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Membrane H^+ Conductance of *Clostridium thermoaceticum* and *Clostridium acetobutylicum*: Evidence for Electrogenic Na^+/H^+ Antiport in *Clostridium thermoaceticum*

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H^+ conductance in de-energized cells of *Clostridium thermoaceticum* and *Clostridium acetobutylicum* was determined from the rate of realkalinization of the medium after an acid pulse. In both organisms, cell membrane proton permeability was increased by fermentation end products and ionophores. In *C. thermoaceticum*, H^+ conductance was increased by Na^+ ions compared with K^+ as counterions. In these cells, addition of Na^+, but not K^+, elicited efflux of H^+; H^+ efflux was stimulated by SCN^- and decreased by various ionophores. We concluded that *C. thermoaceticum* possesses an electrogenic Na^+/H^+ antiporter. In contrast, *C. acetobutylicum* cells did not have an electrogenic Na^+/H^+ antiporter.

Production of organic acids and solvents by clostridia is limited by a number of factors which are not entirely understood (reviewed in references 15 and 22). It is generally agreed, however, that accumulation of toxic end products of fermentation is an important factor limiting further metabolism and growth. The sites of inhibition probably include the cell membrane; the end products are thought to interfere with the diffusion barrier and energy-conserving functions of the membrane by dissipating the proton gradient across the phospholipid bilayer. In agreement with this hypothesis, we have shown previously that the thermophile *Clostridium thermoaceticum* ceases to grow and glycolyze when the internal pH of the cells decreases below pH 5.6 (1). The decrease is the result of acidification of the medium by the end product of fermentation, acetic acid, and dissipation of the transmembrane pH gradient (ΔpH) by the organic acid as it diffuses passively across the cell membrane in its undissociated form. In contrast, in the solventogenic *Clostridium acetobutylicum*, the ΔpH is dissipated by the chaotropic effect on the membrane lipids of the end product butanol (3, 4, 6, 19).

Direct studies of the effects of fermentation end products on the H^+ permeability of these membranes are not possible because in fermenting cells H^+ extrusion by H^+-translocating ATPase obscures H^+ influx (5). We therefore investigated passive H^+ conductance in de-energized clostridia by applying an acid pulse and measuring the rate of H^+ entry into the cells. In this report we show that proton permeability is increased by fermentation end products and ionophores. We also present evidence for an electrogenic Na^+/H^+ antiporter in *C. thermoaceticum*, but not *C. acetobutylicum*, cells.

MATERIALS AND METHODS

Organisms. *C. thermoaceticum* C52 (1) and *C. acetobutylicum* ATCC 4259 were supplied by CPC International, Inc., Moffett Technical Center, Summit-Argo, Ill.

Media and growth conditions. Preparation of the media, inoculation, and anaerobic growth methods have been described previously (1, 19). The carbon source, Maltrin M-100 (3) or glucose, was added to a final concentration of 60 g/liter from a separately deoxygenated and autoclaved solution. The *C. acetobutylicum* cultures were vented daily to relieve the pressure caused by the H_2 and CO_2 produced.

Measurements of ΔpH. The ΔpH was measured as described previously (1, 19).

Measurement of proton movements. H^+ movements were measured with a Radiometer G202C electrode in a custom-made, water-jacketed vessel (Wilbur Scientific, Inc., Boston, Mass.), the contents of which were stirred and sparged with water-saturated, oxygen-free nitrogen which had been passed through an alkaline pyrogallol solution. A separate water-jacketed vessel containing a reference electrode (Radiometer K801) was connected to the main electrode through a salt bridge consisting of 1% agarose, 3.2 mM citric acid, 14.2 mM Na_2PO_4, 70 mM KCl, and 1 mM $MgSO_4$; the pH was 6.5. The glass electrode was connected to a chart recorder through a Radiometer PHM61 meter and voltage back-up device set so that full-scale deflection was 0.2 pH units.

