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Uncoupling by Acetic Acid Limits Growth of and Acetogenesis by *Clostridium thermoaceticum*

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When cells of the anaerobic thermophile *Clostridium thermoaceticum* grow in batch culture and homoferment glucose to acetic acid, the pH of the medium decreases until growth and then acid production cease, at about pH 5. We postulated that the end product of fermentation limits growth by acting as an uncoupling agent. Thus, when the pH of the medium is low, the cytoplasm of the cells becomes acidified below a tolerable pH. We have therefore measured the internal pH of growing cells and compared these values with those of nongrowing cells incubated in the absence of acetic acid. Growing cells maintained an interior about 0.6 pH units more alkaline than the exterior throughout most of batch growth (i.e., $\Delta pH = 0.6$). We also measured the transmembrane electrical potential ($\Delta\psi$), which decreased from 140 mV at pH 7 at the beginning of growth to 80 mV when the medium had reached pH 5. The proton motive force, therefore, was 155 mV at pH 7, decreasing to 120 mV at pH 5. When further fermentation acidified the medium below pH 5, both the ΔpH and the $\Delta\psi$ collapsed, indicating that these cells require an internal pH of at least 5.5 to 5.7. Cells harvested from stationary phase and suspended in citrate-phosphate buffer maintained a ΔpH of 1.5 at external pH 5.0. This ΔpH was dissipated by acetic acid (at the concentrations found in the growth medium) and other weak organic acids, as well as by ionophores and inhibitors of glycolysis and of the H^+-ATPase. Nongrowing cells had a $\Delta\psi$ which ranged from about 116 mV at external pH 7 to about 55 mV at external pH 5 and which also was sensitive to ionophores. Since acetic acid, in its un-ionized form, diffuses passively across the cytoplasmic membrane, it effectively renders the membrane permeable to protons. It therefore seems unlikely that mutations at one or a few loci would result in *C. thermoaceticum* cells significantly more acetic acid tolerant than their parental type.

The anaerobic thermophile *Clostridium thermoaceticum* produces large amounts of acetic acid by glucose homofermentation (7). Since organisms, under pH control conditions, grow faster and produce more acetic acid near neutral pH than in more acid media (32, 33), they can be classified as neutrophiles (28). However, in batch culture without pH control, the pH of the medium decreases as the cells grow and ferment the sugar, until growth and then acetic acid production cease at about pH 5. Several authors have concluded that it is not the low external pH per se that limits growth, but that the limiting factor is the acetic acid produced (32, 33). The undissociated acetic acid is considered more inhibitory than the acetate anion (32, 33), but how the end product exerts its effect is not understood.

It has been realized for a long time that lipophilic compounds can penetrate living cells (26). Undissociated weak organic acids and bases, but not their conjugate ions, diffuse rapidly through lipid membranes, including the cell membranes of clostridia (15). Added in low concentrations, weak acids distribute themselves across the membrane according to the difference in pH between the two sides (ΔpH) and are in fact often used to measure the ΔpH (21, 30). When added in large concentrations, weak acids dissipate the ΔpH, acting as uncoupling agents or protonophores.

Neutrophilic bacteria, which grow optimally at pH near 7, have interiors more alkaline than the medium (reviewed in reference 28). In anaerobes such as streptococci (14, 17) or *C. pasteurianum* (29) the internal pH (pH_{in}) is kept more alkaline than the medium, but decreases as the exterior becomes more acid. These cells generate the ΔpH by extruding H^+ by means of the membrane H^+-ATPase (4, 9, 29) in a process energized by glycolytically generated ATP.

We were interested in determining the mechanism by which acetic acid limits growth and acid production in these cells. Because of the uncoupling activity of weak acids, we postulated that in an acid medium the cytoplasm becomes acidified sufficiently to result in inhibition of cellular functions. Therefore, an investigation of the effects of acetic acid on the ΔpH of *C. thermoaceticum* was carried out. In addition, it was necessary to measure the other component of the proton motive force (PMF), the transmembrane electrical gradient $\Delta\psi$ (23, 24), because ΔpH and $\Delta\psi$ can be interconverted by the exchange of H^+ for K^+ or Na^+ (reviewed in references 18 and 28).

