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Energy Generation by Extracellular Aldose Oxidation in N_2-Fixing *Gluconacetobacter diazotrophicus*

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***Gluconacetobacter diazotrophicus* PAL3 was grown in a chemostat with N_2 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 N_2-fixing *G. diazotrophicus* cultures to increase the efficiency of energy generation.**

Gluconacetobacter (formerly *Acetobacter*) *diazotrophicus* is an endophytic N_2-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 N_2 fixation (13), using glucose of plant origin as the carbon source.

G. 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 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 N_2 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; $NaH_2PO_4 \cdot H_2O$, 1.37 g; KCl, 0.745 g; $MgSO_4 \cdot 7H_2O$, 0.30 g; citric acid, 0.2 g; $FeCl_3 \cdot 6H_2O$, 10 mg; $CaCl_2 \cdot 2H_2O$, 20 mg; and $NaMoO_4 \cdot 2H_2O$, 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 H_2SO_4. 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. N_2-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 dinitrosalicylic 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 C_5 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 N_2 as the nitrogen source, and in the presence of

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TABLE 1. Growth yields, specific rates of consumption, and CO_2 production and carbon balances of *G. diazotrophicus* PAL3 growing in continuous cultures[a]

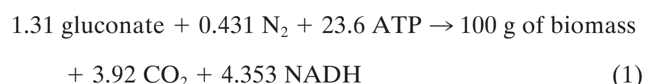
Xylose in culture medium (g/liter)	Y_{GLC} (g/mol)	Q_{GLC} (mmol/g · h)	Q_{XYL} (mmol/g · h)	Q_{CO2} (mmol/g · h)	Q_{O2} (mmol/g · h)	C balance (% C recovery)[b]
0	26.2 ± 2.1 A	1.92 ± 0.08 A		8.39 ± 0.77 A	7.76 ± 0.78 A	96
5	29.2 ± 1.9 B	1.69 ± 0.08 B	0.86 ± 0.14 A	8.40 ± 0.78 A	8.00 ± 1.10 A	106
10	32.4 ± 1.1 C	1.48 ± 0.10 C	1.53 ± 0.20 B	7.35 ± 0.64 AB	7.60 ± 1.09 A	110
20	34.5 ± 1.0 D	1.47 ± 0.07 C	2.74 ± 0.22 C	6.85 ± 0.35 B	7.76 ± 0.50 A	108

[a] 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 two 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.
[b] Carbon recoveries were calculated taking into account the consumption of gluconate and the production of biomass and CO_2.

different concentrations of xylose are shown in Table 1. As xylose concentration in the culture medium increased, growth yields with respect to gluconate (Y_{GLC}) also increased, whereas specific rates of carbon dioxide production (Q_{CO2}) decreased. This increase in Y_{GLC} indicates that more gluconate was incorporated into biomass (i.e., less was completely metabolized to CO_2) as more xylose was oxidized (Q_{XYL} increased) (Table 1). On the other hand, specific rates of oxygen consumption (Q_{O2}) remained almost constant for all conditions, indicating, as expected, that the same amount of energy was required for biomass synthesis. Therefore, the loss of energy production from a diminished gluconate oxidation had to be compensated in some way.

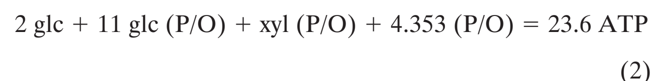
The extracellular aldose oxidation via a PQQ-linked glucose dehydrogenase led certain organisms to an increase in the efficiency of energy generation, which could not be ascribed only to the extra reducing power provided by reduced PQQ (7, 15). To see whether it was also the case for our *G. diazotrophicus* cultures, data from Table 1 were analyzed further by developing a model in which growth is described by a series of reactions accounting for the assimilation of the carbon source into biomass, catabolism, product formation, and respiration. By using the appropriate set of equations it is possible to obtain a relationship between growth and internal metabolic fluxes, which are linked to production and consumption of energy (ATP and reducing power). Based on previous studies of carbon metabolism for *G. diazotrophicus* (1, 2, 7, 10), the growth of this organism fixing N_2 on mixtures of gluconate and xylose could be described by using the set of reactions shown in Table 2.

