Energy metabolism of *Actinobacillus actinomycetemcomitans* during anaerobic and microaerobic growth in low- and high-potassium continuous culture

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Actinobacillus actinomycetemcomitans, a member of the gamma subclass of the *Proteobacteria*, has been implicated as the agent responsible for human periodontitis. In this study, *A. actinomycetemcomitans* 301-b was grown in fructose-limited chemostat cultures under anaerobic [redox potential (E_h) < -400 mV] and microaerobic ($E_h = -200$ mV) conditions to characterize its energy metabolism. Effects of K^+ and Na^+ on growth and metabolism were also examined. In a control medium containing 5.2 mM K^+ and 24 mM Na^+ , the molar growth yield on fructose (Y_{fructose}) of microaerobic cultures was 1.3 times higher than the yield of anaerobic cultures at $D \leq 0.10$ h⁻¹, but the difference in the Y_{fructose} between microaerobic and anaerobic cultures decreased at $D > 0.10$ h⁻¹. When the ATP yield from fermentation was estimated from the amounts of fructose consumed and acetate formed, the value of the microaerobic culture (2.49 mol ATP produced per mol fructose consumed) was lower than the anaerobic value [3.13 mol ATP (mol fructose)⁻¹]. Therefore, ATP production from fermentation could not account for the increase in the Y_{fructose} at $D \leq 0.10$ h⁻¹ and thus additional ATP was expected to be generated via respiration. Assuming that the Y_{ATP} (g cells formed per mol ATP synthesized) was similar between anaerobic and microaerobic cultures, the estimated ATP yield from respiration was between 1.2 and 2.0 mol ATP (mol fructose)⁻¹ below $D = 0.10$ h⁻¹ and decreased to 0.3 mol ATP (mol fructose)⁻¹ when D was increased to 0.19 h⁻¹. Such growth-rate-dependent decreases in the Y_{fructose} and the estimated ATP production from respiration were also observed in a high- Na^+ (5.2 mM K^+ and 106 mM Na^+) culture but not in a high- K^+ (81 mM K^+ and 24 mM Na^+) culture. In the high- K^+ culture, the microaerobic Y_{fructose} was 1.4–2.0 times higher than the anaerobic value and the respiration-derived ATP yield was estimated to be between 1.2 and 1.9 mol ATP (mol fructose)⁻¹ over a wide range of dilution rate. These results suggest that higher concentrations of extracellular K^+ are required for the respiration to occur in rapidly growing cells of *A. actinomycetemcomitans*.

Keywords: fructose metabolism, microaerophile, chemostat culture, potassium ion, periodontopathogen

INTRODUCTION

Actinobacillus actinomycetemcomitans, a member of the gamma subclass of the *Proteobacteria*, is associated with several severe forms of human periodontitis (Slots *et al.*, 1980; Slots & Schonfeld, 1991; Wilson & Henderson, 1995; Zambon, 1985). This bacterium was found at higher proportions in moderate (5–7 mm in

depth) than in deeper periodontal pockets of patients (Dzink *et al.*, 1985). Since moderate pockets exhibit relatively higher oxygen tensions than deeper pockets (Loesche *et al.*, 1983), conditions favourable for the growth of *A. actinomycetemcomitans* probably include moderate levels of oxygen such as those encountered frequently in such moderate periodontal pockets. In our previous study, the microaerophilic growth of *A. actino-*

mycetemcomitans 301-b was characterized in chemostat cultures equipped with a redox control system (Ohta *et al.*, 1996b). When the bacterium was grown at a dilution rate of 0.10 h^{-1} , optimum growth was recorded in microaerobic cultures at culture redox potential (E_h) values of -300 to -200 mV , and growth inhibition occurred under highly aerated conditions ($E_h > -100\text{ mV}$). The recorded growth yield of the microaerobic culture was only about 1.3 times the yield of anaerobic cultures ($E_h < -400\text{ mV}$). One possible explanation for this small increase in the growth yield was an increase in substrate-level phosphorylation at the site of acetate kinase in the fructose fermentation (Ohta *et al.*, 1996b). Alternatively, the occurrence of respiratory-chain phosphorylation was possible, since *A. actinomycetemcomitans* has a respiratory system with oxygen as the terminal electron acceptor (Mannheim *et al.*, 1978).

