Effect of Bicarbonate on the Growth of *Actinobacillus actinomycetemcomitans* in Anaerobic Fructose-limited Chemostat Culture

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The effect of bicarbonate on the growth and product formation by a periodontopathic bacterium, *Actinobacillus actinomycetemcomitans*, was examined in an anaerobic chemostat culture with fructose as the limiting nutrient. The chemostat cultures were run at dilution rates between 0.04 and 0.25 h⁻¹ and the maximum growth yield ($Y_{\text{fructose}}^{\text{max}}$) was estimated to be 40.3 and 61.7 g dry wt (mol fructose)⁻¹ in the absence and presence of bicarbonate, respectively. The major fermentation products in the absence of bicarbonate were formate, acetate, ethanol and succinate, with small amounts of lactate. The addition of bicarbonate to the medium resulted in a marked decrease in ethanol production and in a significant increase in succinate production. Washed cells possessed activity for the cleavage of formate to CO₂ and H₂, which seemed to play a role in supplying CO₂ for the synthesis of succinate in the absence of bicarbonate. The study of enzyme activities in cell-free extracts suggested that fructose was fermented by the Embden–Meyerhof–Parnas pathway. The values of $Y_{\text{ATP}}^{\text{max}}$ and the efficiency of ATP generation (ATP-Eff) during fructose catabolism were estimated and higher values were obtained for the culture in the presence of bicarbonate: 20.2 g dry wt (mol ATP)⁻¹ and 3.0 mol ATP (mol fructose)⁻¹, respectively, versus $Y_{\text{ATP}}^{\text{max}} = 15.1$ and ATP-Eff = 2.7 in the absence of bicarbonate.

INTRODUCTION

Actinobacillus actinomycetemcomitans is a Gram-negative, facultatively anaerobic, small coccobacillus (Phillips, 1984). In the human oral cavity the organism is isolated mainly from dental plaque and periodontal pockets (Slots *et al.*, 1980). *A. actinomycetemcomitans* possesses a number of pathogenic properties, including the ability to produce leukotoxin (Tsai *et al.*, 1984; Ohta *et al.*, 1987), lymphocyte suppressive factor (Shenker *et al.*, 1982*a*), a biologically active lipopolysaccharide (Kiley & Holt, 1980; Iino & Hopps, 1984), and fibroblast inhibitory factor (Shenker *et al.*, 1982*b*). Although clinically significant, little is known about the growth characteristics and basic physiology of the organism other than its requirement for CO₂ for aerobic growth (Thjotta & Sydnese, 1951; Holm, 1954; Slots, 1982) and its fermentation of several carbohydrates (Pulverer & Ko, 1970; Slots, 1982). Among these characteristics, it is of interest to note the CO₂-stimulated growth of *A. actinomycetemcomitans* since the oral environment contains high concentrations of bicarbonate, the hydrated form of CO₂ (Jenkins, 1978). The CO₂-dependent or -stimulated fermentation of carbohydrate has been reported with several facultative or obligate anaerobes, including *Cytophaga succinicans* (Anderson & Ordal, 1961), *Bacteroides ruminicola* (White *et al.*, 1962), *B. fragilis* (Macy *et al.*, 1978), *Actinomyces naeslundii* (Buchanan & Pine, 1967) and *Capnocytophaga ochracea* (Kapke *et al.*, 1980), and with phototrophic, purple, nonsulphur bacteria (Schultz & Weaver, 1982; Hiraishi, 1988*a*). In the metabolism of these bacteria, CO₂ functions by providing, through condensation with phosphoenolpyruvate or pyruvate, C₄ dicarboxylic acids which can serve as acceptors for the electrons generated in carbohydrate degradation.

Abbreviations: ATP-Eff, efficiency of ATP generation; D, dilution rate; PEP, phosphoenolpyruvate.

The purpose of the present study was to examine in more detail the anaerobic, fermentative growth of *A. actinomycetemcomitans* in fructose-limited chemostat cultures, particularly with regard to the effect of bicarbonate.

METHODS

Organism and medium. *A. actinomycetemcomitans* 301-b, used in this study, was isolated from a patient with rapidly progressive periodontitis (Ohta *et al.*, 1986, 1987). The growth medium (AA medium) contained the following ingredients (g l^{-1}): Na_2SO_4 (0.5), KH_2PO_4 (0.2), $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (0.4), NaCl (1.2), NH_4Cl (0.3), KCl (0.3), CaCl_2 (0.11), Bacto yeast extract (2.0), resazurin (0.001), and Tris (6.05). The trace element solution described previously (Ohta & Gottschal, 1988) was also added. After the medium had been adjusted to pH 7.3–7.4 and autoclaved, separately autoclaved solutions of fructose (final concentration 1.8 g l^{-1}) were added to the medium aseptically. To examine the effect of bicarbonate, an autoclaved solution of NaHCO_3 (final concentration 4 g l^{-1}) was added to the medium. This bicarbonate concentration had been found to be saturated by batch culture experiments (unpublished results).

