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Sodium/proton antiport is required for growth of *Escherichia coli* at alkaline pH

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Evidence is presented indicating that *Escherichia coli* requires the Na^+/H^+ antiporter and external sodium (or lithium) ion to grow at high pH. Cells were grown in plastic tubes containing medium with a very low Na^+ content (5–15 μM). Normal cells grew at pH 7 or 8 with or without added Na^+, but at pH 8.5 external Na was required for growth. A mutant with low antiporter activity failed to grow at pH 8.5 with or without Na^+. On the other hand, another mutant with elevated antiporter activity grew at a higher pH than normal (pH 9) in the presence of added Na^+ or Li^+. Amiloride, an inhibitor of the antiporter, prevented cells from growing at pH 8.5 (plus Na^+), although it had no effect on growth in media of lower pH values.

Introduction

The Na^+/H^+ antiporter has been found in many microorganisms [1–4]. This membrane carrier appears to serve at least two different physiological functions: (i) to extrude Na^+ that enters the cell [5,6], and (ii) to acidify the cell interior when the external medium is extremely alkaline [3,7,8]. The first function is of particular importance in organisms that are exposed to very high external Na^+ concentrations, such as those found in sea water or environments of even higher salinity. *Halobacterium halobium* [9] and other halophiles [10] possess high levels of Na^+/H^+ antiporter activity and are capable of extruding sodium against considerable concentration gradients [11]. A dramatic example of the effect of Na^+ entry without compensating extrusion may be seen in Mycoplasma (which lack a cell wall). When such cells are suspended in NaCl solutions in the absence of a metabolizable substrate, NaCl and water enter the cell due to Gibbs-Donnan forces and the cells swell and lyse [12]. In the presence of a substrate, these cells use an ATP-driven proton pump to provide the electrochemical proton gradient for energizing the Na^+/H^+ antiporter and Na^+ that enters is extruded from the cells [13].

The role of the Na^+/H^+ antiporter in pH regulation has been extensively studied in alkalophilic bacteria by Krulwich and collaborators [3]. *Bacillus firmus* RAB grows at pH values between 8.5 and 11.5 and requires 25 mM external Na^+ for optimal growth. A mutant that was isolated which was unable to grow at pH values above 8.5 was found to lack Na^+/H^+ antiporter activity [14]. Similar results were obtained for wild-type *Bacillus alcalophilis* and a nonalkalophilic mutant derived from it [15]. These and other results strongly suggest that when alkalophiles grow at alkaline pH, the cytoplasm remains 1–2 pH units lower than the external medium due to proton entry in exchange for internal Na^+ via the Na^+/H^+ antiporter.

The Na^+/H^+ antiporter of *Escherichia coli* has been studied in several laboratories and is thought to be the primary mechanism for Na^+ extrusion [16–20]. Direct measurements have indicated that the internal Na^+ concentration is less than one-tenth the external concentration, at external concentrations of 4 to 300 mM [21,22]. Growth of *E. coli* on substrates that are cotransported with Na^+, such as glutamate, melibiose and proline, requires considerable antiporter activity to extrude the Na^+ that continuously enters the cell with the

Abbreviations: Mops, 4-morpholinepropanesulfonic acid; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; Bicine, *N,N*-bis(2-hydroxyethyl)glycine.

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substrate. Consistent with this view is the observation of Tsuchiya and Takeda that cells growing in the presence of Na^+ on melibiose as a sole source of carbon and energy have levels of Na^+/H^+ antiporter activity much higher than cells growing on other carbon sources that are not cotransported with Na^+ [23]. The same workers isolated mutants which grew on melibiose in the presence of 10 mM Li^+ [24]. Such mutants were found to show elevated levels of Na^+/H^+ antiport, presumably because the entry of Li^+ with melibiose would lead to Li^+ toxicity, unless the cations were extruded from the cell. Li^+ is a substrate of the Na^+/H^+ antiport.

