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Glucose Metabolism and Gluconic Acid Production by *Acetobacter diazotrophicus*

MARGARET M. ATTWOOD,§ JOHANNES P. VAN DIJKEN, AND JACK T. PRONK*

Department of Microbiology and Enzymology, Kluyver Laboratory of Biotechnology, Delft University of Technology, Julianalaan 67, 2628 BC Delft, The Netherlands

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***Acetobacter diazotrophicus*, a recently isolated nitrogen-fixing acidotolerant acetic acid bacterium, grew well in simple mineral media and exhibited high rates of gluconic acid formation. Glucose oxidation by the organism was less sensitive to low pH values than glucose oxidation by *Gluconobacter oxydans*. Growth and glucose oxidation were not affected by high gluconic acid concentrations. These observations indicate that *A. diazotrophicus* is an interesting organism for the industrial production of gluconic acid. The organism exhibited a high maintenance requirement ($m_S = 1.0$ mmol glucose h^{-1} (g dry weight)$^{-1}$) during glucose-limited growth in chemostat cultures at pH 3.5. Enzyme activities in cell-free extracts suggested that glucose metabolism in *A. diazotrophicus* proceeds exclusively *via* the hexose monophosphate pathway: the enzymes of the Embden-Meyerhof and Entner-Doudoroff pathways could not be detected. Both the phosphorylative and direct oxidative pathways of glucose metabolism appeared to be operative. In addition to a pyridine nucleotide (strictly NAD)-dependent glucose dehydrogenase, *A. diazotrophicus* contained a dye-linked, probably pyrrolo-quinoline quinone (PQQ)-dependent, glucose dehydrogenase. The latter activity seemed to be primarily responsible for gluconic acid formation.**

Bacteria belonging to the genera *Acetobacter* and *Gluconobacter* are well-known for their ability to catalyse the incomplete oxidation of a wide range of organic compounds (1). Some of these bioconversions are applied in industry. For example, *Gluconobacter oxydans* is used on an industrial scale for the production of gluconic acid and ketogluconic acids from glucose. The physiology of *G. oxydans*, and in particular the production of gluconic acid by this organism, has been the subject of many studies.

Various vitamins and amino acids are required for optimal growth of *G. oxydans* in defined media (2). The growth requirements of the organism depend on the culture pH: at pH values below 3.5 growth does not occur in defined media (3). *G. oxydans* contains two enzyme activities which catalyse the oxidation of glucose to gluconic acid: a membrane-associated PQQ-dependent glucose dehydrogenase (EC 1.1.99.17, 4) and a soluble NADP-dependent glucose dehydrogenase (EC 1.1.1.47, 5). It has been demonstrated that the PQQ-linked enzyme plays a major role in gluconic acid production (6, 7).

Recently, a new acidotolerant *Acetobacter* species, called *A. diazotrophicus*, has been isolated from sugarcane roots (8, 9). The organism is capable of utilising molecular nitrogen as a sole source of nitrogen. In contrast to *G. oxydans*, *A. diazotrophicus* grows well in simple mineral media with ammonium salts as a source of nitrogen. The organism is also capable of growth at high sugar concentrations and produces gluconic acid from glucose (8).

The physiological characteristics of *A. diazotrophicus* described in the literature indicate that the organism may be of interest for the industrial production of gluconic acid. Therefore, our research was focused on glucose and gluconic acid metabolism in this organism.

* Corresponding author.

§ Present address: Department of Molecular Biology and Biotechnology, University of Sheffield, Sheffield S10 2TN, United Kingdom.

MATERIALS AND METHODS

Organism and maintenance *A. diazotrophicus* LMG 7603 (ATCC 49037) was obtained from Dr. K. Kersters, Laboratory of Microbiology, Ghent State University, Ghent, Belgium. *G. oxydans* ATCC 621H was obtained from Dr. P. R. Levering, Organon Research Laboratories, Oss, the Netherlands. The organisms were maintained on a solidified medium containing per liter of demineralized water: glucose, 10 g; Difco yeast extract, 10 g; $CaCO_3$, 20 g; and agar, 18 g.

