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Effect of Starvation on Cytoplasmic pH, Proton Motive Force, and Viability of an Acidophilic Bacterium, *Thiobacillus acidophilus*

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The question of whether *Thiobacillus acidophilus* maintains its cytoplasmic pH at values close to neutrality by active or passive means was explored by subjecting the organism to long-term starvation (up to 22 days). Starving cells maintained a ΔpH of 2 to 3 U throughout starvation, although cellular poly-β-hydroxybutyric acid and ATP, the proton motive force, and culture viability were low or not detectable after 200 h. Cells exposed to azide or azide plus *N,N'*-dicyclohexylcarbodiimide immediately exhibited characteristics of cells starved for more than 200 h. Thus, a large ΔpH in *T. acidophilus* was maintained in the absence of ATP, ATPase activity, respiration, significant levels of proton motive force, and cell viability and was therefore not dependent on chemiosmotic ionic pumping. The transition from a metabolically active to an inactive state was accompanied by a large increase in the positive membrane potential, which nearly completely compensated for the ΔpH in the inactive cells. The longevity of the acidophile during starvation was comparable to that reported previously for neutrophiles, and the loss of viability occurred not because of the acidification of the cytoplasm but apparently because of energy depletion.

It is well established that acidophilic bacteria, which grow optimally at a pH of around 3, maintain a near-neutral cytoplasmic pH and a positive membrane potential ($\Delta\psi$) (1, 5–7, 12, 14), but it is not clear whether the ΔpH is maintained actively or passively, and, in fact, the question has engendered some controversy. Krulwich et al. (7), working with *Bacillus acidocaldarious*, found that protonophores and respiratory chain inhibitors stop the proton motive force (Δp)-linked transport functions and that dinitrophenol abolishes ΔpH, and they concluded that the ΔpH in this organism is actively maintained. On the other hand, Hsung and Haug (5) have reported that the ΔpH in *Thermoplasma acidophila* is not affected by treatment with dinitrophenol or azide or even by boiling. They postulated that a passive mechanism is responsible for the maintenance of a ΔpH. Similar results have been obtained with *Thiobacillus ferrooxidans* (1).

We have previously shown that whole cells (10) or spheroplasts (A. Matin and M. K. Matin, J. Gen. Microbiol., in press) of *Thiobacillus acidophilus* in which respiration is inhibited either by an unfavorable external pH or by azide treatment still maintain a large ΔpH. Although this finding strongly suggested that ΔpH in this bacterium is maintained passively, the question was not definitely settled since it remained possible that an H^+ pump energized by cellular ATP continues to function in such cells.

We report here on the effect of starvation on ΔpH and other parameters in *T. acidophilus*. If energy is essential for the maintenance of ΔpH, then this parameter should collapse during starvation. Moreover, we were interested in determining whether, because of the harsh environment in which they thrive, the acidophiles were necessarily more vulnerable to starvation than the neutrophiles. This question is of ecological significance since recurrent starvation conditions are likely to be a fact of life in most natural environments (9).

(A brief preliminary report of this work has been presented [Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, K19, p. 139].)

MATERIALS AND METHODS

T. acidophilus was grown heterotrophically at pH 3.0 in a mineral salts-glucose medium (3, 10). Batch culture- or chemostat-grown cells were harvested by centrifugation (4,100 × *g*, 10 min) and suspended in deionized water at pH 3.0 or in 0.1 M β-alanine buffer at pH 3.0; very similar results were obtained with the two suspending menstruums. The cells were starved at 29°C in an incubator shaker, the evaporation during starvation being compensated for by the addition of sterile distilled water. The pH of the starvation medium increased no more than 0.2 pH units during the course of the starvation when β-alanine was the sus-

pending menstruum; with water as the menstruum, the increase was 0.7 pH unit at day 12 of starvation and 1.0 pH unit at day 22 of starvation.

