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Kinetic control of ethanol production by *Zymomonas mobilis*

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Summary. *Zymomonas mobilis* UQM 2716 was grown anaerobically in continuous culture (D = 0.1/h; 30° C) under glucose or nitrogen limitation at pH 6.5 or 4.0. The rates of glucose consumption and ethanol production were lowest during glucose-limited growth at pH 6.5, but increased during growth at pH 4.0 or under nitrogen limitation, and were highest during nitrogen-limited growth at pH 4.0. The uncoupling agent CCCP substantially increased the rate of glucose consumption by glucose-limited cultures at pH 6.5, but had much less effect at pH 4.0. Washed cells also metabolised glucose rapidly, irrespective of the conditions under which the original cultures were grown, and the rates were variably increased by low pH and CCCP. Broken cells exhibited substantial ATPase activity, which was increased by growth at low pH. It was concluded that the fermentation rates of cultures growing under glucose or nitrogen limitation at pH 6.5, or under glucose limitation at pH 4.0, are determined by the rate at which energy is dissipated by various cellular activities (including growth, ATP-dependent proton extrusion for maintenance of the protonmotive force and the intracellular pH, and an essentially constitutive ATP-wasting reaction that only operates in the presence of excess glucose). During growth under nitrogen limitation at pH 4.0 the rate of energy dissipation is sufficiently high for the fermentation rate to be determined by the inherent catalytic activity of the catabolic pathway.

Introduction

Zymomonas mobilis is a facultatively anaerobic bacterium that ferments glucose to ethanol and carbon dioxide. The fermentation pathway consists of an energy-independent glucose transport system, the enzymes of the Entner-Doudoroff pathway, and pyruvate decarboxylase plus alcohol dehydrogenase (Dimarco and Romano 1985; Viikari 1988). Overall, one molecule of glucose is converted into two molecules each of ethanol and carbon dioxide, and this is accompanied by the net formation of one molecule of ATP.

Although the well-documented ability of *Z. mobilis* to produce ethanol faster than yeast, with the concomitant formation of less cell material, makes this organism an attractive alternative to yeast for the bioproduction of ethanol (Doelle and Doelle 1989), it nevertheless remains imperative in industrial terms to maximise the rate of ethanol formation. In order to do this it is necessary to identify and quantify those metabolic activities that act as major kinetic control points with respect to the rate of fermentation.

Earlier work with *Z. mobilis* has led to suggestions that the fermentation rate is determined by the activities of certain enzymes of the Entner-Doudoroff pathway (Osman et al. 1987) or the concentration of ethanol (Millar et al. 1982; Osman and Ingram 1985). More recently, however, it has been convincingly demonstrated that the fermentation rate is increased during growth under conditions that give rise to "energetic uncoupling" (Lawford and Stevnsborg 1986; Lawford 1988, Lawford et al. 1988). In this paper we describe the effect of pH, nutrient limitation and an uncoupling agent on fermentation rates and enzyme activities of *Z. mobilis*, with a view to quantifying the extent to which various ATP-utilising reactions control the rate of ethanol production.

Materials and methods

Cultivation of bacteria. Z. mobilis UQM 2716 (ATCC 39676) was grown in continuous culture at 30° C in a 2-l laboratory fermentor (Setric Genie Industriel, Toulouse, France) (culture volume 620

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Abbreviations: CCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; qG, rate of glucose consumption (g glucose/g dry wt cells/h); qE, rate of ethanol production (g ethanol/g dry wt cells/h); Y, growth yield (g dry wt cells/g glucose); D, dilution rate