In this study, we examined whether or not respiratory-chain phosphorylation occurs in *A. actinomycetemcomitans*. To this end, several growth parameters, including the specific rate of ATP production from fructose catabolism, were estimated for steady-state cultures at different dilution rates. Our study also focused on the effect of potassium on the ATP production rate. Potassium is influential in bacterial energy metabolism (Huetting *et al.*, 1979) and a predominant cation in oral environments such as the dental plaque and gingival fluid (Tatevossian & Gould, 1976).

METHODS

Bacterial strain and medium. *A. actinomycetemcomitans* 301-b (Ohta *et al.*, 1987, 1996b; Inouye *et al.*, 1990) was used throughout this study. The growth medium (AA medium; Ohta *et al.*, 1989) contained the following ingredients per l: Na_2SO_4 , 0.5 g; KH_2PO_4 , 0.2 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g; NaCl , 1.2 g; NH_4Cl , 0.3 g; KCl , 0.3 g; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.15 g; Bacto yeast extract, 2.0 g; resazurin, 0.001 g; Tris, 6.05 g. The trace element solution described before (Ohta & Gottschal, 1988) was also added. Fructose was chosen as the growth-limiting nutrient because the growth rate of strain 301-b was higher with fructose than with any other sugars (Ohta *et al.*, 1989, 1996a, b). Filter-sterilized solutions of fructose were added to the autoclaved medium aseptically at final concentrations of 6.6–7.5 mM. For the examination of effects of K^+ on growth, KCl in AA medium was increased to 1.5, 3.0, 6.0 or 12.0 g l^{-1} , while for the effect of Na^+ , NaCl in the medium was increased to 3.0 or 6.0 g l^{-1} .

Chemostat culture. The bacterium was grown in a chemostat system as described before (Ohta *et al.*, 1989). For anaerobic cultures, the system was kept under a stream ($200\text{--}300\text{ ml h}^{-1}$) of N_2 , which was freed of traces of oxygen by passage over a gas-purifying column (Gas Clean GC-RX; Nikka Seiko). In the chemostat system the redox potential was measured continuously using a platinum electrode with an Ag/AgCl reference cell and used to control the stirring speed in the culture. The electrode signal was measured with a redox regulator (model F0-11; Tokyo Rikakikai) calibrated against a quinhydrone reference solution at pH 4.0. In this system, manipulation of the air flow in combination with feedback control on the stirring speed made it possible to maintain a

constant redox potential ($\pm 5\text{--}10\text{ mV}$) between approximately -400 mV (anaerobic) and $+300\text{ mV}$ (aerobic; near air saturation), depending on the exact medium composition and the air supply (Ohta & Gottschal, 1988; Ohta *et al.*, 1996b). In this study the redox potential of microaerobic culture was maintained at -200 mV . The temperature of the culture was maintained at 37°C , and the pH was maintained at 7.0 with automatic addition of 2 M NaOH or 2 M HCl . The OD_{660} of cultures was measured in a 1 cm light path cuvette to determine the cell densities. The averaged coefficient of the dry cell weight at OD_{660} was $0.852 \pm 0.055\text{ mg dry wt ml}^{-1}$ per OD_{660} unit (mean $\pm$ SD of 12 runs at dilution rates between 0.04 and 0.25 h^{-1}) (Ohta *et al.*, 1989). The relationship is linear up to 0.6 units of OD_{660} (Ohta *et al.*, 1989). The purity of the cultures was routinely checked on tryptic soy agar (BBL Microbiology Systems) plates.

