APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1996, p. 4576–4579 Vol. 62, No. 12
0099-2240/96/$04.00+0

The Intracellular pH of *Clostridium paradoxum*, an Anaerobic, Alkaliphilic, and Thermophilic Bacterium

GREGORY M. COOK,[1]† JAMES B. RUSSELL,[1,2] ARNO REICHERT,[3] AND JUERGEN WIEGEL[3]*

Section of Microbiology, Cornell University,[1] and Agricultural Research Service, U.S. Department of Agriculture,[2] Ithaca, New York 14853, and Department of Microbiology and Center for Biological Resource Recovery, University of Georgia, Athens, Georgia 30602-2605[3]

Received 4 March 1996/Accepted 9 September 1996

When the extracellular pH was increased from 7.6 to 9.8, *Clostridium paradoxum*, a novel alkalithermophile, increased its pH gradient across the cell membrane (ΔpH, $pH_{in} - pH_{out}$) by as much as 1.3 U. At higher pH values (>10.0), the ΔpH and membrane potential ($\Delta\psi$) eventually declined, and the intracellular pH increased significantly. Growth ceased when the extracellular pH was greater than 10.2 and the intracellular pH increased to above 9.8. The membrane potential increased to 110 ± 8.6 mV at pH 9.1, but the total proton motive force (Δp) declined from about 65 mV at pH 7.6 to 25 mV at pH 9.8. Between the extracellular pH of 8.0 and 10.3, the intracellular ATP concentration was around 1 mM and decreased at lower and higher pH values concomitantly with a decrease in growth rate.

Many bacteria are alkalitolerant, but true alkaliphilic bacteria are predominantly found in the genus *Bacillus* (7, 11, 12). Some anaerobic archaea can grow at alkaline pH values, but none of these bacteria are both highly alkaliphilic and thermophilic (1, 2, 19, 23, 32). Recently, Li et al. (17, 18) described the isolation of a novel group of anaerobic alkaliphilic (eu)bacteria that grew optimally at 55°C and pH 10.1. These isolates extended the combined pH and temperature ranges, allowing growth. One of these organisms, *Clostridium paradoxum*, had doubling times as short as 16 min (18) and is ubiquitously found in sewage sludge from the United States, Europe, and New Zealand.

Alkaliphilic bacteria often have lower intracellular pH (inverted pH gradient [ΔpH]) than the media (14). When the ΔpH is inverted, there is less chemiosmotic potential to drive ATP synthesis, H^+-coupled solute transport, and motility. Nevertheless, oxidative phosphorylation as measured by the phosphorylation potential (ΔGp) remains steady and then increases as the pH rises (13). Several theories have sought to explain this mechanism of ATP synthesis at submaximal proton motive force (Δp). These include a variable H^+/ATP stoichiometry for the alkaliphile synthase and localized proton movement from the proton-translocating respiratory chain to the ATP synthase (13, 31).

In their review of intracellular pH regulation, Padan et al. (24) concluded that bacteria maintain their internal pH between 6.5 and 9.5. *Escherichia coli* (3) and some streptococci conform to this constancy (25), but some acid-tolerant fermentative bacteria can grow with an intracellular pH of less than 5.5 (8, 27, 28). To our knowledge there has never been a report of a bacterium growing with an intracellular pH greater than 9.6 (14, 31), but there have been relatively few measurements for obligate alkaliphiles. Even less is known about the intracellular pH and the energy-transducing processes of anaerobic thermophiles (29). One of the exceptions is *Calorimator* (syn. *Clostridium*) *fervidus*, but this anaerobic thermophile has an energy transduction that appears to be completely dependent on Na^+ as a coupling ion (29).

In this paper, we describe the ability of *C. paradoxum* to grow and to regulate its intracellular pH at external pH values ranging from pH 7.6 to 9.8. A revised pH curve for the growth of this organism is also provided.