ml; D = 0.1/h) under carbon, nitrogen or phosphorus limitation using glucose as the carbon source. The growth medium contained (g/l): ammonium sulphate, 2; magnesium sulphate, 2; dihydrogen potassium phosphate, 2; yeast extract, 2; and glucose, 40–120. During carbon-limited growth the input concentration of glucose was 40 g/l (and the output concentration was < 0.2 g/l). During nitrogen-limited growth the ammonium sulphate was omitted and the input glucose concentration was increased to 100 g/l (the output concentration was > 10 g/l), and during phosphorus-limited growth the potassium dihydrogen phosphate was omitted and the input glucose concentration was increased to 120 g/l (the output concentration was > 20 g/l). In some experiments the uncoupling agent CCCP was added to the input growth medium up to a maximum concentration of 5 μM from a stock solution of 12.5 mM in absolute ethanol. All cultures were stirred at 150 rpm with sparging, and the pH was controlled at either 6.5 or 4.0 using 2 M KOH.

Preparation of culture filtrates. Culture samples (approximately 3 ml) were filtered rapidly through a cellulose acetate filter (0.45 μ pore size; Sartorious, Göttingen, Germany).

Measurement of glucose and ethanol. The concentration of glucose in culture filtrates and input media was measured using a YSI Model 27 Glucose Analyser (Yellow Springs Instruments, Yellow Springs, Ohio, USA) precalibrated with a standard glucose solution (2 g/l). Ethanol concentrations were determined using two procedures. The concentration of ethanol in the culture filtrates was measured by gas chromatography (GC) on a Poropak Q (Alltech Associates, Carnforth, Lancs, UK) column at 160° C in the presence of an internal standard (1.0% v/v isopropanol) using a Varian (Sunnyvale, Calif., USA) 3400 gas chromatograph. Alternatively, the concentration of ethanol in unfiltered culture samples (35 ml) was measured using an ebulliometer (Laboratoires du Jardin Salleron, Paris, France); the boiling point of each sample was compared with that of distilled water, and the concentration of ethanol was calculated using a calibration disc. The ethanol concentrations determined by the two methods did not differ by more than ± 5%.

Detection of fermentation products using HPLC. Samples of culture filtrates (50 μl) were analysed by HPLC (Waters, Millipore Corporation, Mitford, Maryland, USA) at 60° C using an Aminex (Biorad, Richmond, Calif., USA) HPX 87H ion exclusion column (flow rate 0.5 ml/min) and 1 mM phosphoric acid as the eluent.

Preparation of washed cells. Cultures were harvested by centrifugation at 10° C for 10 min at approximately 10000*g* using a benchtop centrifuge (Hettich, Tuttlingen, Germany), then resuspended in 25 mM dipotassium hydrogen phosphate/dihydrogen potassium phosphate buffer pH 6.5 or 4.0, recentrifuged and finally resuspended in the same buffer at 5 g dry wt/l.

Preparation of broken cells. Cells harvested as described above were resuspended in 100 mM TRIS/HCl buffer, pH 7.5, then recentrifuged and resuspended in the same buffer at 5 g dry wt/l. The cell suspension was then sonicated at maximum amplitude for four periods of 15 s each, with cooling on ice in between, using a Branson (Branson Ultrasonics Corp., London, UK) Model 250 Sonifier.

Measurement of cell density. Cell density was determined by appropriately diluting cultures or washed cell suspensions in distilled water and measuring optical density (OD) at 540 nm using a Sequoia-Turner (Sequoia-Turner, Mountain View, Calif., USA) spectrophotometer. Cell density (g dry wt/l) was calculated by multiplying OD by 0.30.

Measurement of glucose consumption by washed cells. Glucose consumption was measured at 30° C in a 2.5 ml reaction mix containing 50 mM glucose (9 g/l), 25 mM phosphate buffer, pH 6.5 or 4.0. The reaction was started by the addition of washed cells (5 mg dry wt). Samples (0.5 ml) were removed from the reaction mix after 0, 10, 20 and 30 min, then diluted with 2.0 ml distilled water and rapidly filtered through a cellulose acetate filter (0.45 μ pore size; Sartorious) to remove the cells. The concentration of glucose in the filtrate was determined using a YSI Model 27 Glucose Analyser. Initial experiments showed that the rate of glucose consumption remained constant for at least 30 min, and therefore discontinuous assays of 30 min duration were carried out thereafter.

