone soya broth (TSB) agar [15 g TSB (Oxoid), 10 g potato starch (Sigma), 20 g bacteriological agar (Oxoid), dissolved in 1 l distilled water and autoclaved]. Spores were harvested as suspensions in 20% (w/v) glycerol (Fisons AnalAR) by scraping
the plates with a sterile pipette. Pooled spore suspension was stored as 2 ml aliquots at -20 °C to provide consistent inocula throughout the experiments.

**Shake-flask fermentations.** Liquid cultures were grown in a defined medium based on previous recipes (Hopwood et al., 1985) and called *Streptomyces* minimal medium (SMM). All chemicals were obtained from Sigma, Fisons or BDH and were AnalR grade where possible. The basal medium contained 3 g NaH$_2$PO$_4$, 1.5 g K$_2$HPO$_4$, 0.6 g MgSO$_4$·7H$_2$O, 0.01 g FeSO$_4$·7H$_2$O and in various combinations, 15 mM NH$_4$SO$_4$, 30 mM NaNO$_3$ or 15 mM L-alanine, glycine, L-aspartate, L-glutamate or L-lysine. After sterilization, the medium was supplemented (1%) with 10 ml 10 mM NaNO$_3$ or 15 mM L-alanine, glycine, L-aspartate, L-glutamate or L-lysine. After sterilization, the medium was stored as 2 ml aliquots at 20 °C to provide consistent inocula.

Inoculum was added at 4 ml per flask.

Culture samples were suction-filtered through predried and preweighed AP25 prefilters (Millipore) and washed with an equal volume of distilled water. The mycelium was dried at 20 °C.

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**Dry cell weight (DCW) determination.** Before cultures were filtered, the pH of samples was tested using pH sticks (BDH). Culture samples were suction-filtered through predried and preweighed AP25 prefilters (Millipore) and washed with an equal volume of distilled water. The mycelium was dried at 100 °C to constant weight. The filtrate was used for HPLC analysis. The statistical accuracy of the DCW and HPLC analyses was investigated by processing 15 samples from a shake-flask culture with a cell density of 0.5 ml min$^{-1}$ to assay simple aliphatic amino acids, also at the same times.

**HPLC analysis.** HPLC (Beckman System Gold) was used to identify medium components and measure their concentrations by comparison with authentic standards. Samples of 0.02 ml filtrate were loaded onto an Aminex HPX-87H column (Bio-Rad) and eluted with 5 mM sulphuric acid (BDH AnalR) at a flow rate of 0.6 ml min$^{-1}$ to measure glucose, pyruvate and 2-oxoglutarate at A$_{200}$. Samples of 0.01 ml filtrate were loaded onto an Ultrasphere ODS C18 analytical column (Beckman Instruments) and eluted with a 95:5 (v/v) mixture of HPLC-grade water and methanol containing 0.05% (v/v) HPLC-grade orthophosphoric acid (all BDH HiPerSolv) at a flow rate of 0.5 ml min$^{-1}$ to assay simple aliphatic amino acids, also at A$_{200}$. Fractions containing medium components and organic acids produced by the organism were collected and used for further tests. Statistical analysis of repeat injections of standards showed HPLC measurements to be accurate within <0.2% for the Ultrasphere column or <0.45% with the Aminex HPX-87H column (standard error of the mean expressed as a percentage of the mean).

**Enzymic assays.** Pyruvate was assayed using Sigma kit number 726, which contained 1-lactate dehydrogenase (EC 1.1.1.27). 2-Oxoglutarate was assayed by a method based on Bergmeyer (1974), using a mixture of glutamate dehydrogenase (EC 1.4.1.3; Boehringer Mannheim), NADH, ADP, imidazole (Sigma), ammonium acetate and EDTA (Fisons AnalR). Each of the assay mixtures was tested against standards before it was used with fermentation samples. Fractions of interest were assayed enzymically against blank control samples, standards and samples from fermentations not seen to produce organic acids.