The starvation mixture was sampled at desired intervals. One portion of the sample was used to determine viability (by plating serial dilutions on 0.025% glucose-mineral salts medium containing 2% agar), cellular ATP levels (by the luciferin-luciferase method recommended by the manufacturer [Lumac, Inc.]), and poly-β-hydroxybutyric acid (by extraction with chloroform), as described previously (9). The second portion was centrifuged and resuspended in 0.1 M β-alanine buffer at pH 3.0 to a standard density; this concentrated suspension was used to measure ΔpH and Δψ. Phase-contrast microscopy showed that the concentrated suspension consisted mainly of intact cells; little, if any, cell debris was detected. Calculations based on the protein content per viable cell showed that the viable-to-nonviable cell ratio in the concentrated suspension was very similar to that in the starvation medium. Thus, there was no enrichment of viable cells in the suspensions actually used in Δψ and ΔpH measurements.

ΔpH and Δψ were measured by the flow dialysis method, as described previously (10), with [^{14}C]aspirin and KS^{14}CN, respectively, as probes; the pH of the reaction medium was 3.0 in all cases. Control experiments, described previously (10), have established that there is no nonspecific binding of either of the probes to the cells. A new control showed that a high concentration of SCN^- (100 μM) led to the collapse of Δψ. Some starvation experiments were repeated with [^{14}C]benzoic acid as a probe for ΔpH determination. The results were very similar to those obtained with [^{14}C]aspirin. Δψ and ΔpH were calculated from the distribution of probe molecules, as described by Ramos et al. (13). Protein was determined by the method of Lowry et al. (8).

RESULTS

The ΔpH, which was −180 mV (3.0 pH units) at the onset of starvation, increased somewhat in the early phase of starvation but then showed a steady decline as starvation continued (Fig. 1). Nevertheless, after 530 h of starvation (ca. 22 days), the cells still possessed ca. 60% of the maximum value of ΔpH observed in this experiment. The positive Δψ increased markedly during the first 200 h of starvation, nearly doubling during this time, but then declined as the starvation continued; at 530 h, the cells possessed a Δψ of +83 mV, which was only slightly higher than the value at the onset of starvation. Since the positive Δψ acts as a counterbalance to the ΔpH, it is instructive to consider the ΔpH-to-Δψ ratio, i.e., the millivolts of ΔpH present across the cells per millivolt of Δψ of opposite polarity. This ratio decreased with time, falling from a value of 2.3 in the nonstarved cells to ca. 1.3 at around 200 h of starvation. The drop in this ratio was paralleled by changes in Δp, which fell from ca. −110 to ca. −30 mV during starvation (Fig. 1).

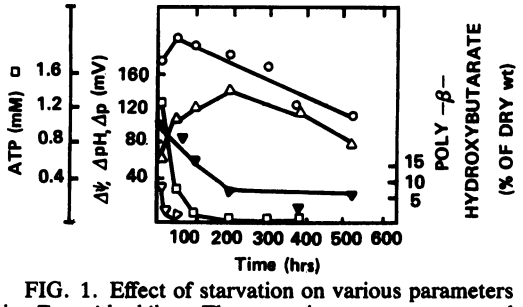

FIG. 1. Effect of starvation on various parameters in *T. acidophilus*. The organism was grown and starved as described in the text. Symbols: ○, ΔpH; △, Δψ; ▼, Δp; □, cellular ATP level; ▽, cellular poly-β-hydroxybutyric acid level. The scale for ΔpH and Δp is in negative millivolts; that for Δψ is in positive millivolts.

Cellular ATP and poly-β-hydroxybutyric acid levels were followed to gauge the energetic state of the cells during starvation. The latter polymer constituted about 10% (wt/wt) of the dry cell biomass of the freshly harvested *T. acidophilus* cells, and it disappeared rapidly during starvation (Fig. 1). Cellular ATP levels also exhibited a rapid, although less precipitous, drop, and by 200 h the nucleotide was no longer detectable. There appeared to be a correlation between the decrease in ATP levels and the drop in Δp (Fig. 1), although the decrease in the latter parameter was less sharp. Similarly, it appeared that an increase in Δψ occurred only as long as ATP was detectable in the cells.

Although a sizable ΔpH was maintained throughout the experiment, the starvation could be divided into two phases with respect to other parameters. The first phase, which lasted for 100 to 200 h, was characterized by detectable levels of cellular ATP, an increasing Δψ, and high values of Δp and the ΔpH-to-Δψ ratio, whereas the second phase, which lasted from 200 h to the end of the experiment, was characterized by the absence of cellular ATP, a gradual decrease in Δψ, and low values of the latter two parameters. It appeared to be possible that the transition from the first to the second phase coincided with the exhaustion of respirable cellular reserves; if so, then the inclusion of a respiratory chain inhibitor in the starvation menstruum should immediately usher the cells into the second phase.