Measurement of enzyme activities in broken cells. Glucokinase and glucose 6-phosphate dehydrogenase ($NAD^+ \pm$linked) activities were measured using minor modifications of established procedures (Bergmeyer 1974; Beardsmore et al. 1982). ATPase activity was determined in a 1 ml reaction mix containing 70 mM TRIS-HCl buffer, pH 7.5, 2 mM magnesium chloride and 5 mM ATP (pH 7.5). The reaction was started by the addition of 0.5 mg broken cells. Samples (0.1 ml) were taken at 1 min intervals for 4 min and added to 5 ml of a mixture containing 1.05% (w/v) ammonium molybdate, 1.25 M HCl and 0.038% (w/v) Malachite Green. The amount of inorganic phosphate released was measured spectrophotometrically as described previously (Dawson and Jones 1982). Phosphatase activity using 5 mM *p*-nitrophenyl phosphate, glucose 6-phosphate, 6-phosphogluconate or phosphoenolpyruvate as substrate was measured as for ATPase activity. All enzyme activities were measured at 30° C.

Results and discussion

The effect of pH and nutrient limitation on growth yields and fermentation rates

Z. mobilis was grown in continuous culture (30° C; D = 0.1/h) under glucose or nitrogen limitation at pH 6.5 or 4.0 (Table 1). The results showed that the highest growth yield (Y; 0.030 g cells/g glucose), and the lowest rates of glucose consumption (qG; 3.34 g/g per hour) and ethanol production (qE; 1.56 g/g per hour) were obtained during glucose-limited growth at pH 6.5. Significantly lower Y, and concomitantly higher qG and qE, were obtained at pH 4.0 or during nitrogen-limited growth. The lowest Y (0.013 g/g) and the highest qG (7.93 g/g per hour) and qE (3.80 g/g per hour) were obtained during nitrogen-limited growth at pH 4.0.

Table 1. The effect of pH and nutrient limitation on the growth of *Zymomonas mobilis* in continuous culture

Parameters	Nutrient limitation			
	Glucose		Nitrogen	
	pH 6.5	4.0	6.5	4.0
Cell density (g/l)	1.135	0.658	1.204	1.110
Growth yield (g/g)	0.030	0.017	0.016	0.013
qG (g/g/g)	3.34	5.83	6.49	7.93
qE (g/g/h)	1.56	2.82	2.90	3.80
Fermentation efficiency (g/g)	0.46	0.47	0.45	0.48

Z. mobilis was grown in continuous culture (30° C; D = 0.1/h) under either glucose or nitrogen limitation at pH 6.5 or 4.0. The results are the average of at least four independent determinations for each growth condition

Z. mobilis grown under phosphorus limitation (D = 0.1/h) at pH 6.5 also exhibited a low Y (0.016 g/g per hour) and high qG and qE (6.20 and 2.90 g/g per hour respectively). It was therefore concluded that the higher fermentation rates exhibited by nitrogen-limited (and phosphorus-limited) cultures reflected the presence of excess glucose rather than nitrogen (or phosphorus) starvation per se. These results were commensurate with those reported earlier from similar types of experiments carried out under slightly different conditions with *Z. mobilis* ATCC 29191 (Lawford and Stevnsborg 1986; Lawford 1988; Lawford et al. 1988).

The efficiency with which glucose was converted into ethanol was in the range 0.46–0.48 for all of the cultures examined, i.e. slightly lower than the theoretical value of 0.51 ($46 \times 2/180 = 0.51$) for glucose dissimilation due to the concomitant formation of cell material. HPLC and GC showed that only trace amounts of fermentation products other than ethanol (and carbon dioxide) were formed.

