

# Dependence on $\text{Na}^+$ of control of cytoplasmic pH in a facultative alkalophile

Debbie McLaggan, Michael J. Selwyn, Alan P. Dawson

*School of Biological Sciences, University of East Anglia, Norwich NR4 7TJ, England*

Received 21 September 1983

Regulation of the cytoplasmic pH of *Exiguobacterium aurantiacum* is dependent on the presence of  $\text{Na}^+$  in the medium. The data suggest that above 500  $\mu\text{M}$  external  $\text{Na}^+$  the cells are able to regulate the cytoplasmic pH by the operation of a  $\text{Na}^+$  cycle involving a  $\text{Na}^+/\text{H}^+$  antiport and a route for rapid  $\text{Na}^+$  entry. Our data indicate that the rate of entry of  $\text{Na}^+$  is subject to control by the cytoplasmic pH via feedback inhibition.

|                                    |                |              |                                   |
|------------------------------------|----------------|--------------|-----------------------------------|
| <i>Exiguobacterium aurantiacum</i> | Cytoplasmic pH | Sodium cycle | $\text{Na}^+/\text{H}^+$ antiport |
|                                    | pH homeostasis | pH jump      |                                   |

## 1. INTRODUCTION

It has been proposed that the  $\text{Na}^+/\text{H}^+$  antiport is essential for cytoplasmic pH ( $\text{pH}_i$ ) homeostasis at alkaline external pH values in *Escherichia coli* [1] and in alkalophilic bacteria such as *Bacillus alcalophilus* [2], with the antiporter causing acidification of the cytoplasm by exchange of internal  $\text{Na}$  ions for external protons. Support for this mechanism of  $\text{pH}_i$  homeostasis has come from the study of a mutant of *Bacillus alcalophilus* which cannot grow when the medium pH is above 9.0 and which lacks the  $\text{Na}^+/\text{H}^+$  antiport [3] as does a mutant of *Escherichia coli* which shows impaired growth at alkaline pH [4]. Unfortunately both mutant organisms show pleiotropic impairment of  $\text{Na}^+$ -linked transport processes [5,6] which weakens the evidence for the essentiality of the  $\text{Na}^+/\text{H}^+$  transporter in  $\text{pH}_i$  homeostasis. Also a route of rapid  $\text{Na}^+$  entry is required for continued operation of the  $\text{Na}^+/\text{H}^+$  antiport, the two processes constituting a  $\text{Na}^+$ -cycle across the cell membrane [7] and this implies a requirement for  $\text{Na}^+$  in the medium. Such a  $\text{Na}^+$  requirement has

not been observed in either *B. alcalophilus* or *E. coli* possibly owing to the universal presence of traces of  $\text{Na}^+$  and a high  $\text{Na}^+$  affinity in the carriers but has been demonstrated in an alkalophilic strain of *Bacillus firmus* [8,9]. It has also been pointed out [7] that if  $\text{pH}_i$  homeostasis is to be independent of the external  $\text{Na}^+$  concentration then both the entry pathway for  $\text{Na}^+$  and the  $\text{Na}^+/\text{H}^+$  antiporter must be controlled, although no experimental evidence exists for such control of  $\text{Na}^+$  entry.

This paper describes investigation of the role of  $\text{Na}^+$  in  $\text{pH}_i$  homeostasis in *Exiguobacterium aurantiacum*, a facultative alkalophile [10] which requires  $\text{Na}^+$  for growth at alkaline pH (unpublished). To investigate pH homeostasis and proton fluxes across the bacterial cell membrane the steady-state situation was perturbed by a rapid change in the external pH, and changes in  $\text{pH}_i$  were measured. This method is similar to the pulse-titration [11] or pH jump which has been used in investigations of growth [4] and other pH-dependent activities [12] in *E. coli*. As noted in [12] the change in  $\Delta\text{pH}$  produced by the sudden change

in external pH will cause an instantaneous change in the rate of proton extrusion processes and an opposite change in the rate of proton entry routes. In consequence  $pH_i$  will alter and the various proton transport processes will be regulated to establish a new steady-state  $\Delta pH$  and  $pH_i$ . The data presented here demonstrate the need for  $Na^+$  in the response of *E. aurantiacum* to a pH-jump and that in the presence of a sufficient external  $Na^+$  concentration the organism exhibits rapid and effective  $pH_i$  homeostasis. These data and those for a  $Na^+$ -concentration jump are consistent with the hypothesis of a  $Na^+$ -cycle being responsible for  $pH_i$  homeostasis and indicate control of the cycle by  $pH_i$ .

