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ORIGINAL PAPER

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Influence of residual ethanol concentration on the growth of *Gluconacetobacter xylinus* I 2281

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Abstract The influence of residual ethanol on metabolism of food grade *Gluconacetobacter xylinus* I 2281 was investigated during controlled cultivations on 35 g/l glucose and 5 g/l ethanol. Bacterial growth was strongly reduced in the presence of ethanol, which is unusual for acetic acid bacteria. Biomass accumulated only after complete oxidation of ethanol to acetate and carbon dioxide. In contrast, bacterial growth initiated without delay on 35 g/l glucose and 5 g/l acetate. It was found that acetyl CoA was activated by the acetyl coenzyme A synthetase (Acs) pathway in parallel with the phosphotransacetylase (Pta)-acetate kinase (Ack) pathway. The presence of ethanol in the culture medium strongly reduced Pta activity while Acs and Ack remained active. A carbon balance calculation showed that the overall catabolism could be divided into two independent parts: upper glycolysis linked to glucose catabolism and lower glycolysis liked to ethanol catabolism. This calculation showed that the carbon flux through the tricarboxylic cycle is lower on ethanol than on acetate. This corroborated the diminution of carbon flux through the Pta-Ack pathway due to the inhibition of Pta activity on ethanol.

Introduction

Acetic acid bacteria are known for their ability to oxidize various alcohols, sugars, and sugar alcohols. These properties are largely used for vinegar production and synthesis of chemicals such as vitamin C (Chotani et al. 2000). Some *Acetobacter* strains produce bacterial cellulose (Ross et al. 1991) and other exopolysaccharides (EPS) such as acetan (de Iannino et al. 1988) or levan (Trujillo et al. 2001). These products find applications in fermented food products as thickening agents, and could have a positive effect on health, like other EPS from food grade bacteria (Hosono 1997; Nagaoka 1994; Oda 1983). Recently, we have shown that *Gluconacetobacter xylinus* I 2281 produces an EPS named gluconacetan, structurally different from those previously reported (Duboc et al. MS submitted).

Results of investigations into *Gluconacetobacter* strains have shown that a complex medium containing glucose must be supplemented with ethanol in order to observe net bacterial growth (Schüller et al. 2000). As in other acetic acid bacteria (Luttik et al. 1997), the biomass yield remained generally low. *G. xylinus* oxidizes ethanol to acetate and is able to further oxidize acetate to carbon dioxide via the tricarboxylic acid (TCA) cycle (Asaï 1968; De Ley et al. 1984). However, preliminary results have hinted at the fact that biomass accumulates only after complete oxidation of ethanol. In fact, ethanol seemed to strongly limit acetate oxidation in *G. xylinus* I 2281.

In general, two independent pathways are involved in acetate catabolism. One is catalyzed by phosphotransacetylase (Pta) and acetate kinase (Ack) and the second involves the acetyl coenzyme A synthetase (Acs) pathway (Saeki et al. 1997). Most studies on regulation of acetate oxidation have used *Escherichia coli*, especially because of its ability to produce and assimilate acetate (Birgitt et al. 1994; Cozzone 1998; Kumari et al. 2000). However, particularly in the case of acetic acid bacteria, little information is available on the influence of ethanol on these pathways.

The influence of residual ethanol concentration on bacterial growth limits the industrial application of *G. xylinus* I 2281. In order to further investigate this phenomenon, the specific activity of the key enzymes Pta, Ack and Acs was assayed during batch fermentations.

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These results were corroborated with carbon balance calculations and showed that carbon flux through the TCA cycle was reduced in the presence of ethanol due to a decrease in carbon flux through the Pta-Ack pathway.

