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The ability of *Escherichia coli* O157:H7 to decrease its intracellular pH and resist the toxicity of acetic acid

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Batch cultures of *Escherichia coli* K-12 grew well in an anaerobic glucose medium at pH 5·9, but even small amounts of acetate (20 mM) inhibited growth and fermentation. *E. coli* O157:H7 was at least fourfold more resistant to acetate than K-12. Continuous cultures of *E. coli* K-12 (pH 5·9, dilution rate 0·085 h $^{-1}$) did not wash out until the sodium acetate concentration in the input medium was 80 mM, whereas *E. coli* O157:H7 persisted until the sodium acetate concentration was 160 mM. *E. coli* K-12 cells accumulated as much as 500 mM acetate, but the intracellular acetate concentration of O157:H7 was never greater than 300 mM. Differences in acetate accumulation could be explained by intracellular pH and the transmembrane pH gradient (ΔpH). *E. coli* K-12 maintained a more or less constant ΔpH (intracellular pH 6·8), but *E. coli* O157:H7 let its ΔpH decrease from 0·9 to 0·2 units as sodium acetate was added to the medium. Sodium acetate increased the rate of glucose consumption, but there was little evidence to support the idea that acetate was creating a futile cycle of protons. Increases in glucose consumption rate could be explained by increases in D-lactate production and decreases in ATP production. Intracellular acetate was initially lower than the amount predicted by ΔpH, but intracellular acetate and ΔpH were in equilibrium when the external acetate concentrations were high. Based on these results, the acetate tolerance of O157:H7 can be explained by fundamental differences in metabolism and intracellular pH regulation. By decreasing the intracellular pH and producing large amounts of D-lactate, O157:H7 is able to decrease ΔpH and prevent toxic accumulations of intracellular acetate anion.

Keywords: *Escherichia coli*, enterohaemorrhagic, intracellular pH, acetate toxicity

INTRODUCTION

Escherichia coli O157:H7 is a highly virulent food-borne pathogen that causes the death of children and immunologically compromised people (Reilly *et al*., 1983). *E. coli* O157:H7 produces a verotoxin that causes diarrhoea, but the mechanism of the entero-haemorrhagic infection is poorly understood (Boyce *et al*., 1995). The O157:H7 serotype has unique antigenic properties, but recent work indicated that it can also be identified by enzymic methods. *E. coli* O157:H7 has little β-glucuronidase, and a low ratio of β-glucuronidase to β-galactosidase activities (Ratnam *et al*., 1988).

Abbreviation: ΔpH, transmembrane pH gradient.

Acetate is often used as a food preservative (Jay, 1989), but *E. coli* O157:H7 appears to be more acid-tolerant than other *E. coli* strains, and it has an unusually strong resistance to acetate (Conner & Kotrola, 1995). The antibacterial effect of acetate has typically been explained by the ability of undissociated acetate molecules to pass through the cell membrane and release protons (Salmond *et al*., 1984). The influx of protons is thought to uncouple growth and drain cellular energy resources by catalytically dissipating proton-motive force (Axe & Bailey, 1995).

Lactic acid bacteria grow at low pH in the presence of acetate, and many of these bacteria are able to decrease intracellular pH when extracellular pH decreases (Kashket, 1987; Russell, 1991). The decline in intracellular pH allows the bacteria to maintain a low

transmembrane pH gradient (ΔpH). Recent work has suggested that low ΔpH is an adaptive mechanism that prevents the accumulation of acetate anions in the more alkaline interior (Russell, 1992).

Standard strains of *E. coli* generate a large ΔpH at low extracellular pH (Padan *et al.*, 1981), but the intracellular pH regulation of *E. coli* O157:H7 has not previously been examined. Based on the observation that many acetate-resistant bacteria let their intracellular pH decline (Russell, 1992), it appeared that *E. coli* O157:H7 might have a different pattern of intracellular pH regulation. The experiments described in this paper were designed to compare the effect of acetate on the intracellular pH of *E. coli* O157:H7 and K-12.

METHODS

Materials. All chemicals were of reagent grade; [*carboxyl*-^{14}C]salicylate, [U-^{14}C]polyethylene glycol, $^{3}H_2O$ and [2-^{14}C]acetate were obtained fom Amersham.

