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Environmental and physiological factors affecting the succinate product ratio during carbohydrate fermentation by *Actinobacillus* sp. 130Z

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Abstract *Actinobacillus* sp. 130Z fermented glucose to the major products succinate, acetate, and formate. Ethanol was formed as a minor fermentation product. Under CO_2-limiting conditions, less succinate and more ethanol were formed. The fermentation product ratio remained constant at pH values from 6.0 to 7.4. More succinate was produced when hydrogen was present in the gas phase. *Actinobacillus* sp. 130Z grew at the expense of fumarate and L-malate reduction, with hydrogen as an electron donor. Other substrates such as more-reduced carbohydrates (e.g., D-sorbitol) resulted in higher succinate and/or ethanol production. *Actinobacillus* sp. 130Z contained the key enzymes involved in the Embden-Meyerhof-Parnas and the pentose-phosphate pathways and contained high levels of phosphoenolpyruvate (PEP) carboxykinase, malate dehydrogenase, fumarase, fumarate reductase, pyruvate kinase, pyruvate formate-lyase, phosphotransacetylase, acetate kinase, malic enzyme, and oxaloacetate decarboxylase. The levels of PEP carboxykinase, malate dehydrogenase, and fumarase were significantly higher in *Actinobacillus* sp. 130Z than in *Escherichia coli* K-12 and accounted for the differences in succinate production. Key enzymes in end product formation in *Actinobacillus* sp. 130Z were regulated by the energy substrates.

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Key words Phosphoenolpyruvate · Metabolic fluxes · Fermentation · Carbon dioxide fixation · Fumarate reduction · Succinate · *Actinobacillus* sp. 130Z · *Escherichia coli*

Abbreviations *DTT* Dithiothreitol · *PEP* Phosphoenolpyruvate

Introduction

Many anaerobic and facultatively anaerobic gram-negative bacteria ferment carbohydrates to a mixture of acids (e.g., formate, acetate, lactate, and succinate) as end products (Zeikus 1980; Krieg and Holt 1984). Phosphoenolpyruvate (PEP) is one of the central intermediates during this mixed acid fermentation. It is either converted into pyruvate, resulting in the formation of the fermentation products acetate, formate, ethanol, and lactate, or it is converted into oxaloacetate, resulting in the formation of the end products succinate and propionate (Macy et al. 1978; Clark 1989). Under anoxic conditions, the flux of PEP towards either oxaloacetate or pyruvate varies greatly among the different mixed-acid fermenting bacteria: *Escherichia coli*, for example, forms succinate only as a minor fermentation product [typically 12 mol/100 mol glucose (Wood 1961)] while *Anaerobiospirillum succiniciproducens* forms succinate up to 120 mol/100 mol glucose (Samuelov et al. 1991). Information on the regulation of succinate production by a variety of environmental factors is limited. The anaerobic formation of succinate follows a pathway involving PEP carboxykinase, malate dehydrogenase, fumarase, and fumarate reductase (Clark 1989; Samuelov et al. 1991). In this pathway, PEP carboxykinase plays a key function in the CO_2 fixation necessary for succinate formation. Recently, catabolic PEP carboxykinase from *A. succiniciproducens* has been purified, cloned, and sequenced (Podkovyrov and Zeikus 1993; Laivenieks et al. 1996). Theoretically, succinate can be formed homofermentatively from glucose:

$$\text{Glucose} + 2\ CO_2 + 4[H] \rightarrow 2\ \text{Succinate} + 2\ H_2O$$

From this equation, it can be seen that additional carbon dioxide and electron donors are necessary to achieve a homofermentative succinate fermentation.

Actinobacillus sp. 130Z, a facultative anaerobe (Guettler and Jain, unpublished results), produces succinate as a major end product at much higher levels than *E. coli*. *Actinobacillus* sp. 130Z has also been shown to produce succinate in concentrations higher than those produced by *A. succiniciproducens* (Samuelov et al. 1991; Guettler et al. 1996). We report here on the following features of *Actinobacillus* sp. 130Z fermentation: (1) the effect of the availability of CO_2, the effect of additional electron donors, and the effect of substrates varying in their oxidation/reduction state on the ratio of fermentation products formed, (2) the growth on fumarate plus hydrogen, and (3) the levels of key enzymes involved in the succinate production from glucose in *Actinobacillus* sp. 130Z and *E. coli* K-12 to assess what accounts for differences in the succinate yields of these micro-organisms.

Materials and methods

Organism and growth conditions

Actinobacillus sp. 130Z (ATCC 55618) is maintained at MBI (Lansing, Mich., USA; Guettler et al. 1996). *Escherichia coli* K-12 (ATCC 19020) was obtained from the ATCC (Rockville, Md., USA). Cells were grown in butyl-rubber-stoppered, 158-ml serum vials containing 50 ml medium with CO_2 as the gas phase, unless stated otherwise. Traces of oxygen were removed by passing the gas over heated (370° C) copper filings. The growth medium contained (in g/l of distilled water): yeast extract (5.0), $NaHCO_3$ (10.0), $NaH_2PO_4 \cdot H_2O$ (8.5), and K_2HPO_4 (15.5). In the fumarate and L-malate reduction studies, 50 mM (final concentration) sodium salts of these acids were added to medium. The pH of the medium was adjusted to 7.5 with sodium hydroxide, which resulted in a pH of 7.0 after autoclaving. Separately autoclaved solutions of carbohydrate (final concentration 10 g/l) were added aseptically to the medium after autoclaving. $Na_2S \cdot 9H_2O$ (final concentration 0.02%) was added in order to establish strict anoxic conditions. Media were inoculated with 0.5% (by vol.) samples of cultures grown in the same medium and incubated at 37° C.

Analysis of fermentation products

The carbohydrate concentration was determined with Anthrone reagent (Seifter et al. 1950). L-lactate and D-lactate were determined enzymatically as described by Gutmann and Wahlefeld (1974) using L-lactate dehydrogenase and D-lactate dehydrogenase, respectively. Formate was determined enzymatically as described by Höpner and Knappe (1971). Acetate and ethanol were analyzed by GLC coupled to a flame ionization detector (FID). The culture broth was deproteinized by adding 30 µl/ml 10 M phosphoric acid and centrifuging the samples for 5 min at 15,000 × *g*. Supernatant (1 µl) was analyzed on a Porapak T 80/100 column (1.8 m × 2 mm i.d.; Alltech, Deerfield, Ill., USA). The oven temperature was 175° C, and the flow of the carrier gas (N_2) was 20 ml/min. The injector and detector temperatures were both 190° C. Succinate and fumarate were analyzed by GLC-FID after ethylation. The dicarboxylic acids were esterified to their ethyl ester by the method of Wang et al. (1994). Ethanol:pyridine (5:1; 500 µl) was added to 1 ml of supernatant. The solution was mixed, 100 µl ethylchloroformate was added, and the solution was vortexed for 10 s. Chloroform (1 ml) was added, and the solution was vortexed for another 10 s to extract the diethyl esters into the chloroform phase quantitatively. The chloroform phase (1 µl) was analyzed on a DB-1701 column (15 m × 0.25 mm i.d.) with a 0.25-µm film coating (J & W Scientific, Rancho Cordova, Calif., USA). The temperature program used was: 2 min at 90° C, from 90 to 180° C at 10° C/min, then 30° C/min to 280° C, and 2 min at 280° C. The nitrogen gas flow was approximately 1 ml/min. The injector and detector temperatures were 220 and 260° C, respectively. Hydrogen was detected by gas chromatography (Thiele et al. 1988).