Materials and methods

Microorganism, medium, and inoculum preparation

Bacterial strain *G. xylinus* I 2281 was kindly provided by the Nestlé Research Center (Lausanne, Switzerland). Stock cultures were stored at −80°C in a 15% w/v skim milk powder and 15% w/v malt extract solution. Cells were re-activated in a 1 l shake flask containing 100 ml defined medium at 30°C for 60 h. The defined medium contained, per liter: 35 g fructose, 5 g acetate, 0.163 g $MgCl_2 \cdot 6H_2O$, 5 g KH_2PO_4, 2 g NH_4Cl, 1.060 g Na_2CO_3, 0.114 g Na_2SO_4, and15 ml trace elements solution. The latter contained (per liter): 1.47 g $CaCl_2 \cdot 2H_2O$, 0.27 g $FeCl_3 \cdot 6H_2O$, 0.085 g $MnSO_4 \cdot H_2O$, 0.024 g $Na_2MoO_4 \cdot 2H_2O$, 0.016 g $CuSO_4 \cdot 5H_2O$, 0.024 g $CoCl_2 \cdot 6H_2O$, 0.024 g $NiCl_2 \cdot 2H_2O$, 0.144 $ZnSO_4 \cdot 7H_2O$, and 4.1 g HCl 25% w/v. The pH was adjusted to 4.0 using 2 *N* KOH and 1 *N* HCl. The medium was sterilized by filtration (0.2 µm, Minisart; Sartorius, Göttingen, Germany).

Bioreactor culture

Growth experiments were undertaken using a 15 l bioreactor (Fermenteur 15 LP; LSL Biolafitte, Saint-Germain-en-Laye, France) with a working volume of 10 l. The bioreactor inoculum was prepared by incubation of 500 ml defined medium, containing glucose as carbohydrate source, in shake flasks for 24 h. The medium for cultivation in the bioreactor contained glucose, ethanol and acetate at concentrations specified in the text. Carbonate was omitted. Temperature was maintained at 30°C and pH at 4.0 by the automatic addition of 2 *N* KOH or 1 *N* HCl. In order to avoid foaming, a level probe activated the addition of a 10 g/l anti-foam solution (Structol J673; Schill and Seilacher, Hamburg, Germany). Aeration rate was maintained at 1 vvm using a thermal mass flow controller (5850E; Brooks Instrument, Hatfield, Pa.) and stirring rate was fixed at 800 rpm. A polarographic pO_2 probe (Infit 765–50; Mettler Toledo, Greifensee, Switzerland) was used to monitor dissolved oxygen, with respect to air-saturated medium.

Substrate and metabolite analysis

Culture samples (approximately 15 ml) were collected using a home-made auto-sampler and kept at 2°C for up to 8 h before handling. For cell dry weight measurements, biomass was recovered from 9 ml culture sample by centrifugation (15 min at 20,000 *g*, 2°C), re-suspension in water and filtration using pre-weighed filters (HT-200; Pall, Ann Arbor, Mich.). Filters were dried for 15 min in a microwave oven (150 W) and re-weighed (Schulze 1995). A correlation was established between dry cell weight and optical density at 600 nm. This correlation was used to estimate biomass concentrations below 0.4 g/l.

Glucose, acetate, ethanol, gluconic acid, 2-ketogluconic acid and 5-ketogluconic acid concentrations were determined by HPLC analysis (1100 series; Agilent Technologies, Palo Alto, Calif.). An ion exchange chromatography column (Supelcogel H 300 mm; Supelco, Bellefonte, Pa.) with a guard column (Superlguard C610H, Supelco) was used at 30°C. A 5 mM sulfuric acid solution in ultrapure water was applied at a constant eluent flow rate of 0.5 ml/min. Glucose, acetate and ethanol were measured using a refractive-index detector. Gluconic acid, 2-ketogluconic acid and 5-ketogluconic acid were detected by a diode array detector at 360 nm.

Gluconacetan was recovered after precipitation of one volume sample with two volumes of industrial ethanol followed by centrifugation (10 min at 4,500 *g* and 4°C). The pellets were washed twice with a 70% ethanol solution, then freeze-dried and weighed (MacCormick et al. 1993).