Measurement of oxygen uptake by washed cells. Bacterial cells were sampled from chemostat cultures, washed twice with 100 mM potassium MOPS buffer (pH 7.0) containing 66 mM NaCl , 5 mM KCl , 2 mM CaCl_2 and 0.5 mM MgCl_2 , and suspended at $34\text{--}50\text{ mg dry wt ml}^{-1}$ in the MOPS buffer. Oxygen consumption by cell suspensions was measured at 37°C in a Hansatech oxygen electrode unit (type DW1). It consisted of a water-jacketed reaction chamber (volume, 1 ml), with a Clark-type oxygen electrode disc set in the floor. The chamber was closed with a stopper. In this unit, the magnetic follower that stirred the cell suspension spun directly above the membrane-covered electrode. The chamber contained the air-saturated MOPS buffer and an appropriate amount of the cell suspension was injected through a hole in the stopper. After the output signal had become constant, the reaction was started by the addition of fructose (final concentration 10 mM). The calibration of the electrode was made on the assumption that the dissolved oxygen concentration in air-saturated water was $217\text{ }\mu\text{M}$ at 37°C (Kielley, 1963).

Chemical analysis. Formate, succinate and ethanol were determined by GLC, and fructose by the enzymic method described before (Ohta *et al.*, 1989). Acetate was determined using an enzyme system consisting of acetyl-CoA synthetase, citrate synthase and malate dehydrogenase (Boehringer Mannheim).

Calculations. The specific rate of ATP production during fructose fermentation ($q_{\text{ATP(fermen)}}$) was calculated by the following equation (Ohta *et al.*, 1989):

$$q_{\text{ATP(fermen)}} = 2q_{\text{fructose}} + q_{\text{acetate}}$$

where q_{fructose} and q_{acetate} are the specific rates of fructose consumption and acetate formation, respectively, and q is expressed in mmol substrate consumed or formed ($\text{g dry wt}^{-1}\text{ h}^{-1}$). The ATP yield from fructose fermentation (mol ATP formed per mol fructose metabolized) was calculated as the ratio of $q_{\text{ATP(fermen)}}$ to q_{fructose} . For the anaerobic cultures, the Y_{ATP} (g cells produced per mol ATP consumed) was estimated by the ratio of the growth yield on fructose (Y_{fructose}) to the ATP yield from fructose fermentation. Based on the discussion by Stouthamer & Bettenhausen (1975), we assumed that the Y_{ATP} was dependent on the growth rate and did not change significantly with a change from anaerobic to microaerobic conditions when medium composition and culture pH were kept constant. Thus, for microaerobic cultures, the specific rates of total ATP production ($q_{\text{ATP(total)}}$) and ATP production through respiratory chain phosphorylation ($q_{\text{ATP(respi)}}$) were estimated by the following equation:

$$\begin{aligned} q_{\text{ATP(total)}} &= q_{\text{ATP(fermen)}} + q_{\text{ATP(respi)}} \\ &= (Y_{\text{fructose}}/Y_{\text{ATP}}) q_{\text{fructose}} \end{aligned}$$

RESULTS AND DISCUSSION

Anaerobic growth

A. actinomycetemcomitans 301-b was grown anaerobically in a fructose-limited chemostat culture and steady states were obtained at dilution rates (D) ranging from 0.04 to 0.20 h^{-1} . In the steady-state cultures, the redox potential values were recorded at -470 to -400 mV and residual fructose concentrations were less than 0.05 mM. The molar growth yield on fructose (Y_{fructose}) was 36.9 g dry wt (mol fructose) $^{-1}$ at $D = 0.04 \text{ h}^{-1}$ and increased to 47.7 g dry wt (mol fructose) $^{-1}$ at $D = 0.20 \text{ h}^{-1}$ (Fig. 1a). The plot of the specific rate of fructose consumption (q_{fructose}) against the corresponding D value yielded a straight line (Fig. 1b), indicating that the q_{fructose} is a linear function of dilution rate. The relationship approximated to:

$$q_{\text{fructose}} = 19.9D + 0.359 \quad (r = 0.999)$$

Assuming the following relationship (Tempest & Neijssel, 1984)

$$q = D/Y^{\text{max}} + m$$

the maximum growth yield (Y^{max}) and the maintenance coefficient (m) were estimated: $Y^{\text{max}}_{\text{fructose}} = 50.3 \text{ g dry wt}$

Fig. 1. Molar growth yield on fructose (a) and specific rate of fructose consumption (b) as functions of the dilution rate in anaerobic (○) and microaerobic (●) fructose-limited chemostat cultures of *A. actinomycetemcomitans* 301-b. ▽, Y_{ATP} calculated for the anaerobic cultures. The growth medium (control medium) contained 5.2 mM K^+ and 24 mM Na^+ .