MATERIALS AND METHODS

Cell growth. *C. paradoxum* JW/YL-7T was grown anaerobically at 55°C in YTG medium with 0.3% glucose as described by Li et al. (18). pH dependence was determined with both pH-uncontrolled batch cultures (160-ml serum vials containing 100 ml of medium under an O_2-free N_2 gas atmosphere) and pH-controlled batch cultures (150-ml fermentor with 75-ml working volume). Growth rates based on measuring the increase in optical density at 600 nm (OD_{600}) in serum bottle cultures were determined in triplicate with 1:20-diluted subcultures and at OD_{600} values below 0.1. Exponentially growing precultures at the test pH values with OD_{600} below 0.2 were used as inocula. The pH-controlled fermentation was performed in a semicontinuous manner, to ensure the use of cultures well adapted to the test pH. The test pH values were first sequentially increased to the maximal pH value for growth and then sequentially decreased until the pH minimum was reached. The pH of a culture was maintained within 0.05 pH unit by addition of sterile anaerobic NaOH. At each new pH value the culture was gradually diluted to about 5% of its OD value and twice allowed to grow up to the late exponential growth phase before the increase in OD_{600} was monitored in two subsequent cultures. Cultures for ΔpH and membrane potential ($\Delta\psi$) determination were grown in batch cultures (with frequent pH adjustment) with YTG medium (18) at pH values ranging from 7 to 10.8. The initial pH was maintained within 0.1 pH unit. pH-adapted cultures were harvested at mid-exponential growth phase (OD_{600}, 0.7), and the intracellular pH was determined as described below. Cultures with an OD of less than 0.7 did not sporulate (microscopic examination), and the cells had more consistent intracellular volumes than those from late-exponential-phase cultures. The ratio of protein to OD was 220 mg of protein $liter^{-1}$ turbidity $unit^{-1}$ as determined by the method of Lowry et al. (20).

pH determination. The pHs of media and cultures were measured at the corresponding growth temperature with a Accumet pH Meter (model 825 MP; Fisher Scientific, Pittsburgh, Pa.) equipped with a combination polymer pH pencil-type electrode (Broadly-James Corp., Santa Ana, Calif.) and a temperature probe. During calibrations of the pH meter and pH measurements, the temperature probe, pH electrode, calibration buffer, and samples were kept in a heated water bath at 55°C or at the corresponding growth temperature. The pHs of the calibration buffers were corrected for temperature according to the values given by the manufacturer. Calibration of the pH meter at room temperature as described by Li et al. (18) caused a significant overestimation of the pH tolerance (e.g., pH 10.3 at 55°C compared to pH 11.2 at room temperature and pH 9.6 compared to pH 10.0, respectively). These biases could be explained by the effect of temperature on the dissociation of the buffer and medium components. The deviation was less at lower pH values and negligible around pH 7.5.

Determination of bioenergetic parameters. Exponentially growing cells (2.0 ml) were washed, resuspended in anaerobic 100 mM Tris-HCl buffer (pH ad-

* Corresponding author. Mailing address: Dept. Microbiology, University of Georgia, Athens, GA 30602-2605. Phone: (706) 542-2651. Fax: (706) 542-2651. Electronic mail address: jwiegel@uga.cc.uga.edu.

† Present address: King's College London, Division of Life Sciences, London W8 7AH, United Kingdom.

justed with NaOH to the desired value). These cell suspensions (nongrowing) were energized by the addition of glucose (0.3%; 15-min incubation at 55°C). Energization caused an increase in gas pressure as felt by the plunger from a hypodermic syringe. Energized cell suspensions (2.0 ml) were transferred to tubes (13 by 100 mm) containing [^{3}H]tetraphenylphosphonium ion ([^{3}H]TPP$^+$) (0.5 μCi; 30 μCi/μmol) and [^{14}C]methylamine (0.5 μCi; 50 μCi/μmole) or containing [1,2-^{14}C]xylose (0.4 μCi; 45 μCi/μmole) and ^3H_2O (4 μCi). At pH values less than 8.0, [7-^{14}C]benzoate (1.0 μCi; 21.8 μCi/μmol) was also used as a pH marker. After incubation for 5 min at 55°C, the cultures were centrifuged through silicon oil (equal-part mixture of Dexter Hysol 550 and 560; Hysol Co., Olean, N.Y.) in microcentrifuge tubes (13,000 × *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 (5 ± 1.8 μl/mg of protein) was estimated from the difference between the partitioning of ^3H_2O and [^{14}C]xylose. The Δψ was calculated from the uptake of [^{14}C]TPP$^+$ according to the Nernst relationship. Nonspecific TPP$^+$ binding was estimated from valinomycin- and nigericin (10 μM each)-treated cells. The ΔpH was determined from the distribution of [^{14}C]methylamine and [^{14}C]benzoate by using the Henderson-Hasselbach equation (26), and *Z*ΔpH was calculated as 65 mV × ΔpH. Δψ and *Z*ΔpH were corrected for extracellular contamination. Intracellular ATP content was determined by the luciferine-luciferase method as previously described (6, 21).

