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A Non-Alkalophilic Mutant of Bacillus alcalophilus Lacks the Na^+/H^+ Antiporter

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SUMMARY: A non-alkalophilic mutant strain of Bacillus alcalophilus grows on L-malate over a pH range from 5.0 to 9.0. The mutant does not exhibit the energy-dependent efflux of Na^+ that has been used to assay a Na^+/H^+ antiporter in the wild type organism. The mutant also fails to transport α-aminoisobutyric acid, at pH 9.0, either in the presence or absence of Na^+; at pH 5.5, the amino acid analogue is taken up by a Na^+-independent mechanism. The properties of the mutant constitute strong evidence that the Na^+/H^+ antiporter is involved in maintaining an acidified cytoplasm in B. alcalophilus.

INTRODUCTION: The obligate alkalophile, Bacillus alcalophilus, grows on L-malate in a pH range from 8.5 to 11.5; the cytoplasmic pH is maintained at or below pH 9.5 (1). Recent evidence indicates that acidification of the cytoplasm, or of the intravesicular space of membrane vesicles, depends upon the presence of Na^+ and upon the activity of a high affinity Na^+/H^+ antiporter (2). The antiporter is $\Delta\psi$-dependent and rapidly translocates more protons into the cell or vesicle than are extruded during respiration. It thus seemed possible that the inability of B. alcalophilus to grow at neutral pH might result from a limit of the capacity of the cytoplasm, below pH 8.5, to buffer the protons entering via the Na^+/H^+ antiporter. If so, mutant strains selected for the ability to grow at neutral pH, should fail to grow at highly alkaline pH, and should lack the antiporter. We report here the isolation of a non-alkalophilic strain of B. alcalophilus

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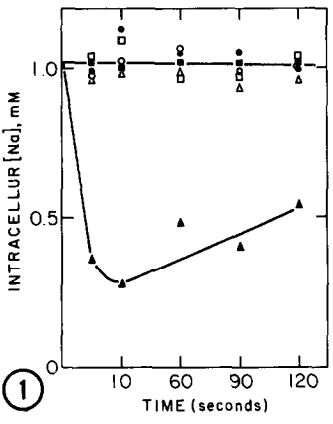
which, indeed, lacks the $\Delta\psi$-dependent Na^+/H^+ antiporter. The mutant strain also fails to exhibit Na^+-dependent uptake of AIB[1].

MATERIALS AND METHODS: Wild type B. alcalophilus (ATCC 27647) was grown on L-malate containing medium, at pH 10.5, as described previously (1). For selection of non-alkalophilic strains, logarithmic phase cells were mutagenized with ethylmethane sulfonate, as described elsewhere (3), grown for one generation time in liquid medium at pH 8.5, and then plated on L-malate-containing medium buffered at pH 6.8 with potassium phosphate and Tris (4). Wild type organisms formed no colonies on such plates even when plated at high concentrations of cells, but colonies were readily obtained after mutagenesis. Over 15 colonies were picked and examined. One of these strains, KM23, will be the subject of this report. Thus far at least six of the other non-alkalophilic strains, isolated from several different mutageneses, exhibit identical properties to KM23.

$^{22}Na^+$ efflux from starved cells was examined essentially by methods used by others working with vesicles (5,6). Cells in the late-logarithmic stage of growth were harvested, washed twice with, and resuspended in, 25 mM potassium phosphate buffer, pH 9.0, to their original density. The suspensions were incubated at 30°C., with shaking, for 3 hours. Starved cells were harvested, and resuspended in 25 mM potassium phosphate buffer, pH 9.0, at approximately 1 mg protein/ml. The suspensions were loaded with 1 mM $^{22}NaCl$ (6,000 CPM/nmol) for 1 hour at 0-4°C. and then warmed to 25°C. Efflux was initiated by addition of 10 mM L-malate. Cells treated with 10 µM valinomycin were preincubated for 10 min. prior to addition of the L-malate. At intervals, 0.2 ml aliquots were filtered (Matheson-Higgens, 0.45 µm filters) and washed with 2 ml of potassium phosphate buffer, pH 9.0. Uptake of AIB (20 µM final concentration) by whole cells was determined in the same buffer, using a filtration assay as described previously (1).