In this report we present evidence that during growth of *C. thermoaceticum* the ΔpH is short-circuited by the acetic acid produced by the cells, but that there is relatively little effect on the $\Delta\psi$. The decreased internal pH thus inhibits a pH-sensitive cellular reaction(s), leading to cessation of growth and fermentation.

MATERIALS AND METHODS

Organisms. The cells used were the parental strain of *C. thermoaceticum* (7) and a mutant strain, C52, that initiates growth at a lower pH than the parental strain. This strain was isolated after selection in pH-controlled continuous culture (3) and was furnished by CPC International Inc., Summit-Argo, Ill. Whereas most of the experiments reported here were carried out with the mutant strain C52, similar results were obtained when the parental strain was used.

Medium and growth conditions. The medium of Ljungdahl and Andreesen (20), modified by Schwartz and Keller (32), was used, with the following changes: $NaHCO_3$ was reduced

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to 10 g/liter; tryptone (Difco Laboratories, Detroit, Mich.) and yeast extract (Difco) were increased to 5 g/liter each; *p*-aminobenzoic acid, biotin, and nicotinic acid were omitted; resazurin was added to 1 mg/liter; and the trace salts were (per liter) 0.5 mg of EDTA, 0.5 mg of $MnCl \cdot 4H_2O$, 10 μg of H_3BO_3, 6.9 μg of $ZnSO_4$, 10 μg of $AlK(SO_4)_2 \cdot 12H_2O$, 2 μg of $NiCl_2$, and 1 μg of $CuCl_2 \cdot 2H_2O$. The pH of the medium was 7.3. The solution was brought to a boil while being vigorously sparged with carbon dioxide which had been deoxygenated by passage through an oxygen-removing, gas-purifying furnace (Sargent Welsh Scientific Co., Springfield, N.J.), until the medium had turned a pale yellow color. Heating was discontinued, and 0.6 g of sodium thioglycolate per liter was added. CO_2 sparging was continued until the medium had cooled to room temperature. The solution was dispensed under anaerobic conditions within an anaerobic glove box (Forma Scientic Co., Marietta, Ohio) into 120-ml serum bottles or 20-ml test tubes (Wheaton Co., Millville, N.J.), fitted with rubber septa; these were crimped with aluminum caps (22). The gas within the anaerobic box consisted of 5% H_2, 5% CO_2, and 90% N_2. The medium was then sterilized by autoclaving and stored within the anaerobic glove box. Inoculations and transfers were done anaerobically with syringes and needles flushed with sterile, deoxygenated carbon dioxide with the aid of a Hungate gassing manifold (1, 12). Separately deoxygenated and autoclaved glucose was added to a final concentration of 2%. A solution containing 0.3% $CoCl_2$ and 0.4% $Fe(NH_4)_2(SO_4)_2$ was sparged with deoxygenated CO_2 for 30 min and filter sterilized within the anaerobic glove box, and 0.1 ml of each solution was added per 10 ml of inoculated culture.

The cells were preserved in 25% glycerol at −15 or −70°C. Stock cultures were grown at 57°C in the above medium and were kept at 4°C for up to 2 weeks. For each experiment cells were inoculated from the stock cultures and grown at 57°C in the same medium. Growth was monitored spectrophotometrically by suitable dilution of the cultures with 0.1 M sodium phosphate, pH 7.0, or with fresh medium and reduction with ca. 1 mg of $Na_2S_2O_4$ to remove the pink color of oxidized resazurin. The intracellular aqueous space in exponential-phase cells suspended in citrate-phosphate buffer (pH 5)–20 mM glucose was 1.27 μl/mg (dry weight) ± 0.21 (n = 10). Stationary-phase cells had an intracellular space of 1.76 μl/mg (dry weight) ± 0.04 (n = 10), as determined by using ^3H_2O to measure the total aqueous space in the cell pellets and subtracting the space occupied by [^{3}H]polyethylene glycol (21).