**Radio-labelled materials.** d-[U-$^{14}$C]Glucose [specific activity > 230 mCi mmol$^{-1}$ (8.51 GBq mmol$^{-1}$)], l-[U-$^{14}$C]alanine [specific activity > 150 mCi mmol$^{-1}$ (5.55 GBq mmol$^{-1}$)] and l-[U-$^{14}$C]aspartate [specific activity > 200 mCi mmol$^{-1}$ (7.4 GBq mmol$^{-1}$)] were obtained from Amersham. Labelled glucose was added to the defined medium to give a final activity of 0.33 mCi ml$^{-1}$ (1.221 × 10$^{4}$ Bq); alanine and aspartate were added to give a final activity of 0.25 mCi ml$^{-1}$ (9.25 × 10$^{3}$ Bq). Fractions of separated fermentation samples were added to 2 ml Ecoscint A scintillant solution (National Diagnostics) and the radioactivity in each was measured with a Liquid Scintillation Counter (model 1900CA; Packard Instruments). Counts from repeat injections of identical fractions varied by <6.2%.

**RESULTS AND DISCUSSION**

**Organic acid production in shake-flask fermentations**

HPLC and enzymic assays showed that _S. lividans_ TK24 excreted exclusively pyruvate and 2-oxoglutarate into the extracellular medium of certain cultures. No other organic acids were detected.

No acids were detected in glucose-ammonium cultures. The pH stayed level (6.0-7.0) throughout. In glucose-nitrate medium, pyruvate and 2-oxoglutarate were detected after 24 h growth (Table 1). The pH fell to 5.0 at 78 h, coinciding with the exhaustion of glucose, the end of rapid growth and the end of excretion of both acids. After 78 h, levels of pyruvate and 2-oxoglutarate decreased, indicating reuse by the organism, and the pH rose to 6.5.

Pyruvate and 2-oxoglutarate were produced in all glucose-amino acid media (Table 1). In glucose-nitrate cultures, the levels of the two organic acids increased and peaked simultaneously, and more pyruvate was excreted than 2-oxoglutarate. In contrast, in all glucose-amino acid media, both acids appeared at 24 h but pyruvate increased more rapidly than 2-oxoglutarate (a typical profile is shown in Fig. 1). After the peak in pyruvate levels, this acid was reused rapidly to exhaustion at a point coinciding with the end of rapid growth. This suggested that pyruvate was used as a carbon source to fuel rapid growth. As in glucose-nitrate cultures, maximum 2-oxoglutarate levels coincided with the end of rapid growth and the exhaustion of glucose. Little 2-oxoglutarate was reassimilated, indicating that it was not utilized as a carbon source for biomass synthesis by the organism. The lowest pH values (around 5.0) were recorded at the pyruvate maxima. The pH rose slightly during pyruvate reuse and the remaining 2-oxoglutarate excretion. Relative to glucose-nitrate medium, pyruvate levels were marginally lower while 2-oxoglutarate levels were higher. This is possibly related to the entry of amino acid carbon skeletons into the tricarboxylic acid (TCA) cycle following deamination. Alanine and glycine skeletons enter at pyruvate while aspartate and glutamate skeletons enter at TCA cycle.
Table 1. Maximum biomass and organic acid levels in shake-flask fermentations using defined media with various carbon and nitrogen sources

The time at which the maxima were recorded is shown in parentheses. Data represent the mean of two to three repeats; the variation was less than 29%.