Fermentation balances were calculated based on the averages of three or more anaerobic cultures. Growth was corrected for residual growth on basal medium. Dry cell mass was computed from a curve relating optical density at 660 nm (OD_{660}) to dry weight as previously described (Lynd et al. 1982). An OD_{660} of 1.0 represented 567 mg dry wt./l.

Preparation of cell extracts

Cultures from mid-to-late exponential phase of growth were harvested by centrifugation at 10,000 × *g* for 10 min under an $H_2:N_2$ (5:95, v/v) atmosphere. After centrifugation, the culture medium was decanted in an anaerobic chamber, and the cell pellet was washed once with 50 mM potassium phosphate buffer containing 1 mM dithiothreitol (DTT; pH 7.0). After centrifugation, the pellet was resuspended in 10 ml of the same buffer, and this suspension was rapidly frozen and stored at -80° C until used.

Anoxic conditions were maintained throughout the entire cell extract preparation procedure, and all manipulations were performed under an $H_2:N_2$ (5:95, v/v) atmosphere. DTT (2 mM) and 0.05 mg/ml deoxyribonuclease were added to the cell suspension. After thorough mixing, the suspension was passed once through an N_2-flushed French pressure cell at 140 MPa. The disrupted cells were collected in an N_2-flushed serum vial. The cells were transferred to an anoxic centrifuge tube and centrifuged at 30,000 × *g* for 20 min. The supernatant was removed, stored under an $H_2:N_2$ (5:95, v/v) atmosphere in rubber-stoppered-sealed vials, and used immediately or stored at –80° C until used. Protein was measured by the method of Bradford (1976), with bovine serum albumin as the standard.

Enzyme assays

Unless stated otherwise, all enzyme activities were measured spectrophotometrically under strict anoxic conditions as described previously (Samuelov et al. 1991). All compounds of the reaction mixture except for the cell extract and the substrate were added to an optical glass cuvette (total volume, 1.7 ml). The cuvette was sealed with a soft rubber stopper and was made anoxic by flushing with N_2 for 5 min. The additions of cell extract and anoxic substrate solutions to the anoxic cuvettes were made with a microliter syringe to give a final liquid volume of 1 ml. All activities were measured by standard or modified assay methods. Enzyme-specific activities were calculated from the linear part of the reaction, and values for activity were determined from a minimum of three separate measurements. All assays were performed at 37° C. The reactions were started by the addition of the substrate, and the rates were corrected for endogenous activity. The wavelength and the millimolar extinction coefficient for NAD^+, NADH, $NADP^+$, and NADPH were 340 nm and 6.23 $cm^{-1}\ mM^{-1}$. The wavelength for methyl viologen and benzyl viologen was 578 nm, and the millimolar extinction coefficients were 9.78 and 8.65 $cm^{-1}\ mM^{-1}$, respectively. Fumarate and phosphoenolpyruvate (PEP) formation were recorded at 240 nm, where their extinction coefficients were 2.53 and 1.50 $cm^{-1}\ mM^{-1}$, respectively. One unit (U) of enzyme activity represents the amount of enzyme catalyzing the conversion of 1 µmol of substrate per min into specific products.

Glucose: PEP phosphotransferase (EC 2.7.1.) was assayed in a reaction mixture containing 0.1 M Tris-HCl (pH 8.4), 10 mM $MgCl_2$, 10 mM PEP, 1.0 mM DTT, 1.0 mM $NADP^+$, 10 mM D-

glucose, 3 U glucose-6-phosphate dehydrogenase, and cell extract. Glucokinase (EC 2.7.1.1) and 6-phosphofructokinase (EC 2.7.1.11) were measured as described previously (Samuelov et al. 1991). Fructose-1, 6-bisphosphate aldolase (EC 4.1.2.13), D-glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12; NAD^+-dependent), glucose-6-phosphate dehydrogenase (EC 1.1.1.49; $NADP^+$-dependent), and 6-phosphogluconate dehydrogenase (EC 1.1.1.43; $NADP^+$-dependent) were measured as described by Lamed and Zeikus (1980). Phosphogluconate dehydratase (EC 4.2.1.12) and 6-phospho-2-keto-3-deoxygluconate aldolase (EC 4.1.2.14) were assayed together as described by Van Dijken and Quayle (1977) except that 0.1 M Tris-HCl (pH 7.5) was used instead of 50 mM glycylglycine (pH 7.5). Enolase (EC 4.2.1.11) was determined as described by Spring and Wold (1975).

PEP carboxykinase (EC 4.1.1.49) was monitored in a reaction mixture containing 0.1 M Mes (pH 6.6), 10 mM $MgCl_2$, 5.0 mM $MnCl_2$, 1.0 mM DTT, 10 mM ADP, 75 mM $NaHCO_3$, 0.3 mM NADH, 20 U malate dehydrogenase, and cell extract. This mixture was incubated for 15 min at 37° C to activate PEP carboxykinase, after which the reaction was started by the addition of 10 mM PEP. PEP carboxylase (EC 4.1.1.31) was detected as described by Terada et al. (1991). L-Malate dehydrogenase (EC 1.1.1.37; NADH-dependent) was assayed as described by Samuelov et al. (1991). Fumarase (EC 4.2.1.2) was determined by monitoring the formation of fumarate at 240 nm. The reaction mixture contained 0.1 M Tris-HCl (pH 7.2), 100 mM L-malate, and cell extract. Fumarate reductase (EC 1.3.) activity was measured in a reaction mixture containing 0.1 M Tris-HCl (pH 7.2), 2.0 mM methyl viologen, 5.0 mM fumarate, and cell extract. Before the reaction was started, sodium dithionite was added to the cuvettes until an A_{578} of approximately 1.8 was reached. Methylmalonyl-CoA carboxytransferase (EC 2.1.3.1) activity was assayed as described by Stams et al. (1984).

