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Effects of growth rate and oxygen tension on glucose dehydrogenase activity in *Acinetobacter calcoaceticus* LMD 79.41

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Abstract. The regulation of the synthesis of the quinoprotein glucose dehydrogenase (EC 1.1.99.17) has been studied in *Acinetobacter calcoaceticus* LMD 79.41, an organism able to oxidize glucose to gluconic acid, but unable to grow on both compounds. Glucose dehydrogenase was synthesized constitutively in both batch and carbon-limited chemostat cultures on a variety of substrates. In acetate-limited chemostat cultures glucose dehydrogenase levels and the glucose-oxidizing capacity of whole cells were dependent on the growth rate. They strongly increased at low growth rates at which the maintenance requirement of the cells had a pronounced effect on biomass yield.

Cultures grown on a mixture of acetate and glucose in carbon and energy-limited chemostat cultures oxidized glucose quantitatively to gluconic acid. However, during oxygen-limited growth on this mixture glucose was not oxidized and only very low levels of glucose dehydrogenase were detected in cell-free extracts. After introduction of excess oxygen, however, cultures or washed cell suspensions almost instantaneously gained the capacity to oxidize glucose at a high rate, by an as yet unknown mechanism.

Introduction

The biochemistry of glucose dehydrogenase, (GDH; EC 1.1.99.17) from *Acinetobacter calcoaceticus* has been studied extensively by Hauge in the early sixties. The enzyme was found to contain an unknown cofactor (Hauge 1960), later identified as PQQ (Duine et al. 1986). A detailed characterization of this enzyme from *A. calcoaceticus* showed that glucose dehydrogenase is a membrane-bound periplasmic enzyme, containing two molecules of PQQ per enzyme molecule (Dokter et al. 1986).

Regulation of the synthesis of the quinoprotein glucose dehydrogenase has mainly been studied in *Pseudomonas* spp. The physiological role of the enzyme is difficult to deduce from such studies due to the presence of alternative

routes of glucose catabolism (Lessie & Phibbs 1984). Glucose dehydrogenase is synthesized constitutively in *Pseudomonas cepacia* (Berka et al. 1984) but is inducible in *Pseudomonas aeruginosa* (Hylemon & Phibbs 1972). GDH activity in *P. aeruginosa* grown on glucose, decreased at low dissolved oxygen tensions (Mitchell & Dawes 1982) and was absent during anaerobic growth on glucose (Hunt & Phibbs 1983). This, however, is not due to the absence of GDH protein, but results from apparent inability of *P. aeruginosa* to synthesize PQQ anaerobically (Van Schie et al. 1984). Whether such a modulation of GDH activity via PQQ synthesis is a general phenomenon in *Pseudomonas* spp. is not known at present. GDH activity in *P. fluorescens* was shown to be affected by several growth conditions. Synthesis of the enzyme is favoured by growth at low temperatures (Lynch et al. 1975a,b), low pH (Quay et al. 1972) or high water activity (Prior & Kenyon 1980).

Although *A. calcoaceticus* LMD 79.41 is unable to grow on glucose or gluconate, glucose is oxidized quantitatively at a high rate to gluconolactone which hydrolyses spontaneously to gluconic acid (Van Schie et al. 1984). Preliminary results indicated that GDH is synthesized constitutively in this organism (De Bont et al. 1984), but that GDH synthesis is affected by growth rate (Visser et al. 1985). In view of the important role of this enzyme in the utilization of glucose as an auxiliary energy source by *A. calcoaceticus* (Van Schie et al. 1987b; Müller & Babel 1986) it was decided to investigate regulation of GDH activity in more detail.

Material and methods

Media and growth conditions

Chemostat cultivation of *Acinetobacter calcoaceticus* LMD 79.41 was performed in Applicon laboratory fermenters with a working volume of 1 l at pH 7.0 and 30° C. Dissolved oxygen was measured with a galvanic oxygen electrode, and controlled at the desired value by the stirring rate. The mineral salts medium was prepared according to Van Schie et al. (1984). It contained one of the following substrates as a carbon and energy source: sodium acetate, 30 mM; sodium p-hydroxybenzoate, 15 mM; alanine 15 mM; sodium adipate 10 mM; sodium succinate 15 mM; ethanol 30 mM. Oxygen-limited cultures were obtained by adjusting the stirring rate to such an extent that approximately 10% of the input concentration of the carbon source was detected in the culture fluid. Batch cultivation was performed in Erlenmeyer flasks of 100 ml, containing 25 ml of mineral medium, on rotatory shakers at 150 rpm and 30° C.