Measurement of proton conductance of cell membranes. The theory and technique for the measurement of proton conductance by perturbation of transmembrane proton equilibrium have been described for mitochondria (12, 13) and bacterial cells (9, 17). For the present study the following procedure was used. After harvesting and washing the cells anaerobically (within the anaerobic glove box as described in reference 1), we incubated a suspension of *C. thermoaceticum* cells (12 to 14 mg [dry weight]/ml) in 300 mM KCl or NaCl for approximately 2 h at 30°C and adjusted the pH to 4, 5, or 6 with anaerobic NaOH or KOH as necessary. *C. acetobutylicum* cells were incubated in 150 mM KCl or NaCl. A 4-ml sample of this suspension was placed in the electrode vessel described above, and 0.1 ml of a 20- to 30-mg/ml concentration of freshly prepared carbonic anhydrase (Sigma Chemical Co., St. Louis, Mo.) was added. When necessary, the pH was adjusted to the desired value, and 0.9% (final concentration) Antifoam C (Sigma) was added. The assay was begun after the pH had stabilized, which indicated cessation of glycolytically generated acid

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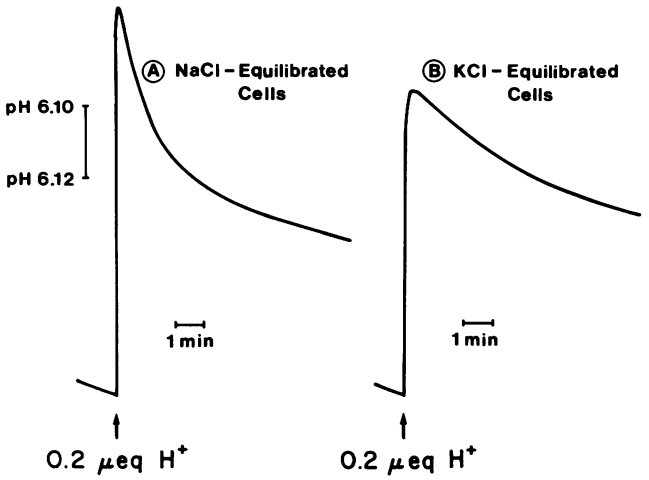

FIG. 1. Proton influx into *C. thermoaceticum* cells after an acid pulse. The medium pH was monitored after addition of HCl, and the rates of realkalinization were calculated to yield H^+ conductance values as described in the text. Upward pen deflection denotes acidification of the medium. *C. thermoaceticum* cells were preincubated in KCl (curve A) or NaCl (curve B).

production, i.e., depletion of energy storage compounds. We added samples of an anaerobic HCl solution (10 or 100 mM) to produce a pen deflection of 0.06 to 0.1 pH units and monitored the subsequent re-equilibration of H^+. Conductance values were calculated from measurements of intra- and extracellular buffering powers and half-times of equilibration (9, 13, 15, 17). To check that the electrode chamber was strictly anaerobic, we incubated cells of *Escherichia coli* ML308-211 anaerobically in the presence of the oxidizable substrate succinate. No H^+ ejection was seen unless an oxygen pulse was administered (cf. references 13 and 18), indicating that the system was anaerobic.

For Na^+-effected H^+ ejection measurements, the cells were suspended in pH 7, 2.5 mM citrate-Tris buffer at a cell concentration of 12 to 14 mg (dry weight)/ml. KSCN (50 mM) was present in the buffer in these experiments except where indicated. Samples of NaCl (final concentration, 5 mM) were added to the suspension in the electrode chamber, and acidification of the medium at pH 6 was monitored.

Materials. Carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone and monensin were bought from Sigma; nigericin was from Calbiochem-Behring, La Jolla, Calif.; pentachlorophenol was from Aldrich Chemical Co., Inc., Milwaukee, Wis.; and tetrachlorosalicylanilide was a gift from F. M. Harold.