Bacterial strains and growth conditions. *E. coli* K-12 (ATCC 12435, J. Lederberg W1485) was provided by Dr Valley Stewart (Ithaca, NY). *E. coli* O157:H7 (ATCC 43895, CDC EDL 933) was originally isolated from raw hamburger implicated in an haemorrhagic colitis outbreak (Reilly *et al.*, 1983). Both bacteria were cultivated in a basal medium containing (per litre): K_2HPO_4, 292 mg; KH_2PO_4, 292 mg; $(NH_4)_2SO_4$, 1·2 g; NaCl, 480 mg; $MgSO_4.7H_2O$, 100 mg; $CaCl_2.2H_2O$, 64 mg; Na_2HPO_4, 5·7 g; cysteine hydrochloride, 0·6 g; yeast extract, 0·5 g; Trypticase (BBL), 1·0 g. The medium was adjusted to pH 5·9 with 6 M HCl. After autoclaving, the medium was continuously purged with O_2-free nitrogen. Concentrated solutions of glucose and sodium acetate were prepared under O_2-free nitrogen and autoclaved separately. Batch cultures were grown anoxically in rubber-stoppered tubes (10 ml, 18 mm diameter) with an excess of glucose (25 mM). Optical density was measured at 600 nm (Spectronic 21D spectrophotometer). Continuous cultures were grown anoxically in pH-controlled, glucose-limited (16·7 mM) chemostats (F1000 fermenter, 360 ml culture vessel, New Brunswick Scientific). Sodium acetate was added to the medium reservoir. Samples were taken after approximately four culture vessel volumes of medium had passed through the culture vessel.

Intracellular pH. Internal pH was determined by an acid distribution method (Reibeling *et al.*, 1975). Growing cultures (0·3 to 0·5 mg protein ml^{-1}) were incubated with [*carboxyl*-^{14}C]salicylate (0·037 MBq, 2·09 GBq mmol^{-1}), [U-^{14}C]polyethylene glycol (0·037 MBq, 2·2 GBq mmol^{-1}), $^{3}H_2O$ (0·148 MBq, 0·13 GBq mmol^{-1}) for 5 min and then centrifuged through silicone oil (50:50 mixture of Dexter Hysol 550 and 556) in a microcentrifuge (13000 ***g***, 5 min). Supernatant samples (20 µl) were removed and bottoms of tubes containing cell pellets were removed with dog nail clippers after freezing. Pellets and supernatants were dissolved in aqueous compatible scintillation fluid. Internal volume [3·2 µl (mg protein)$^{-1}$] was estimated from the difference between $^{3}H_2O$ and ^{14}C-labelled polyethylene glycol.

Intracellular acetate. Cells were incubated as above with [2-^{14}C]acetate (0·37 MBq, 2·03 GBq mmol^{-1}), and intracellular accumulation was estimated by the method of Reibeling *et al.* (1975).

Intracellular potassium. Growing cultures (1 ml) were centrifuged (13000 ***g***, 5 min) through 0·3 ml silicone oil (50:50 mixture of Hysol 550 and 560). The microcentrifuge tubes were frozen (−15 °C), and the pellets were removed with a pair of dog nail clippers. The cell pellets were digested at room temperature for 24 h in 3 M HNO_3, and insoluble cell debris was removed by centrifugation. Potassium was determined by flame photometry (Cole-Parmer model 2655-00 digital flame analyser). Intracellular volume was determined as described above.

Enzyme activities. Exponentially growing cells from a medium containing 60 mM acetate were harvested at pH 5·5 by centrifugation (10000 ***g***, 5 °C, 10 min), washed in Tris/HCl buffer (50 mM, pH 7·4) and sonicated (Branson model 200 sonifier, microtip, maximum output, 5 °C, 10 min, 50% duty cycle). Unbroken cells and cell debris were removed by centrifugation (10000 ***g***, 5 °C, 10 min) and the cell-free extract was kept on ice until assayed. D-Lactate dehydrogenase was assayed by following NADH oxidation as described by Tarmy & Kaplan (1968). Methylglyoxal synthase and glyoxalase were assayed by the method of Cooper & Anderson (1970).

Other analyses. Glucose was analysed by an enzymic method using hexokinase and glucose-6-phosphate dehydrogenase (Bergmeyer & Klotsch, 1965). Protein from NaOH-hydrolysed cells (0·2 M NaOH, 100 °C, 15 min) was assayed by the Lowry method. Fermentation acids were analysed by high-pressure liquid chromatography (Bio-Rad HPX-87H column, 50 °C, 8·5 mM H_2SO_4, 0·5 ml min^{-1}, refractive index detector). The column temperature was increased to 85 °C to separate methylglyoxal from succinate. D- and L-lactate were differentiated by enzymic methods employing L- and D-lactate dehydrogenases (Hohorst, 1965).