<table>
<thead>
<tr>
<th>Medium combination*</th>
<th>Maximum DCW (g l⁻¹)</th>
<th>Maximum pyruvate (mg l⁻¹)</th>
<th>Maximum 2-oxoglutarate (mg l⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose-ammonium</td>
<td>0.9 (48 h)</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Glucose-nitrate</td>
<td>2.1 (72 h)</td>
<td>464 (78 h)</td>
<td>152 (78 h)</td>
</tr>
<tr>
<td>Glucose-alanine</td>
<td>2.8 (72 h)</td>
<td>328 (54 h)</td>
<td>415 (72 h)</td>
</tr>
<tr>
<td>Glucose-glycine</td>
<td>2.4 (96 h)</td>
<td>294 (72 h)</td>
<td>197 (96 h)</td>
</tr>
<tr>
<td>Glucose-aspartate</td>
<td>3.0 (78 h)</td>
<td>301 (72 h)</td>
<td>932 (78 h)</td>
</tr>
<tr>
<td>Glucose-glutamate</td>
<td>3.3 (120 h)</td>
<td>304 (78 h)</td>
<td>638 (120 h)</td>
</tr>
<tr>
<td>Glucose-lysine</td>
<td>2.0 (120 h)</td>
<td>105 (96 h)</td>
<td>238 (120 h)</td>
</tr>
<tr>
<td>Glucose-alanine-ammonium</td>
<td>1.1 (48 h)</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Glucose-aspartate-ammonium</td>
<td>3.3 (54 h)</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Glucose-alanine-nitrate</td>
<td>1.6 (48 h)</td>
<td>490 (72 h)</td>
<td>110 (72 h)</td>
</tr>
<tr>
<td>Glucose-aspartate-nitrate</td>
<td>2.6 (72 h)</td>
<td>726 (72 h)</td>
<td>152 (72 h)</td>
</tr>
</tbody>
</table>

* Glucose at 30 mM, amino acids and ammonium salts at 15 mM and nitrate salts at 30 mM.

Fig. 1. Fermentation data from an S. lividans TK24 shake-flask culture grown in SMM defined medium containing 30 mM glucose and 15 mM alanine. (a) Changes in biomass; (b) changes in carbon and nitrogen sources (■, glucose; ▲, alanine) and organic acids (●, pyruvate; △, 2-oxoglutarate).

intermediates (Stryer, 1995). Lysine skeletons also enter at the TCA cycle, although low acid levels in glucose-lysine medium (Table 1) may reflect the poor use of lysine by S. lividans; in other media, amino acids were exhausted by 78 h.

The very poor growth of S. lividans in glucose-ammonium medium compared to media with other nitrogen sources (Table 1) supports recent observations made by Zhang et al. (1996). Ammonium was present in excess throughout all cultures to which it was added but amino acids (except lysine) were rapidly utilized. This suggests that ammonium is not necessarily preferred as a nitrogen source by S. lividans.

In media with mixed nitrogen sources, organic acid excretion was again prevented by ammonium salts but supported by nitrate salts. In glucose-amino acid-nitrate cultures, the patterns of growth and acid production were almost identical to those in glucose-nitrate cultures, as if the amino acids were absent (Table 1). Acid levels were greater, reflecting the extra carbon input from the amino acids. The pH fell to 5.0 by 72 h in all cultures, coincident with the end of rapid growth. Decreases in pH to 5.0 were accompanied by simultaneous peaks in acid levels and the exhaustion of glucose and aspartate from glucose-aspartate-nitrate medium, or the end of the rapid utilization of glucose and alanine from glucose-alanine-nitrate cultures. In the latter medium, no further substrate was consumed after 54 h, explaining why the biomass peaks were at different times in these two media. After 72 h, the pH rose as acid reassimilation began. Excreted acids were completely reused from glucose-aspartate-nitrate but not glucose-alanine-nitrate cultures.

No acids were excreted in defined media without glucose but containing only amino acids as carbon sources with or without an inorganic nitrogen source. Moreover, the biomass was particularly poor in most of these cultures (data not shown).

