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The intracellular pH of the thermophilic bacterium *Thermoanaerobacter wiegelii* during growth and production of fermentation acids

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Abstract The thermophilic glycolytic anaerobe *Thermoanaerobacter wiegelii* grows over the pH range 5.1–7.7, and no growth is observed below pH 5.0 or above 7.7. When *T. wiegelii* was grown in pH-uncontrolled batch culture, glucose was fermented to ethanol, acetate, and lactate. Small amounts of lactic acid were produced once the external pH reached 6.0, and a fructose-1,6-diphosphate (FDP) activated lactate dehydrogenase (LDH) was detected in cell-free crude extracts. Maximal activation of LDH by FDP was observed at pH 6.2. As the pH of the medium declined from 6.7 to 5.1 due to the production of acetate and lactate, the total protonmotive force (Δp) remained between -110 and -130 mV, and the membrane potential ($\Delta\Psi$) decreased from -104 to -65 mV. This decrease in $\Delta\Psi$ was paralleled by an increase in the chemical gradient of protons ($Z\Delta pH$) from -31 to -62 mV at pH 5.1. Based on these results, *T. wiegelii* maintained a small ΔpH (0.3–0.9 units, inside alkaline) as the medium pH declined and interconverted $\Delta\Psi$ to $Z\Delta pH$ to maintain the total Δp relatively constant. Intracellular potassium decreased from 150 mM at pH 6.70 to 50 mM at pH 5.1, and this represented a 33-mV decline in the transmembrane chemical potential of potassium. The ability to synthesize ATP remained constant as the external pH declined, and therefore metabolic energy per se was not a critical aspect of pH sensitivity.

Key words Thermophile · pH regulation · Fermentation acids · Bioenergetics

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Introduction

Bacteria display a remarkable capacity to survive and grow in extremely hostile environments. Entire groups of organisms have adapted their lifestyles to these extreme environments (e.g., thermophiles, halophiles, acidophiles, alkaliphiles). Bacteria show a remarkable ability to grow over a wide range of pH values from pH 0 to pH 11.0 (Booth 1985). Despite this wide range, it has been shown by measuring the intracellular pH of a number of bacterial species under various growth conditions that bacteria have elaborate mechanisms to maintain a constant neutral intracellular pH (Booth 1985). The level maintained depends on the pH of the environment. For example, acidophiles (i.e., acid-loving bacteria) grow from pH 0 to pH 5.25, but maintain their intracellular pH at 6.5–7.0 (Matin 1999). Bacteria that grow at neutral pH have an intracellular pH of 7.5 (Booth 1985). Alkaliphiles grow between pH 9.0 and pH 11.5, and their intracellular pH is maintained at pH 8.4–9.0 (Krulwich 1986; Krulwich and Guffanti 1989; Cook et al. 1996b; Krulwich et al. 1997). Fermentative bacteria that grow in the presence of fermentation acids adopt a different strategy whereby the pH gradient is kept low to prevent toxic fermentation acids from accumulating and abolishing the ΔpH (Baronofsky et al. 1984; Russell 1991a,b, 1992; Cook and Russell 1994).

Extremely thermophilic bacteria are a heterogeneous group of microorganisms spanning two domains, the Bacteria (or Eubacteria) and Archaea. Despite the increasing knowledge of physiological and genetic aspects of these bacteria, there is still very little known about basic bioenergetic processes in these bacteria. The eubacterial genus *Thermoanaerobacter* ferment hexoses, pentoses, cellobiose, and starch to ethanol, H_2/CO_2, and acetic and lactic acids (Wiegel 1986). The primary metabolism of these bacteria is fermentative, and therefore it was of interest to ascertain the ability of *Thermoanaerobacter wiegelii* to maintain a more or less constant internal pH as does the neutrophilic mesophilic bacterium *Escherichia coli*, or whether these bacteria allow intracellular pH to decline like

other glycolytic anaerobes that produce fermentative end products.

At present, very few studies have been performed regarding the regulation of internal pH in anaerobic thermophilic glycolytic bacteria (Wiegel 1998). A previous study suggested that these organisms did not regulate internal pH (Speelmans et al. 1993). The aim of this study was to determine if *T. wiegelii* was able to maintain intracellular pH homeostasis as the medium pH declined because of the production of fermentation acids.