RESULTS

Growth in batch culture

E. coli K-12 grew well in an anaerobic medium at pH 5·9 (Fig. 1a), and it reduced the final pH to 4·3 (Fig. 1b). Even small amounts of sodium acetate inhibited growth, and there was little increase in optical density or decrease in pH at acetate concentrations greater than 20 mM. *E. coli* O157:H7 was at least fourfold more resistant to acetate than K-12.

Growth in continuous culture

Anaerobic continuous cultures (dilution rate 0·085 h^{-1}) of *E. coli* K-12 grew well at pH 5·9, and yielded 17 mg cell protein per mmol glucose (Fig. 2a). Addition of 20 mM sodium acetate to the medium reservoir had little effect on microbial protein synthesis, but higher concentrations of sodium acetate caused an accumulation of glucose in the culture vessel (Fig. 2b) and a decline in cell protein (Fig. 2a). No growth was detected when the concentration of sodium acetate was greater than 80 mM. *E. coli* O157:H7 was much more resistant to sodium acetate addition than K-12, and it did not leave detectable amounts of residual glucose in the continuous culture (pH 5·9) until the sodium acetate concentration was greater than 100 mM (Fig. 2b); growth persisted until the sodium acetate concentration was 160 mM (Fig. 2a). K-12 had a higher specific rate of glucose consumption than O157:H7, and sodium acetate addition increased the glucose consumption rate of both strains (Table 1). K-12 and O157:H7 converted the

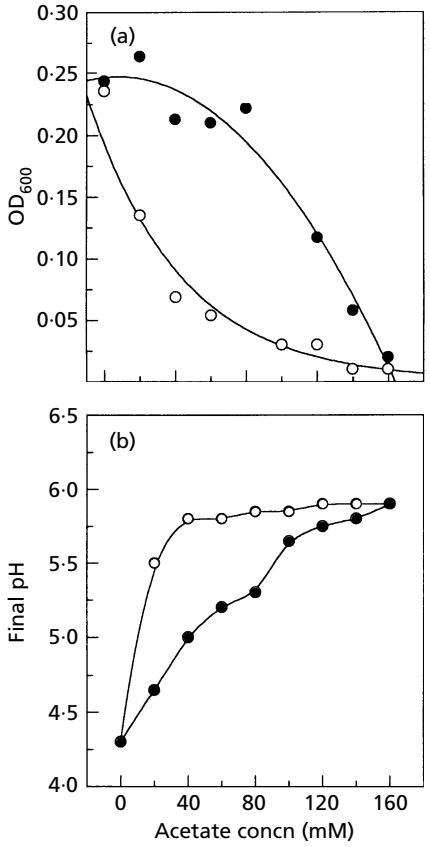

Fig. 1. (a) Growth of *E. coli* K-12 (○) and O157:H7 (●) in anaerobic batch culture for 24 h with excess glucose (25 mM) and increasing amounts of sodium acetate. The initial pH was 5·9. Growth was estimated from the increase in OD_{600}. (b) Final pH. ○, K-12; ●, O157:H7. Measurements were obtained in duplicate from two independent experiments.

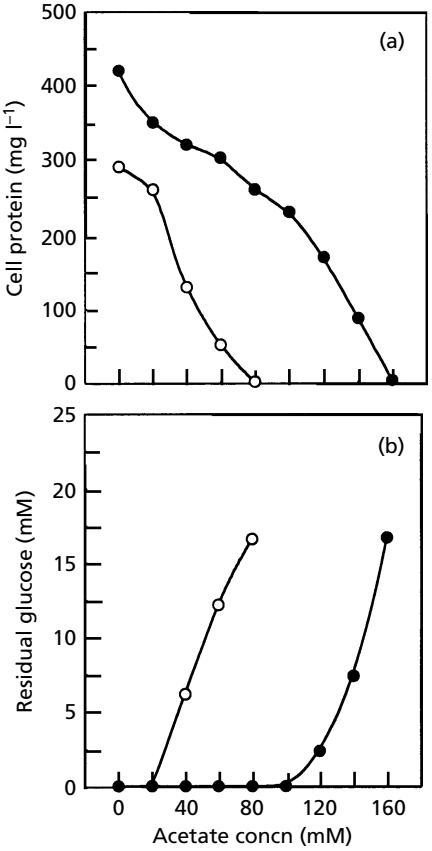

Fig. 2. (a) Growth of *E. coli* K-12 (○) and O157:H7 (●) in anaerobic glucose-limited continuous culture with increasing amounts of sodium acetate at pH 5·9. (b) Accumulation of glucose in the culture vessel. ○, K-12; ●, O157:H7. The medium reservoir had 16·7 mM glucose. Values are the means of replicates of at least two separate experiments.