Pyruvate kinase (EC 2.7.1.40) was assayed as described by Samuelov et al. (1991). Pyruvate formate-lyase (EC 2.3.1.54) was reactivated by a modified method as described by Knappe et al. (1974). Cell extract was incubated for 1 h at 30° C in front of a 60-W lamp in a mixture containing 0.1 M Mops/KOH (pH 8.0), 10 mM potassium oxamate, 0.4 mM $Fe(NH_4)_2(SO_4)_2$, 0.3 mM adenosylmethionine, 0.02 mg/ml FMN, and 9.0 mM DTT. Activity was measured in a reaction mixture containing 0.1 M Mops/KOH (pH 8.0), 0.2 mM CoA, 8.0 mM DTT, 1.0 mM NAD^+, 5.0 mM L-malate, 4 U citrate synthase, 20 U malate dehydrogenase, 20.0 mM pyruvate, and reactivated cell extract. PEP synthase (EC 2.7.9.2) and pyruvate dehydrogenase (EC 1.2.2.2) were measured as described by Cooper and Kornberg (1969) and Snoep et al. (1990), respectively. Pyruvate-ferredoxin oxidoreductase (EC 1.2.7.1) was determined as described by Lamed and Zeikus (1980) and Biesterveld et al. (1994). Pyruvate decarboxylase (EC 4.1.1.1) and pyruvate carboxylase (EC 6.4.1.1) were assayed as described by Lowe and Zeikus (1991) and Schröder et al. (1994), respectively.

Phosphotransacetylase (EC 2.3.1.8) was measured in a reaction mixture containing 0.1 M Tris-HCl (pH 7.8), 0.2 mM CoA, 30 mM NH_4Cl, 1.0 mM DTT, 1.0 mM NAD^+, 5.0 mM L-malate, 4 U citrate synthase, 20 U malate dehydrogenase, 2.0 mM acetylphosphate, and cell extract. Acetate kinase (EC 2.7.2.1) was assayed according to method B as described by Schäfer and Schönheit (1991). Acetaldehyde dehydrogenase (acylating) (EC 1.2.1.10; NADH-dependent) was monitored in a mixture containing 0.1 M Tris-HCl (pH 7.5), 0.3 mM NADH, 1.0 mM DTT, 1.0 mM acetyl-CoA, and cell extract. Alcohol dehydrogenase (EC 1.1.1.1; NADH-dependent) was assayed as described by Samuelov et al. (1991). Formate dehydrogenase (EC 1.2.) activity was measured in a reaction mixture containing 0.1 M potassium phosphate (pH 7.2), 2.0 mM benzyl viologen, 5.0 mM formate, and cell extract. Before the reaction was started, sodium dithionite was added to the cuvettes until an A_{578} of approximately 1.8 was reached. Hydrogenase (EC 1.12.2.1) activity was measured in a reaction mixture containing 0.1 M Tris-HCl (pH 7.8), 2.0 mM benzyl viologen, and cell extract. Sodium dithionite was added to the cuvettes until an A_{578} of approximately 0.2 was reached. The reaction was started by injecting hydrogen gas into the cuvette. Lactate dehydrogenase (EC 1.1.1.28; NADH-dependent) activity was determined in a mixture containing 0.1 M potassium phosphate (pH 6.2), 0.3 mM NADH, 1.0 mM DTT, 10 mM pyruvate, and cell extract.

Malic enzyme (EC 1.1.1.40; $NADP^+$-dependent) was measured in a reaction mixture containing 0.1 M Tris-HCl (pH 8.1), 2.0 mM $MnCl_2$, 2.0 mM NH_4Cl, 1.0 mM DTT, 20 mM Na-arsenate, 1.0 mM $NADP^+$, 10 mM L-malate, and cell extract. Pyruvate formation was confirmed by deproteinizing the samples and analyzing the samples for pyruvate with lactate dehydrogenase. Oxaloacetate decarboxylase (EC 4.1.1.3) was determined in a mixture containing 0.1 M Tris-HCl (pH 7.5), 0.5 mM $MnCl_2$, 1.0 mM DTT, 0.3 mM NADPH, 20 U lactate dehydrogenase, 2.0 mM oxaloacetate, and cell extract.

Citrate lyase (EC 4.1.3.6) was monitored in a mixture containing 0.1 M potassium phosphate (pH 7.2), 2.0 mM $MgCl_2$, 1.0 mM DTT, 0.3 mM NADH, 20 U malate dehydrogenase, 2.0 mM Na_3-citrate, and cell extract. Isocitrate-lyase (EC 4.1.3.1) was measured in a reaction mixture containing 0.1 M potassium phosphate (pH 7.0), 5.0 mM $MgCl_2$, 1.0 mM DTT, 0.3 mM NADH, 20 U rabbit muscle L-lactate dehydrogenase (this enzyme also catalyzes the reduction of glyoxylate), 5.0 mM isocitrate, and cell extract. Succinic semialdehyde dehydrogenase (EC 1.2.1.16; $NADP^+$-dependent) was assayed as described by Jakoby (1962). Aspartate: glutamate transaminase (EC 2.6.1.1) was detected in a mixture containing 0.1 M Tris-HCl (pH 7.5), 10 mM $MgSO_4$, 1.0 mM DTT, 0.3 mM NADH, 2.0 mM α-ketoglutarate, 0.1 mM pyridoxalphosphate, 20 U malate dehydrogenase, 5 mM L-aspartate, and cell extract. Aspartase (EC 4.3.1.1) activity was determined as described by Takagi et al. (1984).

Sorbitol: PEP phosphotransferase (EC 2.7.1.) was assayed in a reaction mixture containing 0.1 M Tris-HCl (pH 8.4), 5.0 mM $MgCl_2$, 10 mM NaF, 10 mM PEP, 1.0 mM DTT, 0.3 mM NADH, 12.5 mM D-sorbitol, 20 U lactate dehydrogenase, and cell extract. Sorbitol kinase (EC 2.7.1.) was measured in a mixture containing 0.1 M Tris-HCl (pH 7.5), 60 mM $MgCl_2$, 1.0 mM DTT, 5.0 mM D-sorbitol, 2.0 mM ATP, 1.0 mM NAD^+, 5.0 mM D-sorbitol, and cell extract; the sorbitol-6-phosphate formed is converted by the sorbitol-6-phosphate dehydrogenase activity present in the cell extract (see results). Sorbitol-6-phosphate dehydrogenase (EC 1.1.1.140; NAD^+-dependent) and sorbitol dehydrogenase (EC 1.1.1.14) were determined as described by Novotny et al. (1984) and Ng et al. (1992), respectively.