RESULTS: The mutant strain KM23 grew on L-malate in medium buffered with potassium phosphate and Tris buffer (4) over a pH range from 5.0 to 9.0 only, with optimal growth between pH 5.5 and 8.5. As shown in Fig. 1, Na^+ efflux was not observed with KM23 cells at pH 9.0, whereas wild type cells under the same conditions exhibited valinomycin-sensitive efflux of Na^+. KM23 similarly exhibited no Na^+ efflux at pH 8.5 (data not shown). KM23 also failed to take up AIB, either in the presence or absence of Na^+, at pH 8.5 or 9.0. Determinations of AIB uptake by KM23 and the wild type strain at pH 9.0 are shown in Fig. 2. At pH 5.5, KM23 took up AIB, in the absence of Na^+, at a rate of 6.3 nmol/min/mg protein, to a steady-state intracellular concentration that was 19-fold the external concentration. Addition of

[1]Abbreviation: AIB, α-aminoisobutyric acid.



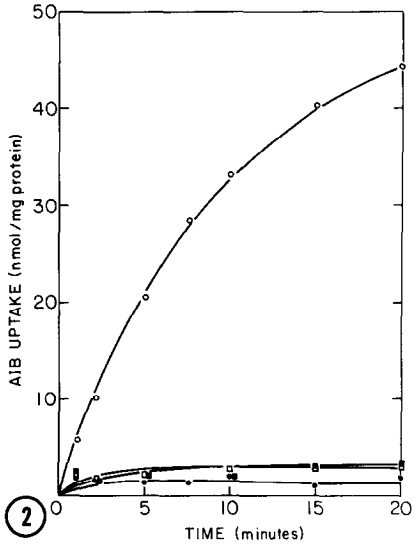


Figure 1. The efflux of ^{22}Na from starved cells of B. alcalophilus and B. alcalophilus KM23. Exponentially-growing cells were washed with 25 mM potassium phosphate, pH 9.0. The organisms were suspended to a final concentration of 0.1 mg protein/ml and starved by shaking for 3 hrs. at 30°C. After starvation, the cell suspensions were concentrated to 1.0 mg protein/ml. B. alcalophilus (closed symbols) and B. alcalophilus KM23 (open symbols) were passively loaded with 1 mM [^{22}Na] Cl by incubation for 1 hr. on ice. After loading with sodium the cells were warmed to 25°C. and separated into aliquots. Cells in one aliquot were heat-killed by placing them in a boiling water bath for 5 minutes (circles), one aliquot was pretreated for 10 minutes at 25°C. with 10 µM valinomycin (squares), and the final aliquot (triangles) was kept at 25°C. for 10 minutes. The reaction was initiated by the addition of 10 mM potassium L-malate, pH 9.0. Samples of 200 µl were removed and rapidly filtered and washed with 2 ml of 25 mM potassium phosphate, pH 9.0. Filtration and washing were achieved within 5 seconds.

Figure 2. The uptake of AIB by B. alcalophilus and B. alcalophilus KM23. Exponentially-growing cells of B. alcalophilus (circles) and B. alcalophilus KM23 (squares) were washed twice with 25 mM potassium phosphate, pH 9.0, and suspended to approximately 0.1 mg protein/ml. Either 10 mM KCl (closed symbols) or 10 mM NaCl (open symbols) was added to the cell suspensions, which were kept aerated by rapid mixing with a magnetic stirring bar at 25°C. Uptake was initiated by the addition of 20 µM [^{14}C] AIB. One ml samples were withdrawn at the times indicated, rapidly filtered and washed with a ten-fold excess of 25 mM potassium phosphate, pH 9.0, containing either 10 mM KCl or NaCl.

various concentrations of NaCl did not affect AIB uptake by the mutant at pH 5.5, but uptake was inhibited completely by 1 µM nigericin and about 30% by 10 µM valinomycin (added 10 min. before AIB).