Sodium and potassium determinations. Cultures (6 ml; OD_{600}, 0.7) were centrifuged through 0.3 ml of silicon oil as described above. The cell pellets and supernatant samples (20 μl) were digested at room temperature for 24 h in 3 N HNO_3, and insoluble cell debris was removed by centrifugation (33,000 × *g*, 4°C, 15 min). Sodium and potassium were determined by flame photometry (model 2655-00 digital flame analyzer; Cole-Parmer Instrument Co., Chicago, Ill.) as previously described (5). Cell pellets were corrected for extracellular contamination.

Glucose transport. To create an artificial sodium motive force (ΔpNa) and Δψ, washed cells (100 mM Tris-HCl buffer, 100 KCl, pH 9.0) from exponentially growing cultures were loaded with potassium (100 mM Tris-HCl buffer, 100 mM KCl, pH 9.0) by valinomycin treatment (10 μM, 30 min, 0°C) and diluted 50-fold into 100 mM Tris-HCl buffer containing 100 mM NaCl (pH 9.0) and additionally [^{14}C]glucose (165 μM; 41 μCi/μmol). Potassium-loaded cells were diluted into either 100 mM Tris-HCl buffer containing 100 mM NaCl and 100 mM KCl to create a ΔpNa$^+$ in the absence of a Δψ or 100 mM Tris-HCl buffer alone to create a Δψ. Controls (no driving force) were loaded with K$^+$ or K$^+$ and Na$^+$ and diluted into K$^+$ or K$^+$ plus Na$^+$, respectively. In each case, [^{14}C]glucose (165 μM; 41 μCi/μmol) uptake was measured as previously described (4). The ATP- and phosphoenolpyruvate (PEP)-dependent phosphorylation of glucose was measured by using toluene-treated cells as described previously (4).

Statistics. All of 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 10%.

RESULTS AND DISCUSSION

pH-dependent growth rate. pH-controlled batch cultures (adapted at pH 9.3 [see Materials and Methods]) grew rapidly on glucose, and the doubling time was as short as 13 min (Fig. 1). Previous work (18) using a pH meter calibrated at room temperature indicated that the optimal pH for growth was 10.1, with growth rates decreasing markedly at pH values less than 8.0 and greater than 10.5. When pH was appropriately corrected for temperature (see Materials and Methods), the trend was similar except that the pH optimum was 9.3 and the upper pH limit for growth was 10.3 (Fig. 1). At pH values greater than 10.0, the cells grew in long filaments which aggregated to large flocs which confounded taking representative samples for growth rate determinations. For pH 9.0 and pH 10.0 the growth rates were corroborated by determining the washout points of cells in continuous cultures. At low cell densities, similar growth rates were observed in pH-uncontrolled batch cultures in serum vials.

Intracellular pH. When washed cell suspensions (nongrowing but glucose energized) obtained from exponentially growing cells were incubated at pH 9.1, the intracellular pH as determined from [^{14}C]methylamine uptake was 7.8 ± 0.3 (Fig. 2). At higher or lower pH values, the ΔpH across the cell membrane was lower, and there was no ΔpH at pH 7 or 10.8. [^{14}C]methylamine uptake declined at pH values less than 8.0, and the ΔpH was confirmed by [7-^{14}C]benzoate uptake. The