Materials and methods

Media and cultivation conditions

Thermoanaerobacter wiegelii strain Rt8.B1 was grown anaerobically in trypticase peptone-yeast extract-glucose (TYEG) medium as previously described (Cook et al. 1996a). Media were prepared anaerobically, adjusted to pH 7 by the addition of 1 N NaOH, and autoclaved at 121°C for 20 min. Carbon sources were all prepared anoxically and added from separately sterilized stock solutions to the desired final concentration. All incubations were at 65°C.

To follow culture growth, 5-ml samples were withdrawn aseptically as required. To each of two 1-ml subsamples, 20 µl of saturated oxalic acid was added to halt metabolism, followed by centrifugation in a benchtop centrifuge (Runne Heidelberg, RS 85-1) at 6500 × *g* for 10 min and storage at −20°C for subsequent analysis. The remaining 3 ml was used to determine cell density by measuring the optical density at 650 nm using a SP6-450 UV/VIS spectrophotometer (Pye Unicam, Cambridge, England) with distilled water as a blank.

Preparation of cell-free crude extracts

Cells grown to the mid-logarithmic phase of growth were harvested by centrifugation at 7500 × *g* for 20 min at 4°C in a Sorvall SS-34 centrifuge (Du Pont, Wilmington, DE, USA). The cells were washed and resuspended in anaerobic MOPS buffer, pH 7.0, containing $MgSO_4$, 20 mM; EDTA, 0.01 mM; and DTT, 0.2 mM. Cells were disrupted by sonication using an Artek 300 series ultrasonic dismembranator. After 5-min sonication (45-s bursts with 1 min cooling on ice) using a standard 0.5-in. titanium tip at a setting of 50%, maximum cell lysis as confirmed by microscopic examination occurred. The sonicate was centrifuged for 45 min at 4°C and 40000 × *g* to remove unbroken cells and cell debris. All cell manipulations were carried out in an anaerobic chamber (Coy, Ann Arbor, MI, USA) containing an atmosphere of 95% N_2/5% H_2 and (NZ Industrial Gases, Wellington, New Zealand).

Lactate dehydrogenase (LDH) (FDP-activated) (EC 1.1.2.3) was assayed in a reaction mixture containing imidazole-HCl buffer, pH 6.2, 100 mM; NADH, 0.25 mM; pyruvate, 10 mM; and fructose-1,6-diphosphate (FDP), 1 mM. LDH activity was measured at 50°C under anaerobic conditions essentially as described by Lamed and Zeikus (1980). Specific activities were determined in a range at which linearity of activity with protein concentration was shown and expressed as the amount of enzyme catalyzing the conversion of 1 nanomole of substrate per minute per milligram of protein. All assays were carried out in triplicate with appropriate controls. Commercial LDH was used as a positive control. All pyridine nucleotide oxidation or reduction reactions were measured at 340 nm ($\varepsilon 340 = 6.22\,mM^{-1}cm^{-1}$).

Protonmotive force and ATP measurements

Protonmotive force and ATP determinations were carried out as previously described by Cook et al. (1993). Exponentially growing cultures were anaerobically transferred with a hypodermic syringe (2.0 ml) to a tube (13 × 100 mm) that contained [^{14}C]methyltriphenylphosphonium iodide (TPP^+) (0.5 µCi, 30–60 Ci/mmol), [7-^{14}C]benzoate (1.0 µCi, 10–25 mCi/mmol), [1,2-^{14}C]polyethylene glycol (1.0 µCi/mg), or ^3H_2O (4.00 µCi, 25 mCi/g). After incubation for 5 min at 65°C, the cultures were centrifuged through 0.35 ml silicon oil [40% mixture of phthalic acid bis(2-ethyl-hexyl ester) and 60% silicon oil (40% part mixture of DC200/200 silicon oil and 60% DC 550)] in microcentrifuge tubes (13000 × *g*, 5 min, 22°C), and 20-µl samples of supernatant were removed. The tubes and contents were frozen (−15°C), and the bottoms (cell pellets) were removed with dog nail clippers. Supernatant and cell pellets were dissolved in scintillation fluid and counted.