Mineral medium *A. diazotrophicus* was grown in a mineral medium containing per liter of demineralized water: $(NH_4)_2SO_4$, 3.0 g; KH_2PO_4, 3.0 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; Na_2SO_4, 0.75 g; $CaCl_2 \cdot 2H_2O$, 0.26 g; $FeSO_4 \cdot 7H_2O$, 11 mg; $ZnSO_4 \cdot 7H_2O$, 0.9 mg; $MnCl_2 \cdot 2H_2O$, 2.0 mg; $CoCl_2 \cdot 6H_2O$, 0.6 g; $CuSO_4 \cdot 5H_2O$, 0.6 mg; $NaMoO_4$, 0.8 mg; H_3BO_3, 2.0 mg; KI, 0.2 mg; EDTA, 30 mg; nitrilo-triacetic acid, 5 mg and silicon antifoaming agent (BDH Chemicals, Poole, Dorset, UK), 25 μl. The medium was adjusted to pH 3.5 with 1 M H_2SO_4 and autoclaved at 120°C. Glucose was autoclaved separately at 110°C. *Gluconobacter oxydans* was grown in glucose-limited chemostat cultures as described previously (7).

Growth conditions Batch cultures were grown in 300 ml Erlenmeyer flasks containing 100 ml mineral medium and incubated on a rotatory shaker at 30°C.

Continuous cultivation was performed in Applikon laboratory fermentors with a working volume of 1 *l*. The pH was automatically titrated with either 1 M KOH or 0.5 M H_2SO_4. The cultures were continuously gassed with water-saturated air (1 *l* min^{-1}) and stirred at 800 rpm. Chemostat cultures were grown at a dissolved oxygen concentration higher than 75% of air saturation (*i.e.* >6 mg O_2 l^{-1}), at 30°C and at pH 3.5.

Controlled batch cultures were performed in the same fermentors. When necessary, batch cultures were gassed with oxygen-enriched air to avoid oxygen limitation.

Culture purity was routinely checked by phase-contrast microscopy and by plating on the complex medium mentioned above.

Measurement of substrate-dependent oxygen consumption Respiration rates of cells were assayed polarographically with a Clark-type oxygen electrode (Yellow Springs Instruments Inc., Yellow Springs, Ohio, USA) at 30°C. Cells were harvested by centrifugation (10 min, $10,000 \times g$) and resuspended in either mineral medium without carbon source (pH 3.5) or in β-alanine buffer (50 mM) containing 5 mM $MgSO_4$. The values presented here have been corrected for the (low) endogenous respiration rates.

Preparation of cell-free extracts Cells were harvested by centrifugation (15 min, $10,000 \times g$, 4°C) and washed with 50 mM potassium phosphate buffer (pH 6.0), containing 5 mM $MgCl_2$. The pellet was resuspended in the same buffer to a final concentration of approximately 0.25 g ml^{-1}. Cells were disrupted by sonication at 4°C in an MSE 150 W sonifier (15 bursts of 30 s with intermittent cooling). Intact cells and debris were removed by centrifugation ($40,000 \times g$, 20 min). The clear supernatant, containing 5 to 11 mg protein ml^{-1}, was used as the cell-free extract.

Enzyme assays Spectrophotometric enzyme assays were carried out at 30°C with freshly prepared extracts. In all assays the reaction rates were linearly proportional to the amount of extract added. Glucose dehydrogenase (PQQ-dependent, EC 1.1.99.17), glucose dehydrogenase (NADP-dependent, EC 1.1.1.47) and hexokinase (EC 2.7.1.1) were assayed as described previously (7). Gluconate dehydrogenase (NADP-dependent, EC 1.1.99.3) was assayed according to van Schie *et al.* (10). Dye-linked gluconate dehydrogenase was measured with the same assay as PQQ-dependent glucose dehydrogenase except that 20 mM gluconate was used as the substrate. Glucose 6-phosphate dehydrogenase (EC 1.1.1.49), 6-phosphogluconate dehydrogenase (EC 1.1.1.44), 6-phosphofructokinase (EC 2.7.1.4), fructose bisphosphate aldolase (EC 4.1.2.13) and the combined activity of 6-phosphogluconate dehydratase (EC 4.2.1.12) and 2-keto-3-deoxy-6-phosphogluconate (KDPG) aldolase (EC 4.1.2.14) were assayed according to van Dijken and Quayle (11).

Analytical procedures

Dry weight The dry weight of cell suspensions was determined by filtration through nitrocellulose filters (pore diameter 0.45 μm, Schleicher and Schüll, Dassel, FRG). After removal of the medium, the cells were washed three times with demineralised water and dried to constant weight at 70°C.