Chemicals

All chemicals were of analytical grade. Enzymes and coenzymes were obtained from Sigma (St. Louis, Mo., USA). Gasses were purchased from AGA Chemicals.

Results

Glucose fermentation products

During fermentation of glucose, *Actinobacillus* sp. 130Z produced acetate, formate, and succinate as the major fermentation products. Small amounts of ethanol were also detected. No L- or D-lactate was detected (not even in non-buffered medium), resulting in a pH of 5.2 at the end of the fermentation, after which growth ceased. Propionate, 2,3-butanediol, 2,3-butanedione (diacetyl), 3-hydroxy-2-butanone (acetoin), acetone, 2-propanol, 1-butanol, butyrate, or caproate formation was not detected.

Actinobacillus sp. 130Z grew at initial medium pH values between 6.0 and 7.4. The optimal pH for growth was 7.0. Remarkably, the pH did not affect the quantities of the fermentation products formed (data not shown). How-

ever, cell growth was affected adversely at low pH, possibly reflecting higher maintenance requirements at lower pH values.

Effect of carbon dioxide on the fermentation balance

The formation of succinate as a fermentation product requires CO_2 fixation. Therefore, the effect of the addition of different amounts of CO_2 to the medium on the D-glucose fermentation balance was studied (Table 1). Exact amounts of carbon dioxide were supplied as poorly soluble magnesium carbonate, which dissolved during the fermentation. No growth was observed at $MgCO_3$ concentrations lower than 5.0 mM. The amount of acetate formed was independent of the CO_2 concentration in the medium. In contrast, the amounts of succinate and ethanol formed were largely affected by the availability of CO_2 (Table 1). A direct relationship was observed between the amounts of CO_2 in the medium and the succinate production. In contrast, ethanol production decreased with an increase in CO_2 in the medium.

When the carbon and the O/R balances of these cultures were calculated using the established values for succinate of 4 mol carbon/mol product and an O/R value of +1 (based on the structure formula of succinate of $C_4H_6O_4$), dramatic deviations from the ideal value of 1.0 were observed. Much more carbon was recovered than had been added in the form of glucose and, in the case of the O/R balance, values of up to 25 were found, indicating the formation of end products that are much more oxidized than reduced. However, the formation of succinate requires the fixation of 1 mol of CO_2 per mol of succinate (see also below), which explains the high carbon recovery values. Furthermore, in contrast to glucose, CO_2 does not have an O/R value of 0 but of +2, resulting in an apparent imbalance of the O/R balance when CO_2 fixation takes place. Since the amounts of CO_2 and HCO_3^- at the beginning and end of a fermentation are difficult to quantify (these concentrations depend on many variables such as pH and pressure), we recalculated the carbon and the O/R balances using values for succinate; these values were corrected for CO_2 fixation related to the formation of succinate. Thus, succinate was counted as having 3 mol carbon/mol product, and a structure formula of $C_3H_6O_2$ [succinate – (CO_2)] was used to calculate an O/R value for succinate of –1. Using these values, a much more balanced fermentation profile was observed (Tables 1–3).

Use of hydrogen and fumarate as electron donors and acceptors

Succinate is a highly reduced fermentation product using four electrons per molecule formed (Clark 1989). Therefore, the effect of the addition of an extra electron donor, i.e., hydrogen, on the glucose fermentation in the presence of high concentrations of CO_2 was studied (Table 2). The addition of hydrogen to the gas phase resulted in a significant increase in the succinate production. This coincided with an electron recovery far higher than 1.0, indicating the incorporation of electrons derived from H_2, and a lower value for the O/R balance, indicating the formation of more-reduced end products.

Potentially, formate can act as a source of both electrons and carbon dioxide. However, the addition of formate to the growth medium did not affect the fermentation balance, nor was it shown to be consumed by glucose-fermenting cultures (data not shown). No formate-hydrogen lyase activity was detectable in *Actinobacillus* 130Z cells.

Table 1 Influence of the availability of CO_2 on the glucose fermentation balance of *Actinobacillus* sp. 130Z. Cells were grown at 37° C in a medium (pH 7.0) containing glucose (10 g/l), yeast extract (5 g/l), $NaH_2PO_4 \cdot H_2O$ (8.5 g/l), K_2HPO_4 (15.5 g/l), and $MgCO_3$ as indicated with N_2 as the gas phase

End products	mol product/100 mol glucose at a CO_2[a] ratio of:			
	10	25	50	100
Cells[b]	108	111	110	107
Succinate	19	32	50	69
Acetate	81	83	82	84
Formate	120	112	102	88
Ethanol	71	56	41	18
Carbon recovered[c]	0.98	1.00	1.01	1.01
Electrons recovered	1.06	1.07	1.08	1.07
O/R balance[c]	0.75	0.78	0.77	0.84
Succinate product ratio[d]	0.13	0.23	0.41	0.68

[a] Mol of CO_2 per 100 mol of glucose
[b] Cell carbon was calculated with $CH_2O_{0.5}N_{0.2}$ (Samuelov et al. 1990)
[c] The balance was corrected for CO_2 fixation (see text)
[d] Equals succinate / [acetate + ethanol]

Table 2 Influence of hydrogen on the glucose fermentation balance of *Actinobacillus* sp. 130Z. Cells were grown at 37° C in a medium (pH 7.0) containing glucose (10 g/l), yeast extract (5 g/l), $NaHCO_3$ (10 g/l), $NaH_2PO_4 \cdot H_2O$ (8.5 g/l), and K_2HPO_4 (15.5 g/l)

End products	mol product/100 mol glucose with a gas phase of:		
	100% N_2	5% H_2, 95% N_2	100% H_2
Cells[a]	113	119	120
Succinate	80	97	112
Acetate	67	63	65
Formate	66	64	54
Ethanol	8	11	12
Carbon recovered[b]	0.95	1.04	1.09
Electrons recovered	1.02	1.13	1.23
O/R balance[b]	0.69	0.54	0.50
Succinate product ratio[c]	1.07	1.31	1.45

[a] Cell carbon was calculated with $CH_2O_{0.5}N_{0.2}$ (Samuelov et al. 1990)
[b] The balance was corrected for CO_2 fixation (see text)
[c] Equals succinate / [acetate + ethanol]

Table 3 Fermentation balances of *Actinobacillus* sp. 130Z grown anaerobically on various carbohydrates. Cells were grown at 37° C in a medium (pH 7.0) containing carbohydrate (10 g/l), yeast extract (5 g/l), $NaHCO_3$ (10 g/l), $NaH_2PO_4 \cdot H_2O$ (8.5 g/l), and K_2HPO_4 (15.5 g/l) with CO_2 as the gas phase