Measurement of PMF. For assays with nongrowing cells, the cells were harvested from batch culture at various growth phases by centrifugation in a microfuge (Fisher Scientific Co., Pittsburgh, Pa.) inside the anaerobic glove box. The pellets were washed and suspended in the indicated buffer. For most experiments the buffer used was citric acid-sodium phosphate mixed in various proportions to achieve the desired pH (6). The reactions were carried out in glass test tubes containing cells at an optical density (625 nm) of 0.65 to 3.0, 20 mM glucose, buffer, and the radioactive probes.

The intracellular pH was determined from the accumulation of the weak acid [7-^{14}C]benzoate (8 μM at 25 Ci/mol, final concentration), [7-^{14}C]salicylate (4 μM at 55 Ci/mol), or [^{3}H]acetate (11 μM at 90 Ci/mol) (28). After a 15-min incubation in the anaerobic glove box at 30 or 57°C in a Vanlab heat block (VWR Scientific, Boston, Mass.), 1.0-ml samples of the cell suspensions were centrifuged through a mixture of silicone oils (35% Fluid 556–65% Fluid 550; Dow-Corning, Midland, Mich.) in a 1.7-ml microfuge tube (13, 14). The microfuge tubes were then removed from the anaerobic chamber. The pellets and samples of the supernatant fluids were counted for radioactivity, as described previously (14).

For $\Delta\psi$ measurements, [^{3}H]tetraphenylphosphonium bromide (final concentration, 1 μM at 1 Ci/mol) was added to the cell suspensions and the experiments were continued as for the ΔpH measurements. The radiolabel in the trapped medium and the nonspecific binding of the probe were measured in each experiment by treating the cells with 5% *n*-butanol or isobutanol, as described previously (14). The counts of such membrane-disrupted cells were subtracted from those of intact cells. The magnitudes of the ΔpH and the $\Delta\psi$ were calculated as described previously (14, 21).

The PMF of growing cells (in vivo) was determined by removing samples of cultures from the Wheaton tubes or bottles, adding the probes and 20 mM glucose (at either 30 or 57°C), and incubating for 15 min. All manipulations were done within the anaerobic glove box. The cells were then processed as described above.

The homoacetate fermentation of the cells was monitored by measuring acetate and other volatile acids by ether extraction and gas chromatography with a Dohrmann gas chromatograph, using standard procedures (11). Similarly, non-volatile acids were measured after methylation and extraction with chloroform.

The radioactive compounds were bought from New England Nuclear, Boston, Mass. The other chemicals were of reagent grade and are available commercially.

RESULTS

PMF of growing *C. thermoaceticum*. During the growth of a typical batch culture at 57°C, cells of *C. thermoaceticum* grew from an initial optical density (at 625 nm) of 0.086 (0.043 mg [dry weight]/ml) to a maximal optical density of 4.0 (not shown). The pH of the medium decreased from pH 7.0 to 5.1. Further incubation resulted in a decrease of the pH to 5.0, whereas the cell density decreased to an optical density of 2.8, due to cell lysis (33). The cells fermented glucose to acetic acid (7); no other organic acids were detected. The acetic acid produced reached 144 mM (of which 45 mM was undissociated acid) at the maximal cell density. At pH 5.0 the total acid produced was 170 mM, of which 62 mM was calculated to be in the un-ionized form. Similar growth and fermentation kinetics have been described previously, including those of pH-controlled batch cultures (33).

Our working hypothesis was that the acetic acid produced by the bacteria stops growth because it acidifies the cytosol. Therefore, to follow the effect of acetic acid production on the internal pH (pH_{in}), we measured the in vivo ΔpH and $\Delta\psi$. The cells presumably were still able to grow when their temperature was kept at 57°C, because all manipulations were carried out within an anaerobic environment. At pH 7.0 the ΔpH was equivalent to about 15 mV (pH_{in} = 7.25) and increased to about 40 mV (pH_{in} = 5.7) at an external pH of 5.0 (Fig. 1). Thus, the internal pH of the cells was more alkaline than that of the medium, but because continuously more acid as growth progressed during the batch culture. Very similar results have been reported previously for the mesophile *C. pasteurianum* (29). The fairly constant ΔpH of 0.6 throughout most of the growth in batch culture of *C. thermoaceticum*, however, collapsed when the medium pH dropped below 5.