Accordingly, *T. acidophilus* cells were starved exactly as in the previous experiment except that 20 mM azide was present in the starvation medium; we have shown previously (10) that at this concentration, azide abolishes respiration in this bacterium. The results (Fig. 2) were entirely in accord with the hypothesis stated above: within a few minutes of exposure to azide, the cells exhibited characteristics of the

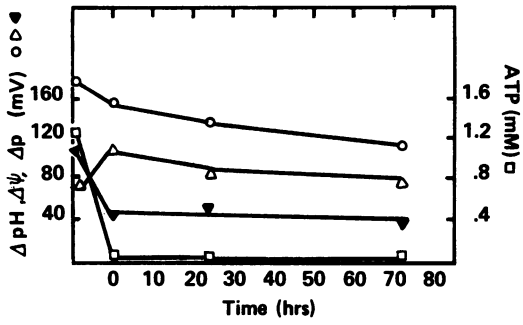

FIG. 2. Effect of incubation in the presence of 20 mM sodium azide on various parameters in *T. acidophilus*. Symbols are as described in the legend to Fig. 1. Values of various parameters before time zero refer to those found in cells before exposure to azide.

second phase of starvation, i.e., no ATP and low values of Δp (ca. −30 mV) and the ΔpH-to-Δψ ratio (not plotted), but yet a sizable ΔpH. The various parameters remained nearly unchanged for the duration of the experiment (72 h). Very similar results were obtained when 10 mM CN^- replaced azide in this experiment.

The results described above suggest that the maintenance of ΔpH in cells in the second phase of starvation (Fig. 1) or in azide-inhibited cells (Fig. 2) is a passive process. The possibility remained, however, that low levels of ATP, not detected by the ATP assay used, played a role in this phenomenon by energizing ATPase-dependent extrusion of protons. We therefore carried out starvation in the presence of azide (20 mM) and *N,N'*-dicyclohexylcarbodiimide (10 μM), which is a known inhibitor of ATPases. Cells starved in this milieu still possessed a ΔpH after 48 h (data not shown); indeed, if anything, cells starved in the presence of azide plus dicyclohexylcarbodiimide possessed a slightly higher ΔpH at 48 h than those shaken in the presence of azide alone (2.4 versus 2.1). This was presumably because dicyclohexylcarbodiimide blocked the passage of protons into the cells through the ATPase.

Since a ΔpH of at least 2 U was maintained during starvation for up to 22 days (Fig. 1), we wondered for how long the culture viability was maintained. In approaching this question, we equated viability with the ability of a cell to form a colony on a 0.025% agar plate after 2 weeks of incubation at 29°C; freshly harvested cells formed colonies under these conditions within 6 days. The cell suspension lost viability with a half-life of ca. 60 h and was essentially completely nonviable by 200 h (Fig. 3). The results suggest that the viability can be maintained only during the first phase of starvation, i.e., when ATP and Δp are present at significant levels, and thus predict that cells suspended in the presence of azide (Fig. 2) would lose viability immediately. This was found to be so (Fig. 3). It is therefore clear that cell viability cannot be maintained in the absence of significant ATP and Δp even though a large ΔpH is present.

DISCUSSION

We have shown here that a ΔpH of 2 to 3 U can be maintained in *T. acidophilus* cells in the absence of ATP, ATPase activity, respiration, and significant levels of Δp, and we therefore conclude that chemiosmotic ionic pumping is not essential for the maintenance of this parameter by the cells. However, our previous work (10), as well as the data presented here, shows that an active metabolic state is necessary to keep the ΔpH uncompensated by an opposing Δψ, thus presumably keeping it available for Δp-requiring functions of the cell. As we have seen, in cells depleted of energy reserves or poisoned by azide, most of the extant ΔpH was compensated by the membrane potential of opposite polarity, with the result that the Δp was maintained at very low values (ca. −30 mV). This residual Δp most likely represented the passive resistance of the cell membrane to H^+ diffusion, as was also suggested by our previous findings (10). Our recent studies show that the active metabolic state is accompanied by an extrusion of H^+ from the cells (E. Zychlinsky and A. Matin, Abstr. Int. Congr. Microbiol., 13th, Boston, 1982, P44:3, p. 126).

