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Effects of carbonylcyanide-*m*-chlorophenylhydrazone (CCCP) and acetate on *Escherichia coli* O157:H7 and K-12: uncoupling versus anion accumulation

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Abstract

Non-growing cells of *Escherichia coli* O157:H7 and K-12 that were incubated anaerobically in sodium phosphate buffer at pH 6.5 consumed glucose at a rate of approximately 8 μmol·(mg protein)$^{-1}$·h^{-1} and had intracellular pH values of 7.3 and 7.5, respectively. The uncoupler, carbonylcyanide-*m*-chlorophenylhydrazone (CCCP), caused a marked decrease in intracellular pH, ATP and potassium of both strains. Low concentrations of CCCP stimulated glucose consumption rate, but higher concentrations were inhibitory. Acetate also caused a decrease in intracellular pH, but it never caused a large decrease in glucose consumption rate. Acetate decreased the intracellular ATP of *E. coli* K-12, but it had no effect on the ATP of O157:H7. Acetate had no effect on the intracellular potassium of *E. coli* O157:H7, and acetate-treated K-12 cells had even more potassium than untreated controls. Based on these results, acetate and CCCP appear to have different effects on *E. coli*. The comparison of *E. coli* O157:H7 and K-12 indicated that intracellular pH, acetate accumulation and intracellular potassium were related. *E. coli* K-12 maintained a higher intracellular pH than O157:H7, accumulated more acetate and had a greater intracellular potassium.

Keywords: *Escherichia coli* O157:H7; Uncoupling; Acetate; Intracellular pH; Potassium

1. Introduction

Escherichia coli O157:H7 is a highly virulent food-borne pathogen that causes the death of children and immunologically compromised people [1]. Conner and Kotrola [2] indicated that O157:H7 was more acid-tolerant than other *E. coli* strains, and it appeared to have an unusually strong resistance to acetate. Continuous experiments indicated that *E. coli* O157:H7 was at least 2-fold more resistant to acetate (pH 5.9) than *E. coli* K-12, and O157:H7 did not leave residual glucose in the culture vessel until the acetate concentration was greater than 120 mM [3].

The antibacterial effect of acetate has typically been explained by the ability of undissociated acetate

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[1] "Proprietary or brand names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by the USDA implies no approval of the product, and exclusion of others that may be suitable."

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molecules to pass through the cell membrane and release protons [4]. The influx of protons is thought to uncouple growth and "drain cellular energy resources by catalytically dissipating protonmotive force" [5]. Axe and Bailey [5] hypothesized that acetate anion would then leave the cell via an electrogenic acetate anion carrier, and this hypothesis was supported by the observation that the intracellular acetate concentration of *E. coli* K-12 was lower than the amount predicted by the transmembrane pH gradient (ΔpH).

Recent work indicated that bacteria with high ΔpH were more acetate sensitive than bacteria with low ΔpH, and these results suggested that acetate toxicity was also being mediated by ΔpH and an accumulation of intracellular acetate anion [6,7]. Standard strains of *E. coli* have large ΔpH at low extracellular pH [8], but *E. coli* O157:H7 decreased its intracellular pH and did not accumulate large amounts of acetate [3]. Because the intracellular acetate concentration of *E. coli* O157:H7 was in equilibrium with ΔpH, it appeared that acetate was not acting as an uncoupler per se [3]. The following experiments sought to compare the effects of CCCP, a synthetic uncoupler, and acetate on *E. coli* O157:H7 and K-12.