## 2. MATERIALS AND METHODS

### 2.1. Organism

*Exiguobacterium aurantiacum* gen.nov., sp. nov. (NCIB 11798) [13], previously referred to as BL77/1, was isolated at 20°C from potato-processing effluent [10]. The strain was maintained both as freeze-dried cell suspensions and on slopes of PPYG medium as in [10].

### 2.2. Growth conditions

The organism was grown overnight at 37°C on PPYG medium [10]. The overnight culture was harvested by centrifugation (12000×g, 8°C, 5 min) and washed twice in 50 mM 2-N-cyclohexylaminoethanesulphonic acid (CHES)-KOH (pH 9.0). The washed cells were diluted to an  $A_{680}$  of about 0.3 (an  $A_{680}$  of 2.25 is equivalent to 1 mg bacterial dry wt/ml) with SD-medium and reincubated at 37°C in an orbital shaker (Gallenkamp) at 250 rev./min for 2 h. The SD-medium contained, per l,  $K_2HPO_4$ , 0.5 g;  $MgSO_4 \cdot 7H_2O$ , 0.2 g; glucose, 5 g; yeast extract, 1 g;  $FeSO_4$ , 40 mg;  $CaCl_2 \cdot 6H_2O$ , 60 mg;  $MnSO_4 \cdot 4H_2O$ , 6 mg;  $Na_2MoO_4 \cdot 2H_2O$ , 0.8 mg;  $CuSO_4 \cdot 5H_2O$ , 8 mg;  $CoCl_2$ , 0.8 mg;  $ZnSO_4 \cdot 7H_2O$ , 8 mg; and 50 mM CHES-KOH buffer (pH 9.0). The culture pH was maintained at pH 9.0 by addition of aliquots of 2 N KOH at 30-min intervals. Exponential phase cells were harvested and washed 3 times in 50 mM CHES-KOH buffer (pH 9.0) containing 1 mM  $MgSO_4$  (CHES was purified by re-crystallization of a saturated solution to reduce the content of

contaminating  $Na^+$ ). The final cell suspension was kept on ice until required.

### 2.3. Measurement of $pH_i$ and $\Delta\psi$

$\Delta pH$  and  $\Delta\psi$  were determined using centrifugation-based assays as in [14]. The transmembrane distributions of the weak base [ $^{14}C$ ]methylamine and the lipophilic cation [ $^{14}C$ ]tetraphenylphosphonium ( $TPP^+$ ) were used to measure  $\Delta pH$  and  $\Delta\psi$ , respectively. Cells at 2 mg dry wt/ml were incubated at 25°C in 50 mM CHES-KOH buffer (pH 9.0) in the presence of 10 mM glucose, 1 mM  $(NH_4)_2SO_4$ , [ $^3H$ ]inulin (100  $\mu M$ ; 3.97 Ci/mmol), and either [ $^{14}C$ ]TPP $^+$  (16  $\mu M$ ; 9.4 Ci/mol) or [ $^{14}C$ ]methylamine (22  $\mu M$ ; 4.6 Ci/mol). The intracellular water volume, as determined in [15] was 2.09  $\mu l$ /mg dry wt cells. The concentration of contaminating  $Na^+$  in the incubations was measured using a flame photometer (EEL Evans Electroselection).

### 2.4. Materials

All chemicals were purchased from BDH (AnalaR), except inulin and CHES which were obtained from Sigma, and yeast extract which was obtained from Pifco laboratories. All radiochemicals were purchased from Amersham International. KOH was of aristar grade.

## 3. RESULTS

### 3.1. Effect of $Na^+$ on $pH_i$ during shifts in $pH_e$

Cell suspensions of *E. aurantiacum* maintained an intracellular pH of 8.3 in the absence of added  $Na^+$  at  $pH_e$  8.85. Addition of KOH to increase  $pH_e$  by 0.55 units to  $pH_e$  9.4 provoked an increase of  $pH_i$  of similar magnitude (fig.1). The increase in  $pH_i$  was initially rapid and then slowed until a new steady state was reached 3 min after the change in  $pH_e$ . In the presence of 1 mM  $Na^+$  the change in  $pH_i$  occasioned by the increase in  $pH_e$  was smaller and the new steady state was achieved almost instantly (fig.1). Thus the presence of  $Na^+$  appeared to allow control of  $pH_i$  to a lower value and to accelerate the approach to the new steady state. When cells were exposed to  $Na^+$  addition and a pH jump simultaneously the effect was to reset  $pH_i$  at a lower value identical to that obtained when  $Na^+$  was present throughout (fig.1). This suggests that  $Na^+$  entry is very rapid since the regulation of  $pH_i$