RESULTS

H^+ conductance of *C. thermoaceticum*. We studied passive proton permeability by adding acid to cells depleted of energy-yielding storage compounds. Under such conditions protons are not pumped outward by ATP-driven pumps since the cells are depleted of ATP and only H^+-coupled porters function to translocate the ions across the membrane. After initial acidification of the medium, the external pH rose over a period of minutes, indicating entry of H^+ into the cells (Fig. 1). The passive proton conductance of *C. thermoaceticum* membranes, calculated from the rate of alkalinization of the medium, ranged from 0.07 to 2.81 μeq of H^+ translocated per s per pH unit per g (dry weight), depending on the assay conditions (Table 1). It should be noted that conductances higher than these cannot be measured with the present method. These conductance values are generally similar to those of other bacterial cells (e.g., see references 9 and 17), indicating that clostridial membranes are relatively impermeable to protons, as has been found for other coupling membranes (10, 11).

TABLE 1. Proton conductance of *C. thermoaceticum*

Assay conditions			Mean (±SEM) proton conductance (μeq of H^+/s per pH unit per g [dry weight]) of cells suspended in[a]:	
pH	Temp (°C)	SCN^- concn (mM)	KCl	NaCl
6.2	30	0	0.07 ± 0.01 (4)	0.36 ± 0.02 (8)
		10		0.48 ± 0.07 (5)
		20		1.02 ± 0.15 (5)
		50	0.19 ± 0.02 (3)	1.51 ± 0.13 (6)
	55	0	0.52 ± 0.10 (5)	1.17 ± 0.29 (5)
5.2	30	0		0.61 ± 0.10 (2)
4.2	30	0	0.28 ± 0.03 (4)	1.58 ± 0.14 (5)
		10	1.02 ± 0.12 (5)	2.21 ± 0.27 (4)
		50	2.81 ± 0.06 (3)	

[a] The values in parentheses indicate the number of experiments, each assayed in three to five replicates, as described in the text. KSCN was added to KCl-equilibrated cells, and NaSCN was added to NaCl-equilibrated cells, at the indicated concentrations.

Effect of Na^+ on the H^+ conductance of *C. thermoaceticum*. Significantly, realkalinization of the medium was faster in NaCl-equilibrated cells (Fig. 1A) than with K^+ as the counterion (Fig. 1B). The stimulatory effects of Na^+ on H^+ permeability were seen also when NaCl was added to KCl-equilibrated cells (Fig. 2). Conductance increased from

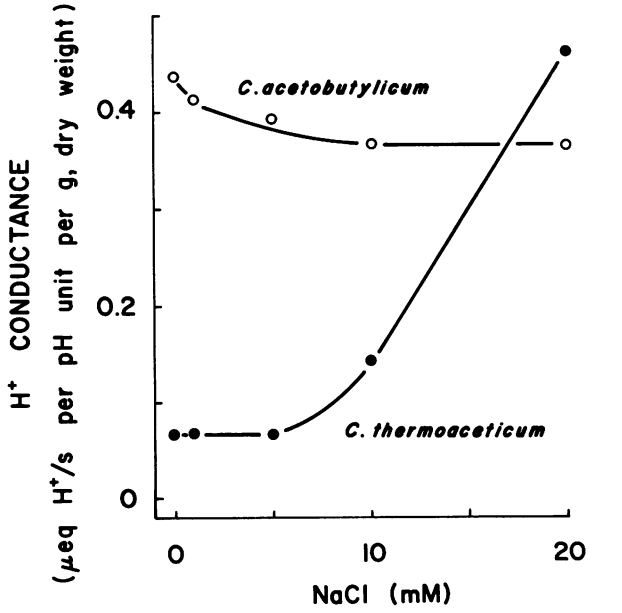

FIG. 2. Effect of NaCl on H^+ conductance. *C. thermoaceticum* cells were preincubated in 300 mM KCl and tested at pH 6.0 to 6.2 at various NaCl concentrations; *C. acetobutylicum* cells were equilibrated and tested at pH 5.0 to 5.2. The experiment was carried out as described in the legend to Fig. 1 and in the text.