It has been presumed that growth of *E. coli* at alkaline pH (pH 8.5) requires Na^+/H^+ antiporter activity [2]. This view predicts that Na^+ should be essential for growth at pH 8.5. The failure to show this requirement experimentally has raised some doubt concerning the role of the Na^+/H^+ antiporter in pH regulation.

In the present study, it is demonstrated that Na^+/H^+ antiporter activity is required for growth of *E. coli* at pH 8.5 and above. This has been accomplished by providing four independent lines of evidence (i) a strict Na^+ requirement for growth at pH 8.5, (ii) amiloride, a known antagonist of Na^+/H^+ antiporter function, prevents growth at pH 8.5, (iii) a mutant with decreased Na^+/H^+ antiporter activity fails to grow at pH 8.5, even in the presence of Na^+ and (iv) cells with elevated antiport activity grow at elevated pH (9) in the presence of Na^+. These results strongly support the importance of the Na^+/H^+ antiporter in pH regulation in *E. coli*.

Materials and Methods

Bacterial strains

The bacterial strains used in this study are listed in Table I.

Growth media

For cultivation of *E. coli* under conditions where the contribution of exogenous Na^+ was evaluated, special precautions were taken to reduce Na^+ to very low levels. This was made necessary due to the contamination of normal growth media by Na^+ from inorganic salts and glass containers. All incubations were carried out in plastic tubes to avoid leaching of Na^+ from glass. Mops and Tricine were used as primary buffers because they were found to be extremely low in Na^+. For growth media at pH 6, 7 and 8, the buffer comprised 100 mM Mops brought to the appropriate pH by the addition of tetramethylammonium hydroxide. The inorganic constituents of the media were: 10 mM KH_2PO_4/2 mM $(NH_4)_2SO_4$/0.002 mM $FeSO_4$. In growth experiments at pH 8.5 and above there is a danger of loss of NH_3 from the growth medium and therefore an alternative nitrogen source was added in the form of metabolizable amino acids. The following four amino acids were added: aspartic acid (0.02%), serine (0.02%), glycine (0.02%) and proline (0.01%). For growth media at pH 8.5, the buffer comprised 100 mM Bicine brought to the appropriate pH with tetramethylammonium hydroxide. The salts and amino acids given above were included. Glucose was added to a concentration of 0.2%. The concentration of Na^+ in the components of the media was determined to be between 5–15 μM by atomic absorption spectroscopy.

TABLE I

Bacterial strains

Cell	Relevant genotype	Ref.
MC4100	F-*ΔlacU169 araD139 flbB5301 rbsR rpsL thi ptsF25 deoC1*	25
HS3051	MC4100 *Ant*-1 [a]	this paper
W3133-2	F-*lac I*$^+$*ΔZY mel A*$^+$*B*(Tr) [b]	26
W3133-2S	F-*lac I*$^+$*ΔZY mel A*$^+$*B*(Li)^r *ant*-up	24

[a] The gene coding for the Na^+/H^+ antiporter is designated *ant*. Elevated activity of the antiporter is depicted as *ant*-up.

[b] The *melB* gene product is temperature-resistant (stable at 37°C).

[c] A mutation in the *melB* gene that results in a melibiose carrier resistant to Li^+ inhibition.