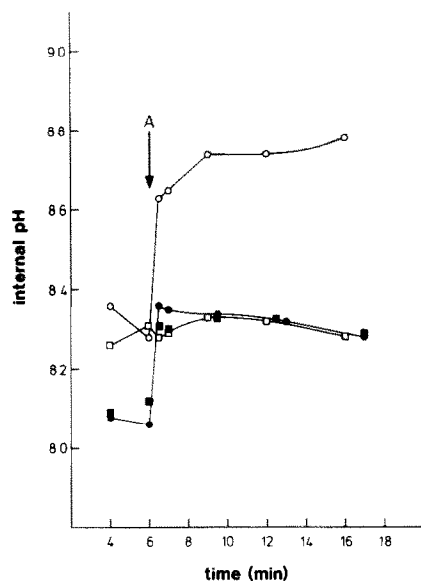


Fig. 1. Effect of  $\text{Na}^+$  on  $\text{pH}_i$  during  $\text{pH}_e$  shift. Cells were incubated at 2 mg dry wt/ml in 50 mM CHES-KOH buffer (pH 8.85) containing 1 mM  $(\text{NH}_4)_2\text{SO}_4$  and 10 mM glucose, in the presence (●, ■) and absence (○, □) of 1 mM NaCl. Samples were taken at intervals and  $\text{pH}_i$  assayed by the transmembrane distribution of [ $^{14}\text{C}$ ]methylamine (22  $\mu\text{M}$ ; 4.6 Ci/mol). (A) The addition of an aliquot (50  $\mu\text{l}$ ) of 2 N KOH (○, ●) or 2 N NaOH (□, ■) which raised  $\text{pH}_e$  to 9.4 and 9.48, respectively. The intracellular pH was again measured at intervals. The external pH was monitored throughout.

requires the lowering of  $\text{pH}_i$  by exchange of internal  $\text{Na}^+$  for external  $\text{H}^+$ .

### 3.2. Effect of jumps in $\text{Na}^+$ concentration on $\text{pH}_i$

Addition of  $\text{Na}^+$  to cells incubated in low- $\text{Na}^+$  medium at high external pH provoked a rapid acidification of the cytoplasm (fig.2). Both the rate and the initial extent of the fall in  $\text{pH}_i$  were dependent on the new external  $\text{Na}^+$  concentration. When addition raised the external  $\text{Na}^+$  to less than 500  $\mu\text{M}$  the final steady state  $\text{pH}_i$  was markedly dependent on  $\text{Na}^+$  concentration; above this concentration the steady state  $\text{pH}_i$  was unaffected by the concentration of  $\text{Na}^+$ . Above 500  $\mu\text{M}$   $\text{Na}^+$ , however, there was a transient over acidification of the cytoplasm, the extent of which was dependent on the  $\text{Na}^+$  concentration (fig.2). Thus above 500  $\mu\text{M}$   $\text{Na}^+$  cells exhibit regulation of  $\text{pH}_i$  which is dependent on  $\text{Na}^+$  flux, but independent of  $\text{Na}^+$  concentration.

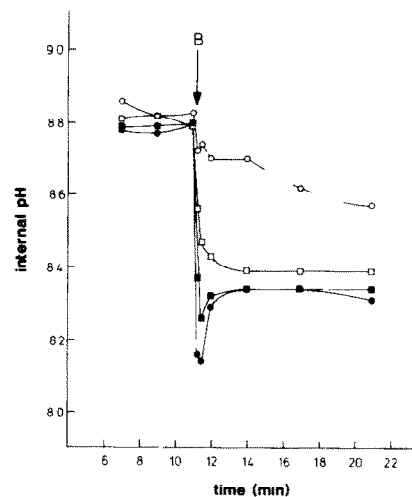


Fig. 2. Effect of  $\text{Na}^+$  concentration shifts on  $\text{pH}_i$ . Cells were incubated in the absence of added  $\text{Na}^+$  at pH 9.0, and KOH was added after 7 min incubation to raise  $\text{pH}_e$  to 9.65 as in fig.1. (B) Addition of  $\text{Na}^+$  (as NaCl): 100  $\mu\text{M}$   $\text{Na}^+$  (○); 500  $\mu\text{M}$   $\text{Na}^+$  (□); 2 mM  $\text{Na}^+$  (■); 10 mM  $\text{Na}^+$  (●).  $\text{pH}_i$  was measured as described for fig.1.