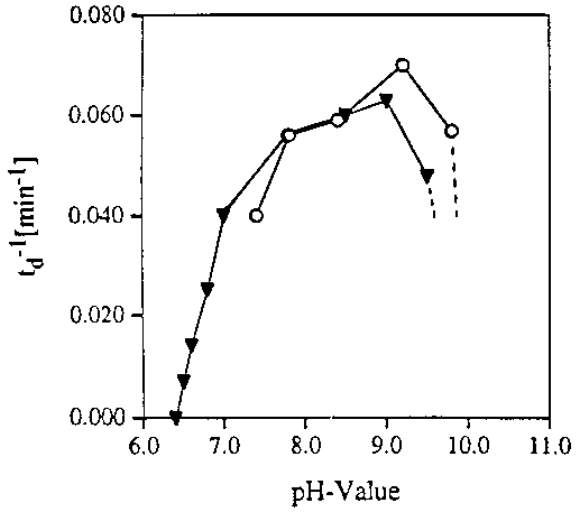

FIG. 1. pH dependence of the doubling time (t_d^{-1}) of *C. paradoxum* at 55°C. The pH profile was obtained with YTG medium in serum bottles (▼) and in a pH-controlled 75-ml (working volume) glass fermentor (○) (see Materials and Methods). At pH values higher than 10.0, the cells grew in long filaments, and these aggregated to large flocs which hindered an exact determination of growth rates by cell counting or OD measurements in withdrawn samples.

ΔpH values for *C. paradoxum* (maximal 1.3 pH units) were less then those reported for *Bacillus firmus* OF4B (2.6 pH units) (31). *B. firmus* OF4 maintains an intracellular pH between pH 7.5 and 9.5, and the intracellular pH is never greater than 9.6, even if the extracellular pH is 11.4 (31).

Δp and Δψ. At an extracellular pH of 9.1 (near the pH for optimal growth) the Δψ was approximately 110 ± 8.6 mV but the Δp was only 35 mV (Fig. 3). When the extracellular pH was significantly greater or less than 9.1, ΔpH and Δψ decreased, but the intracellular ATP pool (using an intracellular volume of 5 ± 1.8 μl/mg of protein) showed little change. The Δp increased as the pH declined and reached a maximum value 65 mV at pH 7.6. From extracellular pHs of 7.8 to 10.3, the intracellular ATP concentration was approximately 1 mM but declined significantly at the more extreme pH values when

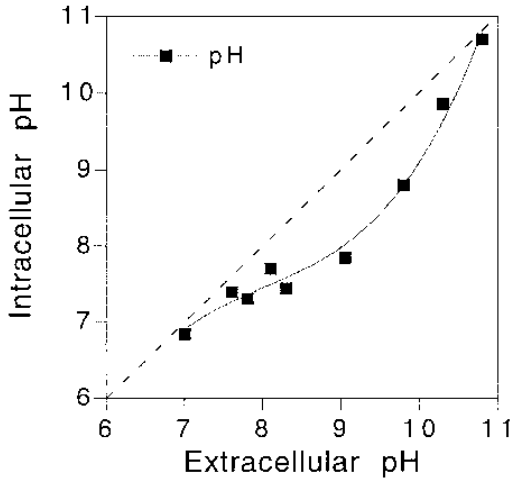

FIG. 2. The effect of extracellular pH on the intracellular pH of *C. paradoxum*. The extracellular pH was adjusted with 2 N KOH. Intracellular pH values were calculated with the data (*Z*ΔpH) shown in Fig. 3 and the Henderson-Hasselbalch equation (26).

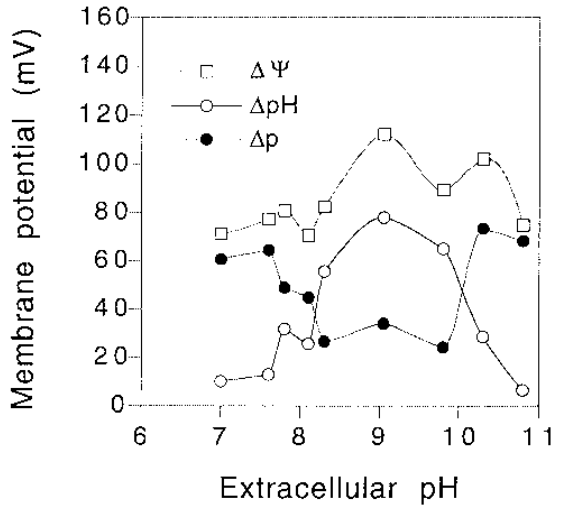

FIG. 3. The effect of changing extracellular pH on the $\Delta\psi$, $Z\Delta$pH, and total Δp of *C. paradoxum*. $\Delta\psi$ was calculated from the uptake of [^{14}C]TPP$^+$ according to the Nernst relationship. The ΔpH was determined from the distribution of [^{14}C]methylamine and [^{14}C]benzoate with the Henderson-Hasselbalch equation (26), and $Z\Delta$pH was calculated as 65 mV $\times$ ΔpH.