The oxygen uptake rate (OUR) and carbon dioxide evolution rate (CER) in the bioreactor off-gas were determined using paramagnetic (series 1100, Servomex, Crowborough, UK) and infrared (series 2500, Servomex) analyzers, respectively. The measured values of oxygen and carbon dioxide were corrected for water vapor according to Duboc and von Stockar (1998).

Cell extracts and enzyme activity analysis

Fermentation broth samples were centrifuged for 10 min at 7,500 *g* and at 2°C. The pellet of biomass was washed twice with cold buffer supplemented with protease inhibitor cocktail tablets (Complete; Roche Diagnostics, Mannheim, Germany). The buffer contained 100 mM triethanolamine, pH 7.6 for Ack analysis; 25 mM Tris HCl with 500 mM $(NH_4)_2SO_4$, pH 8.0 for Pta analysis; or 500 mM KH_2PO_4, pH 7.5 for Acs analysis. Washed biomass samples were stored at −40°C. Cell extracts were prepared by placing 1 ml washed biomass sample in a vibration mill (MM 300; Retsch, Haan, Germany) at 3°C with 2 ml glass beads (0.25 mm diameter; Braun Melsungen, Melsungen, Germany). Two cycles were run at a vibrational frequency of 25 Hz with 1 min intervals. Cell debris was removed by centrifugation (5 min at 10,000 *g*; 2°C). Enzyme activities were determined immediately after cell disruption. The Bradford method (Protein Assay Kit; Bio-Rad, Munich, Germany) was used to determine total protein concentration. Bovine serum albumin served as standard.

Ack activity was determined in the acetyl-phosphate-forming direction according to a method adapted from Bergmeyer (1983) and Rose et al. (1954). The final concentrations were 63 mM triethanolamine, 200 mM sodium acetate, 6.1 mM ATP, 1.9 mM phospho(enol)pyruvate, 6.7 mM $MgCl_2$, 0.11 mM β-NAD(P)H, 35 U pyruvate kinase, 50 U lactic dehydrogenase and 40–60 U myokinase. The decrease of NAD(P)H was monitored spectrophotometrically at 340 nm. One unit of enzyme activity corresponds to the conversion of 1.0 µmol acetate to acetyl phosphate per minute at 30°C and pH 7.6 at initial conversion rate.

Pta activity was assayed by monitoring the formation of acetyl coenzyme A from acetyl phosphate and coenzyme at 233 nm. The assay comprised 3.0 ml 98.5 mM Tris buffer containing 1.6 mM glutathione and 13.3 mM $(NH_4)_2SO_4$. Coenzyme A (0.43 mM) and acetyl phosphate (7.23 mM) were used as substrates according to a method adapted from Klotschz (1969). One unit of enzyme activity corresponds to the conversion of 1.0 µmol coenzyme A to acetyl coenzyme A per minute at pH 7.4 and 30°C at initial conversion rate.

Acs activity was determined according to a method modified from that of Berg (1956). Acetyl coenzyme A reacts with hydroxylamine to form acetyl hydroxylamine. Using $FeCl_3$ as a colorimetric additive, acetyl hydroxylamine was then titrated at 550 nm. In a 153 µl reaction mix, the final concentration was 136 mM KH_2PO_4, 4 mM $MgCl_2$, 9.1 mM ATP, 45 mM KF, 9.1 mM potassium acetate, 9.1 mM reduced glutathione, 0.35 coenzyme A and 182 mM hydroxylamine. One unit of enzyme activity corresponds to the formation of 1.0 µmol acetyl coenzyme A per minute at pH 7.5 and 37°C at initial conversion rate.

The chemicals used in enzymatic assays were supplied by Sigma-Aldrich, Steinheim, Germany. Pta from *Bacillus stearothermophilus* (P-2783), Ack from *E. coli* (A-7437) and Acs from *Saccharomyces cerevisiae* (A-1765) (all from Sigma-Aldrich, Steinheim, Germany) were used to prepare synthetic enzyme solutions.

