M. F. Luna · C. F. Mignone · J. L. Boiardi

The carbon source influences the energetic efficiency of the respiratory chain of N$_2$-fixing Acetobacter diazotrophicus

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Abstract Acetobacter diazotrophicus is a diazotrophic bacterium that colonizes sugarcane tissues. Glucose is oxidized to gluconate in the periplasm prior to uptake and metabolism. A membrane-bound glucose dehydrogenase quinoenzyme [which contains pyrroloquinoline quinone (PQQ) as the prosthetic group] is involved in that oxidation. Gluconate is oxidized further via the hexose monophosphate pathway and tricarboxylic acid cycle. A. diazotrophicus PAL3 was grown in a chemostat with atmospheric nitrogen as the sole N source provided that the dissolved oxygen was maintained at 1.0–2.0% air saturation. The biomass yields of A. diazotrophicus growing with glucose or gluconate with fixed N were very low compared with other heterotrophic bacteria. The biomass yields under N-fixing conditions were more than 30% less than with ammonium as the N source using gluconate as the carbon source but, surprisingly, were only about 14% less with glucose. The following scheme for the metabolism of A. diazotrophicus through the different pathways emerged: (1) the respiratory chain of this organism had a different efficiency of ATP production in the respiratory chain (P:O ratio) under different culture conditions; and (2) N fixation was one (but not the sole) condition under which a higher P:O ratio was observed. The other condition appears to be the expression of an active PQQ-linked glucose dehydrogenase.

Introduction

Acetobacter diazotrophicus is an endophytic diazotrophic bacterium that has been found in large concentration in roots, stems and leaves of sugarcane (Cavalcanete and Döbereiner 1988; Reis et al. 1994). Its presence has also been reported in other sugar-rich plants such as Cameroon grass and sweet potato and more recently in coffee plants (Jimenez-Salgado et al. 1997). This organism is thought to play an important role in providing nitrogen to the infected plants through biological N$_2$ fixation (Sevilla et al. 1998).

A. diazotrophicus grows and fixes N$_2$ at pH values ranging from 2.5 to 7.0 (maximum at pH 5.5) in the presence of a high sugar concentration. The optimal growth is with 10% sucrose but this organism tolerates up to 30% of this sugar (Cavalcanete and Döbereiner 1988; Li and MacRae 1991). Glucose metabolism in this bacterium appears to proceed exclusively via the hexose monophosphate pathway (HMP) since key enzymes of Embden-Meyerhof-Parnas (EMP) and Entner-Doudoroff (ED) pathways could not be detected (Attwood et al. 1991; Alvarez and Martinez-Drets 1995). Further oxidation of the C source proceeds via a complete tricarboxylic acid cycle (Alvarez and Martinez-Drets 1995). Although glucose can be phosphorylated prior to further intracellular oxidation, it was reported that the extracellular oxidation to gluconate plays a major role in the first step of glucose metabolism by A. diazotrophicus (Attwood et al. 1991; Stephan et al. 1991). A pyrroloquinoline quinone (PQQ)-linked glucose dehydrogenase is responsible for the periplasmic conversion of glucose to gluconate (Attwood et al. 1991; Galar and Boiardi 1995).

The growth (biomass) yields of A. diazotrophicus in glucose-limited continuous cultures are low compared to those reported for other bacteria grown aerobically on glucose (Attwood et al. 1991). Low biomass yields of A. pasteurianus are thought to be caused by a low stoichiometry of respiration-coupled proton translocation (therefore a low P:O ratio of the respiratory chain) (Luttik et al. 1997). These authors suggested that, at least in theory, the same explanation applied to the low biomass yields of A. diazotrophicus. Herein, we report that the respiratory chain of this organism can be...
coupled differently (different P:O ratios) depending on the growth conditions, thus affecting the energetic efficiency of growth under N₂-fixing conditions.

Materials and methods

Organism and maintenance

_A. diazotrophicus_ strain PAL3 (LMG 8066 according to the BCCM/LMG culture collection, Gent, Belgium) was kindly provided by Dr. J. Döbereiner (CNPBS/EMBRAPA, Rio de Janeiro, Brazil). This organism was maintained on agar slopes on a potato medium (Stephan et al. 1991). Batch cultures, used as inocula for continuous cultures, were grown as described by Galar and Boiardi (1995).

Mineral medium and continuous cultures

_A. diazotrophicus_ PAL3 was grown in a chemostat using a modified defined minimal medium (LGIM) (Stephan et al. 1991) as follows: glucose (or gluconate), 10.0 g; NaH₂PO₄·H₂O, 1.37 g; KCl, 0.745 g; MgSO₄·7H₂O, 0.30 g; citric acid, 0.2 g; FeCl₃·6H₂O, 10 mg; CaCl₂·2H₂O, 20 mg and NaMoO₄·2H₂O, 2 mg per liter of distilled water. (NH₄)₂SO₄ (2.50 g l⁻¹) was added to this medium for cultures grown with fixed N₂.

