

Bioenergetic Properties and Viability of Alkalophilic *Bacillus firmus* RAB as a Function of pH and Na⁺ Contents of the Incubation Medium

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The bioenergetic properties and viability of obligately alkalophilic *Bacillus firmus* RAB have been examined upon incubation in alkaline and neutral buffers in the presence or absence of added Na⁺. At pH 10.5, cells incubated in the absence of Na⁺ exhibited an immediate rise in cytoplasmic pH from less than 9.5 to 10.5, and they lost viability very rapidly. Viability experiments in the presence or absence of an energy source further suggested that the Na⁺-dependent mechanism for pH homeostasis is an energy-requiring function. The Na⁺/H⁺ antiporter, which catalyzes the vital proton accumulation at alkaline pH, was only slightly operational at pH 7.0; both whole cells and vesicles exhibited net proton extrusion even in the presence of Na⁺. Moreover, cells incubated in buffer at pH 7.0 were actually more viable in the presence of Na⁺ than in its absence. Thus, the inability of *B. firmus* RAB to grow at neutral pH is not due to excessive acidification of the cytoplasm. Rather, the transmembrane electrical potential, $\Delta\Psi$, generated at pH 7.0 was found to be much lower than at alkaline pH. The very low $\Delta\Psi$ compromised several cell functions, e.g., Na⁺/solute symport and motility, which in this and other alkalophiles specifically depend upon $\Delta\Psi$ and Na⁺.

Several lines of evidence from previous work indicate that a Na⁺/H⁺ antiporter plays a vital role in pH homeostasis in obligately alkalophilic bacteria (5, 22, 23, 28). In accord with the chemiosmotic hypothesis of Mitchell (30), cells of alkalophilic bacteria, like mitochondria and other bacterial cells, extrude protons during respiration. In obligately alkalophilic bacilli, this primary proton extrusion is followed by Na⁺-dependent proton accumulation, resulting in a proton gradient (ΔpH), $H^+_{in} > H^+_{out}$, and a Na⁺ gradient, $Na^+_{in} < Na^+_{out}$ (22, 28). The Na⁺/H⁺ antiport activity can thus facilitate the maintenance of a cytoplasmic pH that is no greater than pH 9.5 even at external pHs of 10.5 to 11.5 (5, 9). That a Na⁺/H⁺ antiporter indeed performs this crucial function is evidenced by the presence of the antiport activity in cells and membrane vesicles of obligately alkalophilic *Bacillus alcalophilus* and *Bacillus firmus* RAB and by the absence of the activity in cells and vesicles of the nonalkalophilic mutant derivatives of those species (5, 22, 23, 28). The nonalkalophilic mutants lose the ability to grow above pH 9.0 concomitantly with their loss of the Na⁺/H⁺ antiporter (5, 22, 23, 28). Since the mutants also gain the ability to grow at neutral pH, we have proposed that antiport activity in wild-type alkalophilic cells

may preclude growth by excessive acidification of the cytoplasm at low pH (22, 23). However, this latter suggestion has not been tested in an obligate alkalophile upon shift to neutral pH. Moreover, in an alkaline-tolerant *Synechococcus* species, Kallas and Castenholz (14, 15) recently implicated the failure of some membrane-associated process, perhaps solute transport, rather than a problem with pH homeostasis in the inability to grow at low pH.

In the current study, we have examined the bioenergetic properties and viability of *B. firmus* RAB upon incubation in alkaline and neutral buffers in the presence or absence of added Na⁺. Since Na⁺/H⁺ antiport activity in this species depends upon added Na⁺ (22), the role and effects of the antiporter at various pHs might be inferred by such experiments. The results support the conclusion that the Na⁺/H⁺ antiporter is vitally involved in maintaining a relatively acidified cytoplasm at highly alkaline external pHs. However, they also indicate that the antiporter is relatively inactive at neutral pH and that the inability of obligate alkalophiles to grow at pH 7 may be primarily related to a failure of respiratory activity to generate a high enough transmembrane electrical potential ($\Delta\Psi$) at neutral pH.

MATERIALS AND METHODS

Organisms and growth conditions. *B. firmus* RAB, a soil isolate (5), was grown at 30°C on L-malate-containing medium adjusted with carbonate buffer to pH 10.5 (9) with shaking at 200 rpm on a New Brunswick G25 rotary shaker. Growth was monitored turbidimetrically with a Klett-Summerson colorimeter (no. 42 filter).

Procedures for shift from growth medium to buffers of specific pH and cation content. Cells of *B. firmus* RAB were grown to the mid-logarithmic phase, harvested at room temperature by centrifugation for 3 min at 12,000 × g, washed twice, and suspended in the following buffers at 50 mM concentrations: potassium phosphate or sodium phosphate at pH 7.0; potassium carbonate or sodium carbonate at either pH 9.0 or pH 10.5. The cells were incubated at 30°C on a rotary shaker at 200 rpm, and 0.1-ml samples were removed at various times to determine cell viability or various bioenergetic properties. For determinations of viability, cells were serially diluted into 50 mM sodium carbonate buffer, pH 9.0 or 10.5, plus 20 mM L-malate.

All cells were plated onto plates containing sodium carbonate-L-malate, buffered to pH 10.5. The plates were incubated at 30°C for 24 h. Colonies were counted with a Bactronic colony counter (model C110, New Brunswick Scientific Co.). Care was taken to autoclave 3% agar separately and to mix it with carbonate buffer in a 1:1 dilution at 50°C, a method that avoided decomposition of the agar at high temperature and pH. For qualitative assessment of motility, cells were observed at ×1,000 with a Leitz SM-LUX phase-contrast microscope.

