Energy Generation by Extracellular Aldose Oxidation in N₂-Fixing
Gluconacetobacter diazotrophicus

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Gluconacetobacter diazotrophicus PAL3 was grown in a chemostat with N₂ and mixtures of xylose and gluconate. Xylose was oxidized to xylonate, which was accumulated in the culture supernatants. Biomass yields and carbon from gluconate incorporated into biomass increased with the rate of xylose oxidation. By using metabolic balances it is demonstrated that extracellular xylose oxidation led N₂-fixing G. diazotrophicus cultures to increase the efficiency of energy generation.

Gluconacetobacter (formerly Acetobacter) diazotrophicus is an endophytic N₂-fixing bacterium found in large concentration in sugarcane tissues (4, 12). Its presence also has been reported in other plants (9). This organism is thought to play an important role in providing assimilable nitrogen to the infected plants through biological N₂ fixation (13), using glucose of plant origin as the carbon source.

G. diazotrophicus grows and fixes N₂ at pH values ranging from 2.5 to 7.0 (maximum at pH 5.5) in the presence of high sugar concentrations (11). Glucose metabolism in this bacterium appears to proceed exclusively via the hexose monophosphate pathway, since key enzymes of Embden-Meyerhof-Parnas and Entner-Doudoroff pathways could not be detected (1). The extracellular oxidation of glucose to gluconate is the first step of glucose metabolism in this organism (2, 14). This oxidation (as well as those with other aldoses) is catalyzed by a membrane-bound pyrroloquinoline quinone (PQQ)-linked glucose dehydrogenase (6).

The growth (biomass) yields of G. diazotrophicus in glucose-limited continuous cultures are low compared to those reported for other heterotrophic bacteria grown aerobically under similar conditions (2, 10). We reported previously that the respiratory chain of this organism can be differently coupled (different P/O ratios) depending on the environmental conditions, thus affecting the energetic efficiency of growth (10). In this study we demonstrate that an improved energetic efficiency of growth and N₂ fixation in G. diazotrophicus is linked to the expression of an active aldose oxidation via the periplasmic glucose dehydrogenase.

G. diazotrophicus strain PAL3 (LMG 8066 [Culture Collection Laboratorium voor Microbiologie, Ghent, Belgium]) from Centro Nacional de Pesquisa de Biologia do Solo/Empresa Brasileira de Pesquisa Agropecuaria (Rio de Janeiro, Brazil) was used. The organism was maintained in a potato medium (14). Batch cultures, used as inocula for continuous cultures, were grown as described (6). Chemostat cultures were grown using a modified defined minimal medium (14) as follows: gluconate, 10.0 g; NaH₂PO₄ · H₂O, 1.37 g; KCl, 0.745 g; MgSO₄ · 7H₂O, 0.30 g; citric acid, 0.2 g; FeCl₂ · 6H₂O, 10 mg; CaCl₂ · 2H₂O, 20 mg; and NaMoO₄ · 2H₂O, 2 mg per liter of distilled water. Culture medium contained a fixed gluconate concentration and various concentrations of xylose.

Cultures were performed at 30°C in a 2-liter Bioflo IIe (New Brunswick Scientific Co., Edison, N.J.) fermentor with a working volume of 1.4 liters. Culture pH was controlled at 5.5 ± 0.1 by automatic addition of 0.5 N NaOH or 0.5 N H₂SO₄. Cultures were aerated at a rate of 15 to 20 liter 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. N₂-fixing cultures were obtained as already reported (10). Steady state was considered to be reached when the biomass concentration and the specific rate of oxygen consumption remained almost constant (varied less than 5%). Approximately 10 volume changes were required to reobtain a steady state after any modification in growth conditions.

Biomass dry weight was determined as described by Herbert et al. (8). Gluconate concentrations in media and culture supernatants were assayed using a Boehringer (Mannheim, Germany) test kit (catalog no. 428191). Xylose levels were determined colorimetrically by the dinitrosalicic acid assay for reducing sugars. Oxygen and carbon dioxide concentrations in the gases emitted by the fermentor were determined using a paramagnetic oxygen analyzer (model 1100A; Servomex, Norwood, Mass.) and an infrared carbon dioxide analyzer (model PIR 2000; Horiba, Kyoto, Japan), respectively. Rates of oxygen consumption, carbon dioxide production, and carbon recovery were calculated by a mass balance method (5).

Previous experiments showed that G. diazotrophicus, although not able to grow in a culture medium containing xylose as the sole carbon source, oxidized this pentose to xylonate by the activity of the periplasmic glucose dehydrogenase. Total carbon analyses of the xylose-containing media and the corresponding supernatants resulted in C₅ recoveries of 100%. Thus, this organism was not able to take up xylose or to further metabolize xylonate, as already reported for Pseudomonas putida (7) (data not shown).