Continuous cultures were grown at 30 °C in a 2-1 Bioflo II (New Brunswick Scientific, Edison, N.J.) fermentation unit with a working volume of 1.4 l. The pH was automatically maintained at 5.5 ± 0.1 using either 0.5 N NaOH or 0.5 N H₂SO₄. Foam formation was prevented by automatic addition of an antifoaming agent. Cultures were flushed with air (15-20 l h⁻¹). The dissolved oxygen concentration was continuously measured using an Ingold (Wilmington, Mass.) polarographic probe and maintained at the desired level of air saturation by varying the agitation speed of the impeller. Cultures were considered to be under steady-state conditions when biomass concentration and specific rate of oxygen consumption of the cultures remained almost constant (varied by less than 5%). After modification in growth conditions, between five and ten volume changes were usually required to re-obtain the steady state.

Analyses

Biomass dry weight was determined as described by Herbert et al. (1971). Glucose concentrations in media and culture supernatants were determined with a glucose oxidase enzymatic kit (Wiener, Argentina). Gluconate concentrations were assayed using a Boehringer (Mannheim, Germany) test-kit 428191.

Oxygen and CO₂ concentrations in the emitted gases were determined using a paramagnetic oxygen analyzer (Servomex 1100 A; Norwood, Mass.) and an infrared CO₂ analyzer (Horiba PIR 2000; Japan). Gas flow rates were measured with a bubble flowmeter. Rates of oxygen consumption and CO₂ production were calculated by a mass balance method according to Cooney et al. (1977). C and reductance degree balances were calculated according to Roels (1983). The reductance degree is defined as the number of electrons available for transfer to oxygen on combustion of a compound. For an extensive discussion of the reductance balance see Roels (1983) and de Hoolander (1991).

Results

Continuous cultures

The chemostat cultures of _A. diazotrophicus_ were growth-limited by the availability of the C source. The residual concentration of the C sources employed (glucose or gluconate) in the culture supernatants was below the detection limits of the assays used (see Materials and Methods). In order to check that growth was indeed C-limited, additions were made to the culture vessel of the corresponding C source. When glucose or gluconate was added, an immediate increase in the steady state biomass concentration was observed (data not shown). The dissolved oxygen concentration, after addition of the corresponding C source, was maintained at 1–2% air saturation, as indicated below, in cultures under N₂-fixing conditions.

In N₂-fixing cultures, the oxygen dissolution rate was adjusted to the oxygen consumption by varying the agitation speed of the impeller and maintaining a residual dissolved oxygen concentration of 1–2% air saturation. This procedure avoided inhibition of nitrogenase by oxygen and growth proceeded without oxygen limitation. Cultures washed out when the oxygen concentration was raised to 4–5% air saturation. When ammonium sulfate was used as the N source, the dissolved oxygen concentration was maintained at over 20% air saturation to avoid induction of nitrogenase synthesis.

Under fixed-N conditions, growth yields of _A. diazotrophicus_ PAL3 (Table 1) were not significantly affected by the nature of the C source (glucose and gluconate). On the other hand, under N₂-fixing conditions, biomass yields with gluconate were much lower (34%) than with this C source and ammonium sulfate. This result was predictable, since N₂ fixation is a high energy-demanding process. Surprisingly this was not the case with glucose as the C and energy source. The biomass yields of cultures growing with glucose under conditions of N₂ fixation were only 14% lower than those observed in cultures containing this sugar and ammonium sulfate, in spite of the extra energy expenditure for N₂ fixation (Table 1).

Growth model and calculations

Data from these cultures were analyzed further by developing a model where growth is described by a series of equations accounting for the assimilation of the C source into biomass, catabolism, product formation and reductance balance method according to Cooney et al. (1977). C and reductance degree balances were calculated according to Roels (1983). The reductance degree is defined as the number of electrons available for transfer to oxygen on combustion of a compound. For an extensive discussion of the reductance balance see Roels (1983) and de Hoolander (1991).

Table 1 C and energy balances, and yields (g [dry wt] of cells per mol C source) of *Acetobacter diazotrophicus* PAL3 growing in continuous cultures. Data are the mean of at least five samples from two different continuous cultures in steady state under the same culture conditions

<table>
<thead>
<tr>
<th>Culture condition</th>
<th>Growth yield (g/mol)</th>
<th>C balance</th>
<th>Reductance degree balance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose/NH₃</td>
<td>41.00 ± 2.12</td>
<td>1.05</td>
<td>1.12</td>
</tr>
<tr>
<td>Glucose/N₂</td>
<td>35.19 ± 1.98</td>
<td>0.96</td>
<td>1.05</td>
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<tr>
<td>Gluconate/NH₃</td>
<td>37.33 ± 4.11</td>
<td>1.06</td>
<td>1.12</td>
</tr>
<tr>
<td>Gluconate/N₂</td>
<td>24.63 ± 2.65</td>
<td>0.91</td>
<td>1.02</td>
</tr>
</tbody>
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