In the cultures indicated in Table 1 no NH_3 could be detected in the supernatants (data not shown). Besides that, carbon balances for gluconate showed that no major products other than biomass and CO_2 were produced. Therefore, it can be assumed that the rate of N_2 fixation (Table 2, reaction 5) is directly proportional to the rate of biomass synthesis (Table 2, reaction 1). If so, it follows that all the fixed N_2 was incorporated into the biomass. From this reasoning the synthesis of biomass can be expressed as follows:



$$1.31 \text{ gluconate} + 0.431\ N_2 + 23.6 \text{ ATP} \rightarrow 100 \text{ g of biomass} + 3.92\ CO_2 + 4.353 \text{ NADH} \quad (1)$$

Thus, 23.6 mol of ATP is needed for the synthesis of 100 g of biomass. This amount of ATP must be provided through oxidative phosphorylation of NADH and reduced PQQ (Table 2, reaction 4) and substrate-level phosphorylation (Table 2, reaction 2). NADH (11 mol) and 2 mol of ATP are formed per mol of oxidized gluconate (Table 2, reaction 2). In addition biomass synthesis generates 4.353 mol of NADH (equation 1), and the oxidation of xylose to xylonate generates 1 mol of reduced PQQ (Table 2, reaction 3). Let us call glc and xyl the amount (in moles) of gluconate and xylose oxidized when 100 g (dry weight) of *G. diazotrophicus* cells is produced; then, the ATP balance is



$$2 \text{ glc} + 11 \text{ glc } (P/O) + \text{xyl } (P/O) + 4.353\ (P/O) = 23.6 \text{ ATP} \quad (2)$$

To solve this equation another relation is needed. Let us call *K* the ratio of xylose to gluconate consumption ($K = Q_{XYL}/Q_{GLC}$). According to the stoichiometry described in Table 2, the total amount of gluconate consumed when 100 g of biomass is synthesized is 1.31 + glc, and that of xylose is xyl, so that *K* can be expressed as follows: $K = \text{xyl}/(1.31 + \text{glc})$. Therefore, equation 2 can be written as

TABLE 2. Metabolic reactions describing the energetic metabolism in N_2-fixing *G. diazotrophicus* growing in mixtures of gluconate and xylose

Reaction no.	Reaction	Description
1	$1.31\ C_6H_{12}O_7 + 0.863\ NH_3 + 16.70$ ATP → 100 g of biomass[a] + $3.92\ CO_2 + 6.078$ NADH	Biomass synthesis from gluconate[a]
2	$C_6H_{12}O_7 \rightarrow 6\ CO_2 + 11$ NADH + 2 ATP	Oxidation of gluconate through hexose monophosphate pathway and TCA[b] cycle
3	$C_5H_{10}O_5 \rightarrow C_5H_{10}O_6$ + reduced PQQ	Oxidation of xylose to xylonate via the PQQ-linked glucose dehydrogenase
4	NADH or reduced PQQ + 1/2 $O_2 \rightarrow H_2O$ + (P/O) ATP	Respiration
5	N_2 + 4 NADH + 16 ATP → 2 NH_3 + H_2	N_2 fixation

[a] Formula for biomass: $CH_{1.75}O_{0.43}N_{0.22}$ (from reference 10).
[b] TCA, tricarboxylic acid.

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

Xylose in culture medium (g/liter)	K	Mean exptl Y_{GLC} ± SD (g/mol)	Y_{GLC} (g/mol) calculated using a P/O of[a]	
			0.6	0.75
0	0	26.2 ± 2.1	26.7 ± 0.19*	30.4 ± 0.23
5	0.51	29.2 ± 1.9	27.6 ± 0.16*	31.2 ± 0.19*
10	1.03	32.4 ± 1.1	28.7 ± 0.28	32.1 ± 0.33*
20	1.86	34.5 ± 1.0	30.1 ± 0.32	34.5 ± 0.38*

[a] Values are means ± standard deviations. Standard deviations of calculated yield values were derived from the standard deviations of Q_{XYL} and Q_{GLC} of Table 1 (3). Calculated Y_{GLC} values followed by an asterisk are not statistically different ($P \leq 0.05$) from the paired Y_{GLC} experimental value. P/O, moles of ATP formed per atom grams of O_2 consumed.

$$2 \text{ glc} + 11 \text{ glc (P/O)} + \text{K (glc} + 1.31) \text{ (P/O)} + 4.353 \text{ (P/O)} = 23.6 \text{ ATP} \quad (3)$$

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:

$$Y_{GLC} = 100/(1.31 + \text{glc}) \quad (4)$$

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

Table 3 shows a comparison between experimental and calculated Y_{GLC} using experimental data for K (Q_{XYL} and Q_{GLC} 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 PQQ-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 N_2-fixing conditions as found inside the host plants.

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