The intracellular volume (2.20 µl/mg protein) was estimated from the difference between the partitioning of ^3H_2O and [^{14}C]polyethylene glycol. The electrical potential across the cell membrane ($\Delta\Psi$) was calculated from the uptake of ^{14}C-TPP^+ according to the Nernst relationship. Nonspecific TPP^+ binding was estimated from cells that had been treated with valinomycin and nigericin (10 µM). The ΔpH was determined from the distribution of [^{14}C]benzoate using the Henderson–Hasselbalch equation (Reibeling et al. 1975), and ZΔpH was calculated as 68 mV × ΔpH. $\Delta\Psi$ and ZΔpH were corrected for extracellular contamination.

For intracellular ATP determination, cells from 1 ml of culture were extracted for 20 min with 0.5 ml ice-cold 14% perchloric acid that contained 9 mM ethylenediaminetetraacetic acid (EDTA). After centrifugation (13000 × *g*, 5 min, 22°C), the supernatant (1 ml) was neutralized with 0.5 ml KOH/$KHCO_3$ (1 M each, 0°C). Potassium perchlorate was removed by centrifugation (13000 × *g*, 5 min, 22°C), and the supernatant was assayed for ATP using the firefly luciferine–luciferase method (Lundin and Thore 1975). Neutralized extracts were diluted 50 fold with 40 mM Tris containing 2 mM EDTA, 10 mM $MgCl_2$, and 0.1% bovine serum albumin (pH 7.75). The luciferase reaction was initiated by adding 100 µl of a purified luciferine–luciferase mix to 100 µl of diluted extract according to the supplier's recommendations (Sigma, St. Louis, MO, USA). Light output was immediately measured with a luminometer (model 1250; LKB, Gaithersburg, MD, USA) using ATP as a standard.

Potassium determination and other analyses

Exponentially growing cultures (4 ml) were centrifuged through 0.35 ml of silicon oil as described. The cell pellets and supernatant samples (10 ml) were digested at room temperature for 24 h in 3 N HNO_3, and insoluble cell debris was removed by centrifugation (30000 × *g*, 15 min). The potassium concentration was determined by flame photometry (2655-00 Digital Flame Analyzer; Cole-Parmer, Chicago, IL, USA). Corrections were made for extracellular contamination in cell pellets. The transmembrane chemical potential of potassium (ΔpK^+) was equal to $68 \times \log([K+]_{in}/[K+]_{out})$.

Protein from NaOH-hydrolyzed cells (0.2 M NaOH, 100°C, 15 min) was assayed by the method of Markwell et al. (1978). Glucose, ethanol, and fermentation acids in cell-free supernatant samples were analyzed by high-pressure liquid chromatography as previously described (Cook and Morgan 1994): the sample size was 20 µl, the elutant was 0.01 N H_2SO_4, the flow rate was 0.5 ml/min, and the column temperature was 50°C.

All the experiments were performed two or more times, and the measurements were highly reproducible. The coefficient of variation (standard deviation ÷ mean) was always less than 15%.

Results

The effect of pH on the growth and metabolism of *T. wiegelii*

The effect of initial medium pH on the growth of *T. wiegelii* in batch culture (pH uncontrolled) is shown in Fig. 1. The maximum specific growth rate (0.57 h^{-1}) was observed at pH values from 6.7 to 7.2. No growth was observed below pH 5.1 and above 7.7. When *T. wiegelii* was grown in batch culture, initial pH 6.7, the major end products from glucose fermentation were acetate, ethanol, and lactate (Fig. 2). The production of lactate did not occur until the growth pH decreased below pH 6.0. Under these growth conditions, the carbon recovery was 88% and the ethanol yield was approximately 0.9 (moles of ethanol produced per mole of glucose consumed) (Fig. 2).

Because lactate production did not occur until extracellular pH reached 6.0, the effect of the pH of FDP and buffer on LDH activity was determined. An active NADH-linked LDH that was activated by low concentrations of FDP was detected in cell-free crude extracts. The enzyme functioned only in the direction of pyruvate reduction and required NADH as the coenzyme (data not shown). The affinity of FDP for LDH was dependent on extracellular pH. Maximal activation of LDH was noted at pH 6.2 and was unchanged with FDP concentrations greater than 1 mM. At pH 7.0, half-maximal activation was observed with FDP, and at pH 8.2 no activation was noted (data not shown).