Preparation of right-side-out and everted vesicles. Right-side-out membrane vesicles were prepared from mid-logarithmic phase cells in a variation of the lysosome method of Kaback (13) as described by Mandel et al. (28). Three protease inhibitors, phenylmethylsulfonyl fluoride, *p*-toluenesulfonyl fluoride, and *p*-toluenesulfonic acid, were routinely included, at 1 mM each, in the osmotic shock buffer (22). Vesicles at pH 7.0 were shocked and suspended in 100 mM potassium phosphate buffer–10 mM MgSO₄, pH 7.0. Vesicles at pH 9.0 were shocked and suspended in 100 mM potassium carbonate buffer–10 mM MgSO₄, pH 9.0. Membrane vesicles were everted by their passage through a French pressure cell (8,000 lb/in²) in a modification of the method of Kobayashi et al. (20) as described by Mandel et al. (28).

ΔpH and ΔΨ measurements. The measurement of ΔpH in whole cells was performed in a filtration assay by the method of Zilberstein et al. (39). Cell suspensions of 5 ml (0.1 mg of protein per ml) were incubated for 5 min with vigorous aeration in the presence of either 1.8 μM [¹⁴C]dimethylloxazolidine-2,4-dione (DMO) or 1.9 μM [¹⁴C]methylamine. The reaction was terminated by filtration through glass-fiber filters (Whatman, Inc.; diameter, 25 mm) without washing. Radioactivity was measured by scintillation spectrometry. Control experiments in which 10 μM gramicidin was included in the incubation mixture were conducted for each assay. The value of DMO or methylamine uptake in the presence of gramicidin was subtracted from the experimental value. Previously, gramicidin had been shown to completely collapse the electrochemical proton gradient (Δμ_{H⁺}) in *B. alcalophilus*. Initially, the glass-fiber filter method for whole cells

was validated by parallel measurements of ΔpH in a variation of the flow dialysis method of Friedberg and Kaback (4). The ΔpH probe was allowed to equilibrate across the dialysis membrane, and cells were added to 1.5 mg of protein per ml. A control run containing 10 μM gramicidin was also performed. The ΔpH was calculated by the formula of Waddell and Butler (37). The ΔpH values in whole cells determined by filtration and by flow dialysis corresponded to within 10%.

The ΔpH in right-side-out membrane vesicles was measured by the distribution of [¹⁴C]DMO or [¹⁴C]methylamine in the flow dialysis assay of Ramos et al. (31, 32) as described by Mandel et al. (28). The vesicles were energized by the addition of 20 mM potassium ascorbate plus 0.1 mM phenazine methosulfate (PMS). The ion content of the assay buffer was controlled by pumping either 100 mM sodium salt or potassium salt of phosphate (pH 7.0) or carbonate (pH 9.0) plus 10 mM MgSO₄ through the flow dialysis chamber.

The value for ΔΨ was determined by a filtration assay (9, 33) for whole cells by the accumulation of [³H]tetraphenylphosphonium (TPP⁺). TPP⁺ was added to a vigorously stirred cell suspension (0.1 mg of protein per ml) at a concentration of 5 μM. Counts retained by the filters in the presence of 10 μM gramicidin were subtracted. The ΔΨ was calculated from the Nernst equation: ΔΨ = 58.8 log TPP⁺_{in}/TPP⁺_{out} at room temperature.

ATPase assays. The hydrolysis of ATP by everted membrane vesicles of *B. firmus* RAB was followed by measuring the release of P_i. Vesicles (0.5 mg of protein per ml) were incubated at 30°C in 100 mM phosphate buffer (pH 7.0) or 100 mM carbonate buffer (pH 9.0). Either the sodium or potassium salt of phosphate or carbonate buffer was used. ATP (5 mM) and MgSO₄ (10 mM) were also included in the assays. The reaction was terminated by the addition of trichloroacetic acid, and phosphate was measured by the Josse modification (12) of the method of Fiske and Subbarow (3).

Assays of α-aminoisobutyric acid uptake. The uptake of α-aminoisobutyric acid (AIB) by whole cells was measured by a filtration assay (7). Cells suspended in various buffers were vigorously stirred at 20°C. In some experiments, AIB uptake was driven by endogenous energy sources; in others, 10 mM potassium L-malate was added. The uptake was initiated by adding 500 μM [¹⁴C]AIB and terminated by filtration through HA Millipore filters (pore size, 0.45 μm). The filters were washed with a 10-fold volume of the appropriate buffer. Background values for uptake in the presence of 10 μM gramicidin were subtracted from experimental values.

In some experiments, AIB uptake was driven by a valinomycin-induced potassium diffusion potential. After 1 h of starvation in 50 mM potassium phosphate buffer at pH 7.0, cells were centrifuged at 12,000 × g for 10 min and suspended in 100 mM potassium phosphate buffer (pH 7.0) to a concentration of 10 mg of protein per ml. Valinomycin (10 μM) was added at 30°C. After 10 min, the cells were diluted 200-fold into either 100 mM sodium phosphate (pH 7.0) or 50 mM potassium plus 50 mM sodium phosphate (pH 7.0). The dilution buffers also contained 10 μM valinomycin and 1 mM NaCN. Either 500 μM [¹⁴C]AIB or 5 μM [³H]TPP⁺ was included in the dilution buffer. Samples were removed and assayed for the uptake of AIB or

TPP⁺ by the filtration method (9). No uptake of AIB or TPP⁺ over time was observed when the dilution buffer contained 50 mM potassium phosphate; those control values were taken as binding controls and were subtracted from the experimental values.