Assay of Na^+/H^+ antiporter activity

Two methods were used to measure Na^+/H^+ antiporter activity in everted membrane vesicles. In the first method, the ability of the vesicles to accumulate $^{22}Na^+$ was measured. Cells were grown in Medium 63 containing 0.2% (w/v) potassium succinate as the carbon and energy source. The culture was grown until late log phase and the cells were harvested by centrifugation. Membrane vesicles were prepared by the method of Rosen and Tsuchiya [27]. The cell pellet was resuspended in a buffer consisting of 10 mM Tris-HCl (pH 7.2)/140 mM KCl/10% (v/v) glycerol/0.5 mM dithiothreitol. The cells were passed through a French pressure cell at 4000 lb/in^2. Unbroken cells were removed by centrifugation at 3000 × *g* for 20 min and the vesicles were harvested by centrifugation at 100000 × *g* for 1 h. The vesicles were resuspended in the initial buffer by repeated passage through a syringe equipped with a 26-gauge needle. The final protein concentration was adjusted to 5 mg/ml. The assay mixture contained 0.02 ml vesicles, 0.025 ml of buffer consisting of: 10 mM Tris-HCl (pH 7.2)/140 mM KCl/10% (v/v) glycerol/10 mM $MgSO_4$/50 mM potassium succinate. The reaction was initiated by the additon of 0.005 ml of NaCl containing 0.4 μCi $^{22}Na^+$ at a final concentration of 0.1 mM NaCl. At the appropriate time, the reaction was terminated by the addition of 1 ml of buffer containing 0.2 mg/ml poly(L-lysine) and the vesicles were collected by filtration on a Millipore filter disc and washed with 5 ml of the same buffer lacking

poly(L-lysine). The washed filters were dried under a heat lamp and counted in 5 ml of Aquasol.

For the second Na^+/H^+ antiporter assay, the change in intravesicular pH was monitored by measuring the fluorescence of Acridine orange. Bacteria were grown in Medium 63 [28] containing glycerol as the carbon and energy source. After harvesting at late log phase, the cells were converted to everted vesicles using the method of Ambudkar et al. [29]. The fluorescence of Acridine orange was measured as described.

Results

Isolation of a mutant with lower Na^+/H^+ antiporter activity

In order to evaluate the role of the Na^+/H^+ antiporter in pH regulation, we have attempted to isolate a mutant that had lowered Na^+/H^+ antiporter activity without relying on growth at elevated pH as a phenotype. We have reasoned that a mutant that lacked antiport activity should not be able to grow if large amounts of Na^+ entered the cell and could not be extruded. The melibiose permease of *E. coli* transports this sugar either with a proton or with Na^+ as the cosubstrate. Growth on melibiose in the presence of 100 mM NaCl would force the entry of Na^+ into the cell. We have therefore looked for mutants that were unable to grow on melibiose as the sole carbon source in the presence of 100 mm Na^+. Because the melibiose carrier can use H^+ for cotransport we have considered only those mutants that retained the ability to grow on melibiose as the sole carbon source in the absence of added Na^+. This would avoid the consideration of strains with simple null mutations that affect the activity of the melibiose permease itself.

Strain MC4100 was mutagenized with ultraviolet light [30] and the mutagenized culture was enriched for cells that were unable to grow in M63 melibiose medium that contained 100 mM NaCl [31]. After plating the cells on M63 melibiose plates with or without additional NaCl, it was possible to identify the colonies that did not grow on M63 melibiose plates that contained 100 mM NaCl, but which did grow when no Na^+ was added to the medium. One of these colonies, HS3051, was studied in detail. This strain is able to grow at the same rate on M63 glucose medium either with or without 100 mM NaCl (data not shown). It grows poorly on solid rich medium (e.g., LB) which contains 5–10 mM Na^+.

In order to determine directly the level of Na^+/H^+ antiporter activity in strain HS3051, we prepared everted membrane vesicles and assayed antiporter activity using two assays: one based on the ability of the vesicles to accumulate $^{22}Na^+$ at the expense of the electrochemical proton gradient, and the other based on the Na^+-dependent alkalinization of the vesicles. These results are shown in Fig. 1 and 2. Vesicles prepared from HS3051

