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ORIGINAL PAPER

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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 (Cavalcante 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 (Cavalcante 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 Martínez-Drets 1995). Further oxidation of the C source proceeds via a complete tricarboxylic acid cycle (Alvarez and Martínez-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

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coupled differently (different P:O ratios) depending on the growth conditions, thus affecting the energetic efficiency of growth under N_2-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; $NaH_2PO_4 \cdot H_2O$, 1.37 g; KCl, 0.745 g; $MgSO_4 \cdot 7H_2O$, 0.30 g; citric acid, 0.2 g; $FeCl_3 \cdot 6H_20$, 10 mg; $CaCl_2 \cdot 2H_2O$, 20 mg and $NaMoO_4 \cdot 2H_2O$, 2 mg per liter of distilled water. $(NH_4)_2SO_4$ (2.50 gl^{-1}) was added to this medium for cultures grown with fixed N.

Continuous cultures were grown at 30 °C in a 2-l Bioflo IIe (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 H_2SO_4. 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 CO_2 concentrations in the emitted gases were determined using a paramagnetic oxygen analyzer (Servomex 1100 A; Norwood, Mass.) and an infrared CO_2 analyzer (Horiba PIR 2000; Japan). Gas flow rates were measured with a bubble flowmeter. Rates of oxygen consumption and CO_2 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 Hollander (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 N_2-fixing conditions).

In N_2-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 N_2-fixing conditions, biomass yields with gluconate were much lower (34%) than with this C source and ammonium sulfate. This result was predictable, since N_2 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 N_2 fixation were only 14% lower than those observed in cultures containing this sugar and ammonium sulfate, in spite of the extra energy expenditure for N_2 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

Table 1 C and energy balances, and yields [g (dry wt) of cells per mol of 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

Culture condition	Growth yield (g/mol)	C balance	Reductance degree balance
Glucose/NH_3	41.00 ± 2.12	1.05	1.12
Glucose/N_2	35.19 ± 1.98	0.96	1.05
Gluconate/NH_3	37.33 ± 4.11	1.06	1.12
Gluconate/N_2	24.63 ± 2.65	0.91	1.02

respiration (Roels 1983; de Hollander 1991). By using the appropriate set of equations it is possible to obtain a relationship between growth and product formation with internal metabolic fluxes, which in turn are linked to production and consumption of energy (ATP and reducing power).

Based on previous studies on C metabolism for *A. diazotrophicus* (Attwood et al. 1991; Alvarez and Martínez-Drets 1995; Galar and Boiardi 1995) a simplified metabolic scheme is proposed for this organism (Fig. 1). According to this scheme, growth of *A. diazotrophicus* on glucose and N_2 can be described by using the set of reactions shown in Table 2. In order to facilitate future calculations, C-containing compounds are expressed on a C-mol basis (Erickson et al. 1978).

In Table 2, V1 to V5 indicate the specific fluxes (on a C-mol basis) of the respective reactions. These fluxes can be estimated from the experimental measurements indicated in Table 3 and other calculated specific rates. A mass balance yielded the relationships among fluxes and specific rates indicated in Table 2. (Notice that specific rates of consumption have negative values, whereas specific rates of production have positive values).

In the metabolic scheme of Fig. 1, described by the reactions of Table 2, it was assumed that in *A. diazotrophicus* glucose is oxidized first to gluconate (by a PQQ-linked glucose dehydrogenase) and further metabolized exclusively through the HMP pathway (Attwood et al. 1991; Alvarez and Martínez-Drets 1995). For this pathway, the C conversion efficiency to biomass (CCE) has been reported to be 50% (Babel and Müller 1985). These authors found that the effect of the elemental biomass composition on CCE value was negligible. Therefore, we can assume that this value of CCE is, in principle, valid for any microorganism using this catabolic pathway. Therefore σ in Eq. 2 (Table 2) has a value of 2. A reductance degree balance (Erickson et al. 1978; Roels 1983) yielded a value for h (Eq. 2, Table 2)

