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Glucose metabolic pathways in the anaerobic rumen fungus *Neocallimastix frontalis* EB188

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Primary pathways for glucose metabolism were established in the anaerobic rumen fungus *Neocallimastix frontalis* EB188. This highly capable cellulolytic organism demonstrated a strict anaerobic integration of metabolic pathways. Glycolysis in *N. frontalis* EB188 was coupled to malate dehydrogenase, 'malic' enzyme and specified hydrogenosome reactions. Pyruvate, as in most life forms, was a pivotal compound. The major fermentation products of *N. frontalis* EB188 were acetate, ethanol and lactate, with the concomitant generation of H_2. On the basis of its unique characteristics and streamlined fermentation pathways, it was concluded that *N. frontalis* EB188 should be an important contributor to programs generating energy and selected chemicals from currently intractable biomass.

INTRODUCTION

Fungi are emerging as very important constituents of the rumen ecosystem. They contribute in unique ways to the functions previously reserved for bacteria and protozoa (Orpin, 1976, 1988; Bauchop, 1979; Akin *et al.*, 1983; Gordon, 1985; Lowe *et al.*, 1987).

For example, rumen fungi have been shown to be cellulolytic, and the organism of present interest, namely *Neocallimastix frontalis* strain EB188, secretes cellulase with a high specific activity (Barichievich & Calza, 1990*a*,*b*). In addition, rumen fungi possess the ability to convert glucose into acetate, formate, ethanol and additional compounds (Phillips & Gordon, 1988; Theodorou *et al.*, 1988). Some rumen fungi also possess the hydrogenosome as a special organelle capable of coupling the metabolism of glucose to cellular energy formation and H_2 production (Yarlett *et al.*, 1986).

In the light of what is already known about the special characteristics of *N. frontalis* EB188 (Barichievich & Calza, 1990*a*,*b*), it is of principal interest to establish the primary metabolic pathways of glucose metabolism in this organism. Such studies provide the foundation needed to integrate properly enzyme induction and metabolic control mechanisms, cellulase synthesis and secretion, and energy and selected chemical production.

In the present study we have examined a number of different primary metabolic pathways for glucose metabolism and have determined a characteristic set of such metabolic transformations for the unique rumen fungus *N. frontalis* strain EB188.

MATERIALS AND METHODS

Chemicals

All reagents and auxiliary enzymes were purchased from Sigma Chemical Co. or United States Biochemical Corp. α-Oxo[1-^{14}C]glutarate (59 mCi/mmol), D-[5-^{3}H]glucose (21.1 Ci/mmol), [1-^{14}C]lactic acid (54 mCi/mmol) and [1-^{14}C]pyruvic acid (30 mCi/mmol) were obtained from Amersham International. D-[3,4-^{14}C]Glucose (10.3 mCi/mmol), [1,3-^{14}C]-glycerol (10.5 mCi/mmol) and L-[U-^{14}C]leucine (355 mCi/mmol) were obtained from New England Nuclear. D-[1-^{14}C]-Glucose (50 mCi/mmol), D-[6-^{14}C]glucose (45 mCi/mmol), D-[U-^{14}C]glucose (290 mCi/mmol) and L-[1-^{14}C]ornithine (47 mCi/mmol) were obtained from Research Products International.

Fungal isolation and growth conditions

The fungus *N. frontalis* strain EB188 was isolated from the rumen fluid and characterized as described previously (Barichievich & Calza, 1990*a*,*b*). This organism was used for all studies described here. The Hungate (1969) technique for anaerobic growth and transfer was used with 200 ml round-bottomed-flask cultures. Liquid medium composition was as described by Lowe *et al.* (1985), except that ruminal fluid was not used and the concentrations of yeast extract and Bactotryptone (Difco) were halved. Glucose concentration was 0.2% (w/v). Antibiotics were added as described by Joblin (1981), except that chloramphenicol (10 μg/ml) was also added. Cultures were started by an inoculation of zoospores (200/ml).