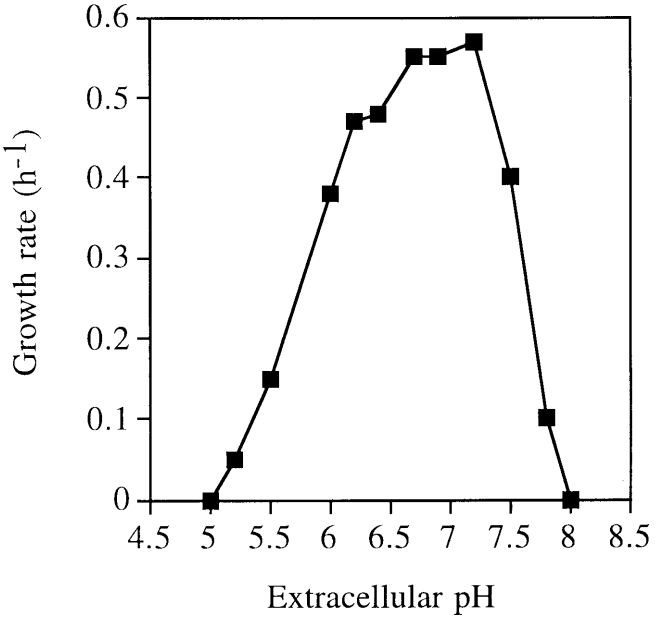

Fig. 1. Effect of extracellular pH on the growth rate of *Thermoanaerobacter wiegelii* in pH-uncontrolled batch culture. TYEG medium was adjusted to the pH being studied using NaOH or HCl, and growth rate was determined in the exponential growth phase

Regulation of intracellular pH in *T. wiegelii*

Thermoanaerobacter wiegelii was able to grow at pH as low as 5.1, and this result suggested this bacterium might be able to regulate internal pH. The regulation of pH homeostasis by *T. wiegelii* was studied by determining the intracellular pH as a function of external pH that was decreased as the culture grew and produced acetic and lactic acids (Fig. 3). As extracellular pH decreased from pH 6.7 to 5.1, an increase in the pH gradient (ΔpH) across the membrane (internal alkaline with respect to external pH) was observed (Fig. 3a). The maximum ΔpH (0.9 units) was observed at pH 5.1, where the internal pH was 6.0 (Fig. 3a). The membrane potential (ΔΨ) was approximately −100 mV at pH 6.7 and decreased with declining pH (Fig. 3b). As ΔΨ decreased, total Δp remained greater than −110 mV, and there was an increase in the ZΔpH. These results indicated that *T. wiegelii* was interconverting ΔΨ to ZΔpH, thus maintaining Δp more or less constant.

The effect of extracellular pH on ATP generation and potassium gradients

To determine what factors may contribute to pH sensitivity of *T. wiegelii* and the inability to grow below pH 5.1, two important cellular functions, ATP synthesis and intracellular potassium, were determined as a function of the growth pH. The ability to synthesize and maintain intracellular ATP constant remained at 1.5–2.0 mM over the pH range 5.1–6.7 (Fig. 4). To elucidate how *T. wiegelii* decreases ΔΨ even though ΔpH increases as external pH declines, the intracellular and extracellular potassium