Preparation of cell-free extracts

Cells were washed with a 100 mM phosphate buffer, pH 7.0 containing 10 mM

$MgSO_4$ and resuspended in 4 ml of the same buffer. Cells were disrupted by sonication at 4° C with an MSE 150 W sonicator for 3 min with intermittant periods of cooling. Whole cells and debris were removed by centrifugation for 20 min at 13.000 × g. The clear supernatants were used as cell-free extracts.

Enzyme assays
Glucose dehydrogenase assays were carried out at 30° C with freshly-prepared extracts using a model 100-60 Hitachi spectrophotometer. In all assays, the reaction was linearly proportional to the amount of extract present. The assay mixture used contained: potassium phosphate buffer, pH 7.0, 100 mM; $MgSO_4$, 10 mM; KCN, 1 mM; DCPIP (2,6- dichlorophenolindophenol), 60 μM and PES (phenazine ethosulphate) 0.3 mM. The reaction was started by addition of glucose to a final concentration of 20 mM. A molar extinction coefficient for DCPIP 18 mM^{-1} was used to calculate enzyme activity (Armstrong 1964).

Measurement of glucose-oxidizing capacity of whole cells
Glucose oxidation by whole cells was assayed by following the rate of oxygen consumption at 30° C with a Clark type oxygen electrode. In the case of oxygen-limited cultures, cells were washed twice at 4° C with 100 mM potassium phosphate buffer pH 7.0 containing 10 mM $MgSO_4$ by centrifugation to remove residual substrate. The reaction was started by addition of 20 mM glucose and oxygen-consumption rates were calculated on the basis of an oxygen concentration in air saturated buffer of 225 μM.

Analytical assays
Glucose was measured with the GOD/PAP method, gluconate with gluconate kinase/6-P-gluconate dehydrogenase, acetate with acetyl-coA-synthetase, citrate synthase and malate dehydrogenase (testcombinations Boehringer). Protein was measured by the Bradford method (Bio-Rad Laboratories) and by a modified Lowry method (Pierce Chemical Company) with bovine serum albumine as a standard, according to the instructions of the manufacturers. A Beckman Model 915B Tocamaster Total Organic Carbon Analyser was used to determine the carbon content of whole culture and culture supernatants, the carbon content of bacteria being obtained from the difference. Bacterial dry weight was calculated assuming a cellular carbon content of 50%.

Chemicals
DCPIP and PES were obtained from Sigma Chemical Co., PQQ (2.7.9-tricarboxy-1H-pyrrolo (2.3.f) quinoline-4.5-dione) was a gift of Dr J.A. Duine from our department (PQQ is now commercially available from Fluka AG, Buchs, Switzerland).

Results

Like the majority of Acinetobacter species *A. calcoaceticus* LMD 79.41 is able to produce acid from several aldose sugars but is incapable of growth on these sugars or on the corresponding aldonic acids (Baumann et al. 1968; Juni 1978). Cells grown in batch culture on acetate, alanine, citrate, glutamate, lactate, malate, or pyruvate and harvested in the stationary growth phase oxidized glucose with activities varying between 35–80 nmol oxygen consumed. $min^{-1} \cdot$ mg dry wt^{-1} (Table 1). The product of glucose oxidation was identified as gluconic acid. During carbon-limited chemostat cultivation on several substrates at a relatively low growth rate of 0.15 h^{-1}, cells possessed a much higher glucose oxidation capacity. Activities varied between 390–775 nmol oxygen consumed $\cdot$ $min^{-1} \cdot$ mg dry wt^{-1} (Table 1). Also these cells quantitatively oxidized glucose to gluconic acid. It can thus be concluded that GDH is synthesized constitutively by LMD 79.41. In order to further investigate the cause of the 10-fold difference in glucose-oxidizing capacity between batch and chemostat cultures, the effect of growth rate on GDH synthesis was investigated.