Fungus incubation with radiolabelled substrates

Fungal mats from 4-day-old cultures were separated into 0.5 mg (wet wt.) pieces with watchmaker's forceps. The pieces were placed into a closed-system incubation vial that provided an efficient way to collect $^{3}H_2O$ and $^{14}CO_2$ continuously (O'Fallon & Wright, 1986). Vials were then incubated at 37 °C for 3 h. At the end of the incubation period the 1.5 ml NaOH trap containing $^{14}CO_2$ or $^{3}H_2O$ was inverted and placed into a 20 ml plastic scintillation vial containing 10 ml of Aqueous Counting Solution (Amersham) and counted in a liquid-scintillation spectrophotometer.

Fungal enzyme preparation

Fungal mats were removed from 4-day-old 200 ml cultures with a platinum inoculation loop (2.5 mm). Four mats were pooled and homogenized in 20 ml of 0.25 M-sucrose/5 mM-Hepes, pH 7.4, with 20 passes of a Potter–Elvehjem homogenizer placed in an ice bath. The enzyme preparation was frozen as 1 ml aliquots at −80 °C. Preliminary studies with fresh fungal preparations showed that the enzymes of interest could be stably frozen at this temperature. Freezing allowed direct comparison of all enzymes in the same batch preparation. When cytoplasmic enzymes were assayed, the homogenate was first centrifuged at 12000 *g* in a microcentrifuge for 10 min.

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Enzyme assays

All assays were conducted under conditions where enzyme activity was linear with respect to both time and protein concentration. The incubation temperature was 37 °C.

Acetyl-CoA synthetase (EC 6.2.1.1) was measured by the method of Patel & Walt (1987). Aconitase (EC 4.2.1.3) was measured by a modification of the method of Anfinsen (1955). The formation of isocitrate from citrate was coupled to $NADP^+$:isocitrate dehydrogenase and monitored by the increase in A_{340}. Alcohol dehydrogenase (EC 1.1.1.1) was assayed by a modification of the method of Bergmeyer (1974*a*) by reduction of acetaldehyde to ethanol. ATP citrate-lyase (EC 4.1.3.8) was assayed by the method of Ivanovsky *et al.* (1980), whereas citrate synthase (EC 4.1.3.7) was determined by the method of Srere (1969). Fumarase (EC 4.2.1.2) was measured by coupling the conversion of fumarate into malate dehydrogenase. NADH formation was monitored at 600 nm by its reaction with phenazine ethosulphate (0.1 mM) and dichlorophenol-indophenol (0.5 mM). The reaction mixture also contained 100 mM-potassium phosphate buffer, pH 7.4, NAD^+ (0.5 mM) and malate dehydrogenase (1 unit/ml). The reaction was started by the addition of sodium fumarate (5 mM). Hydrogenase (EC 1.18.99.1) was assayed by the method of Lindmark & Müller (1973). The differences in A_{600} between reactions run in an H_2 atmosphere or in an N_2 atmosphere provided the measure of hydrogenase activity. α-Oxoglutarate synthase (EC 1.2.7.3) was measured as for pyruvate synthase, except that α-oxoglutarate replaced pyruvate. Lactate dehydrogenase (EC 1.1.1.27) was assayed by the method of Bergmeyer & Bernt (1974). Malate dehydrogenase (EC 1.1.1.37) was determined by the method of Smith (1983), whereas 'malic' enzyme (EC 1.1.1.40) was measured spectrophotometrically with $NADP^+$ as electron acceptor by the method of Lindmark & Müller (1974). NADH:ferredoxin oxidoreductase (EC 1.18.1.3) was assayed by using the protocol described by Yarlett *et al.* (1986), as was NADPH:ferredoxin oxidoreductase (EC 1.18.1.2). NADPH:ferredoxin oxidoreductase (EC 1.18.1.2) was measured by replacing NADH in the assay of NADH:ferredoxin oxidoreductase with NADPH. $NADP^+$:isocitrate dehydrogenase (EC 1.1.1.42) was measured by the method of Bernt & Bergmeyer (1974). Phosphoenolpyruvate carboxykinase (EC 4.1.1.32) was monitored in the direction of oxaloacetate synthesis by using the spectrophotometric assay described by Holten & Nordlie (1965), phosphoenolpyruvate carboxylase (EC 4.1.1.31) was measured as described by Nimmo *et al.* (1986).