The excretion of pyruvate and 2-oxoglutarate is common amongst Streptomyces spp. grown in complex or defined media containing rapidly utilized carbon sources such as glucose, including Streptomyces venezuelae (Ahmed et al., 1984), Streptomyces alboniger (Surowitz & Pfister, 1985),...
Streptomyces penicilis (Dekleva & Strohl, 1987) and Streptomyces coelicolor (Hobbs et al., 1992). We have shown that cultures of S. lividans TK24 in defined glucose-based media excrete between 6 and 18% of the total consumed carbon as organic acids. Acid excretion depends on the presence of a rapidly utilized sugar carbon source, and is influenced by the nitrogen source. Preliminary investigations indicate that acid excretion is also supported by alternative carbon sources (starch, maltose and glycerol) and complex carbon substrates. Ammonium ions suppress acid over-production completely; nitrate and amino acids support it but the levels of pyruvate and 2-oxoglutarate vary, seemingly in accordance with nitrogen source and presence of a rapidly utilized sugar carbon source, and is carbon as organic acids. Acid excretion depends on the ammonium ions. The relationship of the nitrogen source to the activity of enzymes involved in pyruvate and 2-oxoglutarate formation and degradation deserves investigation.

The relationship between organic acid production and carbon-containing substrates

The use of radiolabelled substrates allowed the tracing of the contribution of carbon from glucose and amino acids to organic acids. Five media were tested: d-[U-14C]glucose-nitrate; d-[U-14C]glucose-alanine; l-[U-14C]alanine-glucose; d-[U-14C]glucose-aspartate; and l-[U-14C]aspartate-glucose.

When labelled glucose was combined with nitrate, more than 10% of the carbon from glucose was converted to the mixture of pyruvate and 2-oxoglutarate. In cultures with labelled glucose and amino acids, carbon from both substrates was converted to both organic acids. With glucose and alanine, proportionally more carbon from alanine (27%) was excreted as acids than from glucose (15%). More carbon passed from glucose to pyruvate (9%) than 2-oxoglutarate (6%), while more carbon passed from alanine to 2-oxoglutarate (16%) than pyruvate (11%) (Fig. 2). The same trend was found in labelled glucose-aspartate cultures (data not shown). However, here more carbon passed from glucose (21%) to acids than from aspartate (5%). This result is of interest since the total organic acid concentrations were higher in aspartate than alanine cultures (Table 1). These results suggest that aspartate carbon was used more efficiently than alanine carbon during biomass accumulation, and that more carbon was channelled from glucose to excreted acids in the presence of aspartate.

Alternatively, both pyruvate and 2-oxoglutarate can arise from alanine as well as glucose. Simple transamination of alanine generates pyruvate, which can then enter the TCA cycle giving rise to 2-oxoglutarate. This explains the relatively large amount of alanine carbon flowing to organic acids. Transamination of aspartate gives rise to oxaloacetate, which is beyond 2-oxoglutarate in the TCA cycle. Oxaloacetate can be decarboxylated to pyruvate and CO₂ by oxaloacetate decarboxylase, but this enzyme is not present in all bacteria (it is found in pseudomonads but not Escherichia coli; Saier, 1987). This may explain why relatively little carbon from aspartate is excreted as pyruvate.

These results show the varying influence of nitrogen sources on pyruvate and 2-oxoglutarate excretion by S. lividans TK24. Carbon passes to excreted acids not only from rapidly utilized carbon sources such as glucose, but also from organic nitrogen sources such as amino acids. Preliminary results indicate that this is also true of complex medium constituents such as malt extract and peptones.

Fig. 2. Percentage conversion of 14C from (a) d-[U-14C]glucose (■) to organic acids (○, pyruvate; △, 2-oxoglutarate) and from (b) l-[U-14C]alanine (■) to organic acids (○, pyruvate; △, 2-oxoglutarate) by S. lividans TK24 cultures in defined media containing 30 mM glucose and 15 mM alanine. Percentages were calculated on the basis that the initial c.p.m. in each culture represented 100% of the added glucose or amino acid. The amount of 14C that had passed into excreted organic acids was calculated in each sample.

*Streptomyces coelicolor*
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REFERENCES


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