Fig. 2. Specific production rates of formate (○), acetate (●), ethanol (▽) and succinate (▼) as functions of the dilution rate in anaerobic (a) and microaerobic (b) fructose-limited chemostat cultures of *A. actinomycetemcomitans* 301-b. The growth medium (control medium) contained 5.2 mM K^+ and 24 mM Na^+ .

(mol fructose) $^{-1}$ and $m_{\text{fructose}} = 0.359 \text{ mmol (g dry wt)}^{-1} \text{ h}^{-1}$, respectively.

The main fermentation products from anaerobic fructose-limited chemostat cultures of *A. actinomycetemcomitans* were acetate, formate, ethanol and succinate. Lactate was not detected at any of the dilution rates tested. Carbon recoveries, which were estimated from the ratios of amounts of fermentation-product-carbons to amounts of fructose-carbon consumed, were between 88 and 91%. The specific formation rates of fermentation products as functions of the dilution rate are shown in Fig. 2(a). Acetate was the most abundant product and its formation was proportional to dilution rate. Succinate was the second most abundant product at dilution rates below 0.10 h^{-1} , but its formation did not increase with increasing dilution rate. Increases in the formation of formate and ethanol showed biphasic patterns and the slopes were higher at dilution rates above 0.10 h^{-1} .

Microaerobic growth and oxygen consumption activity

When *A. actinomycetemcomitans* 301-b was grown in microaerobic fructose-limited chemostat cultures maintained at an E_h value of -200 mV, steady states were

obtained at dilution rates ranging from 0.04 to 0.19 h⁻¹. As shown in Fig. 1(a), the recorded Y_{fructose} values below $D = 0.10 \text{ h}^{-1}$ were higher than those of the corresponding anaerobic cultures. However, above $D = 0.10 \text{ h}^{-1}$, the Y_{fructose} value decreased. Increasing the dilution rate further to 0.20 h⁻¹ led to a further decrease in the cell density of culture followed by washout. The relationship between q_{fructose} and dilution rate showed a biphasic pattern (Fig. 1b) and the $Y_{\text{fructose}}^{\text{max}}$ values were estimated to be 65.4 g dry wt (mol fructose)⁻¹ (first phase) and 32.2 g dry wt (mol fructose)⁻¹ (second phase). The first-phase value is 1.3 times higher than the $Y_{\text{fructose}}^{\text{max}}$ of the anaerobic culture but the second-phase value is 64 % of the anaerobic value. In the microaerobic culture, acetate was the sole fermentation product at dilution rates lower than 0.10 h⁻¹ and its formation was not proportional to dilution rate (Fig. 2b). With increasing dilution rate to 0.15 then 0.19 h⁻¹, a proportional increase in acetate formation started simultaneously with the production of formate and ethanol. The estimated carbon recovery was the lowest (40 %) at $D = 0.10 \text{ h}^{-1}$ and ranged from 52 to 71 % at the other dilution rates.

To examine whether or not an induction of respiratory capacity with fructose occurs upon microaerobic culturing, washed cells were prepared from the anaerobic ($E_h = -410 \text{ mV}$) and microaerobic ($E_h = -270$ and -200 mV) fructose-limited chemostat cultures run at $D = 0.10 \text{ h}^{-1}$ then oxygen uptake rates by the two sets of washed cells were compared. Fructose-dependent oxygen uptake activity of the microaerobically grown cells was slightly lower [1.26–1.36 mmol O₂ h⁻¹ (g dry wt)⁻¹ (mean of duplicate determinations)] than that of anaerobically grown cells [1.54 mmol O₂ h⁻¹ (g dry wt)⁻¹], indicating that no induction but a small suppression of respiratory capacity occurred in the microaerobic culture. It was also noted that the measured oxygen uptake rates were significantly low compared with those of washed cells of batch-grown *Escherichia coli* K-12 [about 8 mmol O₂ h⁻¹ (g dry wt)⁻¹] (Ohta & Taniguchi, 1988).