LMD 79.41 grown acetate- limited in chemostat cultures exhibited a growth rate-dependent cell yield (Fig. 1). Below a dilution rate of 0.2 h^{-1} a marked decrease in cell yield was observed. At a dilution rate of 0.025 h^{-1} the cell yield amounted less than 25% of the value obtained at high dilution rates due to a high maintenance requirement. From a double reciprocal plot of cell yield

Table 1. Rate of glucose oxidation by cell suspensions of *A. calcoaceticus* grown on various substrates in batch culture or in carbon- and energy-limited chemostat culture at a dilution rate of 0.15 h^{-1}. The rate of glucose oxidation is expressed as nmol oxygen consumed min^{-1} mg dry wt^{-1}. Cell suspensions of batch cultures were obtained from the stationary phase of growth.

Growth substrate	Chemostat culture	Batch culture
Alanine	480	80
Acetate	390	53
Ethanol	560	ND*
Succinate	410	ND
p-Hydroxybenzoate	775	ND
Adipate	440	ND
Malate	460	33
Citrate	ND	67
Glutamate	ND	51
Lactate	ND	70
Pyruvate	ND	46

*ND = not done

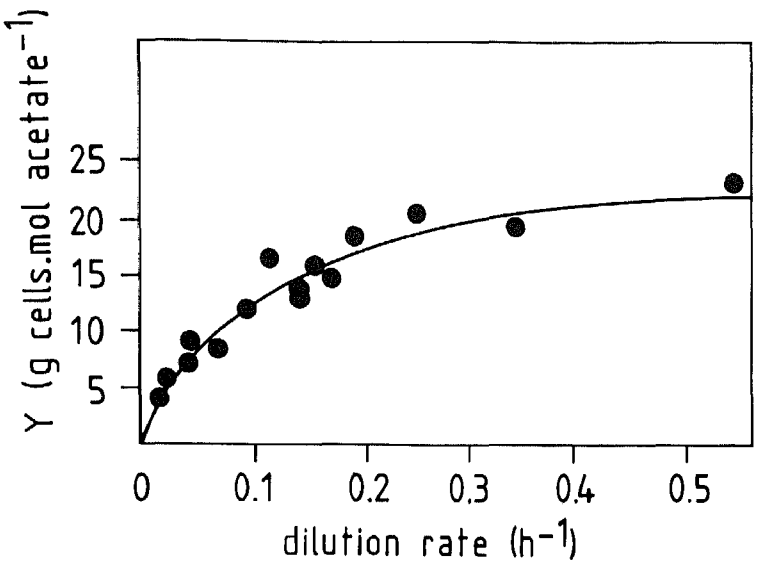

Fig. 1. Biomass concentrations in acetate-limited chemostat cultures of *A. calcoaceticus* as a function of dilution rate. The reservoir concentration of acetate was 30 mM.

versus growth rate (Fig. 2), a maintenance coefficient of 3.2 mmol acetate(g dry wt)$^{-1}$ h^{-1} and a maximum molar growth yield of 26.1 g dry wt (mol acetate)$^{-1}$ was calculated. A similar pronounced effect of growth rate on the cell yield of acetate-grown *Acinetobacter* has been reported by du Preez et al. (1984).

The levels of GDH in acetate-limited chemostat cultures increased with decreasing dilution rate (Fig. 3). Addition of PQQ to the assay mixture had no effect on the enzyme activity indicating the absence of apo-GDH. At a dilution rate of 0.05 h^{-1} GDH activity was approximately 20 times higher than at a dilution rate of 1.0 h^{-1}. The glucose oxidizing capacity of whole cells followed the same pattern as the GDH activities in cell-free extracts (Fig. 3).

During carbon-limited growth on a mixture of acetate and glucose, the sugar was quantitatively converted to gluconic acid (Table 2; Van Schie et al. in preparation). However, when *A. calcoaceticus* was grown on a mixture of acetate and glucose under oxygen-limitation, only traces of gluconic acid were produced. Similar results were obtained during growth on a mixture of succinate and glucose (Table 2) or malate and glucose (results not shown). Increasing the reservoir glucose concentration from 20 to 100 mM had no effect: under oxygen limitation the in situ rate of glucose oxidation of chemostat cultures growing on acetate/glucose mixtures was insignificant. Since a low glucose oxidizing capacity is not necessarily due to the absence of GDH protein, but may result from inhibition of PQQ synthesis (Van Schie et al.