Protein determination The protein content of cell-free extracts was determined by the method of Bradford (12). Bovine serum albumin (fatty acid free, Sigma, St. Louis, USA) was used as a standard.

Substrate determinations Glucose concentrations in media and culture supernatants were measured with the GOD-PAP method (Boehringer, Mannheim, FRG). Gluconate was assayed using Boehringer test-kit 428191 or at by HPLC on an HPX-87H column (300×7.8 mm, Bio-Rad, USA) at room temperature. Sample were eluted with 0.01 N H_2SO_4 at a flow rate of 0.6 ml min^{-1}. Detection was done by means of a Waters 441 UV-detector at 210 nm, coupled to a Waters 741 data module. Peak areas were proportional to concentrations.

RESULTS AND DISCUSSION

Growth characteristics Growth of *A. diazotrophicus* on glucose was initially studied in shake flask cultures. Growth in mineral medium occurred over the pH range 3.0–6.0, in accordance with the results of Gillis *et al.* (9). No growth was detected at pH 2.5 and at pH 7.0. However, some growth could be observed at pH 2.5 in mineral medium supplemented with 0.1% yeast extract.

Growth of *A. diazotrophicus* in chemostat cultures was initially studied at pH 3.5, $T=30$°C, and $D=0.10\ h^{-1}$. To check whether the steady states obtained under these conditions were indeed glucose-limited, the glucose concentration in the influent medium was varied. The observed biomass yields did not differ significantly with the influent glucose concentration ($Y=39.0\pm0.3$ g mol glucose^{-1}). Electron microscopy of thin sections did not reveal any accumulation of storage polymers (results not shown). We therefore concluded that the cultures were indeed carbon and energy-limited. Low concentrations of gluconate (0.27–0.34 mM) and 2-ketogluconate (0.1–0.35 mM) were detected in supernatants of chemostat cultures.

The molar growth yield of *A. diazotrophicus* in glucose-limited chemostat cultures was low compared to the growth yields of other bacteria grown aerobically on glucose (13). To investigate whether the growth yields at a dilution rate of $0.10\ h^{-1}$ were influenced by maintenance requirements (14), growth of *A. diazotrophicus* was studied in glucose-limited chemostat cultures (pH 3.5, $T=30$°C) grown at various dilution rates (Fig. 1). The maximum growth yield and the maintenance coefficient calculated from the experimental data were 51 g mol^{-1} and 1.0 mmol h^{-1} g^{-1}, respectively. The maximum growth yield falls within the range of growth yields reported for aerobic bacteria. The maintenance coefficient calculated from the experimental data is rather high compared to maintenance coefficients observed with other aerobic bacteria (15). The high maintenance requirement during growth at

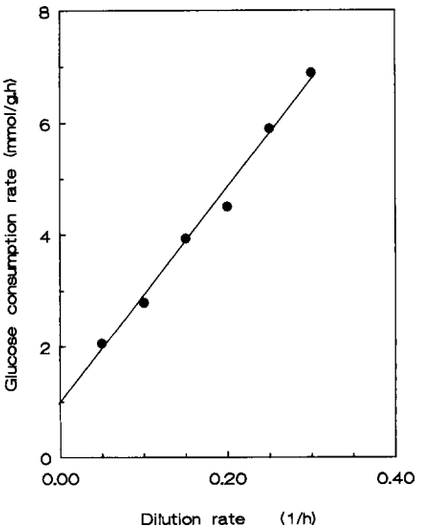

FIG. 1. Specific rates of glucose consumption by glucose-limited chemostat cultures of *A. diazotrophicus*, grown at various dilution rates. Growth conditions: pH 3.5, $T=30$°C, $S_R=20$ mM.

pH 3.5 may reflect an energy-dependent cytoplasmic pH homeostasis mechanism. Increased maintenance requirements at low pH values have also been reported for *E. coli* (16). Experiments with glucose-limited chemostat cultures of *A. diazotrophicus* grown at pH values above 4.0 were complicated by the formation of a viscous extracellular product, probably a polysaccharide (Attwood, unpublished results). Since maintenance energy requirements and energy stress have been implicated as important factors in the regulation of incomplete glucose oxidation by bacteria (17, 18), studies into the growth efficiency of *A. diazotrophicus* may be of interest for the application of the organism for gluconic acid production.