Effect of K⁺ on growth

Our growth medium (AA medium) contained 5.2 mM K⁺, which was higher than the amount (1 mM) just sufficient to meet the cellular requirement of a rapidly growing culture of the facultatively anaerobic, sugar-fermenting *Klebsiella aerogenes* (*K. pneumoniae*) (Tempest & Neijssel, 1984). However, considering that oral environments such as dental plaque and gingival fluid contain relatively high amounts of K⁺ (61.5 ± 13.5 mM for plaque fluid and 17.4 ± 9.0 mM for gingival fluid) (Tatevossian & Gould, 1976), higher extracellular K⁺ concentrations might be required for rapid growth of *A. actinomycetemcomitans*. Therefore, fructose-limited growth of strain 301-b was examined using media containing high K⁺ concentrations. In these media, the Na⁺ concentration was fixed at 24 mM. The Y_{fructose} of the steady-state microaerobic cultures ($E_h = -200 \text{ mV}$) with 81 and 162 mM K⁺ were 1.3 times

Table 1. Effects of K⁺ and Na⁺ on the molar growth yield on fructose in anaerobic ($E_h < -400 \text{ mV}$) and microaerobic ($E_h = -200 \text{ mV}$) fructose-limited chemostat cultures ($D = 0.15 \text{ h}^{-1}$) of *A. actinomycetemcomitans* 301-b

Ion concn (mM)		Y_{fructose} [g dry wt (mol fructose) ⁻¹]*	
K ⁺	Na ⁺	Anaerobic culture	Microaerobic culture
5.2	24	47.2 (2) [100]	51.7 (2) [110]
21	24	47.0 (1) [100]	43.9 (1) [93]
41	24	48.2 (1) [102]	51.1 (1) [108]
81	24	41.5 (1) [88]	65.6 ± 1.5 (3) [139]
162	24	ND	64.8 (2) [137]
5.2	55	52.2 (1) [111]	58.8 (2) [125]
5.2	106	51.5 (2) [109]	56.2 (1) [119]

* Values in parentheses represent the number of independent experiments. For duplicate and triplicate experiments, yield values are the mean and the mean ± SD, respectively. Values in square brackets are percentage values expressed relative to that of the anaerobic culture with 5.2 mM K⁺ and 24 mM Na⁺. ND, Not determined.

higher than the values of the low (5.2 mM) K⁺ culture (Table 1). In contrast, for the anaerobic cultures, increases in extracellular K⁺ concentrations did not result in an increase in the Y_{fructose} . As a control, the effect of Na⁺ on the anaerobic and microaerobic fructose-limited growth (at $D = 0.15 \text{ h}^{-1}$) was also examined; the medium K⁺ concentration was fixed at 5.2 mM. No significant changes in the Y_{fructose} were found for anaerobic or microaerobic cultures with increasing Na⁺ concentration from 24 to 55 then to 106 mM (Table 1).

Growth in high-K⁺ and high-Na⁺ cultures

The effect of growth rate on the fructose metabolism of *A. actinomycetemcomitans* 301-b in a high-K⁺ medium (81.2 mM K⁺ and 24 mM Na⁺) was examined in fructose-limited chemostat cultures. Steady states of both anaerobic and microaerobic cultures were obtained at dilution rates ranging from 0.04 to 0.20 h⁻¹. With increasing dilution rate, the Y_{fructose} of anaerobic cultures increased to a maximum [48.5 g dry wt (mol fructose)⁻¹] at $D = 0.10 \text{ h}^{-1}$ and decreased slightly above $D = 0.10 \text{ h}^{-1}$ (Fig. 3a). In the microaerobic cultures, the Y_{fructose} values were 1.4–2.0 times higher than those of the corresponding anaerobic cultures over the range of dilution rates tested, although a successive small decrease in the yield was observed as the dilution rate increased (Fig. 3a). Growth of *A. actinomycetemcomitans* 301-b was also examined in high-Na⁺ medium (5.2 mM K⁺ and 106 mM Na⁺). Steady states were obtained for both anaerobic and microaerobic fructose-limited chemostat cultures at dilution rates between 0.04 and 0.20 h⁻¹. The maximum Y_{fructose} value of the anaerobic cultures was recorded at $D = 0.15 \text{ h}^{-1}$ [50.0 g