DISCUSSION: The non-alkalophilic mutant strain of B. alcalophilus has gained the ability to grow at neutral and somewhat acid pHs, and lost the ability to grow above pH 9.0. The finding that this mutant does

not exhibit the electrogenic Na^+-efflux, used previously to monitor the high affinity Na^+/H^+ antiporter of the wild type (2), provides new and rather strong evidence for a role of the antiporter in maintenance of an acidified cytoplasm. While this would be a function critical to life at pH 10.5, growth of the wild type species may still show no apparent requirement for Na^+ because the antiporter functions at Na^+ concentrations which cannot be excluded from the medium. A similar role has been proposed for the Na^+/H^+ (5,7,8) and K^+/H^+ antiporters (9,10) in Escherichia coli, but the evidence vis a vis either antiporter is less clear than in B. alcalophilus.

It is notable that the Na^+/AIB symport, whereby AIB is accumulated by wild type cells (1), is absent in the non-alkalophilic mutant. This absence could be a result of the inability of the cell to extrude any Na^+ brought in via the symporter because of the defect in the Na^+/H^+ antiporter. It is possible, in addition or instead, that Na^+-translocating symporters and antiporters have a common "Na^+ subunit", and that subunit is the defective protein in the mutant. Although the data are not shown, addition of 2 μg/ml of monensin to KM23 cells at a variety of low Na^+ concentrations did not facilitate AIB transport at pH 9.0; thus this agent, which catalyzes an electroneutral Na^+/H^+ exchange, cannot substitute for the antiporter to allow expression of Na^+/AIB symport. We are currently studying a series of revertants of KM23 to further examine the possibility that a "Na^+ subunit" exists. It is interesting that while lacking Na^+/AIB symport, the non-alkalophilic strain exhibits a significant level of Na^+-independent AIB transport at pH 5.5. The marked inhibition by nigericin and lesser inhibition by valinomycin suggest that the transport is energized by the protonmotive force. In E. coli, transport of melibiose has been found to occur by a Na^+-symport mechanism in the presence of the cation and by a H^+-symport mechanism in its absence (11). These kinds of observations may relate to some flexible subunit structure of symport systems.

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REFERENCES:

1. Guffanti, A.A., Susman, P., Blanco, R., and Krulwich, T.A. (1978) J. Biol. Chem. 253, 708-715.
2. Mandel, K.G. and Krulwich, T.A. (1979) Abst. XIth Int. Cong. Biochem., p. 449.
3. Wolfson, P.J. and Krulwich, T.A. (1972) J. Bacteriol. 112, 356-364.
4. Guffanti, A.A., Monti, L.G., Blanco, R., Ozick, D., and Krulwich, T.A. (1979) J. Gen. Microbiol. 112, 161-169.
5. Schuldiner, S. and Fishkes, H. (1978) Biochemistry 17, 706-711.
6. Tokuda, H. and Kaback, H.R. (1975) Biochemistry 16, 2130-2136.
7. Schulachev, V.P. (1978) FEBS Lett. 87, 171-179.
8. Padan, E., Zilberstein, D. and Rottenberg, H. (1976) Eur. J. Biochem. 63, 533-541.
9. Brey, R.N., Beck, J.C. and Rosen, B.P. (1978) Biochem. Biophys. Res. Commun. 83, 1588-1594.
10. Beck, J.C. and Rosen, B.P. (1979) Arch. Biochem. Biophys. 194, 208-214.
11. Tsuchiya, T. and Wilson, T.H. (1978) Membrane Biochem. 2, 63-79.