glucose to acetate, formate and ethanol, but sodium acetate addition caused a shift in fermentation end-products. When the rate of glucose consumption increased, glucose was also converted to D-lactate. O157:H7 produced more D-lactate than K-12 (Table 1). Continuous cultures also produced succinate, but this end-product accounted for less than 8% of the glucose consumption. Methylglyoxal was never detected, but the detection limit for this fermentation end-product was approximately 0·5 mM.

Methylglyoxal shunt

E. coli K-12 and O157:H7 both had high D-lactate dehydrogenase activities [1490 and 1250 nmol (mg protein)$^{-1}$ min^{-1}, respectively]. Methylglyoxal synthase and glyoxalase activities [250 and 11 nmol (mg protein)$^{-1}$ min^{-1}, respectively] were also detected and both strains had similar values.

Intracellular pH and acetate accumulation

Continuous cultures of *E. coli* K-12 (pH 5·9) had an intracellular pH of approximately 6·8, and sodium acetate addition did not cause a significant decrease in intracellular pH (Fig. 3a). *E. coli* K-12 accumulated large amounts of acetate, and the intracellular acetate concentration was as high as 500 mM (Fig. 3b). The intracellular pH of *E. coli* O157:H7 declined when sodium acetate was added to the medium reservoir (Fig. 3a), and the intracellular acetate concentration of this strain was never greater than 300 mM (Fig. 3b). *E. coli* K-12 and O157:H7 both had approximately 300 mM intracellular potassium, but K-12 increased its intracellular potassium when the intracellular acetate concentration was greater than 300 mM (Fig. 3c). Intracellular acetate anion concentrations were initially lower than amounts predicted by ΔpH, but the ratio of intracellular to extracellular acetate anion was in close agreement with ΔpH at high acetate concentrations (Fig. 4).

DISCUSSION

Acetate can have either bacteriostatic or bactericidal effects and provides a barrier to the growth of pathogenic bacteria in the gut and in foods. Even low concentrations of acetate (< 50 mM) at mildly acidic

Table 1. Effect of sodium acetate addition to the medium reservoir on specific rates of glucose consumption (q_{Glc}), lactate (q_{Lact}), acetate (q_{Acet}) and ethanol (q_{Eth}) production, and Y_{Glc} of continuous cultures of *E. coli* strains K-12 and O157:H7

Rate units are μmol (mg protein)$^{-1}$ h^{-1}; Y_{Glc} units are g protein (mol glucose)$^{-1}$. These values are the means of three measurements from at least two separate experiments.

Acetate concn (mM)	K-12					O157:H7				
	q_{Glc}	q_{Lact}	q_{Acet}	q_{Eth}	Y_{Glc}	q_{Glc}	q_{Lact}	q_{Acet}	q_{Eth}	Y_{Glc}
0	4·8	0·2	4·0	3·8	17·4	3·4	0·2	2·7	2·2	25·2
20	5·4	0·2	4·0	3·8	15·6	4·0	0·2	3·0	3·4	21·0
40	6·9	2·6	4·5	4·9	12·4	4·4	0·5	3·1	4·1	19·3
60	7·4	3·3	7·1	3·7	11·6	4·7	1·4	2·7	3·9	18·1
80						5·4	2·9	2·4	4·1	15·6
100						6·1	4·5	2·9	3·7	13·8
120						7·1	7·0	2·6	4·3	11·9
140						9·0	11·4	3·8	2·1	9·5

pH (6·0) can inhibit the growth of *E. coli* batch cultures (Wolin, 1969; Wallace *et al*., 1989), but much higher concentrations of undissociated acetate are needed to kill *E. coli* cells, particularly if they have been habituated to acid (Goodson & Rowbury, 1989). Shrirnov and Oktyabr'skii (1988) noted that *E. coli* was more sensitive to acetate when oxygen was not available. *E. coli* is usually grown aerobically with an excess of glucose, but the gastrointestinal tract of mammals is a strictly anaerobic environment that operates as an energy-limited continuous culture system (Miller & Wolin, 1981).