Results

Sequential substrate utilization

A batch fermentation of *G. xylinus* I 2281 on 35 g/l glucose and 5 g/l ethanol is presented in Fig. 1 and reveals three main phases. During the first phase (0–34 h), most ethanol was oxidized to acetate, resulting in a final concentration of 4.4 g/l acetate. Simultaneously, glucose oxidation to gluconic acid was initiated. No detectable growth or gluconacetan production was observed. The second phase (34–44 h) began immediately after ethanol depletion from the medium and was characterized by bacterial growth on acetate and glucose (Fig. 1). Gluconacetan production was initiated and most of the glucose was oxidized to gluconic acid, yielding a final concentration of 30 g/l (Fig. 1). Concomitantly, gluconic acid started to be oxidized to 2-ketogluconic acid and 5-ketogluconic acid. At the end of the second phase, the biomass concentration reached 0.6 g/l and bacterial growth stopped when acetate was exhausted. In the last phase (44–100 h), characterized by constant biomass concentration, oxidation of gluconic acid to 2- and 5-ketogluconic acid continued, resulting in final concentrations of 3.7 g/l and 8.1 g/l, respectively (Fig. 1). Gluconic acid, as sole carbon source, supported neither bacterial growth nor gluconacetan formation (phase 3 on Fig. 1).

In this experiment, acetic acid exhaustion occurred simultaneously with glucose exhaustion. Nevertheless, other experiments (data not shown) illustrated that gluconacetan was not growth-associated, and that it continued to be produced during the third phase of fermentation in the presence of glucose.

During this fermentation, no clear anabolic activity was observed until the complete oxidation of ethanol had been achieved (Fig. 1). Therefore, the overall gluconacetan productivity is drastically affected by the first phase of fermentation.

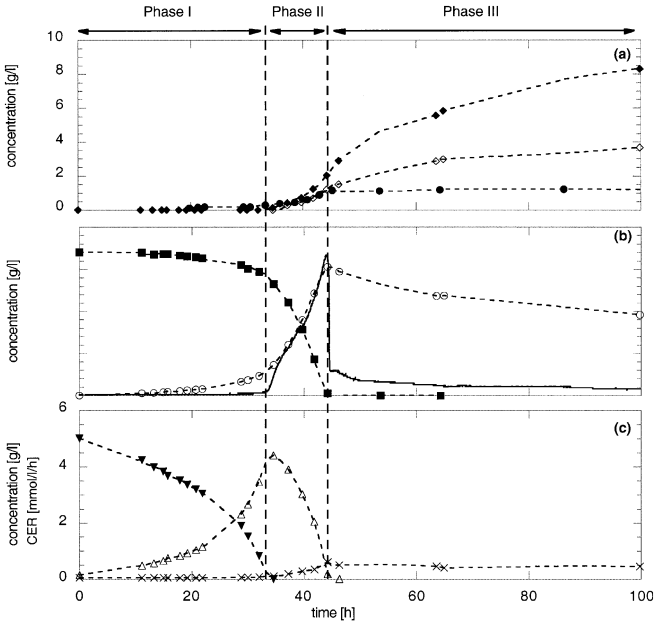

Fig. 1a–c Cultivation of *Gluconacetobacter xylinus* I 2281 in batch culture with glucose (35 g/l) and ethanol (5 g/l). Three different phases can be identified: *Phase I* oxidation of ethanol to acetate, *Phase II* growth on acetate and glucose, *Phase III* acetate and glucose starvation. **a** ♢ 2-Ketogluconic acid (g/l), ● gluconacetan (g/l), ◆ 5-ketogluconic acid (g/l). **b** ■ Glucose (g/l), ◯ gluconic acid (g/l), ▼ ethanol (g/l). **c** *Solid line* carbon dioxide evolution rate (CER; mmol l^{-1} h^{-1}), △ acetate (g/l), × biomass (g/l)