Continuous culture. The anaerobic chemostat cultures were performed as described previously (Laanbroek, 1978; Ohta & Gottschal, 1988). The system was maintained under a stream (300 ml h^{-1}) of anaerobic gases (100% N_2 or 90% $\text{N}_2/10\% \text{CO}_2$) which were freed of traces of oxygen by passage over hot copper filings; N_2 was used for the cultures without the addition of bicarbonate and N_2/CO_2 for the cultures supplemented with bicarbonate. Cultures were kept at 37°C and at pH 7.0 with automatic additions of 2 M-NaOH or 1 M-HCl. The turbidity of cultures was measured in a 1 cm cuvette in an LKB Novaspec 4049 spectrophotometer. The purity of the cultures was checked every day by observing the cells microscopically or inoculating culture samples on trypticase soy broth agar (BBL) and incubating them aerobically; *A. actinomycetemcomitans* exhibited scant growth on this medium in air unless CO_2 was supplemented (Slots, 1982).

Chemical analysis. Acid end products (formate, acetate, lactate and succinate) and ethanol were determined by gas-liquid chromatography (GC) using Hitachi 163 and Shimadzu GC-R1A gas chromatographs. Samples were prepared as described by Holdeman *et al.* (1977). Acid end products were analysed under the following conditions: column, 3 mm $\times$ 2 m glass column packed with 10% Thermon-1000/1% H_3PO_4 on Chromosorb W/HP (80–100 mesh) (Shimadzu); oven temperature, isothermal at 160°C for the analysis of acetate, or at 155°C for lactate and succinate, and at 60°C for formate; carrier gas, N_2 at 50 ml min^{-1} ; injector temperature, 220°C ; detector, flame ionization detector. For the analysis of ethanol, the following conditions were used: column, 3 mm $\times$ 1 m glass column packed with Adsorb P-1 (60–80 mesh) (Nishio Kogyo, Tokyo, Japan); oven temperature, isothermal at 150°C ; carrier gas, N_2 at 20 ml min^{-1} ; injector temperature, 210°C ; detector, flame ionization detector. H_2 and CO_2 were also analysed by GC with a thermal conductivity detector. The gas chromatograph was equipped with either stainless steel columns (3 mm $\times$ 2 m) packed with a 5A molecular sieves (Shimadzu) for the analysis of H_2 or packed with Porapak Q (Waters Associates, Milford, USA) for CO_2 . Fructose was determined by the enzymic method described by Beutler (1984). Cell dry weight was determined by centrifuging 50 ml of culture at $12000 g$ for 15 min and washing the cell pellets with phosphate-buffered saline (PBS; pH 7.0) which contained (g l^{-1}) NaCl (6.8), Na_2HPO_4 (1.2), and KH_2PO_4 (0.7). The pellets were collected on preweighed $0.45 \mu\text{m}$ -pore size nitrocellulose membrane filters (Advantec Toyo, Tokyo, Japan), dried at 105°C and reweighed. The relationship between cell concentration and the OD_{660} in 1 cm optical cuvettes was linear up to 0.6 unit of OD_{660} . The correlation coefficient of cell dry weight with OD_{660} did not vary significantly for cultures run at different dilution rates. Thus, the coefficient was averaged as $0.852 \pm 0.055 \text{ mg dry weight ml}^{-1} \text{ per } \text{OD}_{660} \text{ unit}$ (mean $\pm$ SD of 12 runs) and used to determine the cell density in the culture and washed-cell suspensions.

Washed-cell experiments. The consumption of formate and the concomitant production of CO_2 and H_2 by washed cells were determined. Bacterial cells were taken from the chemostat under a constant flow of N_2 and transferred into a centrifuge bottle filled with N_2 . After centrifugation ($12000 g$, 20 min), the cell pellets were washed twice with a N_2 -gassed solution (T solution) containing 50 mM-Tris/HCl buffer (pH 7.5), 30 mM- KH_2PO_4 , 66 mM-NaCl, 5 mM-KCl, 2 mM- CaCl_2 , 10 mM- $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, and 2 mM-dithiothreitol (DTT). The washed cells were suspended in T solution at 30–50 mg dry wt ml^{-1} and stored in ice until used. Washed-cell experiments were performed anaerobically at 37°C in 8 ml tubes sealed with a butyl rubber septum. After transferring the cell suspension (2–3 ml in T solution, concentrations as indicated in Results), the tube was repeatedly evacuated and filled with N_2 . The reactions were started by the addition of formate via syringes and stopped by adding 0.2 ml of 10% (v/v) H_2SO_4 . The amounts of formate, CO_2 and H_2 were determined by GC.