Phosphoenolpyruvate synthetase (EC 2.7.9.2) was assayed by a combination of the methods of Cooper & Kornberg (1965) and Patel & Walt (1987). The reaction mixture contained Tris/HCl buffer, pH 7.8 (100 mM), $MgCl_2$ (10 mM), ATP (2 mM) and pyruvate (2 mM). After incubation, pyruvate that had not reacted was removed by addition of sufficient NADH and lactate dehydrogenase. When the A_{340} no longer decreased, a mixture of ADP (1.2 mM), KCl (37 mM) and pyruvate kinase (2 units/ml) was added. The decrease in A_{340} after this addition provided a measure of phosphoenolpyruvate synthetase activity. Pyruvate carboxylase (EC 6.4.1.1) was measured by adaptation of reactions described by Asada (1982). An enzyme preparation was incubated with Tris/HCl, pH 7.8 (100 mM), $MnCl_2$ (5 mM), pyruvate (2 mM) and NADH (0.2 mM). After a background level of lactate dehydrogenase activity was established, the pyruvate carboxylase reaction was started by the simultaneous addition of $NaHCO_3$ (10 mM), ATP (5 mM), acetyl-CoA (0.1 mM), biotin (0.1 mM) and malate dehydrogenase (1 unit/ml). Pyruvate carboxylase activity was determined by the change in A_{340} after correcting for lactate dehydrogenase activity. Pyruvate decarboxylase (EC 4.1.1.1) was assayed by the method of Bergmeyer (1974*b*). Pyruvate synthetase (EC 1.2.7.1) was assayed by the method of Lindmark & Müller (1973) in the presence of spinach ferredoxin (33 μg/ml), whereas succinyl-CoA synthetase (EC 6.2.1.5) was measured in the same manner as that of acetyl-CoA synthetase, except that glutathione (10 mM) was added to the reaction mixture and succinate (5 mM) replaced acetate.

RESULTS AND DISCUSSION

Metabolism of radiolabelled substrates

The data obtained on the metabolism of eleven radiolabelled substrates by *N. frontalis* strain EB188 is presented in Table 1. The metabolism of [5-^{3}H]glucose was the highest of any glucose substrates assayed. This result indicated that glycolysis was the primary metabolic pathway for glucose in *N. frontalis* EB188 (Pollard *et al.*, 1981; O'Fallon & Wright, 1987).

The metabolism of [1-^{14}C]pyruvate was also readily demonstrated, indicating that *N. frontalis* EB188 possessed an active pyruvate decarboxylase system. This pyruvate decarboxylase activity was quite specific for pyruvate, as metabolism of the very similar α-oxo acid, α-oxo[1-^{14}C]glutarate, showed only one-tenth the decarboxylation. There was, additionally, significant metabolism of [1-^{14}C]lactate, which indicated that *N. frontalis* EB188 possessed lactate dehydrogenase, which converted [1-^{14}C]lactate into [1-^{14}C]pyruvate and which was subsequently metabolized by the pyruvate decarboxylase. The observed metabolism of [3,4-^{14}C]glucose was consistent with pyruvate decarboxylase activity (Wood *et al.*, 1981).

The metabolism of [1-^{14}C]glucose occurs both in the pentose phosphate pathway and in the tricarboxylic acid cycle, whereas that of [6-^{14}C]glucose occurs primarily in the tricarboxylic acid cycle (Wood *et al.*, 1981; O'Fallon & Wright, 1987). As there was insignificant metabolism of [1-^{14}C]- or [6-^{14}C]-glucose, *N. frontalis* EB188 does not possess an active pentose phosphate pathway or an active tricarboxylic acid cycle.

Table 1. Metabolism of radiolabelled substrates by *N. frontalis* EB188

Fungi were incubated in a metabolic chamber (O'Fallon & Wright, 1986) with medium containing 0.13 mM radiolabelled substrate, 1.7 mM-$CaCl_2$, 4.8 mM-KCl, 1.1 mM-KH_2PO_4, 1.2 mM-$MgSO_4$, 130 mM-NaCl, 0.2% BSA and 25 mM-Hepes, pH 7.5. After 3 h at 37 °C the 1.5 ml NaOH trap was counted for radioactivity in a liquid-scintillation spectrophotometer. Values are means ± S.E.M. for the number of individual incubations given in parentheses.