End products	mol product/100 mol saccharide with the substrates:						
	D-Mannitol	D-Sorbitol	D-Glucose	D-Fructose	D-Arabitol	L-Arabinose	D-Xylose
Molecular formula	$C_6H_{14}O_6$	$C_6H_{14}O_6$	$C_6H_{12}O_6$	$C_6H_{12}O_6$	$C_5H_{12}O_5$	$C_5H_{10}O_5$	$C_5H_{10}O_5$
Cells[a]	104	105	116	116	77	62	68
Succinate	91	75	75	64	103	42	44
Acetate	39	41	88	87	27	51	67
Formate	64	76	78	84	33	33	54
Ethanol	31	48	7	12	10	0	2
C-recovery[b]	0.97	0.97	1.02	0.98	0.99	0.65	0.78
H-recovery	1.00	1.01	1.07	1.04	1.01	0.69	0.81
O/R balance[b]	1.07	1.03	0.88	0.95	1.08	0.79	1.13
Succinate product ratio[c]	1.3	0.84	0.79	0.65	2.78	0.82	0.64

[a] Cell carbon was calculated with $CH_2O_{0.5}N_{0.2}$ (Samuelov et al. 1990)
[b] The balance was corrected for CO_2 fixation (see text)
[c] Equals succinate / [acetate + ethanol]

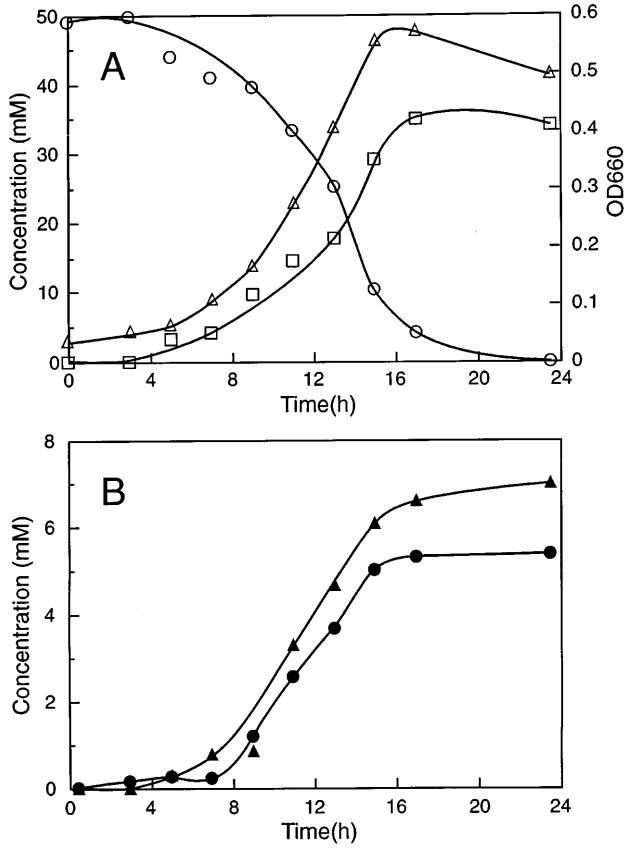

Fig. 1 A, B Growth of *Actinobacillus* sp. 130Z at the expense of fumarate reduction. The medium (pH 7.0) contained Na_2-fumarate (50 mM), yeast extract (5 g/l), $NaHCO_3$ (10 g/l), $NaH_2PO_4 \cdot H_2O$ (8.5 g/l), and K_2HPO_4 (15.5. g/l) with H_2 as the gas phase. **A** Succinate production during growth. △ OD_{660}, ○ fumarate, □ succinate. **B** Accumulation of acetate and ethanol. ▲ Acetate, ● formate

Since *Actinobacillus* sp. 130Z used hydrogen gas as an electron source during glucose fermentation (Table 2), we examined if this bacterium could grow on fumarate plus hydrogen as energy sources. *Actinobacillus* sp. 130Z grew anaerobically on fumarate and hydrogen. In the absence of hydrogen, no growth was observed. Figure 1 shows the time course of growth and end product formation during fumarate reduction. In addition to succinate (Fig. 1 a), small amounts of acetate and formate also accumulated (Fig. 1 b). No ethanol formation was detected. *Actinobacillus* sp. 130Z also grew anaerobically on L-malate and hydrogen. Hydrogen was depleted from the headspace at the end of growth.

Fermentation balances with other-carbohydrates

Since the availability of additional electrons such as hydrogen had a significant effect on the amount of succinate formed (Table 2), more-reduced carbohydrates such as D-sorbitol and D-mannitol were used as the substrate to examine the differences in the fermentation products formed (Table 3). The fermentation balances for several pentoses of different oxidation states were also determined. The use of the more-reduced sugars (D-mannitol, D-sorbitol, and D-arabitol) resulted in the formation of higher amounts of the more-reduced end products succinate and ethanol (Table 3).

Enzyme activities involved in end product formation from glucose in *Actinobacillus* sp. 130Z and *Escherichia coli* K-12

To gain insight as to which enzyme activities are the most important for the formation of the fermentation product succinate, we compared the enzyme activity levels of

Table 4 Comparison of enzyme activities involved in end product formation in *Actinobacillus* sp. 130Z. and *Escherichia coli* K-12 grown anaerobically. Cells were grown at 37° C in a medium (pH 7.0) containing glucose (10 g/l), yeast extract (5 g/l), $NaHCO_3$ (10 g/l), $NaH_2PO_4 \cdot H_2O$ (8.5 g/l), and K_2HPO_4 (15.5 g/l) with CO_2 as the gas phase