2. Materials and methods

2.1. Bacterial strains and fermentation conditions

E. coli O157:H7 (ATCC 43895, CDC EDL 933) was originally isolated from raw hamburger implicated in an hemorrhagic colitis outbreak [1]. *E. coli* K-12 (ATCC 12435, J. Lederberg W1485) was provided by Dr. Valley Stewart, Ithaca, NY. They were cultivated in a basal medium containing (per liter): K_2HPO_4, 292 mg; KH_2PO_4, 292 mg; $(NH_4)_2SO_4$, 1.2 g; NaCl, 480 mg; $MgSO_4 \cdot 7H_2O$, 100 mg; $CaCl_2 \cdot 2H_2O$, 64 mg; Na_2HPO_4, 5.7 g; cysteine-hydrochloride, 0.6 g; yeast extract, 0.5 g; Trypticase, 1.0 g. 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 (37°C) in 500-ml vessels with 25 mM glucose. Optical density was measured at 600 nm. Cells were collected by centrifugation (10,000 *g*, 4°C, 10 min) and resuspended in NaH_2PO_4 buffer (100 mM, pH 6.5). Cell suspensions were incubated anoxically at 37°C in a pH-controlled vessel and 10 mM glucose was added. Sodium acetate and CCCP were added in a stepwise fashion immediately after glucose addition. The acetate- or CCCP-treated cell suspensions were then transferred to rubber-stoppered tubes (10 ml, 18-mm diameter) containing O_2-free nitrogen. The cell suspensions were incubated for 1 h incubation at 37°C.

2.2. Intracellular pH and acetate

Internal pH was determined by an acid distribution method [9]. Cell suspensions (0.3–0.5 (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}), $^3H_20$ (0.148 MBq, 0.13 GBq·mmol^{-1}) for 5 min and then centrifuged through silicone oil in a microcentrifuge (13,000 *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 $^3H_20$ and ^{14}C-polyethylene glycol.

Cell suspensions were incubated as above with [2-^{14}C]-acetate (0.37 MBq, 2.03 GBq·mmol^{-1}), and intracellular acetate accumulation was estimated as previously described [9].

2.3. Intracellular potassium and ATP

Cell suspensions (1 ml) were centrifuged (13,000 *g*, 5 min) through 0.3 ml of silicon oil. 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 N HNO_3, and insoluble cell debris was removed by centrifugation. Potassium was determined by flame photometry. Intracellular volume was determined as described above.

Cellular ATP was extracted with perchloric acid (1 ml culture, 0.5 ml 15% (w/v) perchloric acid, 0°C, 20 min). The extracts were centrifuged, and the super-

natants were neutralized with a mixture of K0H and K_2CO_3 (1 M each). ATP was determined with a LKB luminometer using luciferin-luciferase.

2.4. Chemical analyses

Glucose was assayed enzymatically using hexokinase and glucose-6-phosphate dehydrogenase [10]. Cellular protein from cell suspension pellets was determined by the method of Lowry [11].

2.5. Statistics

All experiments and determinations were performed in duplicate and the coefficients of variation were less than 10%.

3. Results

Cells of *Escherichia coli* O157:H7 and K-12 that were incubated anaerobically in phosphate buffer had a glucose consumption rate of approximately 8 μmol·(mg protein)$^{-1}$·h^{-1}. Cells suspensions that were incubated with only glucose never accumulated more than 20 mM intracellular acetate, but intracellular acetate increased as extracellular acetate was added (Fig. 1). Acetate addition caused a decrease in intracellular pH (Fig. 2a). *E. coli* K-12 accumulated even more acetate than O157:H7 (Fig. 1), and this difference in intracellular acetate could be explained by a higher intracellular pH (Fig. 2a). Low concentrations of acetate enhanced the rate of glucose consumption of *E. coli* O157:H7 and K-12

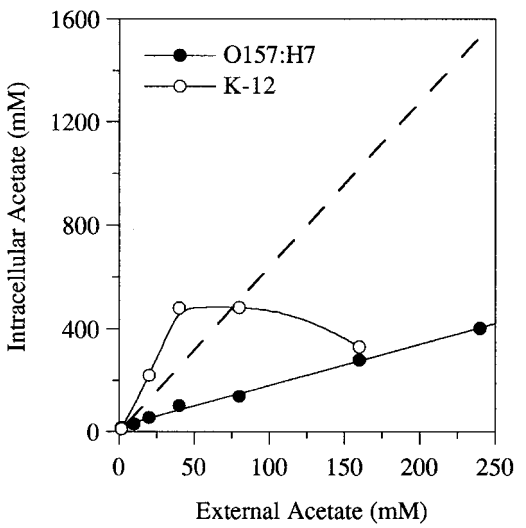

Fig. 1. The effect of extracellular acetate addition on the intracellular acetate concentration of *E. coli* O157:H7 (closed symbols) and *E. coli* K-12 (open symbols). The dotted line shows intracellular acetate as predicted by a constant ΔpH (0.8 units) across the cell membrane. The extracellular pH was 6.5.