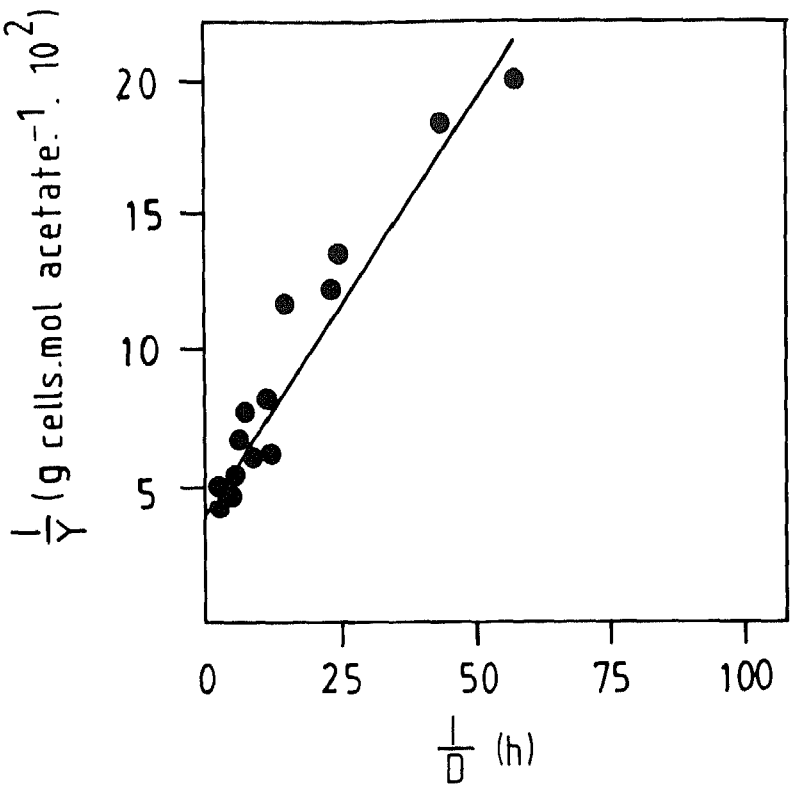

Fig. 2. Reciprocal plot of cell yield versus growth rate of acetate-limited chemostat cultures of *A. calcoaceticus*. Data were obtained from Fig. 1.

1984), cultures were also grown oxygen-limited on acetate/glucose mixtures in the presence of 100 mM PQQ. The introduction of this cofactor in the medium feed, however, did not result in significant glucose oxidation.

In accordance with the behaviour of steady state cultures, cell-free extracts

Table 2. Glucose and gluconate concentrations in chemostat cultures of *A. calcoaceticus* growing at a dilution rate of 0.15 h^{-1} under carbon or oxygen limitation on a mixture of glucose (20 mM) and acetate (30 mM) or succinate (15 mM).

Substrate	Limitation	Glucose (mM)	Gluconate (mM)
Acetate	Carbon	0	19.0
Acetate	Oxygen	18.9	0.9
Succinate	Carbon	0	19.5
Succinate	Oxygen	19.1	0.6