It is clear that in the acidophile it is mainly the positive Δψ and not the ΔpH that fluctuates in response to the energized state of the cell. What

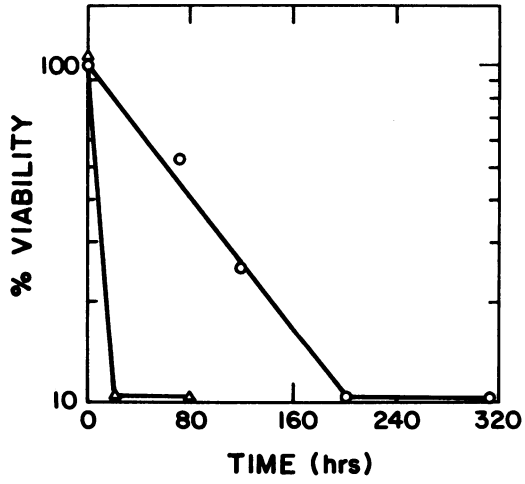

FIG. 3. Effect of starvation on culture viability of *T. acidophilus* starved in the absence (○) or presence (△) of 20 mM sodium azide. The point at time zero corresponds to the viability immediately after the addition of azide.

is the mechanism of this fluctuation? We have suggested previously (10) that the $\Delta\psi$ is generated from both the net diffusion of H^+ and the uptake of cations into the cells, and either or both of these mechanisms could have played a role in the increase in $\Delta\psi$ during the first phase of starvation (Fig. 1). The $\Delta\psi$ increased by over +70 mV during this period without any net change in ΔpH. Thus, if this increase was due to H^+ influx in response to Δp, then it must be assumed that the cell interior contained a significant buffering capacity, and indeed our recent results have revealed a strong pH buffering capacity in *T. acidophilus* cells (E. Zychlinsky and A. Matin, Abstr. Int. Congr. Microbiol., 13th, Boston). On the other hand, if the increase in $\Delta\psi$ involved an uptake of cations or efflux of anions, then it is likely that this ionic movement was energy dependent, since it occurred against the electrical potential and appeared to cease upon the exhaustion of cellular ATP (Fig. 1). Work now in progress is aimed at elucidating the roles of these mechanisms in $\Delta\psi$ generation in *T. acidophilus* during starvation.

It is striking that although the cytoplasmic pH was maintained at physiological values for prolonged periods of time during starvation, cell viability was possible only as long as cellular ATP and Δp values could be maintained at significant levels by the respiration of cellular reserves. The data show that, contrary to the intuitive notion that death during starvation in an acidophile should result from acidification of the cytoplasm, death appears to be caused by the same factor as in neutrophiles, i.e., energy depletion (11).

Vast differences in resistance to starvation stress are exhibited by different neutrophilic bacteria (2), so it is difficult to draw meaningful conclusions as to the relative longevity of acidophiles and neutrophiles during starvation. Nevertheless, it is noteworthy that *T. acidophilus* was more resistant that several neutrophiles. Thus, the two neutrophiles studied previously in our laboratory (9), one of which contained 18% (wt/wt) poly-β-hydroxybutyric acid exhibit a shorter half-life (25 to 50 h) during starvation than *T. acidophilus* (>60 h/ Fig. 3), and, similarly, the neutrophilic *Staphylococcus epidermidis* studied by Horan et al. (4) possesses a half-life of approximately 6 h during starvation. The depletion of cellular energy is also correspondingly faster in the latter organism (4). Thus, acidophiles are not necessarily more vulnerable to starvation than neutrophiles.

We have pointed out previously (10) that the capacity to maintain a positive $\Delta\psi$ for prolonged time periods implies an unusual capacity to hold cellular cations. The results presented here show that this capacity is a completely passive process, since a large $\Delta\psi$ can be maintained in the absence of respiration, cellular ATP and ATPase activity, and, indeed, cell viability.

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