The transport of [¹⁴C]AIB into right-side-out membrane vesicles was measured in a filtration assay as previously described (8, 22). Vesicles were incubated at 30°C under a stream of water-saturated oxygen. The artificial electron donor, 20 mM ascorbate–0.1 mM PMS, was added for 1 min, after which uptake was initiated by the addition of 500 μM [¹⁴C]AIB. The reaction was terminated by filtration and washing with the appropriate buffer. Radioactivity was measured by liquid scintillation counting.

Determination of cellular ATP. The measurements of ATP were made on samples of cell suspensions that were pipetted into 30% perchloric acid as described by Cole et al. (2). After 10 min on ice, the samples were neutralized with KOH. ATP was measured by the luciferin-luciferase assay with a Beckman LS-230 spectrometer, with the coincidence off, as described by Stanley and Williams (36).

Measurements of respiratory rates of whole cells. The uptake of oxygen by cells at various stages of starvation was measured with a Yellow Springs Instruments model 53 Clark-type oxygen monitor, as previously described (25). Both the endogenous rate of oxygen consumption and the rate in the presence of 3.3 mM L-malate were measured and were expressed as nanogram-atoms of oxygen consumed per minute per milligram of cell protein. Protein was determined by the method of Lowry et al. (27) with egg white lysozyme as the standard.

Chemicals. [¹⁴C]methylamine (52.2 mCi/mmol), [1-¹⁴C]AIB (53 mCi/mmol), and [2-¹⁴C]DMO (8.8 mCi/mmol) were purchased from New England Nucle-

ar Corp. [*Phenyl-³H*]TPP⁺ (5,600 mCi/mmol) was obtained from the Nuclear Research Centre-Negev. Ascorbic acid, PMS, gramicidin, valinomycin, and luciferin-firefly tails were from Sigma Chemical Co. All other chemicals were obtained commercially at the highest purity available.

RESULTS

Cell viability. Logarithmic phase cells of *B. firmus* RAB growing on L-malate at pH 10.5 were washed and suspended in buffers containing Na⁺ or K⁺ at pH 10.5, 9.0, or 7.0. Cells incubated in nonnutrient buffer retained greater viability at all three pHs when Na⁺ was present in the buffer (Fig. 1). That the effects were due to the presence of Na⁺ rather than to some inhibitory effect of K⁺ was demonstrated by the use of alternative buffers (data not shown). In the absence of Na⁺, cells lost viability extremely rapidly at pH 10.5 and more slowly at pH 9.0 and 7.0. In the presence of Na⁺, cells retained appreciable viability for 2 h at all three pHs. At pH 7.0, the cells incubated in the presence of Na⁺ remained largely viable during the ensuing 2 h, and the addition of L-malate (either K⁺ or Na⁺ salt) had no effect on the viability (Fig. 1C). At pH 9.0, and even more at pH 10.5, cells incubated in the presence of Na⁺ lost viability between 2 and 4 h of incubation. This loss of viability was probably due to energy depletion, more acutely expressed with increasing pH, since the addition of L-malate at pH 9.0 or 10.5 prevented most of the loss of viability and even

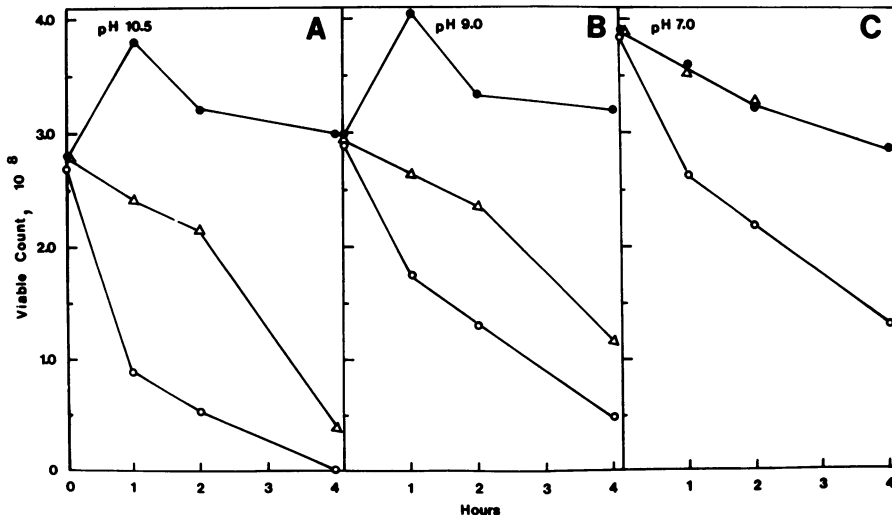


FIG. 1. Viability of *B. firmus* RAB as a function of the pH and Na⁺ content of the incubation medium. Cells grown to the mid-logarithmic phase on L-malate at pH 10.5 were washed and suspended in 50 mM carbonate buffer at pH 10.5 (A) or pH 9.0 (B) or phosphate buffer at pH 7.0 (C). The incubation media contained K⁺ buffer (○), Na⁺ buffer (Δ), or Na⁺ buffer with 10 mM L-malate (●). Cells were incubated at 30°C with shaking; samples were removed for viable cell counts as described in the text.

allowed some transient growth (Fig. 1). Moreover, a series of viability experiments was conducted in which the cells were shifted to buffers containing Na⁺ or K⁺ at the same three pHs in the presence of 50 mM D-glucose, which is transported independently of Na⁺ (A. A. Guffanti, unpublished data). In the presence of both D-glucose and Na⁺, essentially no loss of viability occurred over the incubation period, whereas in the absence of Na⁺, the results with D-glucose were similar to those with L-malate. Thus, in the presence and absence of a utilizable carbon source, cells at pH 10.5 were highly dependent upon the presence of Na⁺ for viability, as anticipated. By contrast, the great viability of cells at pH 7.0, especially in the presence of Na⁺, was surprising. If growth of *B. firmus* RAB at pH 7.0 were precluded by the activity of the Na⁺/H⁺ antiporter, then viability at pH 7.0 should be greater in the absence of Na⁺.