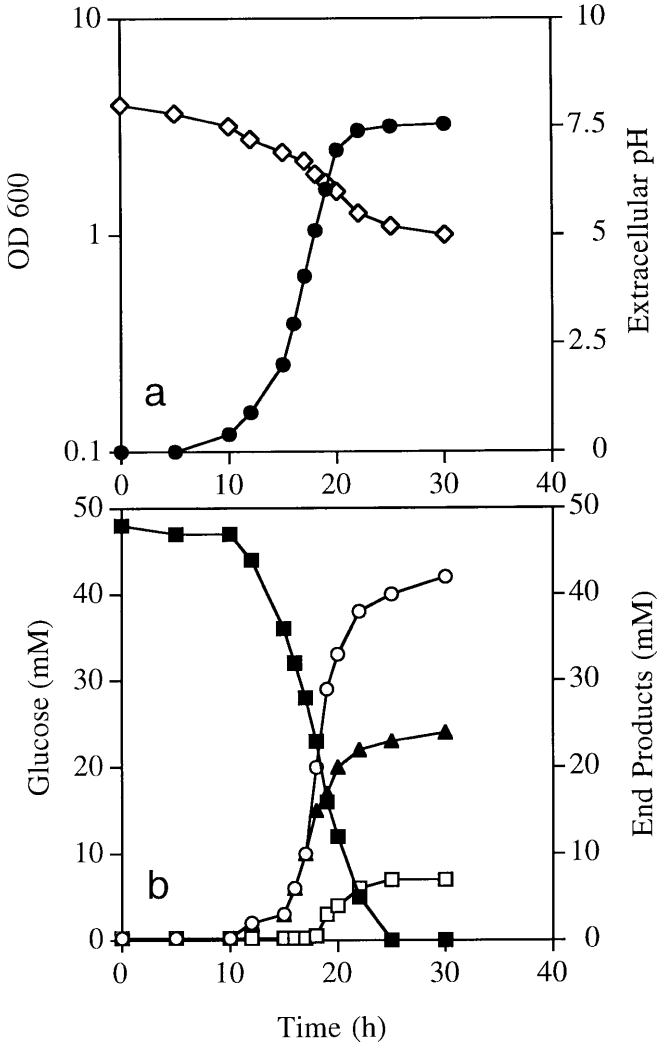

Fig. 2a,b. Changes in external pH (*open diamonds*), acetate (*solid triangles*), ethanol (*open circles*), and lactate production (*open squares*) during growth (*solid circles*) of *T. wiegelii* in TYEG medium (initial pH 6.7). Glucose concentration is shown as *solid squares* (**b**)

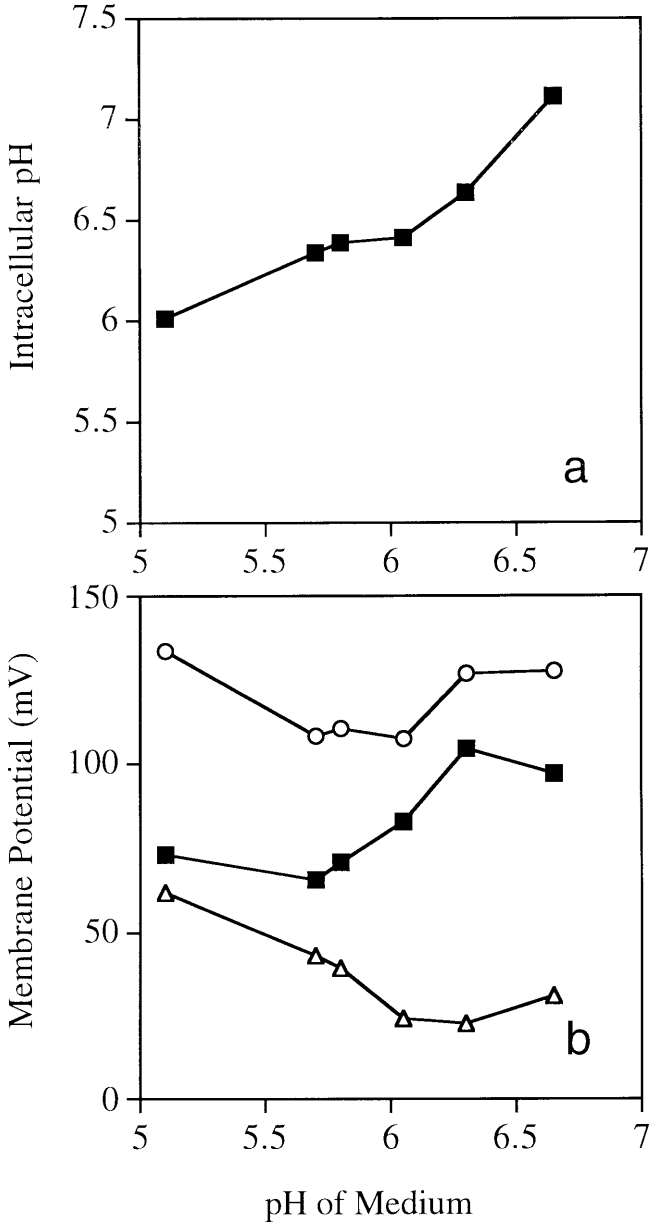

Fig. 3. a Intracellular pH (*solid squares*) in growing *T. wiegelii* as a function of the extracellular pH. **b** Protonmotive force (*open circles*) of *T. wiegelii* during growth in batch culture. The pH of the growth medium decreased during growth of cells, as shown in Fig. 2a. ΔpH (*open triangles*) and ΔΨ (*solid squares*) were measured as the cultures reached the indicated pH values, as described in Materials and methods