Substrate	Activity (pmol of substrate transformed/3 h per 10 μg of fungal material)
α-Oxo[1-^{14}C]glutarate	3.6±0.9 (8)
[1-^{14}C]Glucose	0.5±0.2 (10)
[3,4-^{14}C]Glucose	4.0±1.0 (10)
[5-^{3}H]Glucose	42.9±7.1 (10)
[6-^{14}C]Glucose	0.6±0.2 (10)
[U-^{14}C]Glucose	5.0±0.5 (10)
[1,3-^{14}C]Glycerol	0.1±0.1 (10)
[1-^{14}C]Lactate	5.8±1.9 (10)
[U-^{14}C] Leucine	0.4±0.1 (10)
[^{14}C]Pyruvate	31.4±2.3 (8)
[1-^{14}C]Ornithine	0.2±0.1 (10)

Table 2. Effect of regulatory compounds on the metabolism of glucose by *N. frontalis* EB188

Fungi were incubated under standard conditions (Table 1), except that the designated compounds were added. Activities are expressed as pmol of substrate transformed/3 h per 10 μg of fungal material and are the means ± S.E.M. for the number of individual incubations given in parentheses; N.A., not assayed.

Incubation conditions	Radiolabelled substrate...	Activity		
		[5-^{3}H]Glucose	[1-^{14}C]Glucose	[U-^{14}C]Glucose
Control		61.5±5.3 (10)	1.0±0.2 (8)	5.5±0.6 (8)
Iodoacetamide (2 mM)		19.0±3.0 (10)	N.A.	N.A.
Dinitrophenol (150 μM)		58.6±6.5 (10)	1.0±0.2 (8)	4.1±0.3 (8)
Methyl Viologen (2 mM)		63.2±4.7 (10)	0.9±0.2 (8)	4.8±0.2 (8)

The metabolism obtained for [U-^{14}C]glucose was very close to that obtained with [3,4-^{14}C]glucose. This result further supports what has previously been discussed, namely that glycolysis ends in either pyruvate decarboxylation or lactate formation, with no evidence for metabolism by the pentose phosphate pathway or the tricarboxylic acid cycle. The results in Table 1 also show that [1,3-^{14}C]glycerol, [1-^{14}C]leucine, and [1-^{14}C]ornithine were not metabolized by intact *N. frontalis* EB188.

Effect of iodoacetamide, dinitrophenol and Methyl Viologen on glucose metabolism

Iodoacetamide, an inhibitor of the glycolytic pathway, strongly inhibited the metabolism of [5-^{3}H]glucose, the radiolabelled substrate used to measure glycolytic activity (Table 2). Dinitrophenol, an oxidative-phosphorylation uncoupler, had no effect on glucose metabolism, indicating the lack of such coupling in *N. frontalis* EB188. The lack of stimulation of glucose metabolism by the artificial electron acceptor Methyl Viologen, which would be expected to increase glucose flux through pathways under NADPH or NADH control, e.g., the pentose phosphate pathway, provides further evidence that glycolysis, terminating in pyruvate decarboxylation, is the only pathway leading to decarboxylation of glucose carbon atoms in *N. frontalis* EB188.

Assay for enzymes of selected metabolic pathways

To substantiate further the existence of certain pathways suggested by the radiolabelled-substrate studies, and also to evaluate pathways in which no $^{14}CO_2$ or ^3H_2O would be released, a number of selected enzymes of various pathways were directly assayed. These results are presented in Table 3.

Activities for three selected enzymes of the tricarboxylic acid cycle, namely aconitase, citrate synthase and fumarase were not observed. These data support the previous evidence that strain EB188 does not possess such a cycle. Likewise, specific enzymes for the anaerobic reductive carboxylic acid cycle (Buchanan, 1973) were not observed. These enzymes included acetyl-CoA synthase, α-oxoglutarate synthase, ATP citrate-lyase, pyruvate synthase, phosphoenolpyruvate carboxylase, phosphoenolpyruvate synthase and succinyl-CoA synthase.