Glucose oxidation by intact cells The kinetics of glucose oxidation by intact cells of *A. diazotrophicus* were investigated by measuring the rate of glucose-dependent oxygen consumption at various glucose concentrations. Glucose oxidation by cells from a glucose-limited chemostat culture ($D=0.10\ h^{-1}$, pH=3.5, $T=30°C$) followed Michaelis-Menten kinetics (data not shown). The apparent K_S and V_{max} were 0.5 mM and 350 nmol O_2 min^{-1} mg^{-1}. The K_S is comparable to the substrate saturation constants of PQQ-dependent glucose dehydrogenases from a variety of Gram-negative bacteria (5, 19, 20), suggesting that a similar enzyme system may be involved in glucose oxidation by *A. diazotrophicus*. The observed glucose-dependent oxygen uptake rate is two- to threefold lower than oxygen uptake rates observed with *G. oxydans* grown under similar conditions ($D=0.10\ h^{-1}$, $D=30°C$, pH=5.5; (7). The cell suspensions also oxidized gluconate, with an apparent K_S of 1.4 mM and a V_{max} of 127 nmol O_2 min^{-1} mg^{-1}.

During the industrial production of gluconic acid, product formation leads to a decrease of the culture pH. The use of acidophilic or acidotolerant strains may increase the rate and efficiency of product formation. *A. diazotrophicus* is capable of growth at low pH values (9, this paper). The pH range for glucose oxidation mirrored the pH range for growth (Fig. 2). As expected from the growth characteristics, glucose oxidation by *A. diazotrophicus* was less sensitive to low pH values than glucose oxidation by *Gluconobacter oxydans* (Fig. 2).

Enzymes of glucose metabolism The first step in the metabolism of glucose by Gram-negative bacteria may be a direct oxidation to gluconic acid (21). Alternatively, glucose may be phosphorylated prior to further oxidation. Glucose 6-phosphate formed in the latter reaction may be further metabolized *via* three different metabolic pathways: the Embden Meyerhof pathway or glycolysis, the pentose phosphate pathway and the Entner-Doudoroff pathway. Gluconic acid formed by the direct oxidation of glucose can be phosphorylated and subsequently metabolized *via* the pentose phosphate pathway or the Entner-Doudoroff pathway.

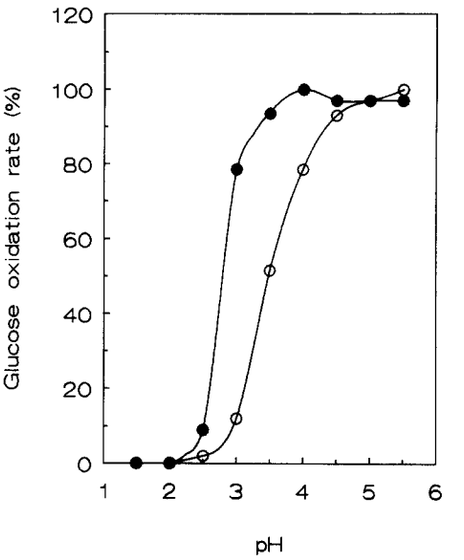

FiG. 2. Effect of pH on glucose-dependent oxygen uptake by *A. diazotrophicus* (●) and *G. oxydans* ATCC 621H (○). *A. diazotrophicus* was pregrown in a glucose-limited chemostat culture ($D=0.10\ h^{-1}$, $T=30°C$, pH=3.5). *G. oxydans* was pregrown in a glucose-limited chemostat culture ($D=0.10\ h^{-1}$, $T=30°C$, pH=5.5) as described previously (7). Oxidation of glucose (20 mM) was assayed with a Clark-type oxygen electrode. 100% activity corresponds to oxygen uptake rates of 360 nmol $O_2 \cdot min^{-1} \cdot mg^{-1}$ and 750 nmol $O_2 \cdot min^{-1} \cdot mg^{-1}$ for *A. diazotrophicus* and *G. oxydans*, respectively.

Cell-free extracts of *A. diazotrophicus* contained high activities of dye-linked glucose dehydrogenase (Table 1). The presence of significant concentrations of PQQ in supernatants of chemostat cultures (10–40 nM; Dr. M. A. G. van Kleeff, pers. comm.) suggests that the dye-linked enzyme activity is due to PQQ-dependent glucose dehydrogenase (EC 1.1.99.17). This enzyme is widely distributed among aerobic, Gram-negative bacteria (22).