Since the ΔpH and the Δψ can be interconverted by the activity of cation/H^+ antiporters, it was of interest to measure both components of the PMF. For example, it is possible that the decrease in ΔpH resulting from the uncoupling activity of acetic acid could be compensated for by an increase in Δψ. The Δψ of growing *C. thermoaceticum* cells during batch growth was about 140 mV at pH 7.0 and decreased to 80 mV at pH 5.0 (Fig. 1). Again, below pH 5 the Δψ decreased precipitously. Thus, the PMF of the cells was about 155 mV at pH 7 and decreased to about 120 mV when the cells reached stationary phase and acidified their medium to pH 5. At pH values below 5, the proton gradient began to collapse, in concert with the cells' inability to grow further.

Interestingly, the values obtained for cells kept at 57°C were not significantly different from those tested at 30°C (Fig. 1). It was therefore possible to measure the H^+ gradient of nongrowing cells at 30°C rather than at the less convenient 55 to 60°C that is necessary for the growth of these thermophiles.

PMF of nongrowing *C. thermoaceticum* cells. To determine whether a component of the medium limits growth and acetic acid production, we compared the PMF maintained by nongrowing cells suspended in buffer with the in vivo PMF described above. Exponential- or stationary-phase cells suspended in citrate phosphate buffer were found to maintain significant pH gradients over a wide range of external pH values (Fig. 2). Stationary-phase cells maintained a slightly more alkaline internal pH (pH_{in} = 7.4) than the exterior at pH 7.0 and a considerably more alkaline pH (pH_{in} = 5.5) than the exterior at pH 3.5. In contrast, exponential-phase cells were not able to generate as high pH gradients at external pH 3.5 and 4.0, but were indistinguishable from stationary-phase cells when the external pH was 4.5 or higher. Since growing cells in batch culture lower the medium pH only to 4.9, at most, the ΔpH of nongrowing cells at a pH below 4.5 is not of significance under the "physiological" condition of growth.

In similar experiments, we measured the magnitude of the Δψ of nongrowing cells incubated in buffer (Fig. 3). Exponential-phase cells maintained a Δψ of 116 mV (interior negative) when the exterior pH was 7, and this decreased to 50 mV at pH 3.5. Cells harvested from stationary phase also

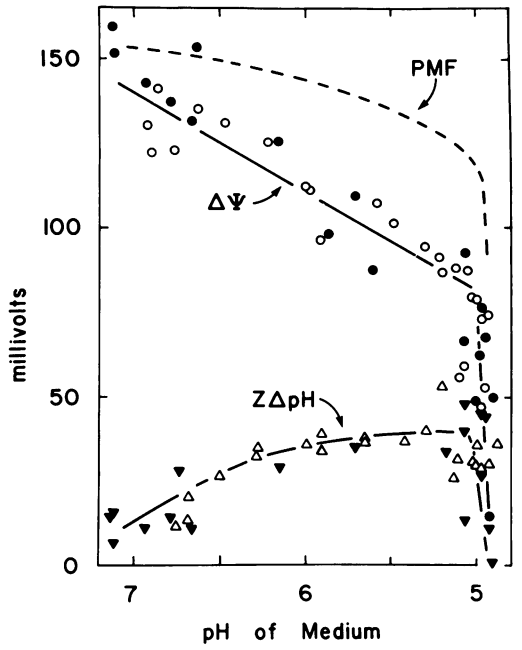

FIG. 1. PMF of *C. thermoaceticum* cells during growth in batch culture. The experiment was performed as described in the text. The pH of the growth medium decreased during growth of the cells, as indicated on the abscissa from left to right. Measurements of ΔpH and Δψ were made as the cultures reached the indicated pH values. Each point is the mean of triplicate determinations, using separate cultures, tested at 57°C (●, ▼) and at 30°C (○, △). *Z*, the factor used to convert ΔpH units to millivolts, is equal to 60 at 30°C and 65 at 57°C. The dashed line representing the PMF is the sum of the regression line through the Δψ points and the ΔpH values.