Fig. 3. Effect of increased extracellular concentration of K^+ (81 mM K^+ and 24 mM Na^+) (a) and Na^+ (5.2 mM K^+ and 106 mM Na^+) (b) on the molar growth yield on fructose in anaerobic ($\circ$) and microaerobic ($\bullet$) fructose-limited chemostat cultures of *A. actinomycetemcomitans* 301-b. ∇ , Y_{ATP} calculated for the anaerobic cultures.

dry wt (mol fructose) $^{-1}$] and the $Y_{fructose}$ decreased slightly at $D = 0.20\text{ h}^{-1}$ (Fig. 3b). The $Y_{fructose}$ of the microaerobic culture at $D = 0.04\text{ h}^{-1}$ was 1.9 times higher than that of the corresponding anaerobic cultures but the $Y_{fructose}$ decreased sharply to the level of the anaerobic cultures with increasing dilution rate (Fig. 3b).

In the anaerobic high- K^+ and high- Na^+ cultures, acetate, formate, ethanol and succinate were major fermentation products. Similar to the product formation profile of the anaerobic control culture, the acetate formation rate was a linear function of dilution rate. The formation of formate and ethanol showed biphasic patterns and increased markedly at higher dilution rates (Fig. 4a, c). Succinate formation was also found but it was not significantly affected by dilution rate. The carbon recoveries for the anaerobic high- K^+ cultures were between 76 and 97% and those for the anaerobic high- Na^+ cultures 88–100%. In the microaerobic high- K^+ culture, acetate formation was predominant over a wide range of dilution rates (Fig. 4b), while in the microaerobic high- Na^+ culture, formation of both acetate and formate was evident (Fig. 4d). The carbon recoveries for the microaerobic high- K^+ cultures were between 67 and 80% and those for the microaerobic high- Na^+ cultures 73–84%.

Fig. 4. Effect of increased extracellular concentration of K^+ (81 mM K^+ and 24 mM Na^+) (a, b) and Na^+ (5.2 mM K^+ and 106 mM Na^+) (c, d) on the specific production rates of formate ($\circ$), acetate ($\bullet$), ethanol (∇) and succinate ($\blacktriangledown$) in anaerobic (a, c) and microaerobic (b, d) fructose-limited chemostat cultures of *A. actinomycetemcomitans* 301-b.

Estimation of ATP production rate during anaerobic growth

For all the steady states of anaerobic chemostat cultures, with the control, high- K^+ and high- Na^+ media, the specific rate of ATP production from fructose fermentation ($q_{ATP(fermen)}$) was calculated and the relationships between $q_{fructose}$ and $q_{ATP(fermen)}$ were examined. As shown in Fig. 5, irrespective of the K^+ and Na^+ concentrations in the medium, plots of the $q_{ATP(fermen)}$ values against the corresponding $q_{fructose}$ values yielded a single straight line with the following approximated equation:

$$q_{ATP(fermen)} = 3.13 q_{fructose} + 0.103 \quad (r = 0.998)$$

This indicates that under the anaerobic condition, the specific rate of fermentation-derived ATP production was not affected by the increases in extracellular K^+ and Na^+ concentrations, and the ATP yield was constant with the value of 3.13 mol ATP per mol fructose.

Estimation of ATP production during microaerobic growth

In the microaerobic control cultures, the specific rate of ATP production from fermentation ($q_{ATP(fermen)}$) was also a linear function of the fructose consumption rate

Fig. 5. Specific rate of ATP production from fermentation as a function of the specific rate of fructose consumption in anaerobic fructose-limited cultures of *A. actinomycetemcomitans* 301-b: control (5.2 mM K^+ and 24 mM Na^+) ($\circ$), high- K^+ (81 mM K^+ and 24 mM Na^+) (∇) and high- Na^+ (5.2 mM K^+ and 106 mM Na^+) ($\square$). The specific rate of ATP production from fermentation ($q_{ATP(fermen)}$) was calculated by the equation $q_{ATP(fermen)} = 2 q_{fructose} + q_{acetate}$. The linear relationship approximates to the equation $q_{ATP(fermen)} = 3.13 q_{fructose} + 0.103$ ($r = 0.998$).