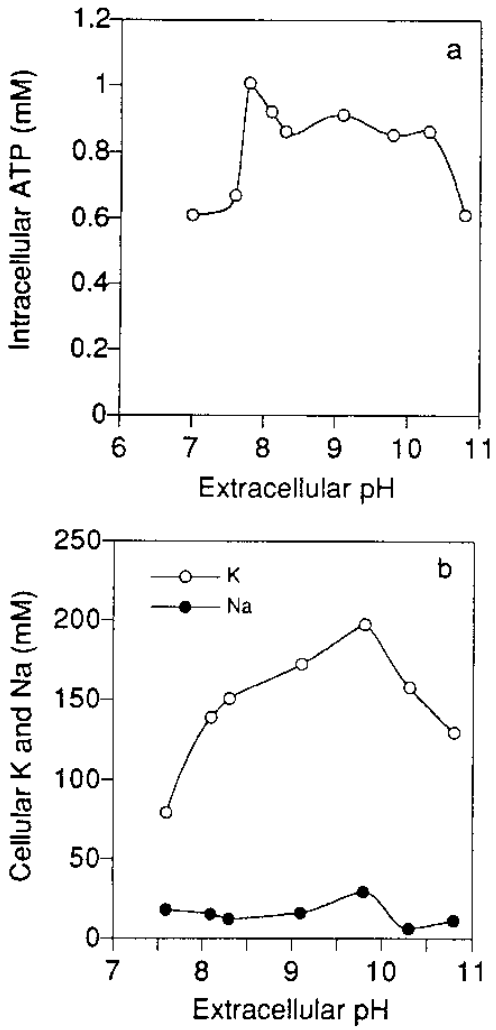

FIG. 4. Intracellular ATP concentration (a) and cellular potassium and sodium concentrations (b) of *C. paradoxum* versus pH. ATP, potassium, and sodium were measured as described in Materials and Methods.

growth decreased (Fig. 4a). The $\Delta\psi$ of *C. paradoxum* was slightly lower than the values (140 to 200 mV) reported for the aerobic alkaliphilic *B. firmus* OF4 (31) but are consistent with values reported for other fermentative bacteria that also generate their $\Delta\psi$ using a F_1F_0-ATPase (4–6).

Acidification of cytoplasmic pH at alkaline extracellular pHs creates special bioenergetic problems. If the ΔpH is reversed ($pH_{in} < pH_{out}$), the $\Delta\psi$ must increase to prevent an overall decline in the total Δp. *C. paradoxum* offset some of the ΔpH ($pH_{in} < pH_{out}$) with an increase in $\Delta\psi$, but the increase in extracellular pH caused a gradual decline in Δp (Fig. 3). At these higher pH values the organism changed from rapidly growing, single motile cells to slowly growing, long nonmotile filaments. In contrast to these observations in the obligately anaerobic *C. paradoxum*, the $\Delta\psi$ of the aerobically growing *B. firmus* cannot counteract cytoplasmic acidification and its Δp decreases markedly (31). Because the energy transduction of *B. firmus* appears to be exclusively proton coupled, it seemed that the proton ATPases were operating at a very low Δp (13, 31). On the basis of the assumption that the phosphorylation potential (ΔGp) of ATP and Δp were in equilibrium, Sturr *et al.* (31) and others (13) hypothesized that the unfavorable energetics of *B. firmus* might be explained by a variable proton stoichiometry. Since the phosphorylation potential ΔGp remained more or less constant in *B. firmus* and its Δp decreased, it appeared that the number of protons (n) per synthesized ATP was increasing (phosphorylation potential = $n \times \Delta$p) dramatically. An alternative explanation involves localized proton circuits. If the ATPase were being driven by a localized (significantly higher) Δp, n would not necessarily increase.

Calorimator fervidus is unable to regulate its internal pH (29, 30). This thermophilic anaerobe depends on Na^+ as a coupling ion for energy transducing processes, uses an F/V-type Na^+-extruding ATPase, and appears to lack other H^+-pumping mechanisms. No Na^+/H^+ antiporter system was detected (30). Other thermophilic anaerobes do show some pH homeostasis. *Thermoanaerobacter wiegelii* maintains a small ΔpH (<0.5 pH unit) as the extracellular pH is decreased from 7.2 to 5.5 (3a).