It was concluded that the in-situ fermentation rate of *Z. mobilis* increased during growth at low pH or in the presence of excess glucose. Depending on whether the fermentation rate is principally determined by the rate at which the catabolic pathway synthesises ATP or the rate at which the ATP so formed is utilised by the cell, this could reflect either (i) an increase in the activity of whichever enzyme constitutes the major kinetic control point in the catabolic pathway (thus increasing the overall activity of the pathway) or (ii) an increase in the rate of ATP turnover (thus lowering the ATP/ADP ratio and consequently increasing the activity of the catabolic pathway by alleviating any allosteric inhibition by ATP and/or by enhancing the availability of ADP for substrate level phosphorylation.

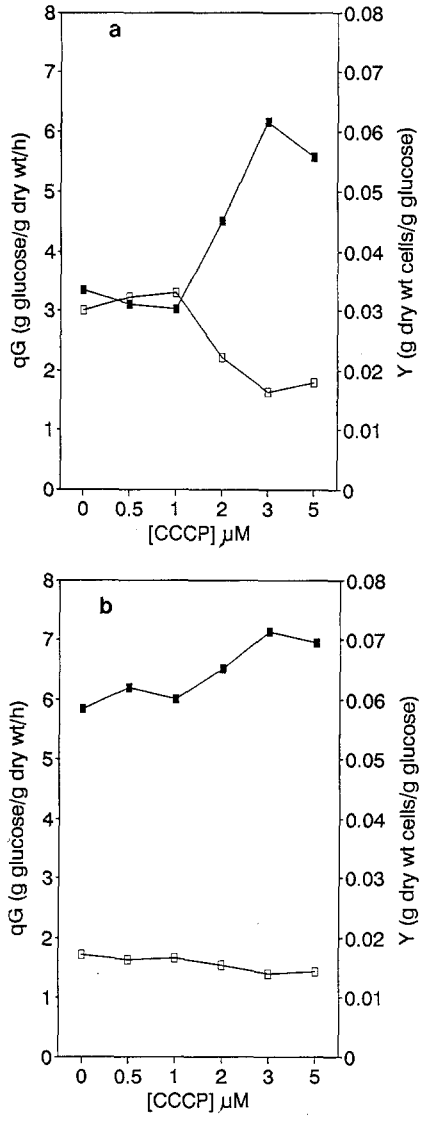

Fig. 1a, b. The effect of the uncoupling agent CCCP on the growth of *Zymomonas mobilis* in glucose-limited continuous culture (30° C; D = 0.10/h). **a** pH 6.5. **b** pH 4.0. Rate of glucose consumption, qG (■); growth yield, Y (□)

The effect of an uncoupling agent on growth yields and fermentation rates

As the likely identity of the major kinetic control point in the catabolic pathway was not known, the hypothesis that the fermentation rate is regulated by the rate of ATP turnover was investigated by growing *Z. mobilis* in glucose-limited continuous culture (D = 0.10/h; pH 6.5) in the presence of the uncoupling agent CCCP (Fig. 1a). The results showed that the qG in the absence of CCCP (3.34 g/g per hour) was not significantly affected by CCCP concentrations up to 1 μM, but increased sharply at higher concentrations to reach a maximum value of 6.15 g/g per hour at 3 μM CCCP; this was mirrored by a decrease in Y to a minimum value of 0.016 g/g. CCCP at 5 μM elicited a small decrease in the qG (and hence a small increase in Y), and caused the glucose concentration in the effluent medium to rise progressively above the normal steady-state value of approximately 0.2 g/l, indicating that the culture was slowly washing out. As uncoupling agents like CCCP act in anaerobes to dissipate the protonmotive force generated by the action of a membrane-bound, proton-translocating ATPase (see Harold 1986), it was concluded that the rate of glucose fermentation by *Z. mobilis* is determined by the rate of dissipation of the protonmotive force (which, in turn, partly determines the rate of ATP turnover within the cell).