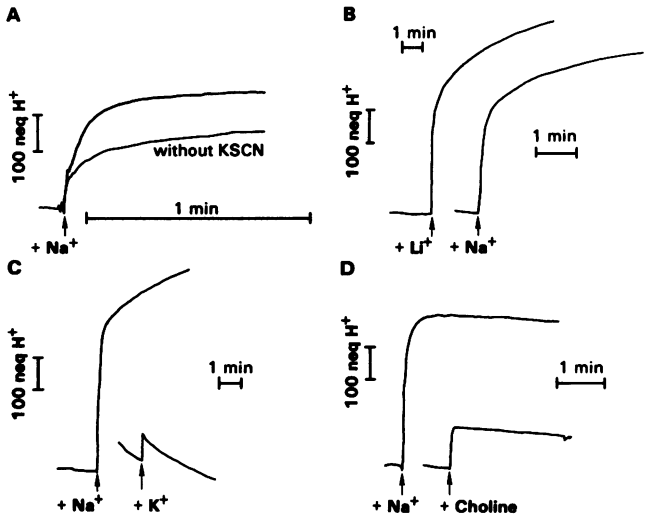

FIG. 3. H^+ efflux elicited by addition of NaCl to *C. thermoaceticum*. Upward pen deflection denotes acidification of the medium. Cells were prepared and measurements were performed as described in the text. Panels: A, NaCl (5 mM) was added, as indicated by the arrow, to cell suspensions containing 50 mM KSCN (upper curve) or 50 mM KCl (lower curve); B, LiCl (5 mM) or NaCl was added; C, NaCl was added to cell suspensions containing 50 mM KCNS, and KCl (5 mM) was added to suspensions containing 50 mM NaSCN; D, NaCl or choline chloride (5 mM) was added.

0.07 to 0.15 and 0.47 μeq of H^+ per s per pH unit per g (dry weight) when 10 and 20 mM NaCl, respectively, was added to the cell suspension. Moreover, when equivalent amounts of KCl were added to NaCl-equilibrated cells, no effect was seen. The passive H^+ conductance of these cells was two- to eightfold higher in NaCl-preincubated cells than with KCl under all of the assay conditions tested (Table 1), suggesting the activity of an Na^+/H^+ antiporter.

H^+ conductance was higher at pH 4 than at pH 6, possibly because of the higher substrate (H^+) concentration in the more acidic medium. H^+ conductance was higher at 55°C, which is close to the optimal growth temperature of 58°C, than at 30°C. This indicated that the membrane was relatively proton impermeable, i.e., was not disrupted, at 30°C, a temperature at which the cells do not grow, in agreement with the unimpaired capacity of the cells to maintain a proton motive force (protonic potential) under these unfavorable conditions (1). Exposure to air increased the H^+ conductance somewhat (1.4- to 2-fold). The H^+ conductance of K^+-equilibrated exponential-phase *C. thermoaceticum* was the same as that of cells harvested at the end of growth (0.069 ± 0.01 [$n = 2$] and 0.061 ± 0.03 [$n = 2$] μeq of H^+ per s per pH unit per g [dry weight], respectively, assayed at 30°C, pH 6.2). Similarly, NaCl-equilibrated cells had the same H^+ conductance when harvested throughout batch culture.

Thiocyanate had a significant stimulatory effect on the H^+ permeability of *C. thermoaceticum* (Table 1), which was seen with both KCl- and NaCl-equilibrated cells and at both pHs 4.2 and 6.2. The effect of SCN^- was as expected (17); in the presence of the permeant anion there is no buildup of charge after the acid pulse that otherwise would inhibit further influx of H^+.

Effect of inhibitors on the H^+ conductance of *C. thermoaceticum*. Additional proton influx routes introduced into the cell membrane would be expected to increase the rates of H^+ entry after a proton pulse. For example, the protonophore carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (5 μM or more) was found to increase the H^+ conductance threefold or more when tested with KCl-equilibrated cells at pH 5.0 to 5.2. At these concentrations, carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone has been shown to decrease the ΔpH of glucose-energized cells (1). Acetate, which also dissipates ΔpH (1), at 10 mM or higher concentrations abolished membrane impermeability to protons since no rise in the external pH was seen after an acid pulse.