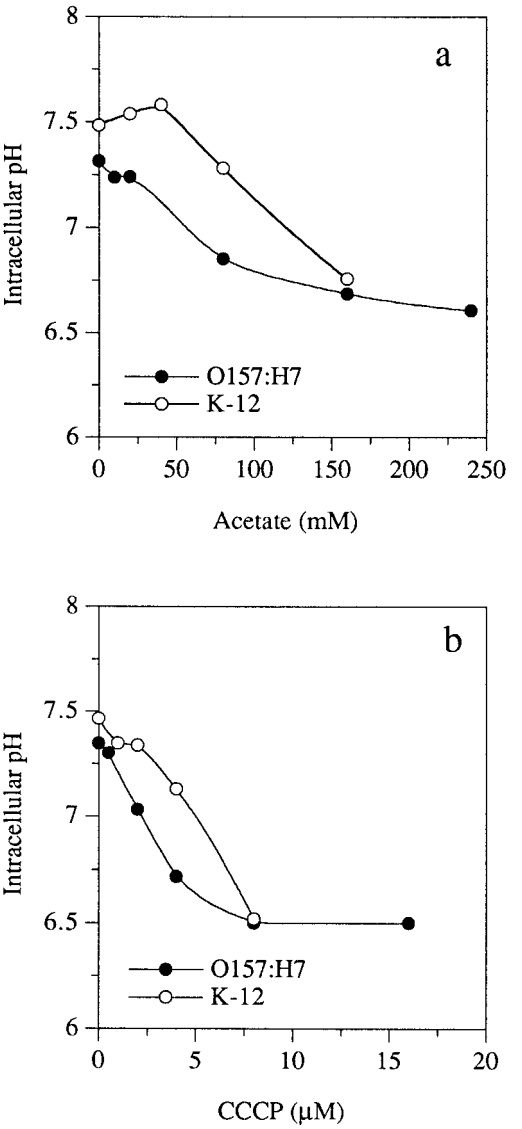

Fig. 2. The effect of extracellular acetate addition (a) or CCCP (b) on the intracellular pH of *E. coli* O157:H7 (closed symbols) and *E. coli* K-12 (open symbols).

(Fig. 3a). Acetate addition had little effect on the intracellular ATP of *E. coli* O157:H7, but even low concentrations of acetate caused a large decrease in the ATP of *E. coli* K-12 (Fig. 4a). Acetate addition had little effect on the intracellular potassium concentration of *E. coli* O157:H7, but it increased the potassium of *E. coli* K-12 (Fig. 5a).

CCCP addition caused a even greater decrease in intracellular pH than extracellular acetate (Fig. 2b versus Fig. 2a), and *E. coli* O157:H7 seemed to be more sensitive than *E. coli* K-12. Low concentrations of CCCP stimulated the glucose consumption rate of *E. coli* O157:H7 and K-12, but concentrations greater than 4 μM caused a marked decline in glucose consumption (Fig. 3b). CCCP caused a decline in intracellular ATP, and both strains seemed to have the same response (Fig. 4b). CCCP caused a marked decrease in intracellular potassium of *E. coli*