Δp_H⁺ patterns. An analysis of the bioenergetic properties of the cells was undertaken, starting with measurements of ΔpH and ΔΨ, which together constitute the Δp_H⁺. The rapid loss of viability at pH 10.5 in the absence of Na⁺ correlated, as expected, with an extremely fast rise in the cytoplasmic pH. By the time cells had been washed with and suspended in potassium carbonate buffer (zero time), the internal pH was already 10.5, i.e., ΔpH = 0 (Table 1). By contrast, the cytoplasmic pH at zero time was <9.5 in cells incubated with Na⁺ at pH 10.5, and it rose just slightly above 9.5 during the first hour of incubation (Table 1). Cells incubated with either Na⁺ or K⁺ at pH 10.5 exhibited apprecia-

ble and comparable ΔΨ values at zero time. There was essentially no change in ΔΨ after 1 h of incubation in the presence of Na⁺. After 1 h of incubation at pH 10.5 in the absence of Na⁺, the largely nonviable cells exhibited a marked reduction in ΔΨ. The addition of L-malate during the assays of ΔpH and ΔΨ to cells previously incubated with Na⁺ in the absence of an energy source could provide an indication of the capacity of those cells to generate a ΔpH and ΔΨ. The addition of L-malate caused an increase in the magnitude of both the ΔpH and ΔΨ produced at zero time and 60 min of incubation in buffer containing Na⁺ at pH 10.5 (Table 1). Potassium L-malate had no effect on the viability, ΔpH, or ΔΨ of cells in buffer containing K⁺ (data not shown), presumably because L-malate uptake is dependent upon Na⁺ (18, 22). On the other hand, the addition of 10 mM NaCl to the assay mixture for the ΔΨ determinations caused an elevation in the ΔΨ produced by the cells incubated in the presence of buffer containing K⁺ (Table 1, numbers in parentheses).

At pH 9.0 in the presence of Na⁺, the ΔpH and ΔΨ values are similar in orientation and magnitude to those found at pH 10.5, except that the ΔpH was relatively smaller after 60 min of incubation than at zero time (Table 1). The increase in cytoplasmic pH over this period still left the internal pH below 9.0, and pH 9.5 appears to be the upper limit of cytoplasmic pH that is compatible with viability (5, 9). Cells that were suspended at pH 9.0 in the absence of Na⁺ actually exhibited a small ΔpH, outside acid, in the beginning of the incubation period (again

TABLE 1. The Δp_H⁺ patterns of *B. firmus* RAB cells as a function of the pH and Na⁺ content of the incubation medium^a

Cation in buffer	Incubation time (min)	pH 10.5				pH 9.0				pH 7.0			
		58.8 ΔpH		ΔΨ		58.8 ΔpH		ΔΨ		58.8 ΔpH		ΔΨ	
		-Mal ^b	+Mal ^b	-Mal	+Mal	-Mal	+Mal	-Mal	+Mal	-Mal	+Mal	-Mal	+Mal
Na ⁺	0	+65	+127	-162	-179	+57	+74	-144	-174	-40	-42	-41	-50
	60	+51	+86	-157	-170	+8	+35	-136	-159	-5	0	-20	-13
K ⁺	0	0	NT ^c	-152 (-169) ^d	NT	-24	NT	-142 (-145)	NT	-41	NT	0 (-11)	NT
	60	0	NT	-50 (-93)	NT	0	NT	-135 (-139)	NT	-29	NT	0 (-9)	NT

^a Cells were grown to the mid-logarithmic phase on L-malate at pH 10.5. They were then washed with and suspended in 50 mM carbonate buffer (pH 10.5 or 9.0) or phosphate buffer (pH 7.0) prepared with the indicated cation. The cell suspensions were incubated for 60 min at 30°C with aeration. Samples were removed at zero time and after 60 min for determinations of ΔpH and ΔΨ as described in the text. No energy source was included in the incubation buffers. All values are expressed as millivolts and are the average of determinations made on at least three independent experiments.

^b Absence (-Mal) or presence (+Mal) of 10 mM L-malate in assay mixtures for assays of ΔpH and ΔΨ of cells incubated with Na⁺.

^c NT, Not tested.

^d Assays of the ΔΨ of cells incubated with K⁺ were conducted both in the absence (no parentheses) and in the presence (numbers in parentheses) of 10 mM NaCl in the assay mixtures.

resulting in a cytoplasmic pH that did not exceed 9.5). Thus, primary proton pumping in the absence of Na^+/H^+ antiporter activity allowed the formation of a conventionally oriented ΔpH under alkaline conditions in which viability was retained at least briefly. The ΔpH dropped to zero after 60 min of incubation, whereas the substantial $\Delta\Psi$ declined relatively little.