Preparation of cell-free extracts. Bacterial cells were collected and washed as described above: PBS supplemented with 2 mM-DTT was substituted for T solution. The washed cells were suspended in N_2 -gassed 50 mM-Tris/HCl buffer (pH 7.5) containing 4 mM-DTT at 10–15 mg dry wt ml^{-1} . The suspension was sonified 8–12 times for 15 s with 2 min intervals using an ultrasonic disruptor (UR-200P; Tomy Seiko, Tokyo, Japan) under a constant flow of N_2 . During sonification, the cell suspensions were kept on ice. The sonicated suspension was

gassed briefly with N₂ and centrifuged at 12000 *g* for 15 min. The supernatant was transferred to sealed vials containing N₂ gas. This supernatant ('cell extract') was kept on ice and used immediately for the enzyme assay. Protein was measured by the method of Lowry using bovine serum albumin as standard.

Enzyme assays. The following enzymes were assayed spectrophotometrically with a Beckman DU-8B UV-visible spectrophotometer. Assays were performed at 25 °C in either 1.0 ml normal open cuvettes or 1.0 ml cuvettes sealed with a butyl rubber stopper. The latter ('anaerobic') cuvettes containing a reaction mixture and N₂ gas were used for the assay of pyruvate formate-lyase (EC 2.3.1.54) and fumarate reductase. 50 mM-Tris/HCl buffer (pH 7.5) was used unless otherwise indicated. The reactions were generally started by the addition of cell extract (between 5 and 100 µl, at a concentration of 0.02–0.2 mg protein ml⁻¹). Reaction rates were calculated from the change in A₃₄₀ due to the reduction of NADP or the oxidation of NADH ($\epsilon = 6.3 \text{ mm}^{-1} \text{ cm}^{-1}$) unless otherwise indicated. Activities were determined in a range of linearity between the reaction rate and the protein concentration.

Glucokinase (EC 2.7.1.2) was assayed by the method of Pilkis (1975). Glucose-6-phosphate isomerase (EC 5.3.1.9) activity was determined by the method of Gracy & Tilley (1975). 6-Phosphofructokinase (EC 2.7.1.11) and 1-phosphofructokinase (EC 2.7.1.56) activities were measured as described by Baumann & Baumann (1975). Fructose-diphosphate aldolase (EC 4.1.2.13) was assayed by the method of Lebherz & Rutter (1975). Enolase (EC 4.2.1.11) activity was determined by monitoring phosphoenolpyruvate (PEP) production at 240 nm ($\epsilon = 1.5 \text{ mm}^{-1} \text{ cm}^{-1}$) according to the procedure of Spring & Wold (1975). Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) activity was measured in the manner of Olive & Levy (1975). 6-Phosphogluconate dehydrogenase (EC 1.1.1.44) was assayed by the method of Bridges & Wittenberger (1975). Phosphoglucomutase (EC 5.4.2.2) and acetate kinase (EC 2.7.2.1) were assayed by the procedures of Bergmeyer *et al.* (1983). 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 & Quayle (1977). The assay for pyruvate kinase (EC 2.7.1.40) was that of Tuominen & Bernlohr (1975). Lactate dehydrogenase (EC 1.1.1.27) was assayed as described by Wittenberger (1975). For attempts to demonstrate pyruvate formate-lyase activity, the assay was performed in anaerobic cuvettes by coupling the reactions to citrate synthase (EC 4.1.3.7) and malate dehydrogenase (EC 1.1.1.37) as described by Knappe & Blaschkowski (1975). For attempts to demonstrate PEP- or pyruvate-carboxylating activities, i.e. PEP carboxylase (EC 4.1.1.31), GDP- (EC 4.1.1.32) and ADP-dependent (EC 4.1.1.49) PEP carboxykinase, and pyruvate carboxylase (EC 6.4.1.1), the assays were carried out as described by Scrutton (1971). Malic enzyme (EC 1.1.1.40) was assayed according to the procedure of Schobert & Bowien (1984). Malate dehydrogenase activity was determined as described by Kitto (1969). Fumarate hydratase (EC 4.2.1.2) was assayed by monitoring the production of fumarate at 240 nm ($\epsilon = 2.53 \text{ mm}^{-1} \text{ cm}^{-1}$) as described by Hill & Bradshaw (1969). Fumarate reductase activity was determined anaerobically with reduced methyl viologen ($\epsilon = 13.0 \text{ mm}^{-1} \text{ cm}^{-1}$ at 600 nm) as the electron donor according to the procedure of Hiraishi (1988*b*).

Chemicals. All enzymes, nucleotides and cofactors were purchased from Boehringer Mannheim, Sigma or Oriental Yeast, Tokyo, Japan.