Conner & Kotrola (1995) indicated that *E. coli* O157:H7 was highly resistant to acetate and was able to survive under aerobic conditions with 27 mM acetate at pH 5·0 for 21 d. Rasmussen *et al*. (1993) reported that O157:H7 did not grow better than other *E. coli* strains under anaerobic conditions in an acidic medium containing acetate, but our results indicated that *E. coli* O157:H7 was much more resistant to sodium acetate than *E. coli* K-12. Continuous cultures of K-12 tolerated more acetate than batch cultures, but O157:H7 was still more resistant.

The antimicrobial effect of acetate has traditionally been attributed to the depression of pH below the growth range and metabolic inhibition by undissociated acid molecules (Jay, 1989). With the advent of the chemiosmotic theory in the 1960s (Mitchell, 1961), it became apparent that highly lipophilic acids and bases could transfer protons across the cell membrane and dissipate proton-motive force. Because undissociated acetate molecules are lipid permeable, the toxicity of acetate has often been explained by 'uncoupling' and decreases in proton-motive force (Salmond *et al*., 1984; Baronofsky *et al*., 1984). Ferguson *et al*. (1995) showed that a pulse dose of acetate could decrease the ΔpH of *E. coli* MJF274, but these short-term experiments did not demonstrate a sustained decrease in ΔpH or proton-motive force.

Axe & Bailey (1995) reported that *E. coli* cells producing acetate had intracellular acetate concentrations that were consistent with an 'uncoupling model', but acetate flux *per se* was not measured. In this model a hypothetical carrier was purported to translocate acetate electroneutrally by an acetate anion/H^+ influx mechanism and electrogenically by an acetate anion efflux mechanism. Because anion efflux would be driven by the membrane potential, the intracellular acetate concentration would be lower than that predicted by ΔpH. The intracellular acetate concentrations of *E. coli* K-12 and O157:H7 were initially lower than that predicted by ΔpH, but the values were in close agreement when the extracellular acetate concentration was high. These latter results indicated that high concentrations of acetate did not 'uncouple' *E. coli*.

Synthetic uncouplers such as 2,4-dinitrophenol increase the specific rate of glucose and ATP consumption (Neijssel, 1977). When *E. coli* K-12 and O157:H7 were grown in continuous culture at pH 5·9, sodium acetate addition caused an increase in the specific rate of glucose consumption, but there was also a change in fermentation end-products. Both strains produced acetate when the extracellular acetate concentration was low; this mixed acid fermentation scheme would provide 3 ATP/glucose (Gottschalk, 1986). Acetate addition caused an increase in D-lactate production. Both strains of *E. coli* have D-lactate dehydrogenase activities (Stokes, 1949; Clark, 1989) and when glucose is converted to D-lactate via the Embden Meyerhof pathway, only 2 ATP/glucose are produced. Teixeira de Mattos *et al*. (1984) noted that *Klebsiella aerogenes* (*K. pneumoniae*) produced D-lactate when glucose was in excess, and that this D-lactate arose from the methylglyoxal shunt. When D-lactate is produced via the methylglyoxal shunt, there is a net loss of 2 ATP/glucose, drastically decreasing ATP production (Cooper, 1984). *E. coli* K-12 and O157:H7 did not produce large amounts of D-lactate until glucose accumulated, and both strains had