($q_{fructose}$) (Fig. 6a). However, the estimated ATP yield value [$2.49 \text{ mol ATP (mol fructose)}^{-1}$] was lower than the anaerobic value [$3.13 \text{ mol ATP (mol fructose)}^{-1}$]. Assuming that the Y_{ATP} was similar between anaerobic and microaerobic cultures, this result did not account for the increased $Y_{fructose}$ values in the microaerobic cultures at lower dilution rates. Hence, it was very likely that the additional ATP was derived from respiration. The Y_{ATP} calculated from the anaerobic cultures increased linearly from $11.7 \text{ g dry wt (mol$

$ATP)^{-1}$ at $D = 0.04 \text{ h}^{-1}$ to $14.9 \text{ g dry wt (mol ATP)}^{-1}$ at $D = 0.20 \text{ h}^{-1}$ (Fig. 1a). Based on the assumption that the Y_{ATP} was dependent of the growth rate and did not change significantly with a change from anaerobic to microaerobic conditions at a fixed growth rate, the specific rates of total ATP production ($q_{ATP(total)}$) and ATP production via respiration ($q_{ATP(respi)}$) were estimated for each steady state culture. As shown in Fig. 6(a), the $q_{ATP(respi)}$ was expected to parallel the $q_{ATP(fermen)}$ until it reached a maximum value at a $q_{fructose}$ of $1.58 \text{ mmol (g dry wt)}^{-1} \text{ h}^{-1}$, and to decrease above this $q_{fructose}$ value. From the ratio of $q_{ATP(respi)}$ to $q_{fructose}$, the respiration-derived ATP yield was estimated to be between 1.2 and $2.0 \text{ mol ATP (mol fructose)}^{-1}$ below $q_{fructose} = 1.58 \text{ mmol (g dry wt)}^{-1} \text{ h}^{-1}$.

In the microaerobic high- K^+ and high- Na^+ cultures, the relationships between $q_{fructose}$ and $q_{ATP(fermen)}$ were also linear and the estimated values of the ATP yield from fermentation were higher than the value of the control culture: 3.14 and $3.34 \text{ mol ATP (mol fructose)}^{-1}$ for the high- K^+ and high- Na^+ cultures, respectively. This suggests that fermentation during microaerobic growth was affected by the extracellular K^+ and Na^+ concentrations. However, the increased ATP yield from fermentation was comparable to the anaerobic value, and again this did not explain the higher $Y_{fructose}$ values in the microaerobic high- K^+ and high- Na^+ cultures. At a $q_{fructose}$ lower than $1.5 \text{ mmol (g dry wt)}^{-1} \text{ h}^{-1}$, the estimated $q_{ATP(respi)}$ did not increase so evidently as in the control culture. However, above $q_{fructose} = 1.5 \text{ mmol (g dry wt)}^{-1} \text{ h}^{-1}$, the $q_{ATP(respi)}$ of the high- K^+ culture increased markedly while that of the high- Na^+ culture decreased (Fig. 6b, c). Therefore, it can be concluded that respiration during microaerobic growth is strongly influenced by extracellular K^+ and Na^+ , and

Fig. 6. Relationships between the specific rate of fructose consumption and the specific rates of total ATP production ($\bullet$), ATP production from fermentation ($\circ$) and ATP production from respiration (∇) in microaerobic fructose-limited cultures of *A. actinomycetemcomitans* 301-b: control (5.2 mM K^+ and 24 mM Na^+) (a), high- K^+ (81 mM K^+ and 24 mM Na^+) (b) and high- Na^+ (5.2 mM K^+ and 106 mM Na^+) (c). The specific rates of total ATP production ($q_{ATP(total)}$) and ATP production via respiration ($q_{ATP(respi)}$) were estimated by the equation $q_{ATP(total)} = q_{ATP(fermen)} + q_{ATP(respi)} = (Y_{fructose}/Y_{ATP}) q_{fructose}$. In the control culture, the Y_{ATP} value at $D = 0.19 \text{ h}^{-1}$ was obtained from the approximated linear relationship between D and Y_{ATP} : $Y_{ATP} = 20.3 D + 10.9$ ($r = 0.996$). The linear relationships approximate to the following equations: for the control culture (a), $q_{ATP(fermen)} = 2.49 q_{fructose} + 0.934$ ($r = 0.998$); for the high- K^+ culture (b), $q_{ATP(fermen)} = 3.14 q_{fructose} + 0.666$ ($r = 0.998$); and for the high- Na^+ culture (c), $q_{ATP(fermen)} = 3.34 q_{fructose} + 0.524$ ($r = 1.000$).