N. frontalis EB188 was shown to possess pyruvate decarboxylase, alcohol dehydrogenase and lactate dehydrogenase activities, allowing for the production of acetate, ethanol and lactate. It was also shown that *N. frontalis* EB188 possessed very high activities of malate dehydrogenase and NADH:ferredoxin oxidoreductase and considerable activities of hydrogenase and NADP$^+$:isocitrate dehydrogenase. Interestingly, NADPH:ferredoxin oxidoreductase activity was only 0.4% that of NADH:ferredoxin oxidoreductase activity.

Table 3. Activities of selected enzymes in cell-free extracts of *N. frontalis* EB188

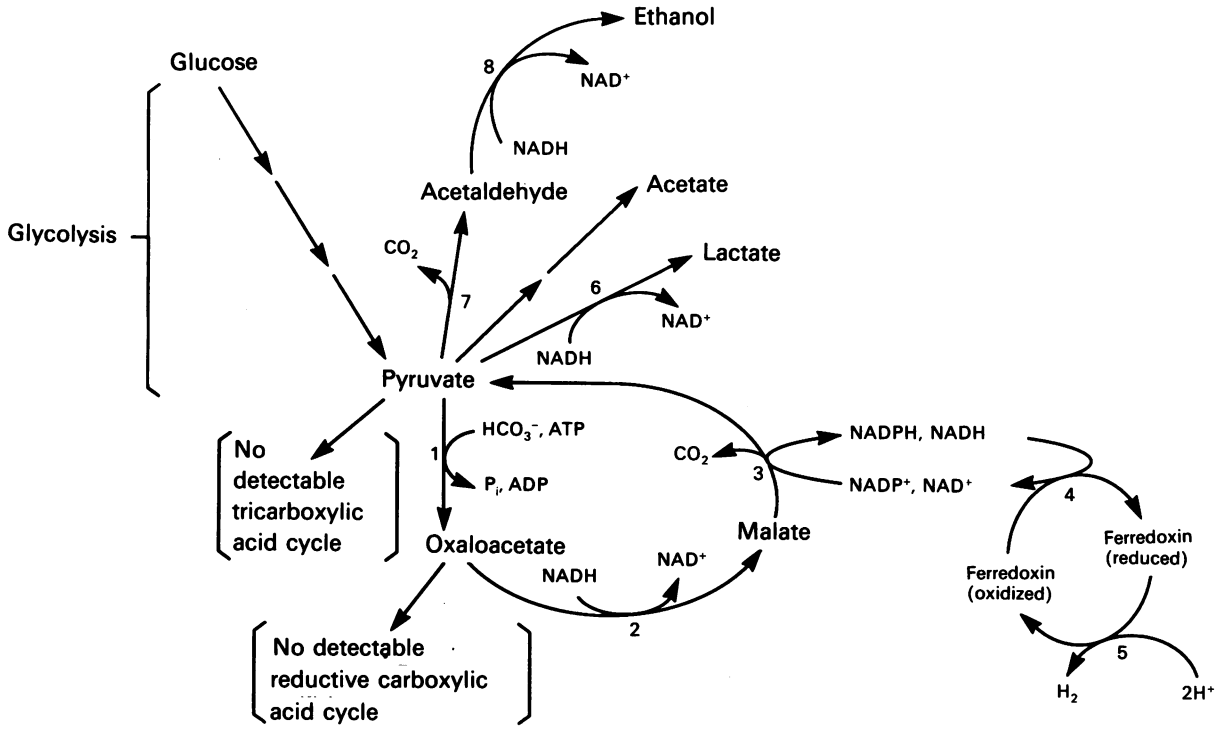
Cell-free extracts of EB188 were assayed for the enzyme activities as described in the Materials and methods section. Values are means ± S.E.M. for the number of individual incubations given in parentheses.