The possibility that the quantitatively similar effects of CCCP and low pH on the fermentation rate reflected a similar mode of action, was investigated by growing *Z. mobilis* under glucose limitation at pH 4.0 in the presence of increasing concentrations of CCCP (Fig. 1b). In contrast to growth at pH 6.5, 3 μM CCCP elicited only small changes in the qG (from 5.83 to 7.13 g/g per hour) and in Y (from 0.017 to 0.014 g/g), but again the culture slowly washed out in the presence of 5 μM CCCP. These results showed that CCCP and low pH exerted largely, but not completely, overlapping effects since in combination they increased the qG by only 0.98 g/g per hour over the rate obtained with CCCP alone (7.13 c.f. 6.15 g/g per hour). Furthermore,

the ability of high concentrations of CCCP to decrease the specific growth rate below 0.10/h is mimicked by the reported failure of *Z. mobilis* to grow at D=0.15/h when the pH was decreased below 3.8 (Lawford 1988). It was concluded, therefore, that the effect of low pH and high concentrations of CCCP is to cause a rapid entry of protons into the cell leading initially to a faster rate of ATP turnover, and subsequently to a catastrophic decrease in the magnitude of the protonmotive force and/or the value of the internal pH. It should be noted that in all of these uncoupling experiments CCCP had no effect on the pattern of fermentation, or the efficiency with which glucose was converted into ethanol. The effect of CCCP on the qG of nitrogen-limited cultures could not be determined since these cultures readily sequestered the CCCP from the growth medium.

Overall, these results suggested that three major processes are responsible for energy dissipation (ATP turnover) in *Z. mobilis:* (i) cell growth (this occurs with all cultures and, assuming that the energy demand is the same as for growth under glucose limitation, is equivalent to a glucose consumption rate of 3.34 g/g per hour at a dilution rate of 0.1/h); (ii) increased proton leakage into the cell during growth at low pH and/or in the presence of CCCP, leading to an increased rate of proton ejection via the membrane-bound ATPase in order to maintain the intracellular pH and/or the protonmotive force at values commensurate with cell growth (at an external pH of 4.0 the internal pH is approximately 5.9, whereas at an external pH of 6.5 there is virtually no difference between the internal and external pH) (Barrow et al. 1985; Kalnenieks et al. 1987). This occurs with cultures grown at pH 4.0 in the absence of CCCP, or at pH 6.5 or 4.0 in the presence of 3 μM CCCP, and is responsible for qGs of 5.83–3.34=2.49 g/g per hour due to low pH, 6.15–3.34=2.81 g/g per hour due to CCCP, and 7.13–3.34=3.79 g/g per hour due to a combination of low pH and CCCP; (iii) ATP wastage during growth in the presence of excess glucose, possible resulting from the induction or activation of some form of futile cycle or bypass reaction (this occurs with nitrogen- and phosphate-limited cultures and is equivalent to a qG of 6.49–3.34=3.15 g/g per hour). The combination of growth, low pH and excess glucose would in theory allow a fermentation rate of 8.98 g glucose/g per hour. However, as the qG during nitrogen-limited growth at pH 4.0 was only 7.93 g/g per hour, it was concluded that the latter reflected the maximum activity of the catabolic pathway.

Glucose consumption by washed cells

The addition of glucose to washed cells elicited substantial qGs (Table 2) that were only slightly lower in cells prepared from cultures grown under glucose limitation at pH 6.5 than in cells prepared from cultures grown under nitrogen limitation at the same pH (3.99 and 4.78 g/g per hour respectively). This confirmed the presence of a growth-independent ATP-wasting reaction, and also indicated that the latter is essentially constitutive (but only operates in the presence of excess glucose). Significantly higher qGs were obtained with washed cells prepared from cultures grown under glucose or nitrogen limitation at pH 4.0 (4.68 and 5.30 g/g per hour respectively), thus confirming the stimulatory effect of low pH on the fermentation rate.