At pH 7.0, the $\Delta\bar{\mu}_{\text{H}^+}$ patterns were very different from those observed at alkaline pH (Table 1). In both the presence and absence of Na^+ , a ΔpH (outside acid) of about two-thirds of a pH unit (equivalent to about -40 mV) was generated. This indicated that the Na^+/H^+ antiporter was inactive or relatively inactive at pH 7.0 whether Na^+ was present or not. The ΔpH declined more in buffer containing Na^+ than it did in buffer containing K^+ during 60 min of incubation. Significantly, no $\Delta\Psi$ was observed at pH 7.0 in cells incubated in the absence of Na^+ unless Na^+ was added to the assay mixture; addition of Na^+ to the assay mixture resulted in the generation of a very small $\Delta\Psi$. The absolute values of $\Delta\Psi$ found in cells incubated in the presence of Na^+ at pH 7.0 (-41 mV at zero time and -20 mV after 60 min) were much lower than at alkaline pH. Notably, the addition of L-malate to the assay mixtures for the ΔpH or $\Delta\Psi$ determinations at pH 7.0 had no effect, just as had been found with viability at this pH. Taken together, the results at pH 7.0 suggest that: (i) the Na^+/H^+ antiporter was not active enough to acidify the cytoplasm at this pH markedly, but it was active enough to account for the Na^+ -dependent elevation of the $\Delta\Psi$ and the slight general reduction in the ΔpH (outside acid); (ii) in the presence of Na^+ the total $\Delta\bar{\mu}_{\text{H}^+}$ produced at this pH was very low, -80 to -90 mV, and consisted almost equally of ΔpH and $\Delta\Psi$, whereas at alkaline pH the total $\Delta\bar{\mu}_{\text{H}^+}$ was slightly higher and had a much higher (fourfold) $\Delta\Psi$ component; and (iii) in the absence of Na^+ no $\Delta\Psi$ was generated, and the total $\Delta\bar{\mu}_{\text{H}^+}$ ($=\Delta\text{pH}$) was a low -41 mV at zero time. The subsequent experiments were conducted to examine and amplify the implications of these findings.

Na^+/H^+ antiport activity. Na^+/H^+ antiport activity was examined with right-side-out vesicles prepared from cells grown in L-malate at pH 10.5. The vesicles were prepared in either potassium carbonate buffer (pH 9.0) or potassium phosphate buffer (pH 7.0). Na^+ -dependent proton movements were followed, upon energization of the vesicles with ascorbate-PMS, by monitoring methylamine uptake (indicative of net proton uptake) and DMO uptake (indicative of net proton extrusion). As reported previously (22), at pH 9.0 energized vesicles took up methylamine (and not DMO) when Na^+ was pre-

sent and took up DMO (and not methylamine) when Na^+ was absent. These data indicate the presence of a Na^+/H^+ antiport activity at pH 9.0 which catalyzes energy-dependent acidification of the intravesicular space only when Na^+ is added. The results at pH 7.0 were quite different (Fig. 2). In the absence of Na^+ , a ΔpH (outside acid) was produced, as shown by the appreciable DMO uptake (Fig. 2, curve A); this had been reported previously (22). Significantly, when Na^+ was present some DMO uptake was still observed (Fig. 2, curve B), correlating with the complete absence of methylamine uptake (Fig. 2, curve C). Thus, as predicted from the findings for the viability and $\Delta\bar{\mu}_{\text{H}^+}$ patterns of whole cells, the Na^+/H^+ antiporter catalyzes energy-dependent acidification of the intravesicular space at alkaline pH but is only slightly active at pH 7.0, effecting a partial reduction in the ΔpH (outside acid).

The activity of the Na^+/H^+ antiporter, although modest at pH 7.0, could account for the small Na^+ -dependent increase in the $\Delta\Psi$ observed at both alkaline and neutral pH, but especially at pH 7.0 (Table 1). We also considered the possibility that some primary Na^+

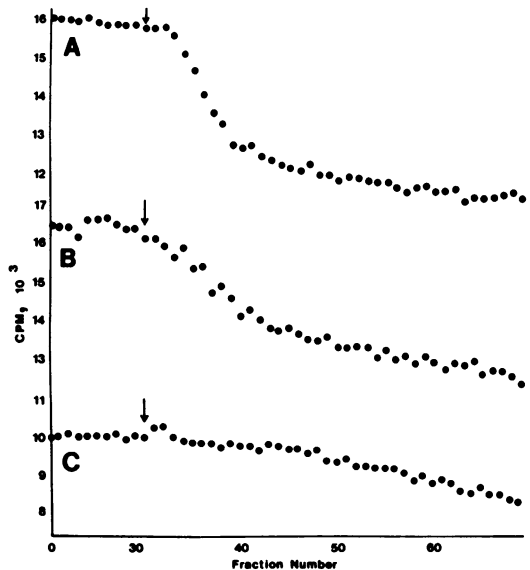


FIG. 2. Proton movements in right-side-out vesicles of *B. firmus* RAB energized at pH 7.0 in the presence or absence of Na^+ . Flow dialysis experiments were conducted as described in the text. The counts flowing out in the dialysate were monitored during the experiments. The energy source, ascorbate-PMS, was added at the points indicated by the arrows. The probes for proton movements were DMO (A and B) and methylamine (C). The cation employed in the dialysis buffer was K^+ (A) or Na^+ (B and C).

pumping mechanism, such as that described by Heefner and Harold (10), might exist. Accordingly, we examined Na⁺-dependent ATP hydrolysis at several pHs. Everted vesicles were incubated with ATP in the presence or absence of Na⁺, and the release of P_i from the ATP was monitored. No Na⁺-dependent ATP hydrolysis was found at pH 8.0 or 9.0, whereas a stimulation of ATP hydrolysis (approximately 60%) by Na⁺ was routinely observed at pH 7.0. It is at least possible, therefore, that some primary Na⁺-dependent pump activity is responsible for part of the pattern of gradients observed at pH 7.0.