The results of the different chemostat cultures of G. diazotrophicus growing with gluconate as the carbon and energy source, with N₂ as the nitrogen source, and in the presence of
TABLE 1. Growth yields, specific rates of consumption, and CO₂ production and carbon balances of *G. diazotrophicus* PAL3 growing in continuous cultures

<table>
<thead>
<tr>
<th>Xylose in culture medium (g/liter)</th>
<th>Y&lt;sub&gt;GLC&lt;/sub&gt; (g/mol)</th>
<th>Q&lt;sub&gt;GLC&lt;/sub&gt; (mmol/g·h)</th>
<th>Q&lt;sub&gt;XYL&lt;/sub&gt; (mmol/g·h)</th>
<th>Q&lt;sub&gt;CO2&lt;/sub&gt; (mmol/g·h)</th>
<th>Q&lt;sub&gt;O2&lt;/sub&gt; (mmol/g·h)</th>
<th>C balance (% C recovery)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>26.2 ± 2.1 A</td>
<td>1.92 ± 0.08 A</td>
<td>8.39 ± 0.77 A</td>
<td>7.76 ± 0.78 A</td>
<td>96</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>29.2 ± 1.9 B</td>
<td>1.69 ± 0.08 B</td>
<td>8.40 ± 0.78 A</td>
<td>8.00 ± 1.10 A</td>
<td>106</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>32.4 ± 1.1 C</td>
<td>1.48 ± 0.10 C</td>
<td>7.35 ± 0.64 AB</td>
<td>7.60 ± 1.09 A</td>
<td>110</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>34.5 ± 1.0 D</td>
<td>1.47 ± 0.07 C</td>
<td>6.85 ± 0.35 B</td>
<td>7.76 ± 0.50 A</td>
<td>108</td>
<td></td>
</tr>
</tbody>
</table>

* Cultures were performed at a dilution rate (growth rate) of 0.050 ± 0.003 h⁻¹. Data are the means ± standard deviations of at least five samples from different continuous cultures in steady state under the same culture conditions. Means followed by different letters are significantly different, with a *P* of ≤0.05.

* Carbon recoveries were calculated taking into account the consumption of gluconate and the production of biomass and CO₂.

TABLE 2. Metabolic reactions describing the energetic metabolism in *N₂*-fixing *G. diazotrophicus* growing in mixtures of gluconate and xylose

<table>
<thead>
<tr>
<th>Reaction no.</th>
<th>Reaction</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.31 C₆H₁₂O₇ + 0.863 NH₃ + 16.70 ATP → 100 g of biomass&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Biomass synthesis from gluconate&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>C₆H₁₂O₇ → 6 CO₂ + 11 NADH</td>
<td>Oxidation of gluconate through hexose monophosphate pathway and TCA&lt;sup&gt;a&lt;/sup&gt; cycle</td>
</tr>
<tr>
<td>3</td>
<td>C₆H₁₀O₄ → C₆H₁₀O₄ + reduced POQ</td>
<td>Oxidation of xylose to xylonic via the POQ-linked glucose dehydrogenase</td>
</tr>
<tr>
<td>4</td>
<td>NADH or reduced POQ + 1/2 O₂ → H₂O + (P/O) ATP</td>
<td>Respiration</td>
</tr>
<tr>
<td>5</td>
<td>N₂ + 4 NAD + 16 ATP → 2 NH₃ + H₂</td>
<td>N₂ fixation</td>
</tr>
</tbody>
</table>

<sup>a</sup> Formula for biomass: CH₁₇O₆N₀.₄₅ (from reference 10).

<sup>b</sup> TCA, tricarboxylic acid.


Table 3. Calculated and experimental growth yields of G. diazotrophicus PAL3 in continuous cultures

<table>
<thead>
<tr>
<th>Xylose in culture medium (g/liter)</th>
<th>K</th>
<th>Yglc ± SD (g/mol)</th>
<th>YGLC (g/mol) calculated using a P/O of*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>26.2 ± 2.1</td>
<td>26.7 ± 0.19* 30.4 ± 0.23</td>
</tr>
<tr>
<td>5</td>
<td>0.51</td>
<td>29.2 ± 1.9</td>
<td>27.6 ± 0.16* 31.2 ± 0.19*</td>
</tr>
<tr>
<td>10</td>
<td>1.03</td>
<td>32.4 ± 1.1</td>
<td>28.7 ± 0.28 32.1 ± 0.33*</td>
</tr>
<tr>
<td>20</td>
<td>1.86</td>
<td>34.5 ± 1.0</td>
<td>30.1 ± 0.32 34.5 ± 0.38*</td>
</tr>
</tbody>
</table>

* Values are means ± standard deviations. Standard deviations of calculated yield values were derived from the standard deviations of QXYL and QGLC of Table 1 (3). Calculated YGLC values followed by an asterisk are not statistically different (P ≤ 0.05) from the paired YGLC experimental value. P/O, moles of ATP formed per atom grams of O₂ consumed.

2 glc + 11 glc (P/O) + K (glc + 1.31) (P/O) + 4.353 (P/O) = 23.6 ATP

(3)

Giving a value to P/O, equation 3 can be solved to calculate glc. Then, growth yield (grams of biomass per moles of consumed gluconate) can be calculated from the following:

\[ Y_{GLC} = 100/(1.31 + glc) \]

(4)

Equations 3 and 4 clearly indicate that YGLC depends on the ratio of xylose to gluconate consumption (K) and P/O.

Table 3 shows a comparison between experimental and calculated YGLC using experimental data for K (QXYL and QGLC from Table 1) and two different P/O ratios. It is shown that, when no xylose was present in the culture medium, the calculated growth yield was close to that obtained experimentally using a P/O of 0.60. However, when the xylose concentration in the culture medium increased to 10 or 20 g/liter, calculated growth yields fitted better with the experimental ones using a higher P/O ratio (i.e., 0.75). Therefore, the higher growth yields of cultures containing xylose cannot be explained only on the basis of the extra reducing power supplied by aldose oxidation via the PQO-linked glucose dehydrogenase; in addition a higher energetic efficiency of the respiratory chain (increase in P/O) must be considered. These results suggest that, although still inefficient in energy generation compared with other heterotrophic bacteria, G. diazotrophicus seems to be able to develop mechanisms leading to an improved production of energy in the presence of aldoses and under N₂-fixing conditions as found inside the host plants.

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REFERENCES