Enzyme	Specific activity [nmol min^{-1} (mg protein)$^{-1}$]	
	Actinobacillus sp. 130Z	*E. coli* K-12
Glucose: PEP phosphotransferase	21	44
Glucokinase	200	89
6-Phosphofructokinase	900	890
Fructose-1,6-diphosphate aldolase	1,800	960
Glyceraldehyde-3-phosphate dehydrogenase	810	850
Glucose-6-phosphate dehydrogenase	120	140
Phosphogluconate dehydrogenase	360	120
Phosphogluconate dehydratase / 6-phospho-2-keto-3-deoxygluconate aldolase	11	12
Enolase	8,300	3,500
PEP carboxykinase	4,700	140
PEP carboxylase	10	140
L-Malate dehydrogenase	2,100	160
Fumarase	8,600	450
Fumarate reductase	1,200	1,100
Methylmalonyl-CoA carboxytransferase	< 1	< 1
Pyruvate kinase	1,100	1,400
Pyruvate-formate lyase	390	1,400
PEP synthase	< 1	< 1
Pyruvate: ferredoxin oxidoreductase	< 1	< 1
Pyruvate dehydrogenase	< 1	< 1
Pyruvate decarboxylase	< 1	< 1
Pyruvate carboxylase	< 1	< 1
Phosphotransacetylase	240	2,100
Acetate kinase	3,200	6,500
Acetaldehyde dehydrogenase (CoA-acetylating)	11	130
Alcohol dehydrogenase	17	75
Formate dehydrogenase	12	5
Hydrogenase	11	93
Lactate dehydrogenase	9	560
Malic enzyme	360	< 1
Oxaloacetate decarboxylase	75	< 1
Citrate lyase	680	< 1
Isocitrate lyase	6	25
Succinic semialdehyde dehydrogenase	< 1	13
Aspartate: glutamate transaminase	130	96
Aspartase	< 5	180

Actinobacillus sp. 130Z with those of *E. coli.* Although the enzymes involved in end product formation in *E. coli* have been reported (e.g., Gray et al. 1966; Brown et al. 1977; Lorowitz and Clark 1982), as far as we know, all key enzymes have not been determined in one study.

The fermentation of glucose by *E. coli* K-12 under the same growth conditions as those for *Actinobacillus* sp. 130Z resulted in the formation of the end products (in parentheses: the mol end product formed/100 mol glucose) succinate (12), acetate (78), formate (152), ethanol (53), and D-lactate (31). Table 4 shows the enzyme activities present in extracts of glucose-fermenting cells of *Actinobacillus* sp. 130Z and *E. coli* K-12. Both micro-organisms contained the key enzymes of the Embden-Meyerhof-Parnas pathway (i.e., 6-phosphofructokinase, fructose-1, 6-bisphosphate aldolase, and glyceraldehyde-3-phosphate dehydrogenase) and those of the pentose-phosphate pathway (i.e., glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase). The levels of these enzyme activities were comparable in both micro-organisms. A very low activity for the key enzymes of the Entner-Doudoroff pathway (i.e., "phosphogluconate dehydratase/6-phospho-2-keto-3-deoxygluconate aldolase") was also detected in both micro-organisms.

Actinobacillus sp. 130Z and *E. coli* K-12 contained almost the same set of enzymes involved in the formation of end products from PEP (Table 4). The major differences were the presence of malic enzyme and oxaloacetate decarboxylase activity in *Actinobacillus* sp. 130Z and the

presence of aspartase activity in *E. coli*. However, the levels of these enzymatic activities varied greatly in these two strains. In *Actinobacillus* sp. 130Z, much higher enzyme activities involved in the conversion of PEP to succinate (e.g., PEP carboxykinase, L-malate dehydrogenase, and fumarase) were present than in *E. coli*. Fumarate reductase activity was comparable in extracts of both organisms. On the other hand, in *E. coli* much higher enzyme activity levels involved in the formation of pyruvate-derived end products (e.g., pyruvate-formate lyase, phosphotransacetylase, acetaldehyde dehydrogenase, and lactate dehydrogenase) were observed than in *Actinobacillus* sp. 130Z. Also the pyruvate kinase and PEP carboxylase activities observed were much higher in *E. coli* than in *Actinobacillus* sp. 130Z (Table 4).

Effect of energy source on enzyme levels involved in end product formation in *Actinobacillus* sp. 130Z

The activity levels of enzymes involved in fermentation product formation were also determined in cell extracts of *Actinobacillus* sp. 130Z grown anaerobically on D-sorbitol and on fumarate and hydrogen (Table 5).

D-sorbitol-fermenting cells contained sorbitol: PEP phosphotransferase [7.9 nmol min^{-1} (mg protein)$^{-1}$] and sorbitol-6-phosphate dehydrogenase activity [1.0 U (mg protein)$^{-1}$]. Sorbitol kinase and sorbitol dehydrogenase activities were not detected, indicating that *Actinobacillus* sp. 130Z metabolizes D-sorbitol via the same pathway as *E. coli* (Lin 1987).

The critical enzyme leading to the formation of succinate in *Actinobacillus* sp. 130Z, PEP carboxykinase, was present at high levels [3–5 U (mg protein)$^{-1}$] in extracts grown on different carbon sources (Tables 4 and 5). Extracts of cells grown in the presence of 5 mM $MgCO_3$ as the sole source of carbon dioxide contained a comparable PEP carboxykinase activity [3.8 U (mg protein)$^{-1}$]. Also high activities of the other enzymes involved in succinate formation from PEP (i.e., malate dehydrogenase, fumarase, and fumarate reductase) were present in cell extracts of glucose- and D-sorbitol-grown cells. In fumarate-reducing cells, a six- to eightfold lower malate dehydrogenase activity was observed as compared to that in the carbohydrate-fermenting cells (Table 5). In contrast, extracts of fumarate-reducing cells contained an elevated hydrogenase activity. Malic enzyme activity and oxaloacetate decarboxylase activity were present in all extracts at comparable levels.

Pyruvate kinase activity was present in equally high levels in all tested cell extracts (Tables 4 and 5). Pyruvate was further degraded via pyruvate formate-lyase. The detection of pyruvate formate-lyase activity could not be coupled to formate dehydrogenase because the cell extract contained high concentrations of formate that interfered with the assay. Calculations showed that the formate concentration in the cell was as high as the formate concentration of the supernatant. Extracts of glucose- and fumarate-grown cells contained low alcohol dehydrogenase and acetaldehyde dehydrogenase activities, while extracts of D-sorbitol-grown cells contained high levels of this enzyme (Table 5).