higher concentrations of extracellular K^+ are required for respiration to occur in rapidly growing cells. In the microaerobic high- K^+ culture, the ATP yield from respiration was estimated to be between 1.18 and 1.91 mol ATP (mol fructose) $^{-1}$ and the yield from fermentation between 3.32 and 4.24 mol ATP (mol fructose) $^{-1}$.

Satisfactory explanations are not yet available for the requirement of K^+ in cells growing rapidly under microaerobic conditions. One possibility is that mechanisms leading to a significant energy loss occur when cells are grown at higher rates in low- K^+ conditions. The most probable mechanism is a K^+ efflux system because a K^+ efflux system (*kefC*) was reported in not only *E. coli* (Bakker *et al.*, 1987) but also *Haemophilus influenzae*: the latter is phylogenetically related to *A. actinomycetemcomitans* (Fleischmann *et al.*, 1995). If this is the case for *A. actinomycetemcomitans*, it will be of interest to examine whether the leakage of K^+ from the cytoplasm is enhanced by exposure of cells to oxygen. The occurrence of a significant energy loss is also very likely for the high- Na^+ culture at higher growth rates because a negative effect of increasing Na^+ on the Y_{fructose} was observed (Fig. 3b). Based on the database of the on-going genome analysis of *A. actinomycetemcomitans* strain HK1651 (B. A. Roe, F. Z. Najjar, S. Clifton, T. Ducey, L. Lewis and D. W. Dyer, University of Oklahoma, Department of Chemistry and Biochemistry, and the University of Oklahoma Health Science Centre, Department of Microbiology and Immunology; <http://www.genome.ou.edu/act.html>), Na^+/H^+ antiporter proteins are expected to be present in this organism, and thus in high- Na^+ conditions, extra energy will be needed to drive the Na^+/H^+ antiporters for keeping intracellular Na^+ concentration low.

K^+ is the major cytoplasmic cation of growing bacterial cells (Silver, 1978). In Gram-negative bacteria such as *E. coli* and *K. aerogenes* (*K. pneumoniae*), intracellular K^+ concentrations are as high as 0.1–0.5 M (Tempest *et al.*, 1966; Kakinuma, 1998). In *E. coli*, K^+ can be taken up via a number of systems that differ in their affinity for K^+ ; there is a constitutive low-affinity system (Trk) and an inducible high-affinity system (Kdp) (Bakker, 1993; Epstein *et al.*, 1993). In *H. influenzae*, the gene encoding the Trk K^+ uptake protein is present but the gene of the Kdp system is absent (Fleischmann *et al.*, 1995). This seems also to be true for *A. actinomycetemcomitans*, based on the database of its on-going genome analysis. The absence of the high-affinity system for K^+ uptake might be disadvantageous for *A. actinomycetemcomitans* in low- K^+ environments. This would be consistent with the fact that the primary ecological niche of *A. actinomycetemcomitans* is a K^+ -rich environment such as dental plaque (61.5 ± 13.5 mM) and gingival fluid (17.4 ± 9.0 mM) (Tatevossian & Gould, 1976). Interestingly, it was reported that in patients suffering a more severe periodontitis, the gingival fluid Na^+ concentrations tended to be lower, and those for K^+ to be significantly higher (Bang *et al.*, 1973). As discussed by Bang *et al.* (1973), in more severe cases of periodontitis

the higher number of degenerating epithelial, connective tissue and blood cells contributes to increasing the K^+ concentration of the exudates by the liberation of their intracellular content. Hence, it may be speculated that both moderate levels of oxygen tension and increased K^+ contents prevail in periodontal lesion sites and these constitute conditions favourable for the growth of *A. actinomycetemcomitans*.

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