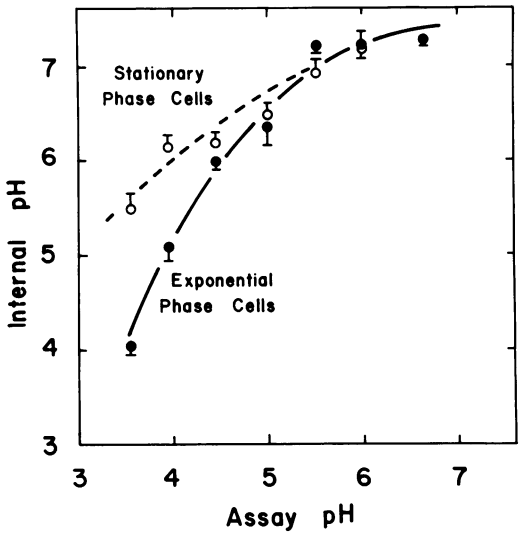

FIG. 2. Effect of external pH on internal pH of nongrowing cells. The cells were harvested at exponential (●) and stationary (○) phases of batch growth, washed, suspended in citrate-phosphate buffer at the indicated pH, and supplemented with 20 mM glucose. The pH gradient was measured at 30°C, as described in the text. Each value is the mean of three to eight cultures, assayed in triplicate; the bars represent the standard errors of the means.

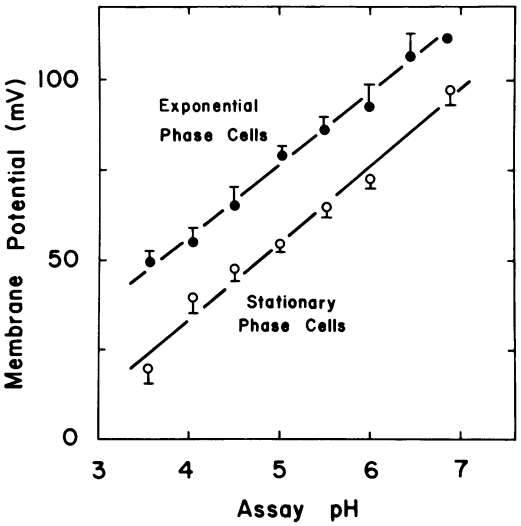

FIG. 3. Effect of external pH on membrane potential of nongrowing cells. The cells were harvested and tested as described in the legend to Fig. 2 and in the text.

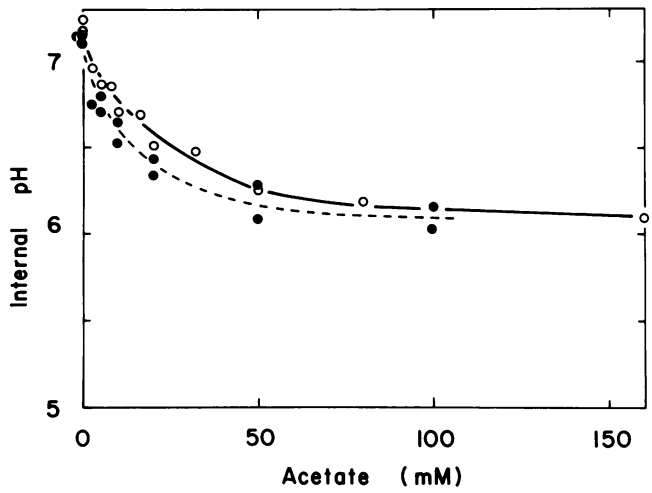

FIG. 4. Effect of acetic acid on internal pH of nongrowing cells at pH 5.5. The experiment was performed with [^{14}C]benzoate (○) or [^{3}H]acetate (●), as described in the legend to Fig. 2 and the text. At pH 5.5, 15.5% of the total acetate is present as the undissociated acid.

had a pH-sensitive $\Delta\psi$, but its magnitude was about 20 mV lower than that of exponential-phase cells throughout the pH range tested. Thus, the phase of growth in batch culture has some effect on the PMF that can be maintained by the cells. It should be noted that because exponential-phase cells had a lower ΔpH but a higher $\Delta\psi$ than stationary-phase cells at pH 3.5, cell shrinkage or swelling as a trivial explanation for the differences is ruled out. Whereas these data were obtained with mutant strain C52, similar results were seen with the parental cells (data not shown).