RESULTS

Growth and product formation in fructose-limited cultures

A. actinomycetemcomitans is able to use several sugars as growth substrates (Pulverer & Ko, 1970; Slots, 1982). The *A. actinomycetemcomitans* 301-b used in this study grew with fructose, glucose, maltose, mannitol and mannose but not with cellobiose, galactose, lactose, raffinose, ribose, rhamnose, sucrose, trehalose and xylose. Fructose was chosen as the growth-limiting nutrient since the growth rate of strain 301-b was higher with fructose than with any other sugars; the specific growth rate, 0.46 h⁻¹ with fructose versus 0.19 h⁻¹ with glucose, in batch cultures was measured using AA medium as a basal medium.

The organism was grown anaerobically in a fructose-limited culture without the addition of bicarbonate at various dilution rates (*D*). Steady states were obtained at *D* ranging from 0.04 to 0.25 h⁻¹ and the amounts of fructose consumed and fermentation products were determined. The major fermentation products were formate, acetate, ethanol and succinate, with minor amounts of lactate. The specific rates of fructose consumption (q_{fructose}) and formation of all products at each steady state were calculated (Table 1). A plot of q_{fructose} against *D* yielded a straight line (Fig. 1). Assuming the following relationship (Pirt, 1975):

$$q_{\text{fructose}} = D/Y_{\text{fructose}}^{\text{max}} + m_{\text{fructose}} \quad (1)$$

the maximum growth yield ($Y_{\text{fructose}}^{\text{max}}$) and the maintenance coefficient (m_{fructose}) were estimated as 40.3 g dry wt (mol fructose)⁻¹ and 0.248 mmol (g dry wt)⁻¹ h⁻¹, respectively.

Fig. 1. Relationship between growth rate and specific rate of fructose consumption (q_{fructose}) of anaerobic fructose-limited chemostat cultures of *A. actinomycetemcomitans* 301-b. Cultures were grown at 37 °C, pH 7.0, in the absence (○) and presence (●) of 48 mM-bicarbonate.

Table 1. Steady-state values for the rates of fructose consumption and product formation in fructose-limited anaerobic chemostat cultures of *A. actinomycetemcomitans* 301-b at various D values in the absence of bicarbonate

The chemostat culture was run at 37 °C, pH 7.0. Specific rates (q) are expressed in $\text{mmol (g dry wt)}^{-1} \text{ h}^{-1}$.

Dilution rate (h^{-1})	0.04*	0.06	0.10*	0.15	0.20*	0.25
No. of runs	2	1	4	1	3	1
Input fructose (mM)	10.3	10.1	9.6–10.1	10.1	10.3	9.6
Residual fructose (mM)	<0.1	<0.1	<0.1	<0.1	<0.1	1.9
q_{fructose}	1.21	1.76	2.67 ± 0.17	4.12	5.07 ± 0.4	6.47
q_{formate}	0.10	0.51	1.47 ± 0.13	4.41	5.03 ± 0.3	10.17
q_{acetate}	0.96	1.43	2.27 ± 0.05	3.46	3.86 ± 0.15	4.47
q_{ethanol}	0.25	0.30	0.96 ± 0.11	2.14	2.84 ± 0.15	3.43
q_{lactate}	<0.01	<0.02	0.03 ± 0.03	0.12	0.15 ± 0.09	2.07
$q_{\text{succinate}}$	0.74	1.18	1.28 ± 0.05	1.24	0.71 ± 0.15	0.32
Y_{fructose} (g mol^{-1})	33.0	34.0	37.5 ± 0.6	36.4	39.4 ± 0.5	38.6
Carbon balance (%)						
Fructose carbon	100	100	100	100	100	100
Product carbon (P)	75	82	82 ± 2	85	71 ± 2	86
Cell carbon (C)†	23	24	26 ± 0	25	27 ± 1	27
Recovery (P + C)	98	106	108 ± 2	110	98 ± 3	113
Redox-balance‡	0.82	0.84	0.84	0.83	0.71	0.74

* The values at these dilution rates are expressed as means or means $\pm$ SD.

† Cell carbon was assumed as 50% of dry cell weight.

‡ Redox-balance $(2 q_{\text{ethanol}} + q_{\text{lactate}} + 2 q_{\text{succinate}})/2 q_{\text{fructose}}$.

