PYRUVATE METABOLISM AND NITROGEN FIXATION IN *AZOTOBACTER*

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1. Introduction

Anaerobic nitrogen-fixing organisms can use sodium pyruvate as an energy source and electron donor for nitrogenase [1,2]. On the other hand NADH or NADPH have been implicated as electron donors to nitrogenase in aerobic nitrogen fixers [3–6] where it has been assumed that the energy requirement was fulfilled through oxidative phosphorylation. Recently, reports [7,8] indicated that sodium pyruvate supported anaerobic ATP production in crude extracts from *Azotobacter vinelandii*, they also confirmed the original observation by Rose et al. [9] that acetate kinase was present in this organism. In view of the emphasis placed by the authors [7,8] on an anaerobic pyruvate metabolism as the energy source and possible electron donor for nitrogenase in *A. vinelandii* and the failure of other investigators to establish the presence of pyruvate-dependent nitrogen fixation in *Azotobacter* [10], we decided to survey species of the *Azotobacteraceae* for acetate kinase and phosphotransacetylase activity; our findings are reported in this communication.

2. Materials and methods

Azotobacter vinelandii, strain O, (NCIB 8789) *Azotobacter chroococcum* (NCIB 8003), *Azotobacter agilis* (NCIB 8636), *Azomonas macrocytogenes* (NCIB 8700), *Beijerinckia indica* (NCIB 8597), *Derxia gummosa* (NCIB 9064) were all purchased from the National Collection of Industrial Bacteria, Torry Research Station, P.O. Box 31, 135 Abbey Road, Aberdeen, AB9 8DG, Scotland. *A. vinelandii*, strain OP, was a gift from Professor P.W. Wilson and a low gum-producing strain of *A. chroococcum* was developed in this laboratory. The *Azotobacter* strains and *A. macrocytogenes* were grown in air in 20-litre pots containing Burk's nitrogen-free basal salt medium [11] supplemented with 20 g of sucrose/l. Sodium acetate (20 g/l), ethanol (20 ml/l) or sodium pyruvate (10 g/l) were also used instead of sucrose; such cells will be referred to as acetate-, ethanol- or pyruvate-grown, respectively. *D. gummosa* and *B. indica* were grown on mannitol (10 g/l) and sucrose (5 g/l) respectively on a low-calcium modified Burk's medium containing in addition in mg/litre: $CaCl_2$ (0.10), $FeSO_4 \cdot 7H_2O$ (0.82), H_3BO_3 (2.32), $CoSO_4 \cdot 7H_2O$ (0.96), $CuSO_4 \cdot 5H_2O$ (0.08), $MnSO_4 \cdot 4H_2O$ (0.08), $Na_2MoO_4 \cdot 2H_2O$ (0.3), $ZnSO_4 \cdot 7H_2O$ (1.74) and nitriloacetic acid (56) at pH 7.6 before autoclaving. *Klebsiella pneumoniae* was grown under nitrogen on Hino and Wilson's medium [12]. Ammonia-grown cultures of *Azotobacter* contained NH_4Cl (3 g/l). The harvesting, disruption and preparation of crude nitrogen-fixing extracts from *Azotobacter* have been described [4]. Biochemicals, creatine phosphokinase and *Escherichia coli* acetate kinase were purchased from Sigma (London) Chemical Co. Ltd., and all salts from British Drug Houses, Poole, Dorset.

2.1. *Enzyme assays*

2.1.1. Nitrogenase

The usual acetylene reduction assay for this enzyme has already been described [13]. As an alternative energy source, acetyl phosphate (20 μmoles) and acetate kinase (2.3 units: 1 unit phosphorylates 1.0 μmole of acetate to acetyl phosphate/min) were used.

Table 1

	(a)*	(b)*			
	Growth substrate	nmoles Acetyl phosphate hydrolysed/ mg protein/ min	nmoles Acetylene reduced/mg protein/min 1	2	3	4
A. vinelandii 'O'	Sucrose	62	79	38	37	6.15
A. vinelandii 'OP'	Sucrose	43	62	18.2	18	5
A. vinelandii	Pyruvate	55	57	28	26	7
A. chroococcum	Sucrose	22	104	13.9	20	12.9
Low gum *A. chroococcum*	Sucrose	8.3	91	5.5	12	5.5
Low gum *A. chroococcum*	Pyruvate	58	32	15.5	14.6	6.6
Low gum *A. chroococcum*	Ethanol	43	122	60.8	49	2.7
A. macrocytogenes	Sucrose	31	57	12	19	7.5
A. agilis	Sucrose	26	4.7	0.9	0.9	0.1
B. indica	Mannitol	7	19	0.9	0.7	0.5
D.gummosa	Sucrose	0	0	0	0	0
K. pneumoniae	Sucrose	264	97	68	58	4.9
A. chroococcum + *A. vinelandii*			66	24.4	23.9	1.8
A. chroococcum + *D. gummosa*			2.6	0.1		0.1