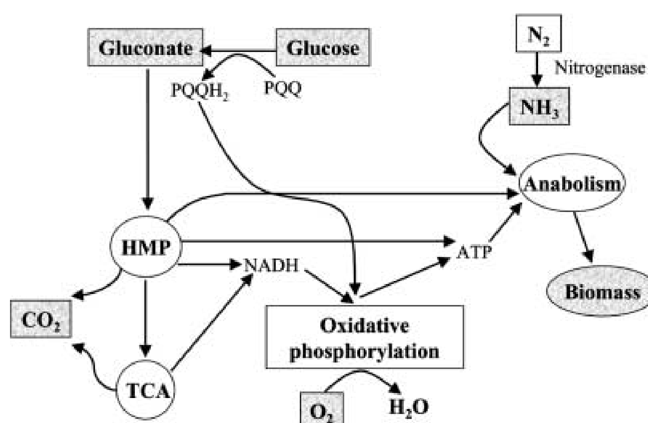

Fig. 1 Simplified metabolic scheme for C metabolism and growth of N_2-fixing *Acetobacter diazotrophicus*; Substances indicated in *shaded boxes* are those that have been measured

of 1.555, which corresponds to the amount (moles) of reducing power produced when 1.0 C-mol of biomass is synthesized from gluconate and ammonia. Finally c, in the same equation, accounts for the moles of ATP needed to synthesize 1.0 C-mol of biomass. Then, $c = 1/Y_{ATP}$.

Electrons from reduced PQQ enter the respiratory chain at the level of cytochrome b (Beardmore-Gray and Anthony 1986). Thus, for a highly coupled respiratory chain, NADH is expected to be more efficient than PQQ in ATP generation. But, for a poorly coupled electron transport chain, as proposed for *A. diazotrophicus* (see below), NADH and PQQ could be considered equivalents in terms of efficiency of energy generation as reported for *Pseudomonas putida* (Hardy et al. 1993). Therefore, in reaction 4 of Table 2, it was assumed that reduced PQQ and NADH yielded the same amount of ATP per pair of electrons transferred to oxygen through the respiratory chain.

Table 2 Reactions describing energetic metabolism in *A. diazotrophicus* (q specific rates of consumption or production of the corresponding substance, μ specific growth rate, D dilution rate, s_1 glucose, s_2 gluconate. The formula for biomass, $CH_{1.75}O_{0.43}N_{0.22}$, with 7% of ash, has been reported for *Klebsiella aerogenes* (Roels 1983). This formula was considered in agreement with the N content of *A. diazotrophicus* biomass, which gave values ranging from 12 to 14.0% w/w. The use, in our calculations, of other biomass formulae already reported for Gram-negative organisms did not significantly modify our results (data not shown)

Metabolic reactions	
1	Oxidation of glucose to gluconate via PQQ-linked glucose dehydrogenase: $CH_2O + PQQ \xrightarrow{V1} CH_2O_{7/6} + 5/30\ PQQH_2$
2	Biomass synthesis from gluconate: $\sigma CH_2O_{7/6} + 0.22NH_3 + cATP \xrightarrow{V2} CH_{1.75}O_{0.43}N_{0.22} + (\sigma - 1)CO_2 + hNADH$
3	Oxidation of gluconate through HMP pathway and TCA cycle: $CH_2O_{7/6} \xrightarrow{V3} CO_2 + 11/6\ NADH + 1/3\ ATP$
4	Respiration: NADH or $PQQH_2 + \frac{1}{2}O_2 \xrightarrow{V4} H_2O + (P/O)ATP$
5	N_2 fixation: $N_2 + 4\ NADH + 16\ ATP \xrightarrow{V5} 2\ NH_3 + H_2$
Mass balances	
6	$qs_1 = -v1$
7	$qs_2 = v1 - \sigma v2 - v3$
8	$\mu = D = v2$
9	$q_{CO_2} = (\sigma - 1)v2 + v3$
10	$q_{O_2} = -\frac{1}{2}v4$
11	$q_{NH_3} = -0.22v2 + 2v5$
12	$q_{N_2} = -v5$
13	$q_{ATP} = -\frac{1}{Y_{ATP}}v2 + \frac{1}{3}v3 + \left(\frac{P}{O}\right)v4 - 16\ v5$

Table 3 Specific rates of C source (q_s), O_2 (q_{O_2}) and N_2 (q_{N_2}) consumption and biomass (μ) and CO_2 (q_{CO_2}) formation of *A. diazotrophicus* PAL3 growing in continuous cultures. Data are the mean of at least five samples from two independent continuous cultures. The standard deviation was never higher than ±2%

Condition	q_s [a]	μ [b]	q_{CO_2} [c]	q_{O_2}	q_{N_2}
Glucose/NH_3	−0.181	0.0484	0.142	−0.153	
Glucose/N_2	−0.200	0.0461	0.145	−0.154	-5.76×10^{-3}
Gluconate/NH_3	−0.193	0.0471	0.158	−0.166	
Gluconate/N_2	−0.316	0.0508	0.237	−0.234	-6.35×10^{-3}