Cellular ATP levels. From the results so far, it is clear that the Na⁺/H⁺ antiporter cannot preclude the growth of *B. firmus* RAB by excessive acidification of the cytoplasm at neutral pH. Why, then, does the alkalophile fail to grow at pH 7.0? The total $\Delta\bar{\mu}_{H^+}$ values of neutral and alkaline pH in the presence of Na⁺ are not very different since the reversed ΔpH at high pH reduces the total electrochemical proton gradient, but the low $\Delta\Psi$ values at neutral pH are striking. Processes that depend upon a $\Delta\bar{\mu}_{H^+}$, such as ATP synthesis (7; T. A. Krulwich and A. A. Guffanti, *Adv. Microb., Physiol.*, in press), might function similarly at neutral and alkaline pH, but $\Delta\Psi$ -dependent processes would be specifically compromised at neutral pH. Indeed, measurements of cellular ATP contents showed that ATP levels generally correlated well with the total $\Delta\bar{\mu}_{H^+}$. Cells shifted to buffer containing either Na⁺ or K⁺ in the absence of an added energy source had cellular ATP concentrations between 2.3 and 3.6 mM at zero time at three different pHs (pH 10.5, 9.0, and 7.0). After 60 min of incubation in medium containing Na⁺, the cellular ATP concentration had increased slightly at pH 10.5 and at pH 9.0 but had decreased by about 50% at pH 7.0. Similar correlations with the $\Delta\bar{\mu}_{H^+}$ were seen in buffer containing K⁺, in which the cellular ATP concentration decreased markedly at pH 10.5, increased slightly at pH 9.0, and decreased by about 50% at pH 7.0. Significantly, the cellular ATP levels at pH 7.0 were the same in buffers containing K⁺ and buffers containing Na⁺, and the ATP levels at 60 min (approximately 1.2 mM) were not dramatically low.

$\Delta\Psi$ -dependent solute transport and motility. We therefore returned to a specific consideration of the low $\Delta\Psi$ at neutral pH. Whereas ATP synthesis is $\Delta\bar{\mu}_{H^+}$ dependent, most solute transport systems in alkalophiles are symport systems in which solute uptake is obligatorily coupled to Na⁺ uptake (1, 8, 9, 17–19, 21, 22), bypassing the problem posed by the chemical gradient of protons in which $H^+_{in} > H^+_{out}$. The Na⁺/solute symporters are dependent on Na⁺

and $\Delta\Psi$. The transport of AIB by whole cells, which was completely dependent upon Na⁺ at all the pHs examined, generally correlated with the $\Delta\Psi$ values (Table 2). In these experiments, the initial rates of AIB uptake and the steady-state levels of accumulation were determined in cells that were shifted to buffer containing Na⁺ or K⁺ at pH 10.5, 9.0, or 7.0. Those cells that were incubated in the absence of Na⁺ were assayed in the presence of added Na⁺ since no AIB uptake was observed in the absence of Na⁺. Cells incubated at pH 10.5 or 9.0 with Na⁺ exhibited excellent AIB uptake both at zero time and at 60 min; the addition of L-malate to the assay mixtures increased the rate and level of uptake. Cells incubated at alkaline pH in the absence of Na⁺ (except during the actual uptake assay) exhibited AIB uptake at zero time that was comparable with that found in the presence of Na⁺. After 60 min, very little uptake was observed at pH 10.5, whereas AIB uptake at pH 9.0 was unaffected, except for a somewhat low steady-state level of accumulation. By contrast, at pH 7.0 there was a marked loss of AIB transport. In fact, appreciable transport was only observed in the presence of Na⁺, and these rates and steady-state levels were quite low (Table 2). Two lines of evidence suggest that solute porters remain functional at pH 7.0. First, establishment of an artificial $\Delta\Psi$ by the generation of a valinomycin-induced potassium diffusion potential in starved cells at pH 7.0 resulted in AIB uptake that was much greater than had been observed in the preceding experiments at the same pH (Fig. 3). Second, as had been shown previously, AIB uptake by membrane vesicles, energized with ascorbate-PMS in the presence of Na⁺, was actually somewhat more rapid at pH 7.0 than at pH 9.0 (1, 22).

Another bioenergetic property that, in alkalophiles, depends upon $\Delta\Psi$ and Na⁺ (11) rather than upon $\Delta\bar{\mu}_{H^+}$ (24, 29) is motility. To ascertain whether Na⁺ was also required for the highly motile *B. firmus* RAB, logarithmic-phase cells were washed with 50 mM potassium carbonate buffer (pH 10.5 or 9.0) or potassium phosphate buffer (pH 7.0). At each pH, two suspensions were prepared and aerated, and 10 mM NaCl was added to one of the suspensions. Motility was ascertained immediately by phase-contrast microscopy, and the $\Delta\Psi$ was measured. The $\Delta\Psi$ values at pH 10.5 and 9.0 were substantial (–143 and –135 mV, respectively) and were no more than slightly elevated by the addition of Na⁺. Motility, however, was observed only in the presence of Na⁺. At pH 7.0, neither substantial $\Delta\Psi$ nor motility was observed either in the presence or absence of Na⁺, although we cannot rule out the possibility that the alkalophilic flagellum is simply nonfunctional at neutral pH.