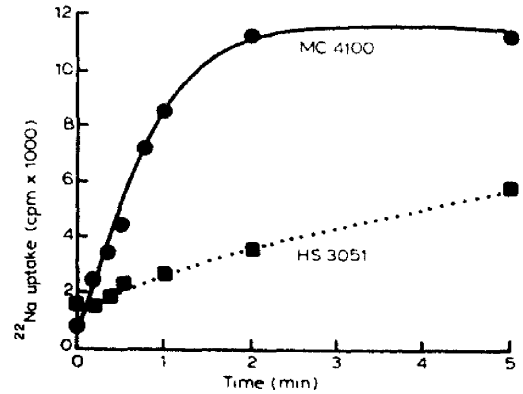

Fig. 1. Uptake of $^{22}Na^+$ by inside-out vesicles in parental strain (MC4100) and mutant (HS3051). The energy source was potassium succinate (25 mM).

grown in minimal medium show a clear defect in Na^+/H^+ antiporter activity in both assays. We conclude that strain HS3051 has a mutation in a gene that controls the activity of the Na^+/H^+ antiporter. Preliminary mapping results indicate that the genetic defect in strain HS3051 is located near the 0–100 min portion of the *E. coli* chromosome (near the arabinose operon). When HfrH was mated with HS3051 and Ara^+ recombinants were selected, more than 80% were able to grow on melibiose in the presence of 100 mM NaCl.

The effect of Na^+ on growth of cells at pH 7 and 8.6

In order to evaluate whether Na^+ is required for growth at alkaline pH, it was necessary to formulate

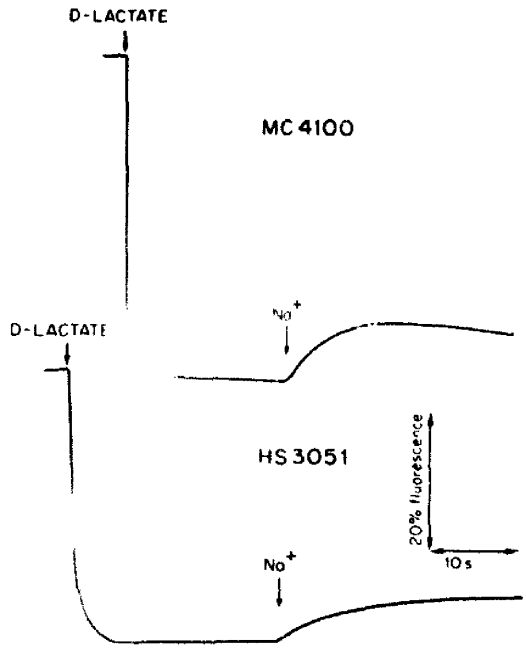

Fig. 2. Na^+-driven alkalinization of vesicles: Acridine orange fluorescence in inside-out vesicles was measured as described in Materials and Methods.