Metabolic inhibition by ethanol

The influence of low residual ethanol concentrations on the growth of *G. xylinus* I 2281 was further investigated in batch cultures containing 35 g/l glucose, 3.7 g/l acetate and 0.3 g/l ethanol (data not shown). In accord with the experiment illustrated on Fig. 1, ethanol was first oxidized to acetate before growth could be detected, and biomass concentration increased only once the ethanol concentration was below 0.02 g/l. In contrast, biomass accumulated with no delay when cells were grown in the absence of ethanol in medium containing 31 g/l glucose and 4.2 g/l acetate (Fig. 2).

To further study the effect of ethanol on cell metabolism, a pulse of 4 g/l ethanol was added to the culture during the exponential growth phase when the acetate concentration had reached 1 g/l (19.8 h, Fig. 2). Figure 2d shows that as soon as ethanol was added to the medium, the carbon dioxide signal fell to a low and constant value of approximately 10 mmol l^{-1} h^{-1}. The added ethanol was immediately oxidized to acetate (Fig. 2c), resulting in an immediate increase in OUR (Fig. 2d). CER remained constant as long as ethanol concentration remained above 0.02 g/l, whereas the OUR increased regularly, driven by ethanol oxidation. Concomitant with the sudden reduction in CER, biomass values remained almost constant. The majority of the pulsed ethanol (87 mmol) was oxidized to acetate (72 mmol). Production of gluconic acid, 2 and 5-ketogluconic acid and gluconacetan was unaffected by the presence of ethanol (Fig. 2). Once the pulsed ethanol had been completely oxidized (27 h), growth on acetate resumed, with equal values of OUR and CER. After complete conversion of ethanol to acetate, the fermentation behaved in a similar way to a batch culture on glucose and acetate (phases 2 and 3, Fig. 1).

Pta inhibition in the presence of ethanol

Four physiological states can be clearly seen in Fig. 2: (i) a first growth phase on acetate; (ii) a second phase due to metabolic restriction by ethanol; (iii) a growth phase on acetate; (iv) a glucose and acetate starvation phase. Cell extracts were prepared from each of the four physiological states and the activities of the key enzymes of acetate catabolism determined. Figure 3 shows the activities of Ack, Pta and Acs in relation to off-gas signals. During the first growth phase all three enzymes were active. During