TABLE 1. Specific enzyme activities in cell-free extracts of *A. diazotrophicus* grown in glucose-limited chemostat cultures ($S_R=20$ mM, $T=30°C$, pH=3.5, $D=0.10\ h^{-1}$ or $0.30\ h^{-1}$)

Enzyme activity	$D=0.10\ h^{-1}$	$D=0.30\ h^{-1}$
Glucose dehydrogenase, dye-linked	2.6	1.7
Glucose dehydrogenase, NAD	0.45	0.16
Glucose dehydrogenase, NADP	<0.005	<0.005
Gluconate dehydrogenase, dye-linked	0.24	0.88
Gluconate dehydrogenase, NAD	0.26	0.39
Gluconate dehydrogenase, NADP	<0.005	<0.005
Glucose 6-phosphate dehydrogenase, NAD	0.86	0.48
Glucose 6-phosphate dehydrogenase, NADP	0.87	0.54
6-Phosphogluconate dehydrogenase, NAD	<0.005	<0.005
6-Phosphogluconate dehydrogenase, NADP	2.0	2.0
Hexokinase	n.d.	0.07
6-Phosphofructokinase	<0.005	<0.005
6-Phosphogluconate dehydratase/2-keto-3-deoxy-phosphogluconate aldolase (combined activities)	<0.005	<0.005

Enzyme activities are expressed as $\mu mol\ min^{-1}$ (mg protein)$^{-1}$.
n.d.: Not determined.

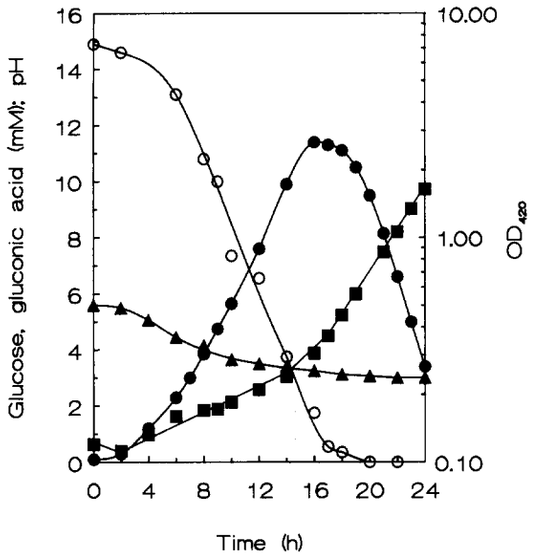

FIG. 3. Gluconic acid formation and growth of *A. diazotrophicus* on glucose in a batch culture without pH control. Growth conditions: $T=30°C$, initial pH 5.6. The culture was inoculated with a 20 ml shake flask culture pregrown on mineral medium plus glucose. Symbols: ■, optical density at 420 nm; ▲, culture pH; ○, glucose concentration; ●, gluconic acid concentration.

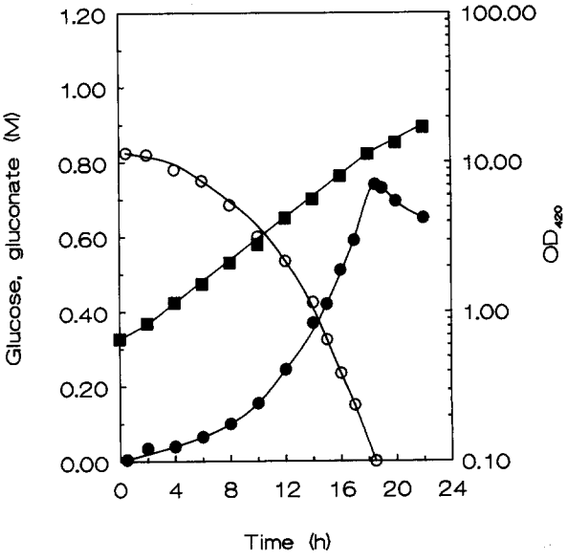

FIG. 4. Gluconic acid formation and growth of *A. diazotrophicus* in a batch culture with pH control ($T=30°C$, pH=3.5). Cells grown in a 1 *l* glucose-limited chemostat culture ($D=0.10\ h^{-1}$, $T=30°C$, pH 3.5, $S_R=20$ mM) were harvested by centrifugation and resuspended in mineral medium supplemented with 0.85 M glucose. Symbols: ■, optical density at 420 nm; ○, glucose concentration; ●, gluconic acid concentration.