ΔpNa and glucose transport. The growth medium used in these experiments typically contained 5 mM potassium ions. When the extracellular pH was increased from 7.6 to 9.8, the intracellular potassium concentration increased from 75 mM to approximately 200 mM and declined at higher pH values (Fig. 4b). Lactic acid bacteria growing at similar external potassium concentrations maintain an intracellular potassium concentration around 600 mM (5). The cells had a sodium ion concentration of approximately 20 mM, and this value was significantly less than that of the growth medium (100 mM) (Fig. 4b). On the basis of these results, we conclude that *C. paradoxum* had a significant ΔpNa.

Neither an artificially generated ΔpNa nor $\Delta\psi$ could drive glucose transport, and these results suggested that *C. paradoxum* has a phosphotransferase system for glucose uptake. Toluene-treated cells had high ATP-dependent phosphorylation of glucose (2,250 nmol min^{-1} mg of protein^{-1}) but little PEP-dependent glucose phosphorylation (<20 nmol min^{-1} mg^{-1} of protein). On the basis of these results, we speculate that *C. paradoxum* transports glucose by a mechanism that does not involve a PEP-dependent phosphotransferase system. To date, a PEP-dependent phosphotransferase system for glucose has not been described for any thermophilic anaerobic

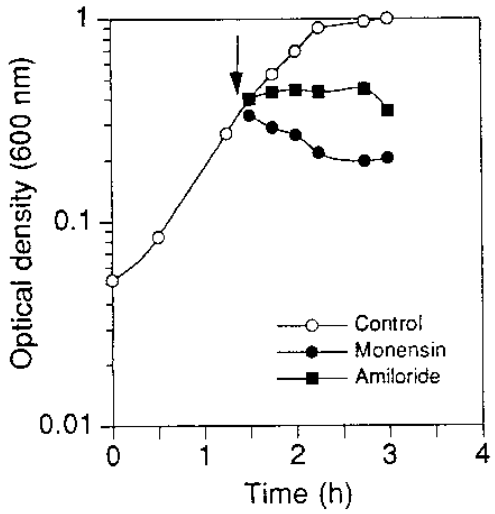

FIG. 5. The effect of monensin (10 μM) or amiloride (200 μM) addition to cultures of *C. paradoxum* growing in YTG medium (18) plus 0.3% glucose. All inhibitors were added at the mid-exponential phase of growth. The arrow indicates addition of inhibitor. Ethanol (100%; 100 μl) was added to the control.

bacterium, but ATP-dependent transport systems for glucose have been reported in other thermophilic anaerobes (4).

Sodium/proton antiport. It is generally accepted that alkaliphilic bacilli use electrogenic Na^+/H^+ antiport systems to regulate intracellular pH at alkaline pHs (9, 12, 15, 16, 22). *C. paradoxum* has an absolute requirement for sodium (18), and growth was inhibited by monensin (Fig. 5), an ionophor that dissipates sodium gradients. The idea that electrogenic sodium/proton antiport was the most likely mechanism of cytoplasmic acidification in *C. paradoxum* was supported by the observation that amiloride, an inhibitor of sodium/proton antiporters (10), also inhibited growth (Fig. 5).

Sturr et al. (31) suggested that the pH-dependent decline in Δp of *B. firmus* would greatly increase proton translocation by the ATP-generating membrane ATPase. This effect would acidify the cytoplasm of an aerobe like *B. firmus*, but the direction of proton translocation in an anaerobe like *C. paradoxum* would be reversed. Booth (3) indicated that alkaliphiles could use either potassium/proton antiporter or potassium/proton symport as a mechanism of cytoplasmic acidification. The intracellular potassium of *C. paradoxum* varied with the extracellular pH (Fig. 4b), but net potassium movement is probably not the only mechanism of proton influx in this bacterium.

This communication represents the first report on the measurements of intracellular pH and Δp in an anaerobic bacterium able to grow optimally at alkaline pHs and elevated temperatures. More detailed studies will elucidate further the energy metabolism and transport processes in this novel bacterium.

ACKNOWLEDGMENTS

This research was supported by a grant from the Department of Energy (DE-FG 09-86 ER-13614).

J.W. expresses thanks for the great hospitality during his stay at Cornell University.

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