H^+ conductance of *C. acetobutylicum*. In contrast to *C. thermoaceticum*, *C. acetobutylicum* cells were equally proton permeable whether equilibrated in KCl or NaCl (e.g., 0.32 ± 0.12 [$n = 4$] and 0.22 ± 0.05 [$n = 3$] μeq of H^+ per s per pH unit per g [dry weight], respectively, for solvent phase cells assayed at pH 5.1). Addition of NaCl to KCl-equilibrated cells (Fig. 2) or addition of KCl to NaCl-equilibrated cells (data not shown) had no effect on H^+ conductance. In contrast to *C. thermoaceticum*, *C. acetobutylicum* cells harvested from the acidogenic (exponential) phase had a significantly higher H^+ conductance compared with cells harvested at stationary phase, when solvent production had started (at pH 5.0 to 5.2, 0.86 ± 0.08 [$n = 4$] μeq of H^+ per s per pH unit per g [dry weight] for acid phase cells and 0.27 ± 0.03 [$n = 11$] μeq of H^+ per s per pH unit per g [dry weight] for cells from the solvent phase [$P < 0.001$]). Butanol (100 mM) added to solvent phase cells raised the H^+ conductance fivefold. This effect is consistent with the dissipation of the protonic potential in the solventogenic phase attributed to the fermentation end product (3, 6, 19).

Na^+-dependent H^+ efflux in *C. thermoaceticum*. Addition of Na^+ to an anaerobic suspension of *C. thermoaceticum* caused a net acidification of the medium (Fig. 3 and 4), implying a net outflow of H^+ from the cells. Li^+ caused a similar response (Fig. 3B). KCl (Fig. 3C) or choline chloride (Fig. 3D) had a much less pronounced effect; the acidification seen was less than 30% of that elicited by NaCl. It was inferred from these results that the cell membrane contained an Na^+/H^+ antiporter. The lack of response to K^+ and choline demonstrated the specificity of this putative antiporter, and use of either Na^+ or Li^+ is a common feature of the known Na^+/H^+ antiporters (8). Na^+-induced efflux of H^+ does not require metabolic energy since the cells were

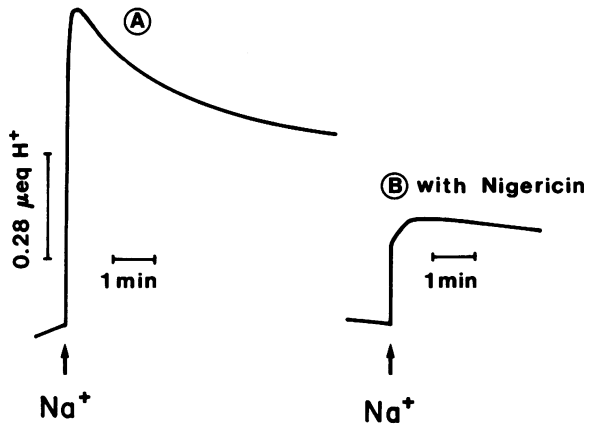

FIG. 4. Effect of nigericin on Na^+-elicited H^+ efflux in *C. thermoaceticum*. The experiment was performed as described in the text and the legend to Fig. 3. Cells were preincubated for 1.5 h with nigericin (20 μg/ml).

suspended without a carbon source for 0.5 to 4 h before assay and did not produce acidic fermentation end products. Also, cells preincubated with potassium arsenate still showed Na^+-dependent H^+ efflux (data not shown). In contrast, *C. acetobutylicum* cells showed no Na^+-dependent H^+ efflux.

If Na^+/H^+ exchange were electroneutral, one would expect no effect of SCN^- on the rate of H^+ efflux (21). If, on the other hand, the movement were electrogenic, the permeant anion SCN^- would relieve the buildup of charge and allow H^+ movement to take place at a faster rate. This was found to occur in *C. thermoaceticum* (Fig. 3A), from which we concluded that the Na^+/H^+ antiporter is electrogenic.