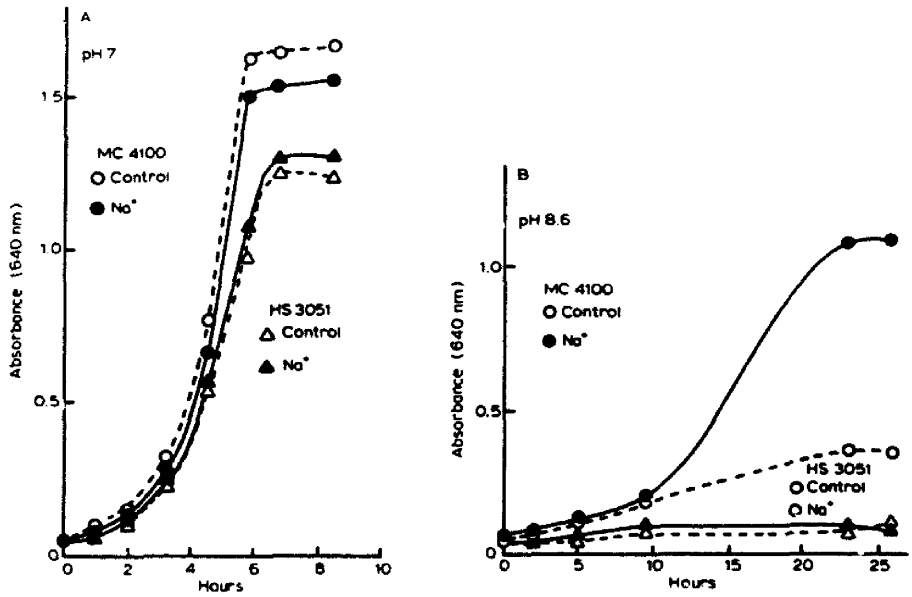

Fig. 3. Effect of added Na^+ on the growth of cells at pH 7 (A) and 8.6 (B). Cells previously grown at pH 7 in low-Na^+ media with glucose as a carbon source were used as the inoculum. Cells were grown in plastic flasks in low-Na^+ media (as described in Materials and Methods). 1 ml aliquots were removed at the times indicated and the optical absorbance at 640 nm was measured.

growth media that contained minimal amounts of Na^+. Most media contain significant amounts of Na^+ which are derived from chemical contamination of inorganic salts and leaching from glass. The low-sodium medium described in Materials and Methods was found to contain only 5–15 μM by atomic adsorption spectroscopy. The wild-type strain MC4100 was grown in this medium at varying pH values with or without added Na^+. At pH 7, the parent (MC4100) and the mutant (HS3051) grew at similar rates and added Na^+ had no effect (Fig. 3A). At pH 8.6, the mutant failed to grow with or without Na^+ (Fig. 3B). The growth of the parent at pH 8.6 was stimulated by the addition of 10 mM Na^+. In addition, we found that Li^+ could substitute for Na^+ without any decrease in the growth yield (data not shown). These results are consistent with the view that growth at an alkaline pH requires the outward movement of Na^+ (or Li^+) through the Na^+/H^+ antiporter.

If the Na^+/H^+ antiporter were required for growth at high pH, one might predict that a cell with elevated levels of the carrier would grow in media of higher pH values, due to enhanced ability to pump protons into the cell. This prediction was borne out by the experiment shown in Fig. 4. A strain (W3133-2S) which shows

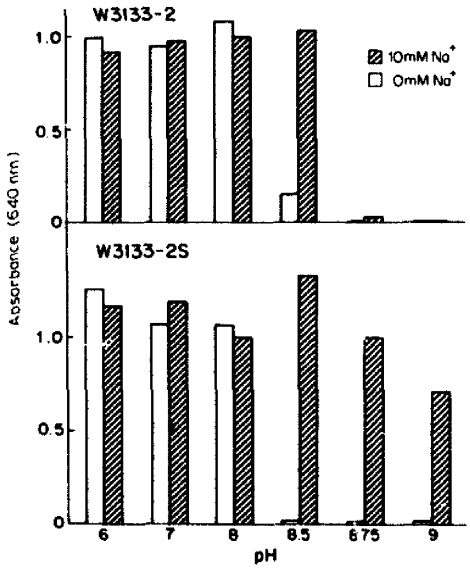

Fig. 4. The growth of normal cells (W3133-2) and cells with elevated antiporter (W3133-2S) at different pH values with or without Na^+. Cells were grown in 0.2% glucose using the low-Na^+ media described in the Materials and Methods. The optical absorbance of the cultures was measured at 640 nm after 24 h growth.

TABLE II

Effect of Na^+ concentration on growth of cells at pH 8.5

Cells were grown for 48 h in the low-Na^+ medium containing 0.2% glucose.

Na^+ concn.	Growth (A_{640})	
	W3133-2 (parent)	W3133-2S (elevated antiporter)
0	0.009	0.008
50 μM	0.008	1.224
100 μM	1.008	1.167
1 mM	1.082	1.099
10 mM	1.056	1.189

TABLE III

Effect of amiloride on growth at pH 7 and 8.5

Cells were grown for 17 h in the low-Na^+ medium containing 0.2% glucose.