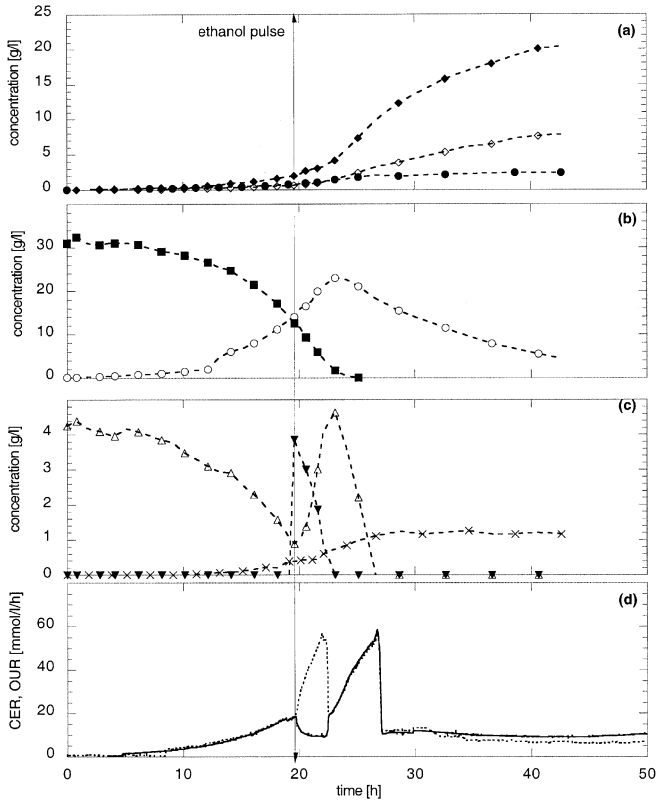

Fig. 2a–d Cultivation of *G. xylinus* I 2281 in batch culture with glucose (31 g/l) and acetate (4.2 g/l). The *arrow* indicates a pulse of ethanol (4 g/l) added to the bioreactor at 19.8 h. **a** ◇ 2- Ketogluconic acid (g/l), ● gluconacetan (g/l), ◆ 5-ketogluconic acid (g/l). **b** ■ Glucose (g/l), ◯ gluconic acid (g/l). **c** ▼ Ethanol (g/l), △ acetate (g/l) × biomass (g/l). **d** *Solid line* CER mmol l^{-1} h^{-1}, *dotted line* oxygen uptake rate (OUR) mmol l^{-1} h^{-1}

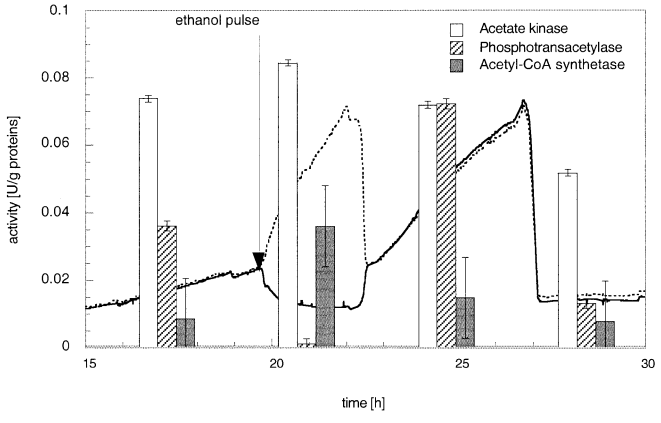

Fig. 3 Activity of acetate kinase (Ack), phosphotransacetylase (Pta) and acetyl-CoA-synthetase (Acs) during a batch cultivation of *G. xylinus* I 2281 with glucose (31 g/l) and acetate (4.2 g/l) as illustrated in Fig. 2. The *arrow* indicates a pulse of ethanol (4 g/l) at 19.8 h. The dynamic response of the culture is represented by CER (*solid line*) and OUR (*dotted line*)

the pulse of ethanol, Pta activity was strongly reduced, Ack activity remained constant and Acs activity seemed to increase slightly. During the second growth phase—after ethanol depletion—all enzyme activities returned to values very similar to those measured during the first growth phase. The activities measured during glucose and acetate starvation (27–30 h) were considered as residual activities from the previous growth phases. Although the Acs pathway is not always mentioned in the metabolic scheme of acetic acid bacteria (Ross et al. 1991), the measured Acs activity seems to confirm the use of this pathway for acetate oxidation. Determination of enzyme activity shows that both the Acs and the Ack-Pta pathways are active during the growth phase, while the Ack-Pta pathway is strongly reduced in the presence of ethanol.

Inhibition of enzyme activities by ethanol was investigated using commercial solutions of Ack, Pta and Acs assayed in the presence of 5 g/l ethanol. The results (data not shown) show that all activities were unaffected by the presence of ethanol. The same result (data not shown) was observed for Pta activity assayed in cell extract in the presence of 5 g/l ethanol. This suggests that inhibition of growth by ethanol is not due to direct inhibition of these enzymes.