*(a) Acetate kinase activity in crude extracts from *Azotobacteraceae* and *K. pneumoniae* and (b) a comparison of nitrogenase activity with different energy donating systems: (1) creatine phosphate (20 μmole), ATP (5 μmole), Mg^{2+} (10 μmole), creatine kinase (100 μg); (2) acetyl phosphate (20 μmole), ATP (2 μmole), Mg (5 μmole); (3) (2) + *E. coli* acetate kinase (2.3 units); (4) ATP (2 μmole), Mg^{2+} (5 μmole). Because activity rates with acetyl phosphate were not linear after 3 min, specific activities were determined from the amount of acetylene reduced in 1 min.

2.1.2. Acetate kinase

The presence of this enzyme was detected by its ability to support acetylene reduction by nitrogenase with added acetyl phosphate. The specific activity in extracts was determined by the rate of acetyl phosphate production from acetate and ATP [14].

2.1.3. Phosphotransacetylase

This was determined by the coenzyme A-dependent disappearance of acetyl phosphate [14].

3. Results

The nitrogenase and acetate kinase activities in crude extracts from all the organisms studied are reported in table 1. Nitrogenase levels in *A. vinelandii*, *A. chroococcum*, *A. macrocytogenes* and *K. pneumoniae* were of the same order. Those of *A. agilis*, *B. indica* and *D. gummosa* are considerably lower (no activity in the case of *D. gummosa*): with these organisms care has to be taken that the pH of the cell suspension does not drop below pH 6. Crude extracts of *B. indica* and *D. gummosa* contained inhibitors of nitrogenase; when these extracts were added to crude extracts of *A. chroococcum* or *A. vinelandii* nitrogenase activity was inhibited. Significant levels of acetate kinase were found only in extracts from *K. pneumoniae* and *A. vinelandii* strains 'O' and 'OP' when the organisms were grown on sucrose. These extracts catalysed high rates of acetylene reduction in the presence of added ATP plus acetyl phosphate compared with ATP alone (table 1b, columns 2 and 4). Extracts from sucrose-grown *A. chroococcum*, in particular, contained high nitrogenase activities but insignificant levels of acetate kinase. Acetate kinase was induced in *A. chroococcum* by using either pyruvate or ethanol as the carbon source but levels were considerably lower than in extracts from *K. pneumoniae*. Crude extracts from sucrose-grown nitrogen-fixing *A. chroococcum* did not inhibit acetate kinase or nitrogenase because: (a) added acetate kinase from

E. coli supported acetylene reduction by the extracts in the presence of acetyl phosphate; (b) such extracts did not inhibit acetyl phosphate-dependent acetylene reduction by crude extracts of sucrose-grown, nitrogen-fixing *A. vinelandii* which contained endogenous acetate kinase; (c) when a mixture of *A. chroococcum* and *A. vinelandii* were disrupted in a French pressure cell the acetate kinase from the *A. vinelandii* remained active. Therefore it is reasonable to deduce that the activity of acetate kinase in nitrogen -fixing *A. chroococcum* when grown on sucrose is insignificant or, at least, inadequate to supply the energy for nitrogen fixation.

Apparent acetate kinase activity as judged by the method of Rose et al. [9] was not always paralleled by the ability of the extract to support acetylene reduction in the presence of acetyl phosphate. This phenomenon was particularly noticeable in extracts from ammonium-grown cells: although they had moderate apparent acetate kinase activity they failed to support acetyl phosphate-dependent acetylene reduction when mixed with crude extracts from sucrose-grown, nitrogen-fixing *A. chroococcum*.

3.1. *Nitrogenase activity by whole cells*

The *Azotobacteraceae* are obligate aerobes and neither grow nor fix nitrogen anaerobically. One reason for this failure could be that nitrogenase activity ceases because of lack of energy which is normally supplied by oxidative phosphorylation. It is feasible that anaerobic ATP production would allow some nitrogenase activity in whole cells under anaerobic conditions. When proper precautions to exclude all traces of oxygen were taken (the assays being conducted in flasks with side arms that contained sodium dithionite solution with benzyl viologen indicator) no significant rates of acetylene reduction were observed with whole cells of *Azotobacteraceae* with any of the media employed (table 2). Attempts to adapt cells to anaerobic nitrogen fixation by exposing to nitrogen in the absence of oxygen for several hours were unsuccessful: such cultures reduced acetylene in the presence of oxygen as readily as controls which had not been pre-exposed to anaerobic conditions, but no significant acetylene reduction was observed without oxygen.