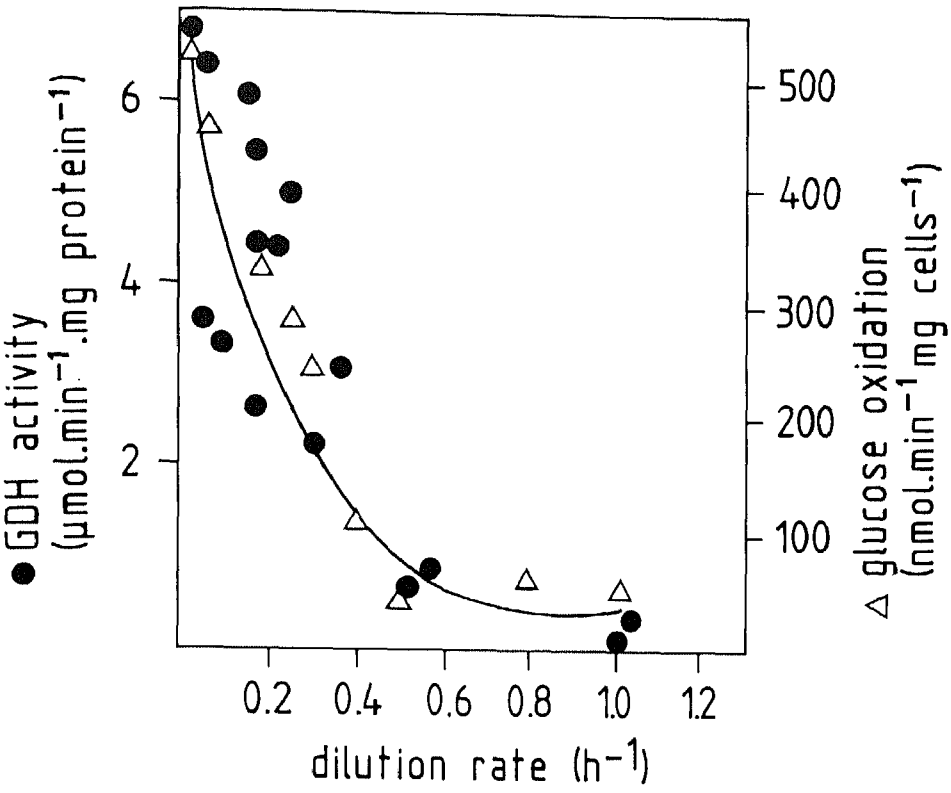

Fig. 3. Effect of dilution rate on GDH activity in cell-free extracts [●] and glucose oxidation capacity of whole cells [△] during growth of *A. calcoaceticus* in acetate-limited continuous cultures.

of oxygen-limited cells exhibited very low GDH activities (Table 3). Addition of PQQ in the assay mixture hardly affected GDH activity: enzyme activities remained less than 10% of that of cells grown acetate-limited at the same dilution rate (Table 3). Unexpectedly, however, a rapid formation of gluconic acid was observed after switching oxygen-limited cultures to conditions of oxygen excess (Fig. 4). The *in situ* rate of glucose-oxidation rapidly increased from 6 to 370 nmol $(min)^{-1}$ mg dry wt^{-1}. Similar results were obtained for oxygen-limited cultures growing on malate or succinate in the presence of glucose. Cell-suspensions from oxygen-limited chemostat cultures which had been washed with cold buffer behaved similarly: after less than 4 min exposure to oxygen during preincubation in the oxygen uptake chamber they showed an instantaneous glucose oxidation, with rates comparable to those of cells grown carbon-limited at the same dilution rate (Table 3). Thus although in oxygen-sufficient cultures the in vitro GDH activity correlated well with the in vivo