[a] q_s is expressed in C-mol substrate (C-mol biomass)$^{-1} \cdot h^{-1}$
[b] μ is expressed in h^{-1}
[c] q_{CO_2}, q_{O_2} and q_{N_2} are expressed in mol C-mol$^{-1} \cdot h^{-1}$

Under N_2-fixing conditions no ammonia could be detected in the culture supernatants (data not shown). Besides that, C and reductance degree balances showed that no other major products, other than biomass and CO_2, were produced (Table 1). Therefore it can be assumed that all fixed N_2 was incorporated into biomass. Thus in Eq. 11, $qNH_3 = 0$, and from Eqs. 8, 11 and 12 we get:

$$q_{N_2} = -0.11\mu \quad (14)$$

In experiments where glucose was used as C source, no accumulation of gluconate in culture supernatants could be detected, hence we can assume that $qS_2 = 0$. Measured and derived values of specific rates for each of the culture conditions are shown in Table 3.

Under steady state conditions there is not a net intracellular accumulation of ATP; therefore $qATP = 0$ (Eq. 13). By introducing Eqs. 6, 7, 8 and 10 into Eq. 13, the ATP balance can be expressed as:

$$\mu \cdot (1/Y_{ATP} + \sigma/3) - 16q_{N_2} + q_{S_1}/3 + 2q_{O_2}(P/O) = 0 \quad (15)$$

(P:O ratio indicates the ATP synthesized per electron pair transferred to the respiratory chain).

Another expression for ATP balance can be obtained by combining Eqs. 6, 8, 9 and 10 with Eq. 13:

$$\mu \cdot (1/Y_{ATP} + (\sigma - 1)/3) - 16q_{N2} - q_{CO2}/3 + 2q_{O_2}(P/O) = 0 \quad (16)$$

Equations equivalent to 15 and 16 can be obtained when gluconate is the C source.

For calculating Y_{ATP} for cultures containing gluconate and ammonium sulfate ($q_{N_2} = 0$), by using either Eq. 15 or Eq. 16, a P:O ratio of 0.5 was used as indicated by Luttik et al. (1997). These authors have reported such a P:O value for *A. pasteurianus* and proposed that the low biomass yields of *A. diazotrophicus* cultures could also be explained by such a low stoichiometry of respiration-coupled proton translocation. Minor changes in the P:O ratio used for Y_{ATP} estimation by Eqs. 15 and 16 did not significantly modify the relative increase of the calculated P:O ratio for cultures grown on glucose and N_2 (calculations not shown). The calculated Y_{ATP} value was 0.237 (Eq. 15) and 0.232 (Eq. 16) C-mol biomass (mol ATP)$^{-1}$. For further calculations an average value of 0.235 C-mol biomass (mol ATP)$^{-1}$ was used. This value for Y_{ATP} was considered to be the same for all culture conditions employed in this study, since in all cases biomass was proposed to be derived from gluconate and ammonia (Table 2). Table 4 shows the P:O ratios calculated for these cultures, through either Eq. 15 or Eq. 16. The calculated P:O ratio for the N_2-fixing culture, with glucose as the C source, was higher than those calculated for the other culture conditions.

Discussion

A. diazotrophicus was able to grow in chemostat cultures using atmospheric N_2 as the N source, provided that the dissolved oxygen concentration was maintained at 1–2% air saturation. Stephan et al. (1991) reported that this organism could not grow in N-free media under air, unless starter N was used. This appears to be due to the growth conditions used, because in our experiments, once N_2-fixing conditions were established (nitrogenase synthesis), *A. diazotrophicus* could be maintained continuously without starter fixed-N.

The continuous culture experiments were conducted maintaining the dissolved oxygen concentration to 1–2 % air saturation (about 0.07–0.14 mg O_2 l^{-1}). This oxygen concentration allows maximum nitrogenase activity (Reis and Döbereiner 1998). When the oxygen dissolution rate was slightly increased, thereby exceeding the oxygen consumption rate, the dissolved oxygen concentration increased continuously, leading to nitrogenase inhibition and culture wash-out. This response of *A. diazotrophicus* N_2-fixing cultures to oxygen agrees with Reis and Döbereiner (1998). These authors observed that nitrogenase of this organism is protected against inhibition by oxygen by high sucrose concentration (10%), but is much more sensitive to inhibition at 1% of sugar (our culture conditions). Our results suggest the lack of respiratory protection of nitrogenase (Dalton and Postgate 1969). If such a mechanism exists, it would be expected that *A. diazotrophicus* cultures

Table 4 Calculated P:O ratios (mol of ATP per atom g O_2) of *A. diazotrophicus* PAL3. Standard deviations of calculated P:O values were calculated from the SD of the different q values in Table 3 (Box et al. 1989)

