Effect of Ca^{2+} and K^{+} on the intracellular pH of an *Escherichia coli* L-form

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The L-form NC7, derived from *Escherichia coli* K12, grew in a complex medium containing 0.2 M- CaCl_2 as osmotic stabilizer, but not at pH values above 7.8. The cessation of growth at alkaline pH was not due to cell death. In complex media containing K^{+} or Na^{+} , the L-form grew over a wide pH range. Growth at alkaline pH was inhibited by 1 mM-amiloride, indicating that $\text{Na}^{+}/\text{H}^{+}$ antiport activity was required for growth at alkaline pH. The internal pH (pH_i) of the L-form in media containing K^{+} , Na^{+} or Ca^{2+} was constant at about 7.8 to 8.0 at external pH (pH_o) values of 7.2 and 8.2. The rates of O_2 consumption by intact cells, lactate oxidation by membrane vesicles from cells grown in Ca^{2+} -containing medium, and cell division were all strongly repressed under alkaline conditions.

Introduction

In general, bacterial cells maintain an internal osmolarity higher than that of the external medium, resulting in turgor pressure which is necessary for cell growth (Epstein & Laimins, 1980). Bacterial L-forms are osmotically fragile, requiring salts such as KCl and NaCl, or carbohydrates such as sucrose, as osmotic stabilizers to survive (King, 1986). In hypertonic environments, ionic pumps are generally thought to extrude salts and water to regulate cell volume and maintain an almost constant intracellular pH (pH_i) (Padan *et al.*, 1981). *Escherichia coli* has three distinct antiport systems that function to extrude cations from the cytosol, namely a $\text{Ca}^{2+}/\text{H}^{+}$ system (Brey *et al.*, 1978; Brey & Rosen, 1979; Rosen, 1987), a $\text{Na}^{+}/\text{H}^{+}$ system (Schuldiner & Fishkes, 1978; Beck & Rosen, 1979; Zilberstein *et al.*, 1982) and a $\text{K}^{+}/\text{H}^{+}$ system (Brey *et al.*, 1980). Although a $\text{Na}^{+}/\text{H}^{+}$ antiporter is thought to be involved in the regulation of pH_i when the external pH (pH_o) is alkaline, it remains unclear whether other

antiport systems are involved in the regulation of pH_i , and how pH_i is regulated in *E. coli*. The L-form NC7, derived from *E. coli* K12, also has three distinct antiport systems ($\text{Na}^{+}/\text{H}^{+}$, $\text{K}^{+}/\text{H}^{+}$ and $\text{Ca}^{2+}/\text{H}^{+}$ antiporters) (Onoda *et al.*, 1989). Recently, we found that the L-form NC7 can grow in the presence of high Ca^{2+} concentrations at neutral pH_o , but not at alkaline pH_o . This paper examines pH_i homeostasis and cation antiport activity in the L-form NC7 at neutral and alkaline pH.

Methods

Organisms and growth conditions. The parent, *Escherichia coli* K12 strain 3301, and the L-form NC7 (Onoda, 1986) derived from it were used. Both strains were grown at 32 °C without shaking in a complex medium (PY) which contained (l^{-1}): 10 g peptone, 5 g yeast extract and 2 g glucose. In addition, KPY, CaPY and NaPY media contained 0.34 M-KCl, 0.2 M- CaCl_2 and 0.34 M-NaCl, respectively. The pH_o in NaPY and KPY media was adjusted to 7.2 with NaOH or KOH. In some experiments, Tris/HCl was used to adjust pH_o . The pH_o in CaPY medium was adjusted to 7.2 or 8.2 with Tris/HCl. Cells were harvested in the exponential phase of growth by centrifugation (2000 g, 15 min), washed once with growth medium supplemented with appropriate osmotic stabilizer, and inoculated into 100 ml of fresh medium in 300 ml flasks. Growth was monitored by measuring the optical density at 600 nm (1 cm path length). The initial OD_{600} was about 0.02. The concentrations of contaminating Na^{+} , K^{+} and Ca^{2+} in PY medium were determined using an Hitachi model 170-40 flame photometer and

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Abbreviations: $\Delta\psi$, membrane potential; ΔpH , transmembrane pH difference; $\Delta\mu\text{H}^{+}$, electrochemical proton gradient; pH_i , internal pH; pH_o , external pH; DMO, 5,5'-dimethyl-2,4-oxazolidinedione; MA, methylamine; TPP, tetraphenylphosphonium; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

were found to be approximately 9.2 mM-Na⁺, 6.5 mM-K⁺ and 0.15 mM Ca²⁺.