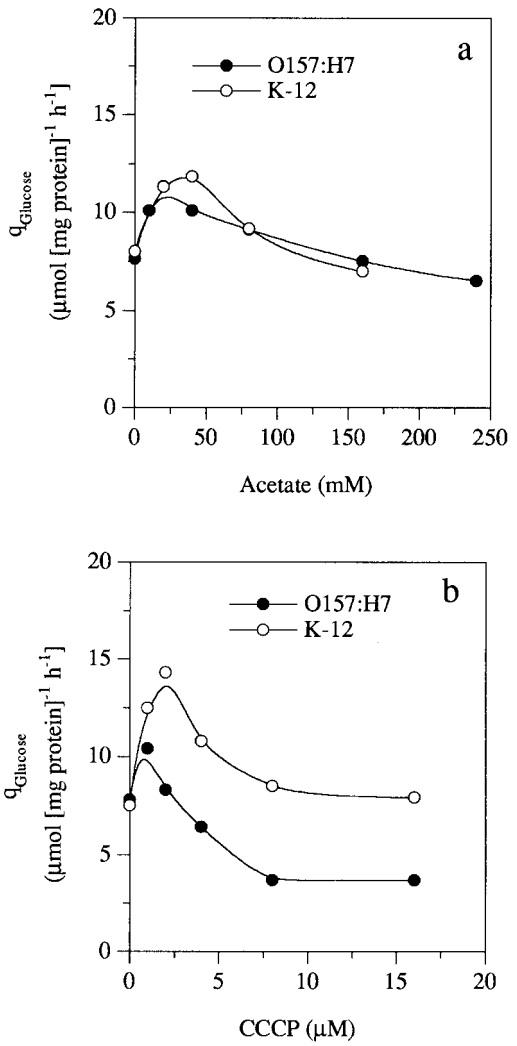

Fig. 3. The effect of extracellular acetate addition (a) or CCCP (b) on the glucose consumption rate ($q_{glucose}$) of *E. coli* O157:H7 (closed symbols) and *E. coli* K-12 (open symbols).

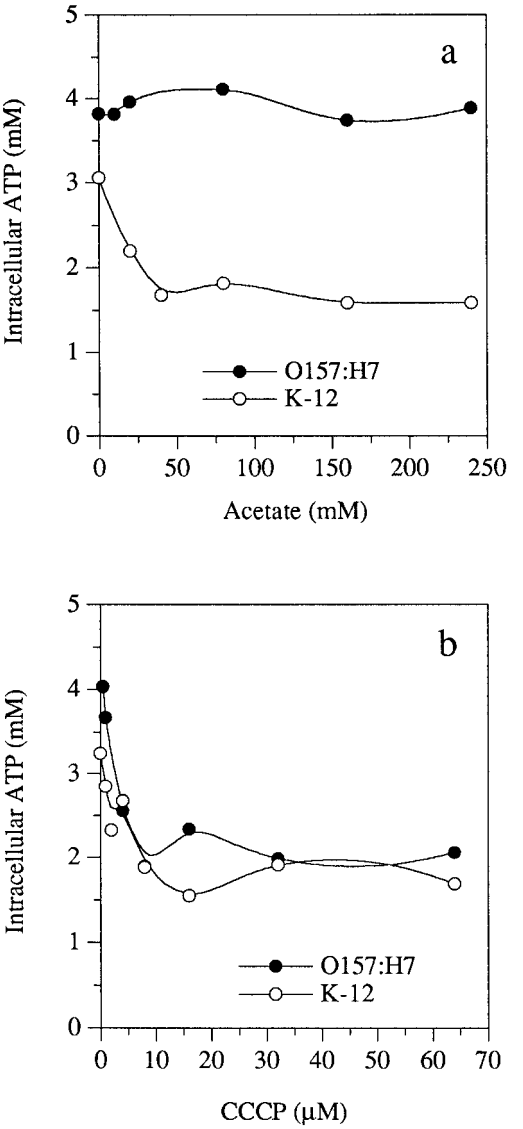

Fig. 4. The effect of extracellular acetate addition (a) or CCCP (b) on the intracellular ATP of *E. coli* O157:H7 (closed symbols) and *E. coli* K-12 (open symbols).