Since these clostridia seemed to be able to generate an H^+ gradient under conditions markedly different from those required for growth, we investigated the stability of the PMF generated by nongrowing cells. Washed cells were incubated under various conditions of temperature and anaerobiosis in citrate-phosphate buffer at pH 5.5 (data not shown). The cells could maintain an internal pH above pH 7.1 for a least 3 h when supplied with 20 mM glucose, but in the absence of a fermentable sugar the pH_{in} decreased to 6.6. This suggests that an energy source is required to generate a proton gradient. Exposure to air decreased the pH_{in} of glucose-fed cells to 7.1, 6.9, and 6.7 after 30, 60, and 120 min of incubation at 30°C, respectively. This dissipation of the ΔpH was accelerated by increasing the temperature to 57°C, as shown by the drop of the pH_{in} of glucose-fed cells to 6.2, 6.0, and 5.9 after 30, 60, and 120 min of exposure to air at the higher temperature.

Effect of acetic acid on H^+ gradient of nongrowing *C. thermoaceticum*. Since acetic acid is the sole end product of glycolysis in these clostridia, it was of interest to measure the effect of this weak acid on the H^+ gradient maintained by nongrowing cells. Addition of acetic acid to cells in pH 5.5 buffer decreased the ΔpH, but even 160 mM total acetic acid (= 24.8 mM undissociated acid) did not abolish it (Fig. 4). In cells incubated in buffer at pH 5.0, 100 mM acetic acid (= 36 mM undissociated acid) decreased the ΔpH to 0.6 (not shown). This concentration is lower than that of 40 to 60 mM for undissociated acetic acid found in the growth medium at the end of batch culture (see above; 33). Even the addition of 200 mM acetic acid at pH 4.4 (= 140 mM undissociated acetic acid) decreased the ΔpH from 1.7 (pH_{in} = 6.1) to only 0.6 (pH_{in} = 5.0). Again, acetic acid was found to have the same effect on the parental cells as on mutant strain C52. In addition, the distribution of [^{14}C]acetate between the cells and the medium was similar to that of [^{14}C]benzoate (Fig. 4) and, in other experiments, differed by <0.2 pH units from the values seen with [^{14}C]salicylate (not shown). This would be expected if acetic acid diffuses across the cell membrane passively, without mediation by a membrane transport carrier. Indeed, this weak acid is commonly used for ΔpH determinations (30), and, to our knowledge, there is no evidence for the existence of acetate transport proteins in bacteria.

The ΔpH of nongrowing cells was also decreased by the ionophores carbonyl cyanide-*p*-trifluoro-methoxyphenyl hydrazone (e.g., 10 μM reduced the ΔpH from 1.7 to 0.85; pH_{out} = 4.5) and nigericin (e.g., 27 μM reduced the ΔpH from 1.5 to 0.4; pH_{out} = 5.5). Moreover, the requirement for metabolic energy for ΔpH generation was demonstrated by the inhibitory effect of *N,N'*-dicyclohexylcarbodiimide, the inhibitor of the H^+-translocating ATPase (e.g., 100 μM *N,N'*-dicyclohexylcarbodiimide reduced the ΔpH to 0.3; pH_{out} = 5.5). The ΔpH of *C. pasteurianum* was shown previously to be sensitive to these inhibitors (29). The various ionophores, as well as the glycolytic inhibitor fluoride (30 mM), also markedly decreased the $\Delta\psi$ of nongrowing *C. thermoaceticum* (data not shown).