Preparation of everted membrane vesicles. The L-forms were grown to stationary phase in KPY, NaPY or CaPY medium, harvested, washed twice and suspended in a buffer containing 10 mM-Tris/HCl, pH 7.2, 140 mM-choline chloride, 5 mM-dithiothreitol and 10% (v/v) glycerol. In other experiments, 0.5 M-choline chloride was used instead of 10% (v/v) glycerol. Everted membrane vesicles were prepared by lysing the cells in a French press. The suspension was centrifuged for 5 min at 12000 *g* to remove unbroken cells and the supernatant solution was centrifuged at 541 000 *g* for 30 min. The pelleted membrane vesicles were suspended in the same buffer.

Fluorescence assay. Changes in Δ pH, the transmembrane pH difference, were estimated from the energy-linked quenching of quinacrine fluorescence. Assays were done in 10 mM-Tris/HCl, pH 7.2 or 8.0, containing 1 μ M-quinacrine, 140 mM-choline chloride, 5 mM-MgCl₂, 0.5 mM-dithiothreitol and 100 to 400 mg ml⁻¹ of membrane protein in a final volume of 2 ml. The salts used were 140 mM-KCl and 140 mM- or 500 mM-choline chloride. On addition of 0.25 mM-CCCP or 0.25 μ M-nigericin, the fluorescence intensity rapidly recovered due to the dissipation of Δ pH. In all experiments, fluorescence was measured using an Hitachi model 850 fluorescence spectrophotometer. For quinacrine excitation was at 420 nm and emission was measured at 500 nm. In other experiments, the steady-state pH_i of the L-forms, equilibrated with either CaPY or KPY medium of pH 7.2 or 8.2, was determined using 9-aminoacridine and the null-point method (Rink *et al.*, 1982) with butanol. Changes in pH_i were monitored by continuous recording of 9-aminoacridine fluorescence with excitation and emission wave lengths of 400 nm and 454 nm, respectively. The cuvette was filled with 2 ml of test solution together with 10 μ M-9-aminoacridine. Cells were pre-incubated in CaPY or NaPY media for 120 min at 32 °C, centrifuged, resuspended in test medium and transferred into a cuvette.

Measurement of $\Delta\psi$ and pH_i. pH_i and the membrane potential, $\Delta\psi$, were estimated from the distribution across the cell membrane of [¹⁴C]DMO, [¹⁴C]MA or [³H]TPP. Cells were grown overnight in CaPY medium, and suspended in CaPY media adjusted to pH 7.2 or 8.2 with Tris/HCl. One ml of a cell suspension (0.1 to 0.2 mg of cell protein) was transferred into tubes containing 2 ml of CaPY medium at the same pH value and 17 μ M-[¹⁴C]MA (35 Ci mol⁻¹) or 17 μ M-[¹⁴C]DMO (35 Ci mol⁻¹) for pH_i measurements, or 5 μ M-[³H]TPP (157 Ci mol⁻¹) for $\Delta\psi$ measurements (1 Ci = 37 GBq). Cells grown in KPY or NaPY medium were also used in experiments for $\Delta\psi$ and pH_i measurements. The assays were the same as those for measurements of $\Delta\psi$ and pH_i in Ca-grown cells, except that the pH values of the KPY and NaPY media were adjusted with KOH and NaOH, respectively. The reaction mixture was incubated for 1 min and filtered through a polycarbonate membrane filter (Jayakumar & Barnes, 1983). The filters were transferred into toluene/Triton scintillation liquid and radioactivity was measured in an Aloka scintillation counter. To calculate the concentration of isotopes within the cells, the intracellular space [10 μ l (mg protein)⁻¹] was estimated by determining the distribution volume of [¹⁴C]inulin (87.6 μ M, 11.4 μ Ci μ mol⁻¹) and ³H₂O (12 μ Ci, 5 mCi ml⁻¹) (Rottenberg, 1979).