Similar to *Gluconobacter* species, cell-free extracts of *A. diazotrophicus* also contained a pyridine nucleotide-dependent glucose dehydrogenase (Table 1). The latter enzyme was strictly NAD-dependent. This is a remarkable difference with the enzyme from *G. oxydans*, which is strictly NADP-dependent (23). The activity of the NAD-dependent glucose dehydrogenase in the cell-free extracts was only 10–17% of that of the PQQ-dependent enzyme. This observation suggests that the PQQ-dependent enzyme plays a major role in the conversion of glucose to gluconic acid, as has been described for *G. oxydans* (6, 7). Cell-free extracts of *A. diazotrophicus* contained significant activities of hexokinase (Table 1), indicating that also the phosphorylative route of glucose metabolism may be operative in this organism.

To investigate which of the central pathways of glucose metabolism are operative in *A. diazotrophicus*, the activity of a number of key enzymes was assayed in cell-free extracts (Table 1). No activity was detected of phosphofructokinase, a key enzyme of the Embden-Meyerhof pathway of glucose metabolism. We were also unable to detect the combined activities of the key enzymes of the Entner-Doudoroff pathway, 6-phosphogluconate dehydratase and 2-keto 3-deoxygluconate aldolase. The *A. diazotrophicus* extract did not inhibit the activity of commercial preparations of the enzymes mentioned above (data not shown). Cell-free extracts of *A. diazotrophicus* did contain high activities of 6-phosphogluconate dehydrogenase. The observed enzyme activities suggest that glucose metabolism in *A. diazotrophicus* proceeds exclusively *via* the pentose phosphate pathway, as has been reported for *G. oxydans* (24). Radiorespirometric experiments are needed to confirm this conclusion.

In addition to the enzyme activities mentioned above, cell-free extracts of *A. diazotrophicus* also contained gluconate dehydrogenase activities (Table 1). Both dye-linked and pyridine nucleotide-dependent gluconate dehydrogenase activities were detected. The latter activity was strictly NAD-dependent, in contrast to *G. oxydans*, which contains both NAD- and NADP-dependent gluconate dehydrogenase activities (25).

Incomplete oxidation of glucose Preliminary experiments with shake flask cultures indicated that gluconic acid accumulated during batch growth on glucose. Growth and product formation were studied in more detail in well-aerated fermentors.

In a batch culture without pH control, growth of *A. diazotrophicus* on glucose followed a biphasic pattern. During the first phase, a net formation of gluconic acid occurred, until approximately 85% of the glucose was converted into gluconic acid (Fig. 3). At this point, when the glucose concentration in the culture had decreased to approximately 1 mM, a net consumption of gluconic acid set in. As a result of gluconic acid formation, the culture pH decreased from 5.6 to approximately 3.5. The culture pH continued to decrease after all glucose had been consumed (Fig. 3). This was probably due to hydrolysis of δ-gluconolactone, the initial product of microbial glucose oxidation (26).

The growth pattern observed in cultures without pH control differed markedly from that reported for *G. oxydans* (3). Growth of the latter organism on glucose also resulted in a rapid decrease of the culture pH. However, in contrast to *A. diazotrophicus*, the resulting acidification of the growth medium completely inhibited gluconic acid formation and growth of *G. oxydans* (3).

According to the literature, *A. diazotrophicus* is capable of growth at high sugar concentrations (8, 9). When an organism is to be used to produce gluconic acid on an industrial scale, it should also be capable of glucose oxidation in the presence of high product concentrations. To check the potential of *A. diazotrophicus* for the production of gluconic acid, the organism was grown as a batch culture with

an initial glucose concentration of 150 g l^{-1} (Fig. 4). The pH of the culture was maintained at 3.5 by automatic titration with 4 M KOH. The specific growth rate in this culture remained 0.17 h^{-1} until all glucose was consumed. At this point approximately 85% of the substrate had been converted into gluconic acid (Fig. 4). This conversion is comparable to that observed with *G. oxydans* (7). HPLC analysis of culture supernatants also revealed the formation of significant amounts of 2-ketogluconic acid (data not shown). After the culture had been left overnight, it turned a dark brick-red, probably as a result of the formation of 2,5-diketogluconic acid (9). This phenomenon, as well as the lower oxidation capacity for glucose, is a disadvantage as compared to gluconic acid production by *G. oxydans*. Only after physiological and/or genetic manipulation, *A. diazotrophicus* may be a candidate for the industrial production of gluconic acid.

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