Table 2
Acetylene reduction by cultures of *Azotobacteraceae*.

		nmoles Acetylene reduced/mg of bacteria/min	
	Carbon source	Air	Argon
A. vinelandii 'O'	Sucrose	1.7	0.06
A. vinelandii 'OP'	Sucrose	9.25	0.1
A. vinelandii	Sodium pyruvate	52	0.1
A. chroococcum	Sucrose	16.4	0.67
Low gum *A. chroococcum*	Sucrose	25	0.2
Low gum *A. chroococcum*	Sodium pyruvate	69	0.26
Low gum *A. chroococcum*	Ethanol	30	0.2
A. macrocytogenes	Sucrose	32.6	0.18
A. agilis	Sucrose	23.4	0.13
B. indica	Mannitol	27	0.51
D. gummosa	Sucrose	17	0.05

Cultures (10 ml) were shaken in 25 ml flasks under air or argon + C_2H_2 (5 ml). Anaerobic conditions were ensured by a solution of sodium dithionite in the side arm with a trace of benzyl viologen as indicator. 0.5 ml gas samples were taken at 5 min, 10 min, 15 min, 1, 2 and 3 hr.

3.2. *Phosphotransacetylase*

This enzyme was detected in all the extracts tested. However, assays were unsatisfactory because of the relatively high rates of acetyl phosphate disappearance in the absence of added coenzyme A.

4. Discussion

The evidence in this paper confirms the observation that phosphotransacetylase and acetate kinase are present in *A. vinelandii* [7–9] but it eliminates the possibility that pyruvate metabolism, through acetyl phosphate, offers an important source of ATP production for nitrogenase function in the *Azotobacteraceae*. The evidence against this is 2-fold: (a) there was very little, if any, acetate kinase activity in most of the *Azotobacteraceae* tested and even where positive results occurred, activity was considerably lower than that in crude extracts from *K. pneumoniae*, an anaerobic nitrogen fixer; (b) the ability to produce ATP under anaerobic conditions [8] should, theoretically, allow *Azotobacter* whole cells

to reduce acetylene anaerobically; they failed to do this when rigid precautions were taken to exclude oxygen. This suggests that *Azotobacter* produce little or no energy anaerobically, a conclusion supported by the observation that pool ATP in *A. vinelandii* drops by 75% under anaerobic conditions [15] and under such conditions ATP utilisation should be minimal since both growth and nitrogen fixation stop. The most probable explanation for the lack of nitrogenase activity under anaerobic conditions is the lack of ATP normally provided by oxidative phosphorylation. Other explanations are possible: they involve the complex mechanisms for controlling glycolysis and electron flow in *Azotobacter*. For instance, reduced pyridine nucleotides, which build up in the absence of oxygen, inhibit glucose 6-phosphate-, 6 phosphogluconate-, isocitrate- and pyruvate-dehydrogenase [16, 17]. Such inhibition could prevent the production of ATP anaerobically from sodium pyruvate. Alternatively, a high ADP/ATP ratio could inhibit nitrogenase function despite some anaerobic production of ATP. On the other hand, continuing nitrogenase function or the production of poly-β-hydroxybutyrate [17] would minimise the NAD(P)H/NAD(P) ratio. There may be a critical ratio of NAD(P)H/NAD(P) which inhibits electron flow to nitrogenase and which is only achieved under anaerobic conditions since the presence of traces of oxygen allows nitrogenase to function.

Acetate kinase is produced in pyruvate-grown or ethanol-grown *A. chroococcum*. This suggests that pyruvate metabolism can involve this enzyme. The possibility exists that some ATP is produced by pyruvate and acetyl phosphate metabolism in *Azotobacter* under aerobic conditions but the absence of acetate kinase in extracts from sucrose-grown *A. chroococcum* and the low activity of this enzyme in other *Azotobacteraceae* makes it unlikely that this is a general method of producing ATP for nitrogen fixation in these bacteria.

In addition to the apparent difference in acetate kinase content between sucrose-grown *A. vinelandii* and *A. chroococcum*, other differences occur between these species: (1) the former strain grows readily on acetate while the latter in our hands did not, (2) *A. chroococcum* experienced a long lag phase when grown with pyruvate whereas *A. vinelandii* did not. These properties may be useful diagnostic differences between the two species.

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