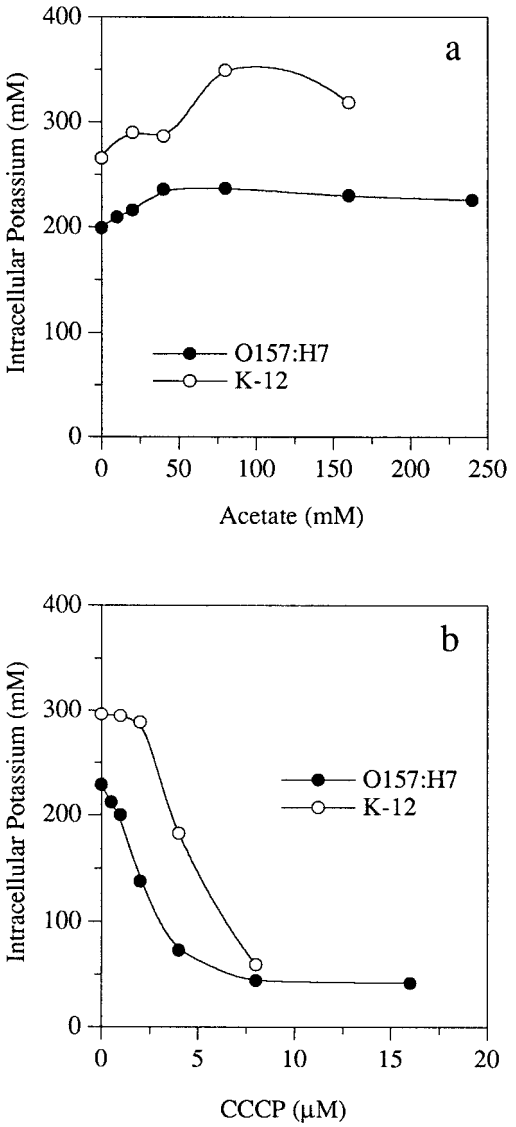

Fig. 5. The effect of extracellular acetate addition (a) or CCCP (b) on the intracellular potassium of *E. coli* O157:H7 (closed symbols) and *E. coli* K-12 (open symbols).

O157:H7 and K-12 (Fig. 5b) and O157:H7 was more sensitive than K-12.

4. Discussion

The antimicrobial activity of acetate and other volatile fatty acids has generally been ascribed to the undissociated species, the influx of acid, and metabolic uncoupling [4]. The notion that acetate might be acting as an uncoupler was consistent with the observation that low concentrations of acetate and CCCP both stimulated the glucose consumption rates and caused a decrease in intracellular pH. High concentrations of CCCP caused a greater decline in glucose consumption than acetate, and his difference could be explained by differences in intracellular pH. CCCP caused a greater decline in intracellular pH than acetate. *E. coli* O157:H7 let its intracellular pH decline to a greater extent than of *E. coli* K-12, and previous work indicated that *E. coli* O157:H7 was able to tolerate a greater decline in intracellular pH than *E. coli* K-12 before growth was inhibited [3]. Both of these results suggest that the two strains have different patterns of intracellular pH regulation.

Metabolic uncouplers often cause a decease in intracellular ATP, and this effect can be explained by the activity of membrane bound ATPases [12]. When uncouplers facilitate the influx of protons, the membrane bound ATPase become more active, and additional ATP is consumed. CCCP and acetate were both able to decrease the intracellular ATP of *E. coli* K-12, but only CCCP was able to decrease the intracellular ATP of *E. coli* O157:H7. Acetate decreased the intracellular pH of *E. coli* O157:H7, but it had virtually no effect on ATP. Because *E. coli* O157:H7 did not have a higher glucose consumption rate than *E. coli* K-12, it did not appear that acetate was acting as an uncoupler per se.

Low affinity potassium transport in *E. coli* is driven by Trk, a symporter that is driven by protonmotive force [13], and previous work with *E. coli* indicated that uncouplers like CCCP cause an almost immediate efflux of intracellular potassium [14]. CCCP was able to decrease the intracellular potassium of both *E. coli* strains, but acetate never caused a decrease in intracellular potassium. Acetate even caused a significant increase in intercellular potassium of *E. coli* K-12. These results indicated that CCCP and acetate were having distinctly different effects.

The ability of acetate to decrease intracellular pH has often been viewed as an undesirable consequence of acetate influx and dissociation [5], but work with lactic acid and other acid-resistant fermentative bac-

teria indicated that declines in intracellular pH might be an adaptive and even beneficial response [7]. When intracellular pH and ΔpH decline, less acetate anion accumulates intracellularly. *E. coli* O157:H7, the strain that let its intracellular pH decline the most, never accumulated large amounts of acetate anion, but *E. coli* K-12, the strain that maintained the higher intracellular pH, accumulated as much as 480 mM acetate (Fig. 1).

The importance of acetate as an intracellular anion and osmolyte has often been ignored [7]. *E. coli* K-12 accumulated more acetate than *E. coli* O157:H7, and virtually all this acetate would have been dissociated. Acetate addition caused an increase in the intracellular potassium concentration of *E. coli* K-12, and intracellular potassium was nearly as highly as the acetate anion concentration. *E. coli* O157:H7, the bacterium that maintained a lower intracellular pH, had less intracellular acetate anion and potassium.

Acknowledgments

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