Protein determination. Protein was determined by using the Bradford protein assay (Bio-Rad). Bovine albumin was used as a standard.

Chemicals. Nigericin and CCCP were supplied by Sigma. EGTA, quinacrine and 9-aminoacridine were from Nacalai tesqu. [¹⁴C]MA, [¹⁴C]DMO and [¹⁴C]inulin carboxyl were products of American Radio Labeled Chemicals. [³H]TPP and ³H₂O were supplied by Amersham. All other chemicals were reagent grade and obtained from commercial sources.

Results

Effects of calcium on growth

Previous work demonstrated that growth of the L-form NC7 in NaPY or KPY medium was calcium-dependent (Onoda & Oshima, 1988), with optimal growth at 0.2 mM-Ca²⁺ in KPY and 1.0 mM-Ca²⁺ in NaPY medium, respectively. In this study, the L-form was able to grow at high CaCl₂ concentrations. The optimum concentration of calcium for growth in PY medium was between 0.1 and 0.2 M and growth was markedly reduced at concentrations above 0.3 M (Fig. 1). In PY medium containing relatively low concentrations of CaCl₂ (0.15 to 0.2 mM), growth occurred only in the presence of 0.2 M-sucrose, implying that the osmolality of the medium was insufficient to osmotically protect the L-form. The relationship between OD₆₀₀ and the amount of cell protein is shown in Fig. 1. No significant growth was observed over a wide range of Mg²⁺ concentrations in the presence or absence of 0.2 M sucrose (Fig. 1). The effects of a combination of Mg²⁺ and Ca²⁺ on growth of the L-form were also examined. In this case, because the MgPY medium used in this experiment contained about 0.2 mM contaminating Ca²⁺, external calcium was removed by chelating with 0.2 mM-EGTA. Addition of 0.2 mM-Ca²⁺ to MgPY medium resulted in stimulation of growth, but in the absence of Ca²⁺ no growth was observed. These results imply that Ca²⁺ plays an important physiological role other than as an osmotic stabilizer.

Effects of pH_o on growth

In KPY medium, the L-form grew over the pH range 6.0 to 9.0 (Fig. 2a), and acidified the medium during growth (Fig. 2b). Similar results were also obtained in NaPY medium containing 1.0 mM-Ca²⁺ (data not shown). In CaPY medium the L-form grew well at a pH_o of 7.8 or less and caused acidification of the medium. However, it did not grow above pH 7.8 and acidification of the medium was not observed.

Effect of amiloride on cell growth in KPY and NaPY media

Amiloride is a potent inhibitor of the Na⁺/H⁺ antiporter (Mochizuki-Oda & Oosawa, 1985). When the L-form was incubated in KPY or NaPY medium over the pH range 6.5 to 8.6, growth was inhibited by amiloride at alkaline pH (Fig. 3). This result indicated that Na⁺/H⁺ antiport activity plays an important role in growth of the L-form at alkaline pH in KPY and NaPY media. In *E. coli*, the Na⁺/H⁺ antiporter and external Na⁺ are

Fig. 1. Effect of salt concentrations on growth. Cells were grown at 32 °C in NaPY medium containing Ca^{2+} or Mg^{2+} at various concentrations. Sucrose was added to the cultures at a final concentration of 0.2 M. All PY media were treated with 0.2 mM-EGTA to remove contaminating Ca^{2+} and salts of K^+ , Na^+ , Ca^{2+} and Mg^{2+} were added as osmotic stabilizers. The OD_{600} was measured after 48 h incubation. Cells were grown in PY medium containing CaCl_2 at various concentrations (●), CaCl_2 at various concentrations plus 0.2 M-sucrose (○), MgCl_2 at various concentrations (■) and MgCl_2 at various concentrations plus 0.2 M-sucrose (□). △, Protein content ml^{-1} in PY medium containing CaCl_2 at various concentrations.