A substrate amount of bicarbonate (0.4%, 48 mM) was added to the fructose-limited medium and the chemostat culture was run at D values between 0.04 and 0.23 h^{-1} (Table 2). The q_{fructose} also varied linearly with D (Fig. 1). The estimated values of $Y_{\text{fructose}}^{\text{max}}$ and m_{fructose} were 61.7 g dry wt mol^{-1} and 0.747 $\text{mmol (g dry wt)}^{-1} \text{ h}^{-1}$, respectively. These values were significantly higher than those in the absence of bicarbonate. Consequently, it is expected that there is a significant difference between the efficiency of energy conservation or of energy utilization for biomass

Table 2. Steady-state values for the rates of fructose consumption and product formation in fructose-limited anaerobic chemostat cultures of *A. actinomycetemcomitans* 301-b at various *D* values in the presence of 48 mM bicarbonate

The chemostat culture was run at 37 °C, pH 7.0. Specific rates (*q*) are expressed in mmol (g dry wt)⁻¹ h⁻¹.

Dilution rate (h ⁻¹)	0.04	0.06	0.11	0.15	0.20*	0.23
No. of runs	1	1	1	1	2	1
Input fructose (mM)	9.9	9.9	9.9	9.9	8.0, 9.9	8.0
Residual fructose (mM)	<0.1	<0.1	<0.1	<0.1	<0.1	2.4
<i>q</i> _{fructose}	1.28	1.72	2.60	3.23	4.02	4.37
<i>q</i> _{formate}	0.81	1.50	2.73	3.71	6.43	10.01
<i>q</i> _{acetate}	1.20	1.64	2.43	2.94	3.86	4.59
<i>q</i> _{ethanol}	0.12	0.14	0.16	0.23	1.12	2.58
<i>q</i> _{lactate}	0.04	<0.02	<0.03	<0.03	0.04	0.66
<i>q</i> _{succinate}	1.21	1.59	2.51	2.77	2.61	1.71
<i>Y</i> _{fructose} (g mol ⁻¹)	31.1	34.8	40.7	46.5	49.8	52.6
Carbon balance (%)						
Fructose carbon	100	100	100	100	100	100
Product carbon (P)	108	110	111	109	113	126
Cell carbon (C)†	22	24	28	32	34	37
Recovery (P + C)	130	134	139	141	147	163
Redox-balance‡	1.05	1.01	1.03	0.93	0.93	1.06

* The values at this dilution rate are expressed as means.

† Cell carbon was assumed as 50% of dry cell weight.

‡ Redox-balance $(2 q_{\text{ethanol}} + q_{\text{lactate}} + 2 q_{\text{succinate}})/2 q_{\text{fructose}}$.

formation during the growth in the absence and presence of bicarbonate. As shown in Table 2, a marked decrease in *q*_{ethanol} and a significant increase in *q*_{succinate} were evident at *D* below 0.15 h⁻¹ with the addition of bicarbonate. However, *q*_{succinate} decreased with *D* at *D* above 0.15 h⁻¹. This phenomenon was not due to bicarbonate-limitation because the amount of bicarbonate consumed was roughly estimated as 52–78% of that added based on the carbon recovery data. Furthermore, the culture was kept under a stream of anaerobic gas containing 10% CO₂.

The accumulation of residual fructose was observed at *D* values of 0.23–0.25 h⁻¹ (Tables 1 and 2), although these *D* values were approximately half the maximum growth rate. Possibly, this was due to either the low apparent affinity for fructose (*K_s*) or the limitation by some other nutrients. If the former was the case, the *K_s* value was estimated to be 1.6–2.4 mM from the Monod equation (Pirt, 1975). At present we do not have satisfactory data to decide whether the growth is limited by some other nutrient at *D* values of 0.23–0.25 h⁻¹.

CO₂ formation during fructose catabolism

As in the mixed acid producers such as *Escherichia coli* (Doelle, 1975), it is expected that the cleavage of pyruvate to acetyl-CoA and formate and that of formate to CO₂ and H₂ occurs in *A. actinomycetemcomitans*. The former reaction is catalysed by pyruvate formate-lyase and the latter by formate hydrogen-lyase complex (i.e. formate dehydrogenase and hydrogenase). To demonstrate these activities, hypophosphite, a formate analogue and a potent inhibitor of pyruvate formate-lyase (Thauer *et al.*, 1972; Hiraishi, 1988a), was added to a steady state of the fructose-limited chemostat culture run at *D* = 0.20 h⁻¹ in the absence of bicarbonate and changes in the cell density and the amount of the end products were then followed. The results shown in Fig. 2 indicate that hypophosphite partially inhibited growth and blocked the production of acetate, ethanol and formate but stimulated that of lactate. The washout rate of acetate plus ethanol was equal to *D*, clearly indicating that pyruvate formate-lyase was the major enzyme catalysing the cleavage of pyruvate in the fructose catabolism.