concentration was determined. The intracellular potassium concentration was approximately 150 mM at pH 6.7, and this declined rapidly below pH 6. At pH 5.1, the intracellular potassium concentration had decreased to 50 mM (Fig. 4). Extracellular potassium concentration remained more or less constant at 20 mM over this pH range. The ΔpK^+ (transmembrane chemical potential of potassium) was 60 mV at pH 6.7, and this decreased to 27 mV at pH 5.1.

Discussion

Thermoanaerobacter wiegelii is a thermophilic glycolytic anaerobe that metabolizes glucose via the Embden-Meyerhof-Parnas pathway and carries out a heteroethanol fermentation (Cook and Morgan 1994; Cook et al. 1996a). Minor amounts of lactic acid are produced by *T. wiegelii*, but only after the extracellular pH decreases to 6.0 or below. The lactate dehydrogenase of *T. wiegelii* was activated by fructose-1,6-diphosphate (FDP), and this effect was pH dependent with maximal activation occurring at pH 6.2. Similar pH effects of FDP on lactate dehydrogenases have been reported in *Thermoanaerobium brockii* HTD4 (Lamed and Zeikus 1980) and *Thermoanaerobacter ethanolicus* JW 200 (Carreira et al. 1982).

It has been widely demonstrated that fermentative microorganisms maintain a small ΔpH of 0.5–1.0 units and typically grow until the external pH reaches 5.0 (Reibeling et al. 1975; Baronofsky et al. 1984; Herrero et al. 1985;

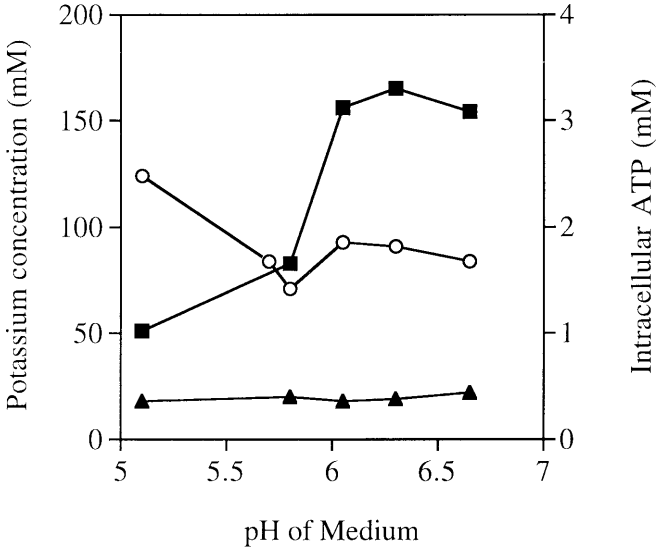

Fig. 4. Effect of medium pH on intracellular ATP (*open circles*) and potassium (*solid squares*). Extracellular potassium concentration is shown by *solid triangles*

Menzel and Gottschalk 1985; Miyazaki et al. 1992; Russell 1991a,b, 1992; Cook and Russell 1994). For producers of weak acids such as acetic and butyric, the decrease in ΔpH is not surprising because the undissociated forms of these fermentation acids equilibrate readily across the cytoplasmic membrane whereas their ionized forms apparently are impermeable (Russell 1992). Consequently, large concentrations of weak acids dissipate the ZΔpH component of the Δp. For example, in the absence of lactic acid, glucose-energized cells of *Lactococcus lactis* maintain a ΔpH of 2 units at pH 5.0 (Poolman et al. 1987), but in the presence of lactic acid the ΔpH is only 0.5 units (Cook and Russell 1994).