TABLE 2. AIB transport by *B. firmus* RAB cells as a function of the pH and Na⁺ content of the incubation medium

Cation in buffer	Incubation time (min)	AIB uptake ^a											
		pH 10.5				pH 9.0				pH 7.0			
		Initial		Steady state		Initial		Steady state		Initial		Steady state	
		-Mal ^b	+Mal ^b	-Mal	+Mal	-Mal	+Mal	-Mal	+Mal	-Mal	+Mal	-Mal	+Mal
Na ⁺	0	28	57	65	87	28	56	64	84	9	11	19	22
	60	33	51	52	54	29	51	51	60	<9	<9	<9	<9
K ⁺ ^c	0	29	NT ^d	47	NT	30	NT	59	NT	<5	NT	<4	NT
	60	<9	NT	<10	NT	31	NT	20	NT	<5	NT	<4	NT

^a Cells were washed and suspended in buffers containing Na⁺ or K⁺ at the indicated pHs as described in the legend to Fig. 1 and in Table 1, footnote a. No energy source was added to the incubation medium. At zero time and after 60 min, samples were removed and assayed for AIB uptake in reaction mixtures containing 0.5 mM AIB. Both the initial rates (in nanomoles per minute per milligram of protein) and steady state levels (mM) of AIB accumulation were determined. The values are averages of determinations from at least three independent experiments.

^b Absence (-Mal) or presence (+Mal) of 10 mM L-malate in the assay mixture.

^c For assays of AIB uptake by cells incubated in K⁺, 10 mM NaCl was added to the assay mixtures; in the absence of added Na⁺, no uptake was observed.

^d NT, Not tested.

The $\Delta\Psi$ requirement for motility was further examined in experiments with cells that were washed with and suspended in 50 mM potassium carbonate buffer at pH 9.0. Before the addition of 10 mM NaCl, cell suspensions were incubated for 2 min with no addition, 10 μ M valinomycin, or 10 μ M gramicidin. Upon the addition of NaCl, the cells that had been incubated with no additions exhibited motility and a $\Delta\Psi$ of -148 mV. The valinomycin-treated and the gramicidin-treated cells exhibited neither motility nor $\Delta\Psi$.

Respiratory rates. On the basis of the foregoing, the most likely impediment to growth of *B. firmus* RAB at neutral pH was the low $\Delta\Psi$ found at that pH. We wondered whether the low $\Delta\Psi$ values resulted from the depression of respiration. Endogenous respiratory rates were determined for cells that had been washed and incubated in buffer containing Na⁺ or K⁺ at pH 10.5, 9.0, or 7.0. The endogenous respiratory rates of cells incubated in the presence of Na⁺ at zero time were the same at all three pHs. After 60 min of incubation, the respiratory rates had declined considerably, especially at pH 7.0. Cells incubated in buffer containing K⁺ showed respiratory rates at pH 10.5 that were lower by almost 50% at zero time and by almost 100% at 60 min than respiratory rates observed in cells incubated with Na⁺. At pH 9 and 7.0, the cells incubated in buffer containing K⁺ exhibited respiratory rates similar to those observed with cells incubated with Na⁺. Thus, incubation at pH 7.0 did not produce the immediate, dramatic effect on oxygen uptake that was apparent on the $\Delta\Psi$ level; nor was the respiratory rate of cells at pH 7.0 greater in the presence of Na⁺ than in its

absence, although $\Delta\Psi$ and viability were both greater.

DISCUSSION

The effects of incubating obligately alkalophilic *B. firmus* RAB at pH 10.5 in the absence of Na⁺ are consistent with our earlier proposal (5, 22, 23, 28) that a Na⁺/H⁺ antiporter plays a vital role in pH homeostasis at alkaline pH. Indeed, there even appears to be Na⁺-dependent acidification of the cytoplasm at pH 9.0, although the elimination of Na⁺ was less devastating at the less alkaline pH. A similar role for a Na⁺/H⁺ antiporter in pH homeostasis in *Escherichia coli* has been proposed (39). Since the Na⁺/H⁺ antiporter in the alkalophiles is $\Delta\Psi$ -dependent (5, 22, 23, 28), its function should be energy consuming; this may account for the increasingly rapid loss of viability in the absence of a utilizable energy source with increasing pH.

The results at pH 7.0 clearly support the conclusions of Kallas and Castenholz (14, 15) that the failure of alkalophiles to grow at neutral pH cannot be attributed to excessive acidification of the cytoplasm. In the present study, *B. firmus* RAB was quite viable at pH 7.0 in the presence of Na⁺. At pH 7.0 in the presence of Na⁺, both cells and vesicles generated a Δ pH, outside acid. This indicates that the antiporter no longer catalyzed net proton accumulation, although the vesicle studies do suggest a much more modest Na⁺-dependent inward flux of protons. It is unlikely, at least in vesicles, that the apparently modest antiporter activity is completely secondary to a problem with the production of $\Delta\Psi$. The use of ascorbate-PMS allows a