Table 2. The effect of uncoupling agent CCCP on the rate of glucose consumption by washed cells of *Z. mobilis*

Uncoupling agent	Nutrient limitation			
	Glucose		Nitrogen	
	pH 6.5	4.0	6.5	4.0
	Rae of glucose consumption (g/g/h)			
−CCCP	3.99	4.78	4.68	5.30
+CCCP	5.95	6.32	5.82	6.63

Z. mobilis was grown in continuous culture (30° C; D=0.1/h) under glucose or nitrogen limitation at pH 6.5 or 4.0. Washed cells were assayed for glucose consumption in the presence or absence of 5, 10 and 20 μM CCCP at the pH of growth, and the highest activity attained was taken to be the maximum rate. The results are the mean of at least four independent experiments

In order to examine the effect of CCCP on the qG by washed cells, it was necessary to determine the concentration of CCCP required to elicit maximum stimulation (Fig. 2). The qG by washed cells prepared from a glucose-limited culture (D=0.1/h; pH 6.5) was maximally stimulated (from 3.99 to 5.95 g/g per hour) by 10 μM CCCP; the optimum concentration of CCCP was therefore approximately three times higher than for growing cultures. This stimulation was reversed by higher concentrations of CCCP, which presumably stimulated the proton-translocating ATPase to such an extent that the intracellular concentration of ATP fell to

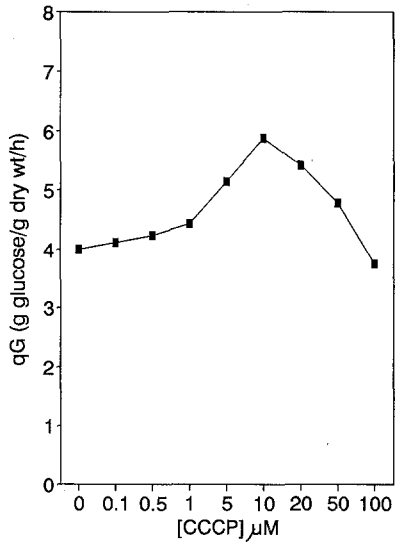

Fig. 2. The effect of CCCP on the rate of glucose consumption at pH 6.5 of washed cells of *Z. mobilis* prepared from a glucose-limited culture (30° C; D=0.10/h; pH 6.5)

a level at which the fermentation rate was limited by the activity of glucokinase. The increase in qG elicited by CCCP was significantly higher than that caused by low pH in washed cells prepared from either carbon-limited cultures (1.96 c.f. 0.79 g/g per hour) or nitrogen-limited cultures (1.14 c.f. 0.62 g/g per hour).

Although the results with washed cells generally supported the earlier conclusions drawn from experiments with continuous cultures, the activity of the putative futile cycle or bypass reaction was higher than predicted, whereas the effects of low pH and CCCP on qG were lower than predicted. These discrepancies may simply have reflected the inevitably different conditions under which the two types of experiments were carried out. Indeed, measurements of ethanol production by washed cells using gas chromatography indicated that the fermentation efficiency was only 0.28–0.30 compared with 0.46–0.48 in continuous cultures. HPLC failed to reveal the presence of other fermentation products, but the possibility that the organism converts some glucose into an unidentified product, the formation of which requires ATP (see for example Linton and Rye 1989) cannot be ruled out and would explain the discrepancy between the measured and predicted rate of ATP wastage.

Enzyme activities of cells grown under various conditions in continuous culture

In order to investigate the biochemical mechanisms via which fermentation rates were increased by exposure to low pH, CCCP or excess glucose, the activities of selected enzymes were assayed in broken cells of *Z. mobilis* (Table 3). The activity of the energy-independent glucose transport system was not measured. However, the activity of the second enzyme, glucokinase, was approximately four times higher in cells grown under glucose limitation than under nitrogen limitation (excess glucose). This indicated that glucokinase was strongly derepressed under these conditions (presumably to compensate for the low concentration of glucose in the growth medium and hence, in the absence of accumulation, within the cell). In contrast, the nature of the growth-limiting nutrient had no significant effect on glucose 6-phosphate dehydrogenase activity (NAD^+-linked). Neither enzyme activity was affected by the pH of growth.