Table 5 Regulation of PEP metabolic enzymes by growth substrate in *Actinobacillus* sp. 130Z. Cells were grown at 37° C in a medium (pH 7.0) containing yeast extract (5 g/l), $NaHCO_3$ (10 g/l), $NaH_2PO_4 \cdot H_2O$ (8.5 g/l), and K_2HPO_4 (15.5 g/l) with CO_2 as the gas phase. In the case of D-sorbitol, this medium was supplemented with 10 g/l D-sorbitol and CO_2 was used as the gas phase. In the case of fumarate reduction, this medium was supplemented with 50 mM Na_2-fumarate and H_2 was used as the gas phase

Enzyme	Specific activity (nmol min^{-1} [mg protein]$^{-1}$) in extracts of cells grown on:	
	D-Sorbitol	Fumarate + H_2
Enolase	9,100	5,500
PEP carboxykinase	3,300	3,000
PEP carboxylase	12	< 1
Malate dehydrogenase	2,900	360
Fumarase	13,000	9,500
Fumarate reductase	960	1,200
Pyruvate kinase	1,200	1,000
Pyruvate-formate lyase	630	450
Phosphotransacetylase	340	350
Acetate kinase	3,400	2,800
Acetaldehyde dehydrogenase (CoA acetylating)	430	18
Alcohol dehydrogenase	2,700	9
Formate dehydrogenase	10	37
Hydrogenase	12	130
Lactate dehydrogenase	10	100
Malic enzyme	370	250
Oxaloacetate decarboxylase	100	60
Citrate lyase	1,100	380
Isocitrate lyase	3	17
Succinic semialdehyde dehydrogenase	1	< 1
Aspartate: glutamate transaminase	150	92
Aspartase	< 5	13

Discussion

This report describes the effect of various environmental conditions on the succinate fermentation ratio and the enzyme activity levels involved in end product formation in *Actinobacillus* sp. 130Z. The availability of CO_2 and the oxidation/reduction state of carbohydrates fermented affected the succinate product ratio as can be predicted based on carbon and electron balances. *Actinobacillus* sp. 130Z uses hydrogen as an electron donor during both glucose fermentation and anaerobic growth on fumarate. The levels of enzyme activities present in *Actinobacillus* sp. as compared to those in *Escherichia coli* provide a biochemical explanation for the differences in succinate yields. Key enzymes (hydrogenase, malate dehydrogenase, and alcohol dehydrogenase) are regulated as can be predicted from the end product profile formed after growth on the energy substrates D-sorbitol and fumarate plus hydrogen.

Actinobacillus sp. 130Z fermented a wide range of carbohydrates (Table 3; Guettler and Jain, unpublished results). So far, the fermentative metabolism of only one other *Actinobacillus* sp., *A. actinomycetemcomitans*, has been studied, although enzyme activities responsible for oxaloacetate synthesis have not been reported (Ohta et al. 1989). High activities of key enzymes of both the Embden-Meyerhof-Parnas and the pentose-phosphate pathways were detected in *Actinobacillus* sp. 130Z (Table 4), indicating that these pathways are simultaneously active during hexose fermentation by this micro-organism. Both these pathways are also active simultaneously during glucose fermentation in *E. coli* and in *A. actinomycetemcomitans* (Table 4; Ogino et al. 1980; Ohta et al. 1989). Very low activities of the enzymes involved in the Entner-Douderhoff pathway were detected, suggesting that this pathway does not play a major role during hexose fermentation.

Phosphoenolpyruvate (PEP) is a central intermediate during mixed acid fermentation (Macy et al. 1978; Clark 1989; Samuelov et al. 1991). Anaerobically, PEP is converted by two enzymes in *Actinobacillus* sp. 130Z, PEP carboxykinase and pyruvate kinase (Tables 4 and 5). PEP carboxykinase is a CO_2-fixing enzyme that converts PEP to oxaloacetate. Since *Actinobacillus* sp. 130Z does not contain methylmalonyl-CoA carboxytransferase activity (Table 4), which is in agreement with this micro-organism's not forming propionate as an end product, succinate is the only fermentation product formed via this pathway. Pyruvate kinase converts PEP to pyruvate, which in *Actinobacillus* sp. 130Z is converted to the end products acetate, formate, and ethanol. Both PEP carboxykinase and pyruvate kinase appeared to be constitutive in *Actinobacillus* sp. 130Z (Tables 4 and 5). The presence of a constitutive PEP carboxykinase in this micro-organism is remarkable since up to now only an inductive expression of catabolic PEP carboxykinases has been reported, with the levels of PEP carboxykinase responding to the medium pH (Samuelov et al. 1991) and the availability of CO_2 (Caspari and Macy 1983). PEP carboxykinase in *E. coli* is controlled by catabolite repression (Shrago and Shug 1969).

The flux of PEP in either pathway in *Actinobacillus* sp. 130Z is, thus, not influenced by the enzyme levels of these PEP-converting enzymes since both enzymes are constitutive. However, the in vivo activity of these enzymes is determined by the intracellular concentrations of the different substrates of these enzymes. Since PEP carboxykinases have a high K_M for HCO_3^- (15–20 mM; Podkovyrov and Zeikus 1993), the metabolic fate of PEP is expected to be severely affected by the availability of HCO_3^-. Indeed, in the presence of limiting concentrations of CO_2, *Actinobacillus* sp. 130Z formed less succinate and more ethanol (Table 1). The formation of the fermentation product ethanol from PEP requires the incorporation of the same amount of electrons as does the formation of succinate, but the biosynthesis of ethanol does not require the fixation of CO_2 (Clark 1989). Surprisingly, the pH of the medium, which has a large effect on the CO_2/HCO_3^- ratio, did not affect the ratio of fermentation products formed (data not shown). This is in contrast to the fermentation balance of *Anaerobiospirillum succiniciproducens*, in which a low medium pH results in an increase of the flux of PEP towards more succinate formation (Samuelov et al. 1991). However, the external pH affects the internal CO_2 concentration only when it also affects the cytoplasmic pH. Two classes of micro-organisms can be recognized in this respect (Booth 1985). Most aerobic and facultatively anaerobic bacteria stringently regulate the internal pH. For instance, the cytoplasmic pH of *E. coli* is unaffected by large variations in the medium pH (Booth 1985). On the other hand, many strictly anaerobic bacteria show a decrease of the cytoplasmic pH with a decrease in the medium pH (Gottwald and Gottschalk 1985; Goodwin and Zeikus 1987). This difference in regulation of the cytoplasmic pH might, therefore, explain the differences in the effect of the medium pH on the ratio of fermentation products formed by the facultatively anaerobic *Actinobacillus* sp. 130Z and the strictly anaerobic *A. succiniciproducens.*

When electron sources are able to serve as a substrate, the incorporation of additional electron sources in the growth medium should result in the formation of higher amounts of the more-reduced fermentation products succinate and/or ethanol to recycle the NAD(P)H formed. *Actinobacillus* sp. 130Z consumed electrons from hydrogen during glucose fermentation (Table 2). Under these conditions, higher amounts of the fermentation product succinate were formed, while hardly any more ethanol was formed (Table 2). However, when more electrons were added to the medium in the form of more-reduced substrates than glucose, formation of increased concentrations of succinate or ethanol or both depended on the growth substrate (Table 3). For instance, the fermentation of D-sorbitol resulted in the formation of higher amounts of ethanol, which coincided with higher acetaldehyde dehydrogenase and alcohol dehydrogenase activity levels in extracts of these cells (Table 5). However, the amount of succinate produced was not affected when compared with

glucose (Table 3). On the other hand, with D-arabitol as the substrate, the production of succinate, but not of ethanol, was significantly increased (Table 3). These results suggest that not only the availability of reducing equivalents but also the form in which these electrons are available affects the formation of succinate.