Amiloride concn. (mM)	pH	Na^+ at 10 mM	Growth (A_{640})
0	7	–	1.216
0.125	7	–	1.415
0.250	7	–	1.313
0.500	7	–	1.246
1.000	7	–	0.250
0	8.5	–	0.041
0	8.5	+	0.990
0.125	8.5	+	1.002
0.250	8.5	+	0.910
0.500	8.5	+	0.038
1.000	8.5	+	0.027

high levels of antiporter [24] was capable of growing at pH 9.0 in the presence of Na^+. The concentration of Na^+ required by these two strains to permit growth at pH 8.5 was next investigated. Strain W3133-2 required 100 μM Na^+ or more to grow at the alkaline pH (Table II). On the other hand, the strain with elevated antiporter (W3133-2S) required only 50 μm Na^+ for growth at pH 8.5. This phenomenon is similar to that observed by Krulwich et al. [32] in a mutant of *B. firmus* which showed elevated levels of antiporter and a reduced Na^+ requirement for growth at alkaline pH.

Amiloride, an antagonist of Na^+/H^+ antiporter inhibits growth at pH 8.5

In order to carry out an independent test of the idea that the Na^+/H^+ antiporter is required for growth at alkaline pH, we took advantage of amiloride, a known antagonist of Na^+/H^+ antiporter activity [33]. If Na^+/H^+ antiporter activity were required for growth at alkaline pH but not at neutral pH, one would expect that amiloride would prevent growth at alkaline pH but have little or no effect at neutral pH. Table III shows the effect of amiloride on the growth of MC4100 at pH 7 and 8.5. Growth of the bacteria at pH 7 is only partially inhibited by amiloride at the highest concentration tested (1 mM), whereas, at pH 8.5, the growth inhibition is much more severe at both 1 mM and 0.5 mM amiloride.

Discussion

The ability to regulate the pH inside the cytoplasm has been studied in many different types of cell. In animal cells and in alkalophilic bacteria there is a convincing body of evidence that the Na^+/H^+ antiporter is required for pH regulation. In *E. coli*, the involvement of the Na^+/H^+ antiporter in pH regulation has been suggested by several workers [2,7,34] and there have been reports of mutants with decreased antiporter activity that do not grow at alkaline pH [17,35]. In one case, the mutation was mapped to the *rpoA* gene which encode the α-subunit of RNA polymerase; its relation to Na^+/H^+ antiport activity is unclear [34]. In another report, Ishikawa et al. [35] have reported the isolation of a mutant with properties similar to those of the HS 3051 mutant described in this study.

The proposed role of the Na^+/H^+ antiport in pH regulation predicts that the presence of Na^+ would be essential for growth of cells at high pH. Data in this paper provide experimental evidence to support this prediction. *E. coli* are able to grow at pH 8.5 only in the presence of added Na^+. The concentration of Na^+ required for growth of normal cells at pH 8.5 was found to be 100 μM while only 50 μM Na^+ was required for growth of the Tsuchiya mutant with elevated antiport activity. The factor that limits growth at high pH is apparently the antiporter, since a cell with elevated activity of this carrier (in the presence of Na^+) is capable of growth at a higher pH (9) than that of normal cells. Conversely, the inhibition of the carrier by amiloride or by mutation prevents the cell from growth at high pH with or without Na^+.

Recently, a DNA fragment has been identified by Goldberg et al. [36] that increases the level of Na^+/H^+ antiporter activity when it is present on a high-copy plasmid. In addition, it allows cells to grow in the presence of 100 mM Li^+ (presumably by using the Na^+/H^+ antiporter to pump out the toxic Li^+ cation which leaks into the cell). This gene has been referred to as the *ant* gene. Karpel et al. [37] have recently sequenced the *ant* gene which is presumed to code for the Na^+/H^+ antiporter or one of its subunits. This gene maps to the same general location of the chromosome as the mutation in HS3051. It will be interesting to compare the phenotypes of strains that contain null mutations in the *ant* gene with that of strain HS3051.

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