Fig. 2 shows that the washout rate of formate (0.27 h⁻¹) was higher than *D* (0.20 h⁻¹), suggesting the presence of a formate-decomposing reaction. This reaction was confirmed by

Fig. 2. Changes in the amounts of fermentation products and cell density of *A. actinomycetemcomitans* 301-b growing in a fructose-limited chemostat culture after the addition of 20 mM-sodium hypophosphite to the culture. The culture was grown at 37 °C, pH 7.0, in the absence of bicarbonate. The amounts of formate (○), acetate plus ethanol (●), lactate (□), succinate (■) and cell density (△) were determined every 2 h after the addition of hypophosphite. The dashed line represents the theoretical change in the amount of hypophosphite.

washed-cell experiments (Table 3). The washed cells prepared from a fructose-limited chemostat culture run in the absence of bicarbonate consumed formate with the concomitant production of CO₂ and H₂, indicating the presence of formate hydrogen-lyase complex activity. The amount of CO₂ produced was nearly equal to that of the formate consumed [91 nmol CO₂ (mg dry wt)⁻¹ versus 94 nmol formate (mg dry wt)⁻¹ in 30 min incubation at 37 °C] although the amount of H₂ produced was lower [55 nmol H₂ (mg dry wt)⁻¹] than the amount of formate consumed. The activity of formate cleavage was measured as 3.0 nmol min⁻¹ (mg dry wt)⁻¹ with washed cells from a culture at $D = 0.25 \text{ h}^{-1}$. With washed cells from a culture at lower D (0.10 h⁻¹), a higher activity was obtained: 44 nmol min⁻¹ (mg dry wt)⁻¹ (mean of duplicate determinations).

Enzyme activity of *A. actinomycetemcomitans*

Enzyme activities involved in fructose catabolism were determined in cell extracts prepared from cultures run at D values of 0.04 h⁻¹ and 0.25 h⁻¹ in the presence of bicarbonate (Table 4). Cell extracts contained the key enzyme activities of the Embden–Meyerhof–Parnas pathway, i.e. 6-phosphofructokinase, fructose-diphosphate aldolase, enolase and pyruvate kinase. They also contained glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activities, which are involved in the pentose phosphate pathway. No activities were detected for the key enzymes of the Entner–Doudoroff pathway, i.e. phosphogluconate dehydratase and 6-phospho-2-keto-deoxygluconate aldolase.

All attempts to detect pyruvate formate-lyase activity in cell extracts were unsuccessful although its presence in growing cells was shown from the results presented in Fig. 2. Attempts

Table 3. Activity of the cleavage of formate to CO_2 and H_2 in washed cells of *A. actinomycetemcomitans* 301-b grown in fructose-limited culture at a $D=0.25 \text{ h}^{-1}$ in the absence of bicarbonate

Washed cells were incubated at 37°C for 30 min under strictly anaerobic conditions.

Amount of cells (mg dry wt)	Amount (μmol) of			
	Formate		CO_2 formed	H_2 formed
	Added	Used		
0	29.1	0	0	0
16	27.8	1.4	1.6	1.0
32	28.0	3.2	2.6	1.5
32	0	0	0.1	0.2

Table 4. Enzyme activities in cell extracts of *A. actinomycetemcomitans* 301-b grown anaerobically in fructose-limited chemostat culture with 48 mM bicarbonate at D values of 0.04 and 0.25

Enzyme	Dilution rate...	Specific enzyme activity [$\text{nmol min}^{-1} (\text{mg protein})^{-1}$]	
		0.04	0.25
Glucokinase		38	36
Phosphoglucomutase		292	371
Glucose-6-phosphate isomerase		1310	1120
1-Phosphofructokinase		120	137
6-Phosphofructokinase		78	107
Fructose-diphosphate aldolase		192	147
Enolase		1030	1330
Pyruvate kinase		67	90
Glucose-6-phosphate dehydrogenase		63	48
6-Phosphogluconate dehydrogenase		76	52
Lactate dehydrogenase		811	500
Acetate kinase		2990	6290
Malate dehydrogenase		1500	772
Fumarate hydratase		1620	1140
Fumarate reductase		1090	512

were also unsuccessful to detect the enzyme activities for the carboxylation of PEP or pyruvate, i.e. PEP carboxylase, GDP- and ADP-dependent PEP carboxykinase and pyruvate carboxylase. NADP-dependent malic enzyme could be detected which required monovalent cations; NH_4^+ was the most effective (data not shown). All of the other activities for the formation of succinate, i.e. malate dehydrogenase, fumarate hydratase and fumarate reductase activities, were easily detected. These enzyme activities in the cell extracts from a culture at $D = 0.25 \text{ h}^{-1}$ were significantly lower than those at a lower D (0.04 h^{-1}). By contrast, the acetate kinase activity at $D = 0.25 \text{ h}^{-1}$ was about twice as high as that at the lower D . Thus, the decrease in $q_{\text{succinate}}$ in a culture with increasing D over 0.15 h^{-1} (Table 2) was explained by the decrease in the enzyme activity for the succinate-forming pathway.