Since acetic acid partially dissipated the ΔpH in cells incubated in a citrate-phosphate buffer, we tested a number of other buffer systems (Table 1). Late exponential-phase cells incubated at pH 5.5 maintained the highest ΔpH in citrate-phosphate buffer of the systems tested, with a pH_{in} of about 7.2. The internal pH was slightly lower in citrate, malate, and Tris-citrate buffers and lower still in fumarate, malonate, succinate, and maleate buffers, presumably because of uncoupling by these dicarboxylic acids. Phthalate, 2-[*N*-morpholino]ethanesulfonic acid, and dimethylglutarate also were effective in dissipating the ΔpH. The uncoupling activity of maleate was also seen when the acid was added to cells in citrate-phosphate buffer: increasing its concentration to 5, 50, and 200 mM decreased the ΔpH from 1.68 to 1.56, 1.24, and 1.03, respectively.

Uncoupling effect of acetic acid. That acetic acid exerts an uncoupling effect in growing cells was demonstrated by an

TABLE 1. ΔpH maintained by nongrowing *C. thermoaceticum* at pH 5.5[a]

Buffer	ΔpH
Citrate-phosphate	1.68
Citrate	1.51
Malate	1.42
Tris-citrate	1.18
Fumarate	0.84
Malonate	0.60
MES	0.42
Succinate	0.35
Maleate	0.35
Phthalate	0.23
Dimethylglutarate	0.17

[a] The cells were harvested during late exponential phase, washed, suspended in various buffers, and supplemented with 20 mM glucose. The uptake of [^{14}C]benzoate after 15 min of incubation at 30°C was measured as described in the text. Most of the buffers were prepared by adjusting 50 mM solutions of the acids with NaOH to give a pH of 5.5. The citrate-phosphate buffer consisted of 43 mM citric acid and 114 mM Na_2HPO_4 (6) and the Tris-citrate buffer consisted of 50 mM Tris base adjusted to pH 5.5 with citric acid. MES, 2-(*N*-Morpholino)ethanesulfonic acid.

TABLE 2. ΔpH of stationary-phase cells incubated in various media[a]

Treatment of cells	pH_{out}	ΔpH
Cells kept in growth medium	4.9	0.6
Suspended in citrate-phosphate buffer	4.9	1.4
Suspended in fresh medium, HCl added to lower the pH	4.8	1.6
Suspended in fresh medium, acetic acid added to lower the pH	5.1	0.5

[a] Stationary-phase cells were harvested and suspended as described above and in the text.

experiment in which cells were grown, harvested, and suspended in fresh medium which had been acidified to pH 5 with either HCl or acetic acid (Table 2). Cells in HCl-acidified medium were able to maintain a ΔpH of 1.6, like cells suspended in citrate-phosphate buffer. In contrast, cells in the acetic acid-acidified medium had a ΔpH of 0.5, equal to the ΔpH measured in vivo.

To assess the effect of acetic acid on the PMF of growing cells, we calculated the expected PMF from the ΔpH and Δψ of nongrowing cells incubated in citrate-phosphate buffer, and supplied with glucose, in the absence of acetic acid. These cells, harvested from exponential phase, were able to generate a PMF of 140 mV at pH 7 (ZΔpH, 24 mV; Δψ, 116 mV [Z = factor used to convert ΔpH units to millivolts]) and, when harvested at stationary phase, one of 143 mV at pH 5 (ZΔpH, 88 mV; Δψ, 55 mV), in the absence of acetic acid. However, growing cells had a PMF of 155 mV at pH 7 (ZΔpH, 15 mV; Δψ, 140 mV) and one of 120 mV at pH 5 (ZΔpH, 40 mV; Δψ, 80 mV). Thus, there was relatively little effect of batch growth phase on the magnitude of the PMF, but the relative contributions of ΔpH and Δψ changed during growth, which is the usual effect of the external pH on these parameters (13, 27). In addition, there was also an increase in the Δψ that partially compensated for the decrease in ΔpH caused by the uncoupling effect of acetic acid.