Effect of ionophores on Na^+-elicited H^+ efflux in *C. thermoaceticum*. It was expected that ionophores would short-circuit the putative Na^+/H^+ antiporter of *C. thermoaceticum*, and several ionophores tested were found to decrease the H^+ efflux elicited by Na^+ addition. At pH 5 in the presence of SCN^-, Na^+-elicited H^+ was abolished when cells were preincubated for 1.5 h with 1 mM pentachlorophenol; 200 μM pentachlorophenol or less had no effect (data not shown). Similarly, preincubation for 1.5 h with 500 μM tetrachlorosalicylanilide inhibited H^+ extrusion, whereas a 100 μM concentration of the protonophore had no effect. Monensin (20 μg/ml) (data not shown) and nigericin (20 μg/ml) (Fig. 4), which exchange Na^+ or K^+ for H^+ (14), significantly reduced Na^+-elicited H^+ efflux.

Effect of ionophores on ΔpH. Another function of the membrane, the ability to ferment *C. thermoaceticum* to maintain a ΔpH, has been shown to be sensitive to ionophores. ΔpH-dissipating effects on *C. thermoaceticum* of the uncoupling agent carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone and nigericin have been reported previously (1). In further experiments with cells fermenting glucose in pH 5.1 buffer, the ΔpH was essentially dissipated by tetrachlorosalicylanilide (50 μM), pentachlorophenol (200 μM), and nigericin (20 μg/ml). These concentrations of inhibitors are lower than those that affect Na^+/H^+ antiporter activity. It should be noted that passive proton permeability measurements require de-energized cells to prevent re-extrusion of H^+, whereas the ΔpH is only established and maintained in energized cells. Thus, the experimental conditions are of necessity different for the two assays.

DISCUSSION

The low permeability of bacterial cell membranes to hydrophilic solutes and electrolytes allows them to fulfill their role of bioenergetic coupling membranes (5, 9, 11). Low H^+ permeability is essential for the maintenance of protonic potential (proton motive force), which in turn energizes a number of H^+-driven solute transport systems (16). In cells starved of energy-yielding substrates, ATP-powered or proton motive force-driven systems would not be active, and only passive proton conductance would be seen, such as H^+/cation exchange systems. Among the routes by which H^+ ions enter and exit from the cell are a variety of Na^+/H^+ porters (reviewed in reference 8).

The two clostridial species examined differed with respect to the effect of growth stage in batch culture on passive H^+ permeability; in *C. thermoaceticum*, exponential- and stationary-phase cells had the same H^+ permeability, but in *C. acetobutylicum*, membranes from acidogenic-phase cells were more H^+ permeable than those from solventogenic-phase cells. This change in passive proton permeability may be due to differences in phospholipid composition, with the resulting differences in membrane fluidity, that have been reported for acidogenic- and solventogenic-phase *C. acetobutylicum* cells (2, 20).

As expected from previous experiments with growing or fermenting cells in which energy-requiring ion transport systems are active, fermentation end products increased the passive H^+ conductance of de-energized clostridial membranes. Thus, acetic acid increased the H^+ conductance of *C. thermoaceticum*, whereas butanol increased it in *C. acetobutylicum*. As expected, H^+ conductance was increased in cells treated with a variety of ionophores. It is likely that increased membrane proton permeability effected by the end products ultimately limits growth and fermentation.

An unexpected finding was that *C. thermoaceticum* cells possess electrogenic Na^+/H^+ antiporter activity. The evidence includes the following: (i) greater H^+ conductance when *C. thermoaceticum* cells were preincubated in NaCl compared with KCl (preincubation would allow the Na^+ or K^+ ions to equilibrate across the cell membrane and act as counterions for H^+); (ii) efflux of H^+ on addition of Na^+ or Li^+ but not K^+ or choline; (iii) inhibitory effects of ionophores on Na^+-effected H^+ efflux; and (iv) stimulation of Na^+-elicited H^+ efflux by SCN^-, which dissipates transmembrane potential gradients. Cells of *C. acetobutylicum* do not possess an Na^+/H^+ antiporter by these criteria.

ACKNOWLEDGMENTS

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