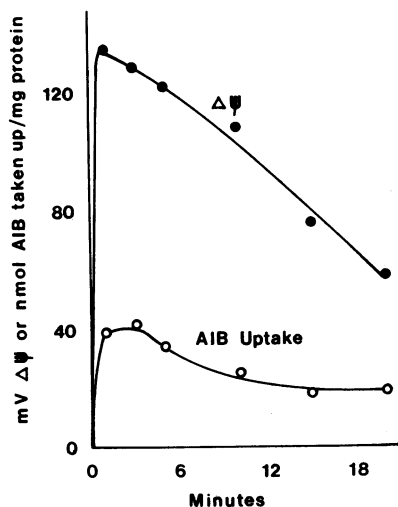


FIG. 3. AIB uptake (○) and $\Delta\Psi$ (●) of cells by *B. firmus* RAB at pH 7.0 upon generation of a valinomycin-induced potassium diffusion potential. Cells of *B. firmus* RAB were starved and then were treated with valinomycin in buffer containing K⁺ as described in the text. Upon a rapid 200-fold dilution of the cells into sodium phosphate buffer, uptake of TPP⁺ and AIB were monitored. Control cells were diluted into buffer containing 50 mM sodium phosphate and 50 mM potassium phosphate; the sodium was included so that AIB uptake would be observed if a $\Delta\Psi$ should be generated. No uptake of AIB or TPP⁺ over time was found in the controls.

sufficient $\Delta\Psi$ to support excellent AIB and L-malate transport by vesicles at pH 7.0 (22).

In whole cells, however, the low $\Delta\Psi$ generated by natural respiratory chain substrates at pH 7.0 seems most likely to account for the failure of alkalophiles to grow at that pH and may underlie their greater viability at pH 7.0 in the presence of Na⁺. The presence of Na⁺ apparently allows somewhat higher $\Delta\Psi$ values to be produced. At pH 7.0, this effect of Na⁺ could reflect the modest activity of the Na⁺/H⁺ antiporter (partially converting the ΔpH to $\Delta\Psi$), or it could relate to the Na⁺-dependent stimulation of ATP hydrolysis, or it could do both these things. It should be noted that the reliability of TPP⁺ as a completely accurate probe of the $\Delta\Psi$ remains, along with all indirect methods, controversial (e.g., 38). However, many validating studies have been reported (e.g., 6, 16, 35), and in the experiment at pH 7.0 (Fig. 3), there was an excellent correspondence between the $\Delta\Psi$ measured with TPP⁺ and the $\Delta\Psi$ that theoretically should have been produced. In any event, the dramatic differences in the present study between the $\Delta\Psi$ at pH 7.0 and at alkaline pH is more important than the absolute values of the difference.

Why would a very low $\Delta\Psi$ preclude growth of

obligate alkalophiles at pH 7.0 when the total $\Delta\bar{\mu}_{H^+}$ is not appreciably lower than at high pH? Unlike conventional bacteria, for which the $\Delta\bar{\mu}_{H^+}$ is a central energetic currency for motility and solute transport as well as for ATP synthesis, the alkalophiles use a sodium motive force for the former two processes (Krulwich and Guffanti, in press). Thus, the presence of a ΔpH (outside acid) would be irrelevant for Na⁺/solute symport systems, which would rely upon the meager $\Delta\Psi$. The failure of L-malate to stimulate transport or to affect the viability or the $\Delta\bar{\mu}_{H^+}$ at pH 7.0 may thus relate to the poor transport of this Na⁺-dependent solute at neutral pH. The consequences of the dependence of cells upon the $\Delta\Psi$ per se are apparently palpable even in nonnutrient buffers since Na⁺ and a somewhat higher $\Delta\Psi$ increased the viability of cells in buffer at pH 7.0. Presumably some maintenance function (perhaps, for example, transport of trace ions from the buffer) is $\Delta\Psi$ dependent. The specific problem encountered by a Na⁺-coupled cell in the face of a low $\Delta\Psi$ provides an explanation for the ability of nonalkalophilic mutants of *B. firmus* RAB (or *B. alcalophilus*) to grow at pH 7.0. Concomitant with the mutational loss of the Na⁺/H⁺ antiporter, such strains change the coupling ion for solute symporters from Na⁺ to protons (1, 8, 22, 23). A similar pleiotropy has been found in *E. coli* mutants (34, 40), and investigators of both systems have proposed that a direct relationship (perhaps, for example, a common Na⁺-translocating subunit) exists between Na⁺-coupled symporters and antiporter (8, 23, 34, 40). If the Na⁺-coupled nature of the alkalophiles, particularly with respect to solute transport, is indeed what precludes growth at pH 7.0, then the ability of the nonalkalophilic mutant to grow at pH 7.0 could be explained by its having become a H⁺-coupled cell which could utilize the entire $\Delta\bar{\mu}_{H^+}$ available at pH 7.0.

Finally, it is of interest that the low $\Delta\Psi$ values generated at pH 7.0 do not reflect a grossly compromised respiratory rate, as reflected by the rate of oxygen consumption by cells using endogenous substrates. Studies of the respiratory chain of *B. alcalophilus* (26) suggest that at pH 7.0, the flow of electrons through all the carriers of the chain might be prevented, or at least altered, because of pH-dependent shifts in the midpoint potentials of only some of the respiratory chain components. We are currently studying the structure and function of the respiratory chain in *B. firmus* RAB. It will be of interest to see whether the properties of the redox carriers again indicate that at pH 7.0 coupling sites might be lost but respiratory rates might remain high. Measurements of H⁺/O ratios are also being attempted at pH 9.0 and pH 7.0 to ascertain directly whether a subtle kind of

respiratory failure accounts for the low $\Delta\Psi$ values at pH 7.0.

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