	Activity (nmol/min per mg of protein)
Alcohol dehydrogenase	6±2 (8)
Hydrogenase	100±26 (8)
Lactate dehydrogenase	67±11 (10)
Malate dehydrogenase	13440±2010 (10)
'Malic' enzyme	336±42 (10)
NADH:ferredoxin oxidoreductase	3560±453 (10)
NADPH:ferredoxin oxidoreductase	16±4 (10)
NADP$^+$:isocitrate dehydrogenase	125±21 (10)
Pyruvate carboxylase	14±4 (10)
Pyruvate decarboxylase	18±3 (10)

It can be noted that the carboxy group which pyruvate carboxylase adds to pyruvate to generate oxalacetate is the same carboxy group in malate that is decarboxylated by 'malic' enzyme to generate pyruvate. Under our experimental conditions this carboxy group would not be radiolabelled and therefore no $^{14}CO_2$ will be produced by 'malic' enzyme when the initial radiolabelled substrate was [U-^{14}C]glucose. Thus the 'malic' enzyme reaction would go undetected in the experiments of Tables 1 and 2 (but not in the assay of Table 3). On the other hand, any formate production would have been observed by a difference in $^{14}CO_2$ production from [U-^{14}C]glucose and [3,4-^{14}C]glucose. No real difference was observed, implying that formate is not a significant fermentation product in *N. frontalis* EB188. Interestingly, formate production is also low in *N. patriciarum* (Yarlett *et al.*, 1986), and in various strains of *Neocallimastix* sp. formate production is actually used as an index for growth (Lowe *et al.*, 1987).

Construction of metabolic scheme

Integrating the evidence in Tables 1, 2 and 3, which was arrived at by evaluating the flux of various radiolabelled substrates, and independently by assaying enzymes of designated pathways, leads to the construction of Scheme 1 for the metabolism of glucose by *N. frontalis* EB188. In essence, glycolysis in *N. frontalis* EB188 is coupled to malate dehydrogenase, 'malic' enzyme and specified hydrogenosome reactions. Pyruvate, as in most life forms, is a pivotal compound. The major fermentation



Scheme 1. Primary metabolic conversion of glucose into acetate, ethanol and lactate by *N. frontalis* EB188

Numbers designate the following enzymes: 1, pyruvate carboxylase; 2, malate dehydrogenase; 3, 'malic' enzyme; 4, NADH (NADPH):ferredoxin oxidoreductases; 5, hydrogenase; 6, lactate dehydrogenase; 7, pyruvate decarboxylase; 8, alcohol dehydrogenase.

products of *N. frontalis* EB188 are formulated to be acetate, ethanol and lactate, with the concomitant generation of H_2.

Despite a similarity in basic concept, our metabolic diagram (Scheme 1) differs in several important respects from that in the commendable work of Yarlett *et al.* (1986) in studies with *N. patriciarum*. Namely, the phosphoenolpyruvate carboxykinase (rxn 1) and pyruvate kinase (rxn 10) reactions described by Yarlett *et al.* (1986) are not likely to occur in the directions shown (Metzler, 1977; Wood *et al.*, 1981), the latter in fact constituting a futile cycle with its direction towards glycolysis. In place of phosphoenolpyruvate carboxykinase, which we did not detect, we observed pyruvate carboxylase activity, which would carboxylate pyruvate to form oxaloacetate. A lack of observable pyruvate synthetase activity eliminates this reaction from our scheme, and the very high level of NADH:ferredoxin oxidoreductase supplants the activity of NADPH:ferredoxin oxidoreductase.

In general terms of energy production in *N. frontalis* EB188 the pyruvate produced by glycolysis is converted via pyruvate carboxylase into oxaloacetate, which in turn is reduced by malate dehydrogenase to malate. This malate is transported into the hydrogenosomes (Yarlett *et al.*, 1986), where hydrogenosomal enzymes generate energy by coupling 'malic' enzyme oxidation of malate to electron transfer via NADH:ferredoxin oxidoreductase, ferredoxin and hydrogenase to proton reduction, with the concomitant generation of H_2.

Combining the unique streamlined fermentation pathway described herein with additional properties such as secretion of cellulase with exceptionally high specific activity (Barichievich & Calza, 1990*a*,*b*) should make *N. frontalis* EB188 an important contributor to a viable program for generating energy substrates and selected chemicals from currently intractable biomass.

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