DISCUSSION

Typically, bacteria maintain a cytoplasmic pH closer to neutrality than the external medium (reviewed in references 9, 19, and 28). There seems to be a minimum internal pH that is compatible with growth. Such a "critical" pH differs among species. In *Escherichia coli* cells, which maintain a constant internal pH (13, 27), growth seems to require a pH_{in} of 7.6 to 7.8. In three species of the acidophile *Lactobacillus*, growth ceased when the pH_{in} was below 4.4 (J. J. Baronofsky and E. R. Kashket, unpublished data). In the neutrophile *C. thermoaceticum*, inhibition of metabolism and cessation of growth occurred when the cytosol was acidified below pH 5.5 to 5.7. Since nongrowing cells were able to maintain a ΔpH as high as 2.5 at low external pH, it is not the external pH of 5 per se that inhibits growth of these cells in batch culture.

The factor responsible for acidification of the cytoplasm is the acetic acid produced by the cells. The sensitivity of the cells to acetic acid increases as the medium pH decreases (32, 33), until growth ceases when the external medium reaches about pH 5. Under those conditions the PMF of the cells collapses, presumably because metabolism cannot supply the ATP required for proton extrusion by the H^+-ATPase. With this interpretation of the mechanism of growth inhibition by acetic acid it is not necessary to distinguish between inhibitory effects of the free acid and the acetate anion (cf. reference 33).

It is generally accepted that acetic acid traverses the bacterial cytoplasmic membrane by diffusing passively through the hydrophobic region of the membrane. This conclusion is based primarily on the absence of evidence for a membrane carrier for this molecule, such as acetate transport-deficient mutants or substrate-saturable transport kinetics. However, the existence of a specific transport protein cannot be ruled out solely because a compound is able to cross membranes without carrier mediation. For example, ammonia permeates easily through cell membranes, yet there are specific transport proteins for this solute in fungi and in some bacteria (8, 16). Nevertheless, the very similar ΔpH values that are calculated with a number of nonmetabolizable weak acids is taken as evidence that these compounds are accumulated passively (30; this paper).

An interesting finding is that the thermophile *C. thermoaceticum* generated a proton gradient under conditions less stringent than those required for growth. For example, at 30°C and at 55 to 60°C, the cells had the same PMF, yet they could not grow at the lower temperature. This suggests that the growth reaction(s) that is temperature sensitive is not that required for generation of the PMF. Also, the ΔpH of *C. thermoaceticum* was relatively resistant to dissipation in the presence of oxygen, even though these cells are obligate anaerobes. It is probably for this reason that in *C. pasteurianum* a proton electrochemical gradient could be demonstrated, even though the cells were briefly exposed to air during preparation or sampling (2, 5, 29).

The sensitivity of *C. thermoaceticum* to acetic acid is not incompatible with evolutionary selective pressures, since in their natural habitat these cells would probably be found with other, acetate-consuming organisms. Under such conditions acetic acid would not accumulate and there would not be a decrease in the internal pH.

Acid and solvent-producing clostridia, which may offer economically attractive sources for chemicals currently derived from fossil fuels, have elicited renewed interest recently (32, 34). One focus of current research is the physiological factors that limit synthesis of the end product (10). A strategy for developing increased acetic acid production by *C. thermoaceticum* cells includes the isolation of mutants under selection conditions that favor acetic acid-tolerant cells (31). For economic practicality, it is necessary to obtain strains that not only achieve increased final total acetic acid concentrations, but also do so at as low a medium pH as possible (31). In principle, a number of mutations leading to acetic acid tolerance can be envisioned. For example, although mutant cells with membrane lipids sufficiently altered to be significantly more impermeable to acetic acid might be obtainable, that the acetic acid is generated inside the cell and must exit makes such strains unlikely. Similarly, a proton-driven active transport system for acetic acid efflux, like the lactate exporter in streptococci (25), could function to alkalinize the cells' interior, but there seems to be little hope of acquiring such membrane transport proteins de novo. A third possibility, also improbable, would be simultaneous, multiple mutations in the enzymes that catalyze the rate-limiting reactions of metabolism and growth in the direction of increased resistance to acid pH. We conclude, therefore, that isolation of cells significantly more tolerant to acetic acid than those presently available is not likely.

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