Discussion

Batch culture experiments (Fig. 1) revealed that oxidation of ethanol led first to acetate accumulation with no biomass formation. Both membrane-bound and cytosolic dehydrogenases are involved in this process (Deppenmeier et al. 2002; Matsushita et al. 1994; Takemura et al. 1993). Acetate oxidation began only after depletion of ethanol, allowing biomass accumulation with a maximum specific growth rate (μ_{max} =0.14 h^{-1}) that corresponds well with values reported for other *xylinus* strains (Naritomi et al. 1998). An unfavorable effect of ethanol on growth, compared to acetate, has already been reported for several *Acetobacter* strains (Saeki et al. 1997). Similarly, the steady state biomass concentration of *Acetobacter xylinus* subsp. *sucrofermentans* BPR3001A decreased when the ethanol concentration in the medium feed was higher than 15 g/l under continuous operation (Naritomi et al. 1998). However, in contrast to our results, these studies (Naritomi et al. 1998; Saeki et al. 1997) reported bacterial growth in the presence of ethanol.

The ethanol pulse experiment shown in Fig. 2 allowed further investigation of the effect of ethanol on acetate catabolism. The metabolic states can be described using specific yields. For the calculation of yields (Table 1), it was assumed that gluconacetan and glucose oxides were synthesized from glucose and not from acetate (Weinhouse and Benziman 1974). Furthermore, it was assumed that the CO_2 produced resulted only from ethanol and acetate catabolism. Finally, the biomass yield was zero in the presence of ethanol. To resume these calculations assume that the overall catabolism can be divided into

Table 1 Yields (C-mol/C-mol) of metabolites produced from glucose by *Gluconacetobacter xylinus* I 2281 before and during a pulse of ethanol (experiment shown in Fig. 2). *EPS* Exopolysaccharides

	Gluconate	2-Keto gluconate	5-Keto gluconate	EPS	Carbon balance
Before ethanol pulse	0.76	0.04	0.14	0.05	0.99
During ethanol pulse	0.79	0.03	0.11	0.04	0.97

two independent parts: upper catabolism relating to glucose consumption (described in Table 1) and lower catabolism relating to ethanol oxidation into acetate and CO_2 (0.87 C-mmol/C-mmol and 0.17 C-mmol/C-mmol, respectively). The validity of these assumptions is supported by the excellent carbon balance recovery for upper (0.99, Table 1) and lower (0.87+ 0.17=1.04) catabolism. It also appears that glucose and gluconacetan yields are not affected by the presence of ethanol (Table 1). Moreover, bacterial growth on glucose without acetate is tremendously reduced.

According to a study using *A. xylinus*, ethanol is not a gluconeogenic substrate (Weinhouse and Benziman 1972). Consequently, as seen in Fig. 2, CO_2 can only be produced in the TCA cycle. Thus, it may be concluded that the TCA cycle is operating during growth inhibition by ethanol, albeit at a lower level.

This observation is in accordance with the enzyme activities assayed during the ethanol pulse (Fig. 3). Although the Pta-Ack pathway is blocked due to a decrease in Pta activity, the Acs pathway seems to be active during all of the different physiological states. Qualitatively, the capacity for acetyl coA synthesis (via Acs and Ack/Pta pathways) is higher before the ethanol pulse and after ethanol exhaustion than while ethanol is present. Furthermore, the results reported here agree well with the enzyme activities measured in batch fermentations of *Acetobacter racens* (Saeki et al. 1999). In this latter study, Ack activity remained constant during the fermentation while Pta activity decreased during growth on a complex medium containing glycerol, glucose and ethanol. Nevertheless, in contrast to our results, Acs activity was reported to be lower at the beginning of the batch fermentation when ethanol was still present. However, *A. racens* was able to grow on a medium containing ethanol only, which is not the case for *G. xylinus* (Saeki et al. 1999).