Table 3. Enzyme activities in broken cells of *Z. mobilis*

Enzymes	Nutrient limitation			
	Glucose		Nitrogen	
	pH 6.5	4.0	6.5	4.0
	Enzyme activities (nmol/min/mg)			
Glucokinase	664	738	153	186
Glucose 6-phosphate dehydrogenase	2510	2074	2316	2156
ATPase	108	177	107	167
p-Nitrophenyl phosphatase	72	63	63	64

ATPase activity was not significantly affected by the nature of the growth-limiting nutrient, but was increased by at least 50% during growth at pH 4.0 compared with pH 6.5. This suggested that the enzyme was significantly derepressed during growth at low pH in order to compensate for the increased leakage of protons into the cell under these conditions. The maximum ATPase activity following growth at low pH was equivalent to a glucose consumption rate of 1.84 g/g per hour, i.e. very close to the value predicted from the washed cell experiments, although again substantially lower than predicted from work with continuous cultures.

Several enzymes involved in the fermentation of glucose by *Z. mobilis* catalyse reactions in which either ATP acts as phosphoryl group donor (the conversion of glucose to glucose 6-phosphate by glucokinase) or ADP acts as a phosphoryl group acceptor (the conversion of 1,3-*bis*phosphoglycerate to 3-phosphoglycerate by phosphoglycerate kinase, and of phosphoenolpyruvate to pyruvate by pyruvate kinase). ATP wastage could therefore be effected by the ability of an appropriate phosphatase to act in combination with another enzyme to form an ATP-hydrolysing futile cycle (e.g. glucokinase plus glucose 6-phosphatase; 6-phosphogluconate phosphatase plus gluconate kinase) or to bypass a site of substrate level phosphorylation (e.g. 1,3-*bis*phosphoglycerate phosphatase or phosphoenolpyruvate phosphatase). These possibilities were tested by assaying broken cells for phosphatase activity with glucose 6-phosphate, phosphoenolpyruvate and 6-phosphoglycerate as substrates (no 1,3-*bis*phosphoglycerate was available). No significant phosphatase activity was detected in either the presence or absence of added glucose (50 mM). All of the broken cells exhibited low levels of phosphatase activity when *p*-nitrophenyl phosphate was used as the substrate, but again the activities were not increased by the addition of glucose. These results therefore failed to support the hypothesis that ATP wastage in the presence of excess glucose is catalysed by a phosphatase-mediated futile cycle or bypass reaction. At this stage, however, we cannot rule out the possibility that the putative phosphatase hydrolyses, 1,3-*bis*phosphoglycerate and/or is activated by an intermediate of the glucose fermentation pathway rather than by glucose itself.

The physiological basis of ATP wastage during the growth of *Z. mobilis* at low dilution rate in the presence of excess glucose (i.e. when growth is limited by the availability of anabolic substrates such as nitrogen or phosphorus) is probably similar in principle to that which also occurs in many aerobic bacteria (Cornish et al. 1988; Linton 1990). This probably reflects the inability of the organism to regulate the rate of glucose uptake into the cell such that it does not exceed the rate at which glucose needs to be metabolised in order merely to support growth at the imposed submaximal growth rate (i.e. mainly to drive the synthesis of cell material). The potentially deleterious consequences of taking up

glucose too rapidly can therefore be overcome by increasing the fermentation rate and thus disposing of the excess glucose as ethanol and carbon dioxide. The additional ATP so formed must also be turned over; hence the need for an ATP-wasting futile cycle or bypass reaction of the general type that has been predicted for, but as yet only occasionally demonstrated in, other organisms (see Tempest and Neijssel 1984).

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