Culture Condition	P:O (Eq. 15)	P:O (Eq. 16)	Culture condition	P:O (Eq. 15)	P:O (Eq. 16)
Glucose/NH_3	0.58 ± 0.100	0.56 ± 0.099	Gluconate/NH_3	0.50 ± 0.086	0.49 ± 0.086
Glucose/N_2	0.78 ± 0.065	0.79 ± 0.063	Gluconate/N_2	0.50 ± 0.045	0.52 ± 0.044

would have increased the respiration rate, in order to match the increased oxygen dissolution, at the expense of lowering the biomass yields (Boiardi 1994). Nevertheless further research is necessary to clarify the mechanism/s of protection of nitrogenase against oxygen, if any, in *A. diazotrophicus*.

In cultures grown with ammonium sulfate, either glucose or gluconate-limited, the growth yields for both C sources were comparable, but lower than commonly reported for other heterotrophic bacteria (Heijnen and van Dijken 1992). The lower biomass yield obtained with gluconate (37.3 vs 41.0 g mol^{-1}, Table 1) is expected since this C source is more oxidized than glucose. Low biomass yields, correlated with high maintenance requirements, were observed by Attwood et al. (1991) in continuous cultures of *A. diazotrophicus* with glucose as the C source. In another study Luttik et al. (1997) reported that the low growth efficiency of *A. pasteurianus* under C limitation could be ascribed to a low stoichiometry of respiration-coupled proton translocation and proposed the same explanation for the low biomass yields of *A. diazotrophicus*.

Biomass yields of N_2-fixing cultures, with gluconate as C source, were even much lower than those obtained with ammonium (Table 1). This result was expected due to the extra energy required for N_2 fixation. This was also reflected by a higher oxygen consumption rate (Table 3). However, the same did not happen when glucose was used as the C source; biomass yields were much less affected by N_2 fixation. Moreover, N_2-fixing cultures with glucose had specific rates of oxygen consumption similar to those grown with ammonium (Table 3). As specific oxygen consumption is a measure of the energy required for biomass synthesis (Calhoun et al. 1993), it seems that cultures grown on glucose and N_2 used similar amounts of energy to produce biomass and to fix N_2 than cultures with ammonium to synthesize only biomass. Therefore N_2-fixing cultures with glucose were, in some way, more efficient in C source utilization than ammonium-grown ones, and than N_2-fixing cultures with gluconate. For this, two possibilities may be considered: (1) N_2-fixing cultures with glucose were more efficient in biomass synthesis (i.e. more biomass could be synthesized with a given amount of ATP), therefore increasing Y_{ATP}, or (2) these cultures showed a higher energetic efficiency of the respiratory chain (therefore increasing the P:O ratio). According to the metabolic scheme proposed (Fig. 1, Table 2), biomass is synthesized from gluconate and ammonium, irrespective of the C or N source utilized for growth. Hence, it seems highly unlikely that the Y_{ATP} would have been modified under the different growth conditions used in this study. It is commonly accepted that differences in biomass yields of organisms grown under similar conditions are a reflection of the efficiency of their energy-generating systems (Calhoun et al. 1993). Applying the growth model described above, we were able to calculate the P:O ratio for *A. diazotrophicus* growing under the conditions used in this study (Table 4). It appears that *A. diazotrophicus* growing with glucose and N_2 increased the P:O ratio, probably by rechanneling the electron flow through a more efficient branch of the respiratory chain. The use of alternative branches of the respiratory chain as an adaptation to different environmental conditions is commonly found in bacteria (Stouthamer 1979) and particularly expressed in other diazotrophs to cope with high differences in oxygen concentration (Liu et al. 1995; Hennecke 1998). Low oxygen concentration conditions, leading to N_2-fixing cultures, were not enough for the expression of a more efficient branch of the respiratory chain in *A. diazotrophicus*, since cultures growing with gluconate did not have an increased P:O ratio as was observed with glucose (Table 4). The only difference between glucose and gluconate metabolism is the extracellular conversion of the former into the latter via the PQQ-linked glucose dehydrogenase. The finding that extracellular aldose oxidation via a PQQ-linked glucose dehydrogenase may lead to an increase in the efficiency of energy generation, which could not be ascribed to the extra reducing power provided by reduced PQQ, has been reported in other organisms (van Schie et al. 1987; Hardy et al. 1993). In *A. diazotrophicus*, both the expression of an active glucose dehydrogenase and N_2-fixing conditions were necessary to direct the electron flow through a more efficient branch of the respiratory chain.

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