Fig. 2. Effect of pH on cell growth. Cells were cultured in media with the indicated initial pH values at 32 °C, and the OD_{600} was measured after 48 h incubation (b) in KPY medium (●) or CaPY medium (○). The pH of the media was measured at the end of the growth experiments (a) for KPY medium (▲) and CaPY medium (△).

Fig. 3. Effect of amiloride on cell growth. Cells were cultured in NaPY and KPY media at the indicated initial pH values at 32 °C and the OD_{600} was measured after 48 h incubation in NaPY (b) and KPY (d) media with (●) and without (○) 1 mM-amiloride. Amiloride was added to NaPY and KPY media at zero time and 24 h after incubation, respectively. The pH of the media was measured at the end of the growth experiments in NaPY (a) and KPY (c) media with (▲) and without (△) amiloride. CaCl_2 (1 mM) was added to all NaPY media.

required for growth at alkaline pH (McMorrow *et al.*, 1989), so NaCl was added to CaPY medium at pH 8.0 at a final concentration of 50 or 100 mM. At a pH_0 of 8.0 in CaPY medium, no growth was observed in the presence of 50 or 100 mM-NaCl. Similar results were obtained when KCl was added at the same concentrations as NaCl.

Oxygen consumption at pH_0 7.2 and 8.2

Growth of the L-form, was inhibited completely by the presence of a protonophore (10 μM -CCCP) in KPY and NaPY media (Onoda *et al.*, 1988) and in CaPY medium, (data not shown). This indicated that in the presence of hypertonic calcium, the L-form required a protonmotive force for growth, which was dissipated by the protonophore. Extrusion of protons by the respiratory chain and/or the H^+ -ATPase should result in the generation of a ΔpH . Therefore, oxygen consumption by intact cells in media at pH 7.2 and 8.2 was examined (Table 1). Oxygen consumption by intact cells in KPY and NaPY media was almost the same at both pH 7.2 and 8.2. In CaPY medium, however, O_2 uptake by intact cells was less at

Fig. 4. Formation of a ΔpH and antiporter activities in right-side-out membrane vesicles. ΔpH formation and activities of cation/proton antiporters were measured by fluorescence quenching. The assay medium contained: 10 mM-Tris/HCl (pH 7.2 or 8.2), 140 mM-choline chloride, 5 mM-MgCl₂, 0.5 mM-dithiothreitol, 1 μM quinacrine, and about 100 μg of membrane vesicle protein ml⁻¹. Quenching was initiated by addition of Tris/1 mM-ATP (a) or Tris/10 mM-DL-lactate (b), respectively. At the times indicated by arrows, either CaCl₂, KCl or NaCl were added to give a final concentration of 1 mM for Ca²⁺ and 10 mM for K⁺ and Na⁺.

Table 1. Effect of Ca²⁺ and K⁺ on the rate of oxidation of glucose at pH 7.2 and 8.2 by the *E. coli* L-form NC7

The incubation medium was NaPY, KPY or CaPY plus 10 mM-glucose, adjusted to the indicated pH.

Medium	O ₂ uptake [nmol min ⁻¹ (mg protein) ⁻¹]	
	pH 7.2	pH 8.2
NaPY	465	510
KPY	503	584
CaPY	379	133

pH 8.2 compared to pH 7.2. This indicated that in CaPY medium the respiration-driven H⁺ extrusion was strongly suppressed under alkaline conditions.

Cation transport systems

The pH_i of everted K- or Ca-vesicles (membrane vesicles from KPY- or CaPY-grown cells) was monitored by changes in fluorescence of quinacrine. When the membrane vesicles were energized with Mg-ATP at

pH 7.2 or 8.2, the fluorescence was rapidly quenched, reflecting the formation of a ΔpH across the membrane of the everted vesicles (Fig. 4a). When K-vesicles were energized a ΔpH was formed (acid interior), which was slightly lower at pH 8.2 than at 7.2. Three distinct cation/proton antiporter activities were also detected at both pH values. Similar results were obtained with Na-vesicles. In the energized Ca-vesicles, fluorescence quenching was lower at pH 8.2 than at pH 7.2. A rapid exchange of Ca²⁺ for H⁺ was observed at both pH values, while the Na⁺/H⁺ and K⁺/H⁺ antiporter activities appeared to be lower at pH 8.2 than at pH 7.2. Addition of DL-lactate to the vesicles decreased the fluorescence quenching (Fig. 4b). When Ca-vesicles were energized with DL-lactate, only slight quenching of fluorescence was observed at pH 8.2 compared to pH 7.2. However, the formation of a ΔpH in K-vesicles following addition of DL-lactate was similar at both pH values.