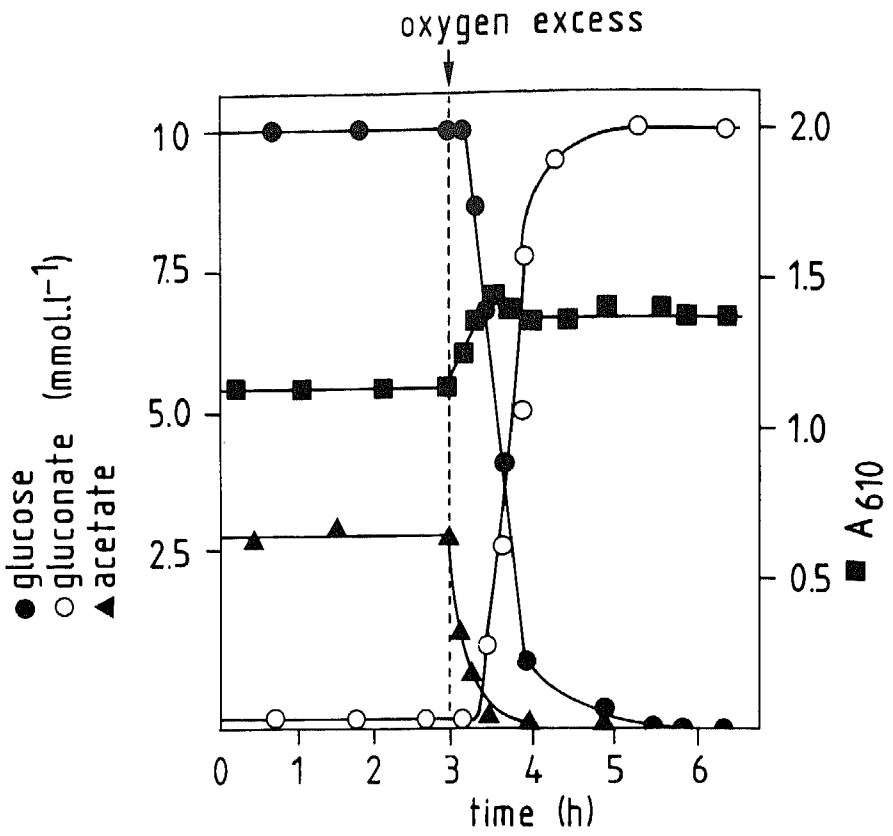

Fig. 4. Transient state behaviour of *A. calcoaceticus* after a shift to oxygen excess of an oxygen-limited chemostat culture growing on a mixture of acetate and glucose at D = 0.15 h^{-1}; ■, optical density; ●, glucose concentration: ○, gluconate concentration and ▲, acetate concentration in the culture.

Table 3. Glucose oxidation capacity of washed cell suspensions and glucose dehydrogenase activities in extracts cells of *A. calcoaceticus* grown in a chemostat culture at a dilution rate of 0.15 h^{-1} under oxygen or carbon limitation on acetate in the presence or absence of glucose. Glucose oxidation capacity of whole cells is expressed as nmol oxygen consumed min^{-1} mg dry wt^{-1} and activities of cell free extracts are expressed as μmol DCPIP reduced min^{-1} mg $protein^{-1}$.

Carbon source	Growth limitation			
	Oxygen		Carbon	
	Whole cells/cell extracts		Whole cells/cell extracts	
Acetate	340	0.16	390	2.7
Acetate + glucose	320	0.14	395	3.0

glucose-oxidizing capacity (Fig. 3), this did not hold for oxygen-limited cultures. In cells grown under oxygen limitation, the glucose oxidizing capacity (as assayed under oxygen excess) was much higher then anticipated on the basis of the in vitro GDH activities (Table 3).

Discussion

The ability of *Acinetobacter calcoaceticus* LMD 79.41 to oxidize glucose was independent of the growth substrate during both batch and carbon-limited chemostat cultivation. However, both the glucose oxidation capacity of whole cells and GDH levels in extracts of cells of acetate-limited chemostat cultures increased with decreasing growth rate (Fig. 3). A similar relation between growth rate and enzyme activity was recently found for the quinoprotein methanol dehydrogenase in *Methylophilus methylotrophus* (Greenwood & Jones 1986). According to the terminology of Harder & Dijkhuizen (1983) these enzymes can thus be classified as "repressible constitutive enzymes". Derepression of the synthesis of enzymes at low growth rates, especially those which take part in the initial degradation of a catabolic substrate, is a common phenomenon (Matin 1981; Harder & Dijkhuizen 1983). Apparently, the intracellular pool size of the repressor is in equilibrium with the extracellular concentration of the growth-limiting substrate, thus causing an inverse relationship between enzyme levels and growth rate.

It has been suggested that the physiological rationale behind increasing levels of an enzyme, that initiates a catabolic pathway, with decreasing growth rate must be sought in kinetics. By increasing the amount of enzyme an organism would compensate for the decrease in concentration of its substrate in the environment (Harder & Dijkhuizen 1983). This explanation does not apply to the regulation of GDH synthesis in *A. calcoaceticus* since the growth rate-dependent enzyme synthesis (Fig. 3) occurred in the absence of glucose.

The regulation of GDH synthesis in *P. aeruginosa* has also been interpreted as a response to extracellular substrate concentration, though in a different context. In *P. aeruginosa* an alternative pathway of glucose metabolism (with a higher substrate affinity) is present. In this organism induction of GDH by glucose (and accidentically by glycerol and gluconate) is regarded as an adaptation to nutrient excess (Whiting et al. 1976; Dawes 1981). This explanation also does not hold for *A. calcoaceticus* since in this organism only one glucose-metabolizing system is present (i.e. the low affinity GDH). Moreover, in *A. calcoaceticus* GDH synthesis is not induced by only a few substrates but derepressed on a large number of substrates (Table 1). Rather, the fact that *A. calcoaceticus* cannot grow on glucose or gluconate suggests that the rationale behind the regulation of GDH in this organism must be sought in its possible