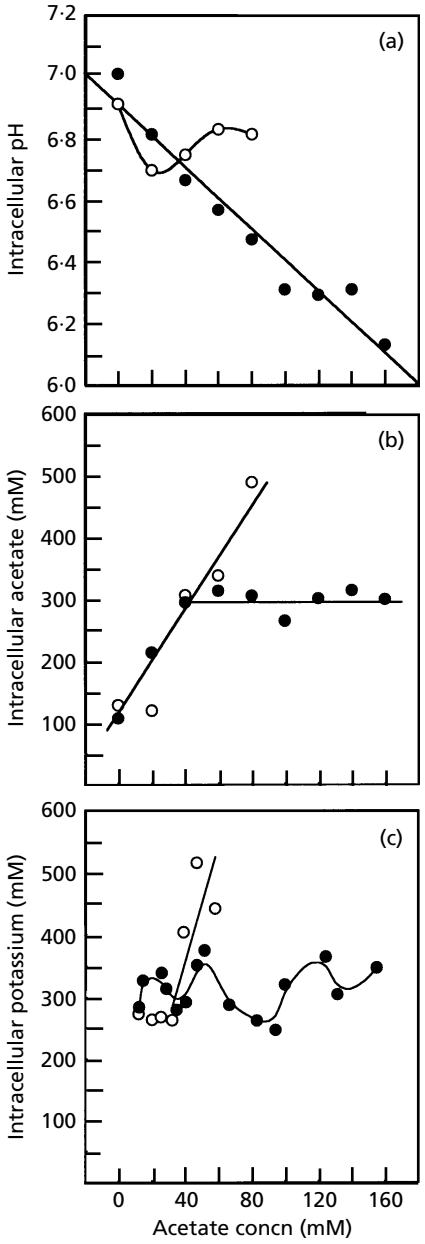

Fig. 3. Intracellular pH (a), acetate (b) and potassium (c) in *E. coli* K-12 (○) and O157:H7 (●) grown in anaerobic glucose-limited continuous culture at pH 5·9 with increasing amounts of sodium acetate in the medium reservoir.

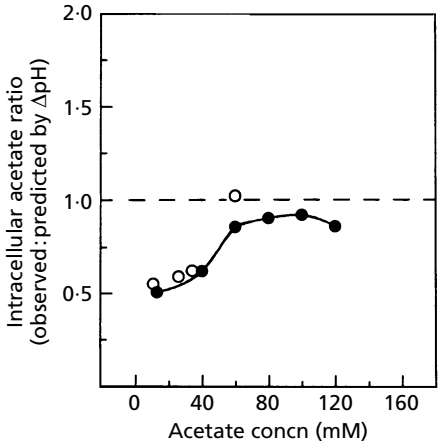

Fig. 4. Ratio of observed and predicted intracellular acetate anion concentrations at different concentrations of extracellular acetate. Predicted values were derived from ΔpH and the Henderson–Hasselbalch equation. A value of 1·0 indicates perfect agreement. ○, K-12; ●, O157:H7.

methylglyoxal synthase and glyoxalase activities. Because glycolysis and the methylglyoxal shunt have the same labelling patterns (Cooper, 1984), we could not differentiate between D-lactate arising from the methylglyoxal shunt and D-lactate dehydrogenase. If only 30% of glucose had been diverted through the methylglyoxal shunt, the specific rate of ATP consumption would have remained constant.

Aerobic cultures of *E. coli* maintain an intracellular pH of approximately 7·6 over a wide range of extracellular pH (Padan *et al.*, 1981), but Kashket (1983) indicated that anaerobic batch cultures of *E. coli* had an intracellular pH of 7·0 at an extracellular pH of 6·0. When *E. coli* K-12 and O157:H7 were grown in anaerobic continuous cultures at pH 5·9, the intracellular pH was approximately 6·8. K-12 always maintained a ΔpH of approximately 0·9 units, but O157:H7 decreased its ΔpH from 0·9 to 0·2 units as acetate increased. Because the high intracellular acetate anion concentrations were in close agreement with those predicted by ΔpH, it appeared that ΔpH was the principal driving force for acetate accumulation. Differences in ΔpH caused a marked difference in accumulation of acetate across the cell membrane. The intracellular acetate concentration of O157:H7 never exceeded 300 mM, but K-12 accumulated as much as 500 mM. *E. coli* K-12 and O157:H7 both accumulated potassium when sodium acetate was added, with the intracellular potassium concentration matching that of the intracellular acetate anion.

The experiments with *E. coli* K12 and O157:H7 indicated that bacteria can have different strategies for dealing with acetate. Bacteria with large ΔpH (e.g. *E. coli* K-12) accumulate large amounts of intracellular acetate anion, and this accumulation appears to cause an increase in intracellular potassium. *E. coli* has a variety of potassium efflux systems that regulate osmotic pressure, but potassium efflux causes cytoplasm acidification (Ferguson *et al.*, 1995). Decreases in intracellular pH and ΔpH protect the cell from acetate anion and potassium accumulation, but this adaptation is only beneficial if the cell has a metabolic system that can tolerate low pH. In *E. coli* O157:H7 the decrease in intracellular pH from 7·0 to 6·1 was accompanied by a marked increase in glucose consumption and a decline in glucose yield (Table 1).

Further work is clearly needed to explain the intracellular pH regulation of *E. coli* O157:H7, but methylglyoxal shunt activity could be important. *E. coli*

O157:H7 produced more D-lactate, had a higher glucose consumption rate and accumulated less potassium than K-12, and recent work has indicated that *E. coli* MJF274 has a methylglyoxal-sensitive, glutathione-activated potassium channel that acidifies the cytoplasm (Ferguson *et al.*, 1995).

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