In this study, *T. wiegelii* grew over the pH range 5.1–7.7 and maintained a ΔpH gradient of 0.3–0.9 units (inside alkaline with respect to external pH). Speelmans et al. (1993) have investigated pH regulation in the neutrophilic thermophilic bacterium *Caloramator fervidus*. In contrast to *T. wiegelii*, *C. fervidus* has a very narrow pH range for growth (pH 6.3–7.7) and does not appear to regulate intracellular pH, hence its inability to grow below pH 6.0 (Speelmans et al. 1993). Energy transduction in this organism is completely dependent on sodium ions for energy coupling. Whether this is a general property of other thermophilic glycolytic anaerobes is not known because very few studies have been performed. *T. wiegelii* does not appear to be dependent on a sodium gradient for solute transport (Cook et al. 1993, 1994), but growth of *T. wiegelii* is inhibited by less than 1 μM monensin, suggesting that a sodium gradient is essential for some aspect of the microorganisms growth (unpublished results).

Other studies have also reported that some thermophilic fermentative bacteria have the capacity to regulate internal pH. Nochur et al. (1992) demonstrated that *Clostridium thermocellum* has a ΔΨ for cellobiose-grown cells of −130 to −150 mV, and the ΔpH gradient in these cells was 1.7 (internal pH, 8.5) at an external pH of 6.8. Growing cells of the thermophilic anaerobe *Clostridium thermoaceticum* maintained a ΔpH gradient of 0.6 units (interior alkaline) throughout most of batch growth when fermenting glucose to acetate (Baronofsky et al. 1984). Acidification of the medium below pH 5.0 by acetic acid collapsed both the ZΔpH and ΔΨ. To cope with low pH, Terracciano et al. (1987) have demonstrated the presence of a Na^+/H^+ antiporter for eliciting the efflux of H^+ in *C. thermoaceticum*. *T. wiegelii* is relatively insensitive to the Na^+/H^+ antiporter inhibitor amiloride, suggesting that this antiporter may be absent in this bacterium. The mechanism that *T. wiegelii* uses to maintain intracellular pH more alkaline than the external pH is unknown, but Girbal et al. (1994) have demonstrated that *Clostridium acetobutylicum* grown under acidogenic growth conditions maintained a small ΔpH (interior alkaline) at pH 6.5 and that alkalization of the cytoplasm involved hydrogenase activity.

In this study, *T. wiegelii* grew until the extracellular pH was less than 5.1, and the ΔpH at this point was 0.9 units. The maintenance of intracellular pH near neutrality when faced with decreasing external pH requires changes in the ΔpH that is a component of the Δp. One mechanism bacteria employ to modify their ΔpH while maintaining Δp constant is to make compensatory changes in the electrical potential (ΔΨ), which may be accomplished by the use of various cation-transport systems. For example, *Streptococcus faecalis* and *E. coli* are able to interconvert ΔpH for ΔΨ by electrogenic K^+ transport (Bakker and Mangerich 1981). In these bacteria, as the pH declines, electrogenic K^+ uptake causes a depolarization of the membrane, which allows the cell to pump more protons into the medium.

Results presented here demonstrated that, as external pH decreased, ΔpH increased and ΔX decreased. The mechanism for this interconversion is unknown in *T. wiegelii*, but it was also noted that intracellular K^+ concentration decreased as external pH declined. To maintain Δp constant under acidogenic (glucose-grown) or alcohologenic (glucose- or glycerol-grown) conditions, *C. acetobutylicum* interconverts ΔX to ΔpH by making compensatory changes in K^+ distribution (Girbal et al. 1994). Under acidogenic conditions, *C. acetobutylicum* maintains internal pH alkaline with respect to external pH, and the intracellular K^+ concentration is approximately 97 mM. Growth under alcohologenic conditions causes a reversal of the ΔpH (interior acidic), and the intracellular potassium concentration decreases to 53 mM. The authors propose that this lowering of internal K^+ may represent a novel mechanism to reduce the energy demand on the cell to accumulate K^+ against its chemical gradient. In *T. wiegelii*, the ΔpK^+ decreased from 60 to 27 mV as the medium pH declined, but this may simply be a reflection of changing culture conditions such as different stages of growth, increasing concentrations of ethanol and fermentation acids, as opposed to a specific response attributed solely to external pH. Intracellular ATP concentration remained constant during growth of *T. wiegelii* as the growth pH declined, and therefore it did not appear that

metabolic energy per se was the critical aspect of pH sensitivity.

In conclusion, like their mesophilic counterparts, fermentative thermophilic bacteria such as *T. wiegelii* also maintain a small ΔpH in the presence of fermentation acids. It will be important to elucidate how these bacteria regulate their intracellular pH under these growth conditions.

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