Based on the enzyme activity measurements, acetate metabolism may be described as follows (Fig. 4): during the growth phase, both the Ack-Pta and Acs pathways are active in *G. xylinus*. Acetate is oxidized through the TCA cycle producing the major part of the measured carbon dioxide [$Y_{(CO2/acetate)}$ 1.01 C-mol/C-mol]. In the presence of ethanol, the activity of Pta falls to zero; thus, the Ack-Pta pathway is blocked but the Acs pathway is still active and the TCA cycle can be fueled. Nevertheless, according to the CO_2 profile, the flux through the TCA cycle is reduced. This is in agreement with results obtained with *A. racens* in which Saeki et al. (1999) showed that the activities of TCA enzymes were indeed lower on ethanol than on acetate. Thus, in acetic acid bacteria, ethanol could affect a large pool of enzymes involved in acetate oxidation and the TCA cycle.

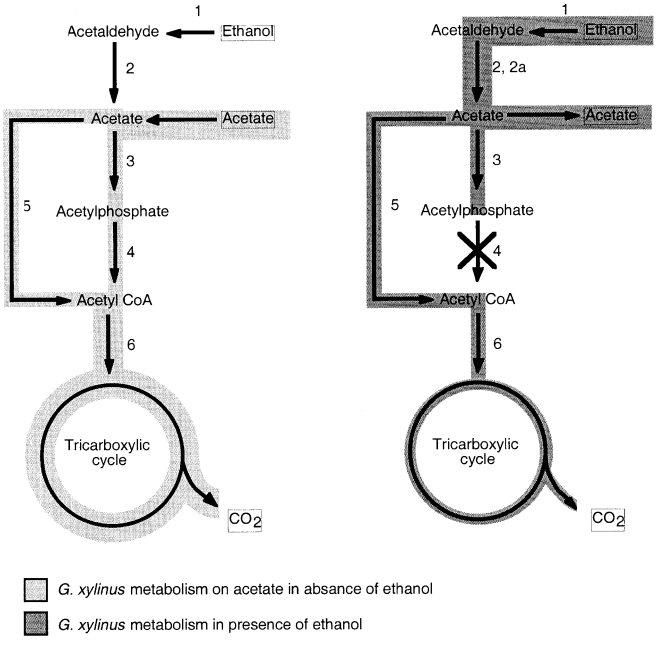

Fig. 4 Proposed pathways for ethanol metabolism in *G. xylinus* during exponential growth phase and during ethanol oxidation into acetate. *1* Alcohol oxidase, *2* acetaldehyde dehydrogenase [NAD(P)], *2a* acetaldehyde dehydrogenase (FAD), *3* Ack, *4* Pta, *5* Acs, *6* citrate synthase. Components inside the boxes represent extracellular compounds

During the ethanol pulse, biomass formation was undetectable. This growth inhibition may result from the low carbon flux through the TCA cycle and a low acetyl coenzyme A synthesis, which usually provides essential building blocks for lipids and amino acid synthesis. Moreover, the accumulation of acetylphosphate due to the inhibition of Pta in the Ack-Pta pathway, may act as a global signal, as hypothesized in *E. coli* and related bacteria, for metabolic regulation (reviewed in McCleary et al. 1993) and cell division (Prüss 1998).

After ethanol exhaustion, the CER profile immediately reverted to the value observed before the pulse of ethanol. The rapid kinetics of the fall in CER are in agreement with a regulation due to inhibition. Nevertheless, the mechanism of inhibition of Pta activity in the presence of ethanol is still not clear. It is unlikely that the decrease in metabolic activity results from a direct inhibitory effect of ethanol on Acs, Pta and Acs activities since none of these enzyme activities was affected by the presence of 5 g/l ethanol in standard assay mixtures. However, many other

mechanisms, such as post translational modification of the enzyme or synthesis of inhibitors driven by ethanol, might be involved in the depletion of Pta activity.

This study shows that the presence of residual ethanol strongly reduces the carbon flux through the TCA cycle. The rapid and reversible reduction of carbon flux suggests an inhibition induced by ethanol. This observation correlates with depletion in Pta activity and undetectable bacterial growth of *G. xylinus* I 2281 in the presence of ethanol.

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