DISCUSSION

The addition of bicarbonate to the fructose-limited medium resulted in a significant increase in the $Y_{\text{fructose}}^{\text{max}}$ value (Fig. 1). To interpret this increase, this discussion focuses mainly on (1) the catabolic pathway of fructose and (2) the estimation of the efficiency of ATP generation during fructose fermentation and of $Y_{\text{ATP}}^{\text{max}}$ in the absence and presence of bicarbonate.

Fig. 3. Probable pathway of fructose catabolism in *A. actinomycetemcomitans* 301-b. Solid lines indicate the enzyme activities which were demonstrated and dashed lines indicate those not yet determined. F-1-P, fructose-1-phosphate; F-1,6-BP, fructose-1,6-bisphosphate; GAP, glyceraldehyde-3-phosphate; DHAP, dihydroxyacetone-phosphate; PEP, phosphoenolpyruvate; Acetyl-CoA, acetyl-coenzyme A. (1) PEP:fructose phosphotransferase system; (2) 1-phosphofructokinase; (3) fructose-bisphosphate aldolase; (4) triose phosphate isomerase; (5) glyceraldehyde-3-phosphate dehydrogenase, 3-phosphoglycerate kinase, and phosphoglyceromutase; (6) enolase; (7) PEP carboxylase; (8) pyruvate kinase; (9) pyruvate carboxylase; (10) lactate dehydrogenase; (11) malate dehydrogenase; (12) pyruvate formate-lyase; (13) formate hydrogen-lyase complex; (14) fumarate hydratase; (15) fumarate reductase; (16) phosphate acetyltransferase; (17) acetate kinase; (18) acetaldehyde dehydrogenase and alcohol dehydrogenase.

The composition of metabolic end products (Tables 1 and 2), the growth inhibition with hypophosphite (Fig. 2), and enzyme activities involved in fructose catabolism (Tables 3 and 4) suggest a fructose fermentation pathway for *A. actinomycetemcomitans* as shown in Fig. 3. Fructose-6-phosphate formation from fructose and ATP could not be detected in cell extracts. Instead, 1-phosphofructokinase activity was detected (Table 4), suggesting that the initial step in fructose catabolism is the formation of fructose 1-phosphate, probably catalysed by the PEP:fructose phosphotransferase system. The presence of this system was partly confirmed by measuring the rate of the fructose-dependent conversion of PEP to pyruvate with permeabilized cells according to the method of Kornberg & Reeves (1979); the rate was $4.1 \text{ nmol pyruvate formed min}^{-1} (\text{mg cell protein})^{-1}$ in cells from a fructose-limited chemostat culture run at $D = 0.1 \text{ h}^{-1}$ in the absence of bicarbonate (data not shown). Fructose 1-phosphate is catabolized primarily by the Embden-Meyerhof-Parnas pathway to yield PEP or pyruvate, which may then be either carboxylated to oxaloacetate or converted to acetyl-CoA.

All attempts to detect any activity for PEP carboxylase, GDP- and ADP-dependent PEP carboxykinases and pyruvate carboxylase in cell extracts were unsuccessful. The identification of enzyme activities involved in the carboxylation of PEP or pyruvate by cell extracts is often difficult because of the possible mutual interference of these activities (Schobert & Bowien, 1984). Therefore, to identify the activity, it will be necessary to perform radiometric assay using labelled bicarbonate. Difficulty was also encountered in demonstrating pyruvate formate-lyase activity in cell extracts, possibly because of its hypersensitivity to oxygen as described with the enzymes of *E. coli* (Knappe & Blaschkowski, 1975) and *Streptococcus mutans* (Yamada *et al.*, 1985). However, the exclusive function of pyruvate formate-lyase in the cleavage of pyruvate to formate and acetyl-CoA is strongly suggested in anaerobically grown cells from the results shown in Fig. 2.

ATP was possibly gained at four different steps catalysed by phosphoglycerate kinase (EC 2.7.2.3), pyruvate kinase, acetate kinase and fumarate reductase. The coupling of fumarate reduction and electron-transport-linked phosphorylation in this organism is expected from the work of Mannheim *et al.* (1978). They reported that *Actinobacillus* and *Pasteurella* organisms including *A. actinomycetemcomitans* are capable of using both aerobic respiration and desmethylmenaquinone-mediated fumarate respiration as energy-yielding pathways.