Actinobacillus sp. 130Z grew at the expense of fumarate and L-malate reduction (Fig. 1). In contrast to *Wolinella succinogenes*, which also grows at the expense of fumarate reduction (Kafkewitz and Goodman 1974; Kröger et al. 1992), *Actinobacillus* sp. 130Z does not grow at the expense of aspartate and asparagine reduction, coinciding with the absence of aspartase activity in *Actinobacillus* sp. 130Z (Table 4). Cells grown on fumarate contained a six- to eightfold lower malate dehydrogenase activity level as compared to cells grown at the expense of carbohydrate fermentation (Table 5). This suggests that high malate dehydrogenase activity levels are essential for the production of large amounts of the end product succinate during carbohydrate fermentation.

Actinobacillus sp. 130Z also contains high malic enzyme and oxaloacetate decarboxylase activities (Tables 4 and 5). It is not clear if these activities are catalyzed by two separate enzymes or by one protein (Guagliardi et al. 1988). The oxaloacetate and pyruvate branches of end product formation are linked by the action of these two enzymatic activities. That one or both of these enzymes play a role in vivo can be concluded from the significant amounts of acetate and formate produced during growth at the expense of fumarate reduction (Fig. 1 b).

The formation of oxaloacetate from PEP can be catalyzed by two enzymes: PEP carboxykinase and PEP carboxylase. While in *Actinobacillus* sp. 130Z the PEP carboxykinase activity is 480-fold higher than the PEP carboxylase activity, in *E. coli* the levels of these two enzyme activities are similar. Moreover, PEP carboxykinase activity is 34-fold higher in *Actinobacillus* sp. 130Z than in *E. coli*. Also the levels of the enzymes malate dehydrogenase and fumarase present in the succinate formation pathway are, respectively, 13- and 19-fold higher in *Actinobacillus* sp. 130Z than in *E. coli*. The fumarate reductase activity was comparable in both micro-organisms. High levels of these four enzymes involved in succinate formation were also present in *A. succiniciproducens* (Samuelov et al. 1991), suggesting that high levels of these four enzyme activities are necessary for the formation of succinate. Recently Millard et al. (1996) have overproduced either PEP carboxykinase or PEP carboxylase in *E. coli* at levels four fold higher than the level of PEP carboxykinase activity present in *Actinobacillus* sp. 130Z. Remarkably, only the overproduction of PEP carboxylase resulted in increased succinate yields, while the overproduction of PEP carboxykinase did not result in an enhanced succinate production (Millard et al. 1996).

The phosphotransferase system responsible for carbohydrate import also competes for intracellular PEP (Patnaik and Liao 1994). Extracts of *Actinobacillus* sp. 130Z and *E. coli* contain both glucokinase and glucose: PEP phosphotransferase activity (Table 4). However, in *E. coli* it has been established that only the phosphotransferase is responsible for the uptake of glucose (Fraenkel 1987). *Actinobacillus* sp. 130Z probably also uses the phosphotransferase system as the major import system for carbohydrates, as is indicated by the fact that extracts of D-sorbitol-fermenting cells contain only sorbitol phosphotransferase activity. This suggests that the competition for PEP is not a major factor affecting the flux of PEP to succinate.

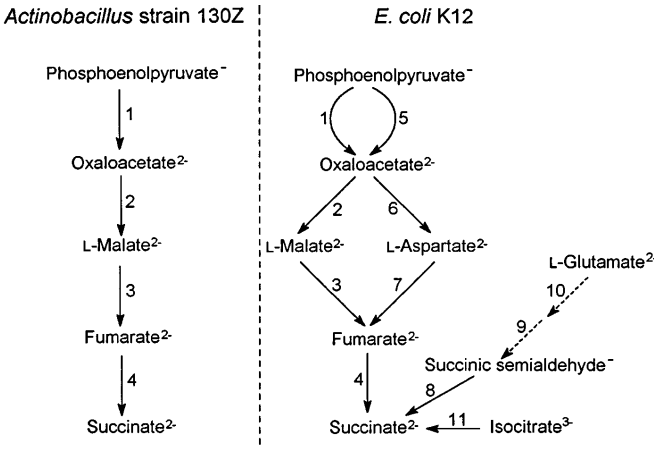

Fig. 2 Pathways for the formation of the fermentation product succinate in *Actinobacillus* sp. 130Z and *Escherichia coli* K-12: *1* PEP carboxykinase, *2* malate dehydrogenase, *3* fumarase, *4* fumarate reductase, *5* PEP carboxylase, *6* aspartate: glutamate transaminase, *7* aspartase, *8* succinic semialdehyde dehydrogenase, *9* γ-aminobutyrate: glutamate transaminase, *10* glutamate decarboxylase, and *11* isocitrate lyase

Figure 2 summarizes the pathways used for succinate formation in *Actinobacillus* sp. 130Z and *E. coli* K-12. From the levels of the enzyme activities present in fermenting cells of *Actinobacillus* sp. 130Z (Tables 4 and 5) it can be concluded that this micro-organism forms succinate mainly via one single pathway (Fig. 2). On the other hand, in *E. coli* as many as six pathways involved in the formation of succinate can be recognized (Fig. 2; Table 4). *E. coli* contains similar levels of PEP carboxykinase and PEP carboxylase (Table 4), both of which convert PEP into oxaloacetate. *E. coli* also contains high aspartase and aspartate transaminase activities at levels comparable to those of fumarase and malate dehydrogenase (Table 4). These two enzymatic activities form an alternative pathway for the formation of fumarate in *E. coli* (Fig. 2). Besides these four pathways, succinate can also be formed by the action of the enzymes succinic semialdehyde dehydrogenase and isocitrate lyase (Table 4; Fig. 2). That at least one of these last two enzymes indeed contributes to in vivo succinate formation in *E. coli* can be concluded from the study of Alam and Clark (1989), who have found that a fumarate-reductase-negative *E. coli* mutant still produces succinate at concentrations of 15–40% as compared to the concentration of the wild-type. These data suggest that succinate is formed as a side-product during carbohydrate fermentation in *E. coli*.

This study shows that the levels of CO_2, the source of electrons, and the levels of the enzymes involved in the

different pathways determines the succinate product ratio. Since *Actinobacillus* sp. 130Z is able to use hydrogen as an electron source, this strain, unlike *E. coli* or *A. succiniciproducens*, may allow homo-succinate fermentation.

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