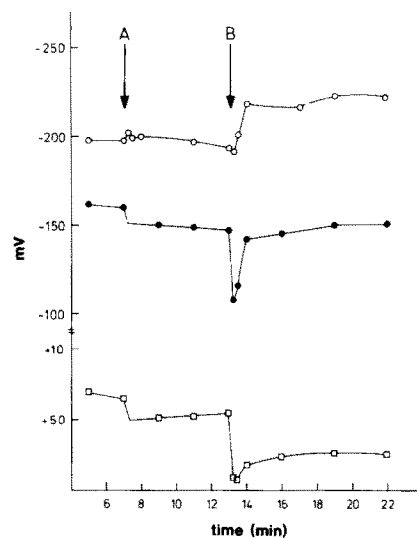


Fig. 3. Effect of  $\text{Na}^+$  on  $\Delta\psi$ . This was a parallel incubation to that described in fig.2 except that the  $\Delta\psi$  was monitored from the transmembrane distribution of [ $^{14}\text{C}$ ]TPP $^+$  (16  $\mu\text{M}$ ; 9.4 Ci/mol) as described in section 2. (A) Addition of 2 N KOH, raising  $\text{pH}_e$  from 9.0 to 9.6. (B) Addition of 10 mM  $\text{Na}^+$  (as NaCl) (○). The results from fig.2 of the effect of 10 mM  $\text{Na}^+$  on  $\text{pH}_i$  are shown in mV (□). The total protonmotive force,  $\Delta p$  ( $\Delta\psi - 59 \text{ pH}$ ) was calculated from the sum of the above measurements (●).

### 3.3. Effect of $\text{Na}^+$ and $\text{pH}_e$ on the membrane potential

The membrane potential ( $\Delta\psi$ ) of cells incubated in the absence of added  $\text{Na}^+$  was not affected by an alkaline pH shift with KOH (fig.3). Subsequent addition of  $\text{Na}^+$  (10 mM) and the consequent reduction of  $\text{pH}_i$  resulted in an increase in  $\Delta\psi$  of 25 mV. This increase in  $\Delta\psi$  followed the restoration of  $\text{pH}_i$  to a more acidic value and compensates for the decrease in the  $\Delta\text{pH}$  component of the protonmotive force. Overall the protonmotive force remained constant (fig.3). A caveat must be placed against these data,  $\text{TPP}^+$  was used as a probe of  $\Delta\psi$  and may be unreliable in some circumstances [16]. Despite this reservation it does appear that  $\Delta\psi$  increases after the  $\text{Na}^+$ -dependent restoration of  $\text{pH}_i$  to a more acidic value.

## 4. DISCUSSION

The results presented here show that external  $\text{Na}^+$  is essential for acidification in  $\text{pH}_i$  homeostasis in *E. aurantiacum* (fig.1,2), these being the first observations which directly demonstrate the effects of  $\text{Na}^+$  on  $\text{pH}_i$  regulation in an alkalophilic bacterium. Furthermore, the time scale of the responses shows that the entry pathway for  $\text{Na}^+$  must allow rapid entry of  $\text{Na}^+$  at a rate comparable to, or greater than the rate of the  $\text{Na}^+/\text{H}^+$  antiport.

Below 500  $\mu\text{M}$  external  $\text{Na}^+$  it appears that the rate of  $\text{Na}^+$  entry is the limiting factor in both the speed of response to a changed  $\text{pH}_e$  and in the maintenance of a steady-state  $\text{pH}_i$  lower than  $\text{pH}_e$  but above 500  $\mu\text{M}$  external  $\text{Na}^+$  the bacterium is able to respond rapidly and maintain effective  $\text{pH}_i$  homeostasis.