The activity for the cleavage of formate to CO₂ and H₂, possibly formate hydrogen-lyase complex activity, was revealed with washed cells (Table 3). Thus, it is expected that CO₂ is produced from formate and then fixed for the succinate synthesis in the culture in the absence of bicarbonate. The specific rate of CO₂ and H₂ formation ($q_{\text{CO}_2 + \text{H}_2}$) in a steady-state culture can be estimated from the following equation:

$$q_{\text{CO}_2 + \text{H}_2} = q_{\text{acetate}} + q_{\text{ethanol}} - q_{\text{formate}} \quad (2)$$

To compare rates of CO₂ formation and succinate synthesis in the culture, the ratio of $q_{\text{succinate}}$ to $q_{\text{CO}_2 + \text{H}_2}$ was calculated. The value ranged from 0.7 to 1.0 at D below 0.15 h⁻¹ and was 0.4 at $D = 0.20$ h⁻¹. From these calculations, it seems that at D below 0.15 h⁻¹, the CO₂ production was nearly coupled to the CO₂ fixation. At D above 0.20 h⁻¹, this was not the case because of the lowered enzyme activities for the succinate-synthesis pathway (Table 4) and the lowered activities for the cleavage of formate from 44 nmol min⁻¹ (mg dry wt)⁻¹ in washed cells from the culture at $D = 0.10$ h⁻¹ to 3 nmol min⁻¹ (mg dry wt)⁻¹ at $D = 0.25$ h⁻¹. In addition, the $q_{\text{CO}_2 + \text{H}_2}$ value at $D = 0.25$ h⁻¹ was negative. A satisfactory interpretation for this is not available at present.

As in mixed acid producers such as *Klebsiella aerogenes* (*K. pneumoniae*) (Streekstra *et al.*, 1987), the fructose catabolism by *A. actinomycetemcomitans* is summed up in the following overall-equations:

Although there are two alternative pathways for the succinate synthesis via oxaloacetate from either PEP or pyruvate, the same overall equation as (6) is given for the two pathways. Pyruvate is formally regarded as a major precursor metabolite for the biosynthesis of building blocks (Ingraham *et al.*, 1983). This implies that biomass formation is associated with ATP formation in glycolysis during the formation of pyruvate (Streekstra *et al.*, 1987). Thus, it can be defined that the specific rate of ATP synthesis (q_{ATP}) equals $2 q_{\text{fructose}} + q_{\text{acetate}}$. The values of q_{ATP} were calculated at each steady state and the plots of q_{ATP} against D yielded a linear relationship (Table 5). Again, by using equation (1), the maximum yield referred to moles of ATP synthesized [$Y_{\text{ATP}}^{\text{max}}$, g dry wt (mol ATP synthesized)⁻¹] was estimated to be 15.1 and 20.2 in the absence and presence of bicarbonate, respectively. The $Y_{\text{ATP}}^{\text{max}}$ value of 15.1 is close to the value of 14.0 for the

Table 5. Growth parameters of *A. actinomycetemcomitans* 301-b grown anaerobically in the fructose-limited chemostat culture in the absence and presence of bicarbonate

The equations are calculated by estimating the first and second regression coefficients from the data presented in Tables 1 and 2. q is expressed in mmol substrate consumed or formed (g dry wt)⁻¹ h⁻¹ and Y in g dry wt (mol substrate)⁻¹.

Parameter	In the absence of bicarbonate	In the presence of bicarbonate
q_{fructose}	$0.248 + 24.8 D (0.998)^*$	$0.747 + 16.2 D (0.996)^*$
$Y_{\text{fructose}}^{\text{max}}$	40.3	61.7
q_{ATP}	$0.985 + 66.4 D (0.997)^*$	$2.047 + 49.4 D (0.998)^*$
$Y_{\text{ATP}}^{\text{max}}$	15.1	20.2
ATP-Eff†	2.7	3.0

* Correlation coefficient shown in parentheses.

† The efficiency of ATP generation is calculated as the ratio of $Y_{\text{fructose}}^{\text{max}}$ to $Y_{\text{ATP}}^{\text{max}}$, mol ATP formed (mol fructose metabolized)⁻¹.

glucose-limited anaerobic chemostat culture of *Aerobacter aerogenes* (*Klebsiella pneumoniae*) possessing a similar branched fermentation pathway (Stouthamer & Bettenhausen, 1975). The efficiency of ATP generation [ATP formed (mol fructose metabolized)⁻¹] during fructose fermentation can be calculated as the ratio of $Y_{\text{fructose}}^{\text{max}}$ to $Y_{\text{ATP}}^{\text{max}}$ and values of 2.7 and 3.0 are obtained for the cultures in the absence and presence of bicarbonate, respectively. From these parameters, it can be concluded that bicarbonate stimulates not only the efficiency of ATP formation during fructose catabolism but also the efficiency of the utilization of this energy for biosynthesis (i.e. $Y_{\text{ATP}}^{\text{max}}$). Further interpretation, in particular for the increase in $Y_{\text{ATP}}^{\text{max}}$, requires biochemical studies with labelled bicarbonate on the exact CO₂ fixation pathway.

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