function as an auxiliary energy-generating system (De Bont et al. 1984). Indeed, it has recently become clear that GDH-mediated glucose oxidation generates a membrane potential which is sufficiently high to drive solute transport and ATP synthesis (Van Schie et al. 1985, 1987b). From in vivo studies it was evident that glucose oxidation contributes significantly to the energy budget of the cell (Gommers et al. 1986). Therefore, to our opinion, regulation of GDH in *A. calcoaceticus* is directed by the energy-status of the cell: a high ratio of catabolism over anabolism, due to maintenance energy requirement, triggers derepression of GDH whereas at growth rates near μ_{max} (low catabolism/high anabolism) GDH is repressed. Also in *Klebsiella aerogenes*, cultivation conditions which require a high catabolic activity lead to enhanced GDH levels (Hommes et al. 1985).

When *A. calcoaceticus* was grown oxygen-limited on a mixture of acetate and glucose, the glucose oxidation system was not operative as reflected by the steady state glucose and gluconate concentrations in the culture (Table 2). This is in accordance with the low GDH activity in cell-free extracts under these growth conditions (Table 3). Growth in the presence of PQQ did not alter gluconate formation and addition of PQQ to sonic extracts did not enhance the low GDH activity. Also in *Pseudomonas aeruginosa* GDH activity was very low both during oxygen-limited growth (Mitchell & Dawes 1982) and during anaerobic growth with nitrate as the electron acceptor (Hunt & Phibbs 1983). Evidence has been presented, however, that during anaerobic growth this organism synthesizes a GDH apo-enzyme which can be reactivated with PQQ (Van Schie et al. 1984, 1987a). From the results presented herein it is clear that *A. calcoaceticus* LMD 79.41 did not synthesize apoenzyme during oxygen-limited growth, since PQQ did neither stimulate glucose oxidation in sonic extracts nor in whole cells. These results suggest that, similar to the quinoproteins glycerol dehydrogenase (Ameyama et al. 1985) and methanol dehydrogenase (Duine et al. 1980) oxygen-limited growth results in a repression of enzyme synthesis (Flickinger & Perlan 1977; Greenwood & Jones 1986). However, the results presented in Table 3 and Fig. 4 indicate a different situation with respect to GDH activity in *A. calcoaceticus*. When an oxygen-limited culture, growing on acetate in the presence of glucose, was shifted to oxygen excess, a rapid appearance rate of glucose oxidation capacity became apparent (Fig. 4). Similarly, cell suspensions, washed with (cold) buffer, immediately oxidized glucose at a rate identical to that of cells precultivated under oxygen excess at the same dilution rate (Table 3). In particular the latter observation indicates that this increase in glucose oxidation is not due to de novo synthesis of GDH. This would imply a very rapid enzyme synthesis during the washing of cells at 4°C. It thus seems as if during oxygen-limited growth a cryptic glucose-oxidizing system is present, which only becomes functional at high oxygen tensions and which cannot be detected in vitro. With

respect to the apparent in vivo modulation of GDH activity by the dissolved oxygen tension it is of interest that this parameter has a profound effect on the cytochrome composition of *A. calcoaceticus*. During oxygen-limited growth cytochromes b and d increase whereas cytochrome o remains constant (Ensley & Finnerty 1980). Electron-flow during growth at a low dissolved oxygen tension is predominantly via cytochrome d due to its higher affinity towards oxygen (Jones & Poole 1985). The recently proposed model of the electron transport chain of *A. calcoaceticus* in which no interaction exists between electrons derived from NADH and $PQQH_2$, (Beardmore-Gray & Anthony 1986) may explain, at least in part, the preferential oxidation of the reduction equivalents from acetate in cultures grown under oxygen limitation. If the terminal cytochrome oxidase d can exclusively be used by electrons from NADH, generated in acetate oxidation, then the electron flow from glucose would be blocked, due to the low affinity of cytochrome o towards oxygen. Increasing the dissolved oxygen concentration would restore electron transfer from glucose to oxygen via cytochrome o.

Alternatively, GDH activity may be modulated during oxygen-limited growth by a change in kinetic properties. It has recently been shown that the activity of the quinoprotein methanol dehydrogenase is modulated by a so-called modifier protein which alters the kinetic constants of the enzyme (Page & Anthony 1986). If such a mechanism would regulate GDH activity in *A. calcoaceticus* it should involve an oxygen (or redox potential) sensitive binding of modulator to GDH protein.

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