The  $\text{Na}^+$ -jump experiments provide further information about the entry pathway and its control. Authors in [7], in discussing the need for control of both halves of the  $\text{Na}^+$  cycle in order to provide  $\text{pH}_i$  homeostasis suggested control of the  $\text{Na}^+$  entry pathway. The overshoot of  $\text{pH}_i$  shown in the  $\text{Na}^+$  jump experiments (fig.2) rules out internal  $\text{Na}^+$  or external pH as the controlling parameter as either would provide instantaneous control of the  $\text{Na}^+$  entry pathway. It would appear that it is the cytoplasmic pH ( $\text{pH}_i$ ) which controls the  $\text{Na}^+$  entry pathway since, on account of the buffer capacity of the cytoplasm and the need for protons to enter

via the  $\text{Na}^+/\text{H}^+$  antiport, adjustment of  $\text{pH}_i$  will lag behind  $\text{Na}^+$  entry and during this lag, excess  $\text{Na}^+$  ions will enter before the  $\text{Na}^+$  entry pathway is shut-off by the lowered  $\text{pH}_i$ . The excess internal  $\text{Na}^+$  causes the overshoot in lowering  $\text{pH}_i$  as it leaves via the  $\text{Na}^+/\text{H}^+$  antiport. Subsequently, the stress of high external pH, as well as respiration and ATPase driven proton jumps will operate to raise  $\text{pH}_i$  as the oscillation settles to the steady-state  $\text{pH}_i$  with the  $\text{Na}^+/\text{H}^+$  antiport also regulated to a low level of activity. Control of both sectors of the  $\text{Na}^+$  cycle not only limits  $\text{Na}^+$  entry and hence maintains the internal  $\text{Na}^+$  concentration at a low level, but will minimize the steady state rate of  $\text{Na}^+$  cycling thus also minimizing the energy requirement for  $\text{pH}_i$  homeostasis.

However, the mechanism thus proposed leaves two problems unanswered, firstly that of overall electrical charge balance and secondly, the source of energy for driving the cycle. Authors in [7] suggested charge balance by anion entry but the alternative of cation efflux is also worth considering. Of the internal cations,  $\text{K}^+$  is the most likely candidate, and if the  $\text{K}^+$  efflux were linked to  $\text{Na}^+$  entry on an antiporter this could provide part of the energy for driving the  $\text{Na}^+$  cycle, alternatively either of the sodium transport steps could be energised by ATP hydrolysis.

## ACKNOWLEDGEMENTS

We wish to thank Dr B.M. Lund for her help and advice and Dr I.R. Booth for his encouragement and valuable discussions during this work. D.M. holds an SERC CASE award.

## REFERENCES

- [1] Padan, E., Zilberstein, D. and Rottenberg, H. (1976) Eur. J. Biochem. 63, 533-541.
- [2] Guffanti, A.A., Susman, P., Blanco, R. and Krulwich, T.A. (1978) J. Biol. Chem. 253, 708-715.
- [3] Krulwich, T.A., Mandel, K.G., Bornstein, R.F. and Guffanti, A.A. (1979) Biochem. Biophys. Res. Commun. 91, 58-62.
- [4] Zilberstein, D., Agmon, V., Schuldiner, S. and Padan, E. (1982) J. Biol. Chem. 257, 3687-3691.
- [5] Zilberstein, D., Ophir, I.J., Padan, E. and Schuldiner, S. (1982) J. Biol. Chem. 257, 3692-3696.

- [6] Guffanti, A.A., Cohn, D.E., Kaback, H.R. and Krulwich, T.A. (1981) *Proc. Natl. Acad. Sci. USA* 78, 1481-1484.
- [7] Booth, I.R. and Kroll, R.G. (1983) *Biochem. Soc. Trans.* 11, 70-72.
- [8] Krulwich, T.A., Guffanti, A.A., Bornstein, R.F. and Hoffstein, J. (1982) *J. Biol. Chem.* 257, 1885-1889.
- [9] Kitada, M.K., Guffanti, A.A., and Krulwich, T.A. (1982) *J. Bacteriol.* 152, 1096-1104.
- [10] Gee, J.M., Lund, B.M., Metcalf, G. and Peel, J.L. (1980) *J. Gen. Microbiol.* 117, 9-17.
- [11] Mitchell, P. and Moyle, J. (1962) *Biochem. J.* 104, 588-600.
- [12] Slonczewski, J.L., Macnab, R.M., Alger, J.R. and Castle, A.M. (1982) *J. Bacteriol.* 152, 384-389.
- [13] Collins, M.D., Lund, B.M., Farrow, J.A.E. and Schleifer, K.H. (1983) *J. Gen. Microbiol.*, in press.
- [14] Kroll, R.G. and Booth, I.R. (1981) *Biochem. J.* 198, 691-698.
- [15] Stock, J.B., Rauch, B. and Roseman, S. (1977) *J. Biol. Chem.* 252, 7850-7861.
- [16] Zaritsky, A., Kihara, M. and Macnab, R.M. (1981) *J. Membr. Biol.* 63, 215-231.