Changes in pH_i and $\Delta\psi$ in L-form cells after pH_o transitions

DMO and MA distribution in the L-form were examined at pH_o values of 7.2 and 8.2, respectively, after

Fig. 5. pH_i of cells after a pH shift. The L-form was incubated at 32 °C for 120 min in (a) CaPY medium at pH 7.2 or pH 8.2, and (b) in NaPY medium containing 1 mM- CaCl_2 at pH 7.2 or pH 8.2. Samples from the above were added to cuvettes containing 2 ml of media at different pH values plus 5 μM -9-aminoacridine. The change of 9-aminoacridine emission at 454 nm (excitation 364 nm) upon addition of 1% (v/v) butanol (arrowed) was monitored.

incubation for 120 min in KPY, NaPY or CaPY medium. In cells suspended in all media at pH_o 7.2 or 8.2, the pH_i values remained constant at 7.8 to 8.0 (Table 2). After incubation in each medium at different pH_o values, changes in ΔpH were observed in all media at a pH_o of 8.2. Because of the reversed ΔpH , the total values for the electrochemical proton gradient ($\Delta\tilde{\mu}\text{H}^+$) at pH_o 8.2 were low (Table 2). Thus, in all cases, similar patterns were obtained for $\Delta\tilde{\mu}\text{H}^+$.

Measurements of pH_i by the null-point method

We also determined the pH_i of the L-form NC7 equilibrated with either NaPY or CaPY medium at

Table 2. Effect of NaCl, KCl and CaCl_2 on $\Delta\tilde{\mu}\text{H}^+$ and its components in the *E. coli* L-form NC7

Δ values are expressed as $\text{value}_{\text{internal}} - \text{value}_{\text{external}}$.

Cation	pH_o	pH_i	$\Delta\psi$ (mV)	ΔpH (mV)	$\Delta\tilde{\mu}\text{H}^+$ (mV)
Na^+	7.2	7.79	-94	-35	-129
	8.2	7.89	-96	19	-77
K^+	7.2	7.92	-142	-42	-184
	8.2	7.98	-148	13	-135
Ca^+	7.2	7.73	-87	-31	-118
	8.2	7.81	-95	23	-72

pH 7.2 and 8.2 using 9-aminoacridine. When the pH_i of a bacterium is lower than the pH_o , the fluorescence of 9-aminoacridine decreases on mixing with intact cells (Deamer *et al.*, 1972). Cells were incubated in medium at pH 7.2 or 8.2 for 120 min and resuspended in 2 ml of the test media at different pH values. When the pH_i of the cells was lower than pH_o , a quenching of the fluorescence was expected. No fluorescence change was expected when the pH_i was the same or higher than pH_o . By determining this 'null-point' value, we could estimate pH_i . When cells were incubated in CaPY medium at pH 7.2 or 8.2, quenching of the fluorescence was observed when pH_i was lower than pH_o (Fig. 5), indicating that the estimated value of pH_i was 7.6 to 7.8. The pH_i of cells incubated in NaPY medium at a pH_o of either 7.2 or 8.2 was 7.6 to 7.8.

Morphology of cells cultured in alkaline CaPY medium

The non-growing L-form in alkaline CaPY medium showed many large cells, which seemed to be the result of disturbed cell division. When the L-form was incubated for 12 h in CaPY medium at pH_o 8.2 and shifted to 7.2, growth resumed, implying that the cells remained viable for at least 12 h under the non-permissive conditions. Thereafter, the cells continued to grow at the rate characteristic of pH 7.2.

Discussion

Many neutrophiles maintain a constant pH_i at around 7.5 over a wide range of pH_o values (Booth, 1985). The Na^+/H^+ antiporter has been shown to be part of the pH homeostasis mechanism in *E. coli* and alkaliphilic *Bacillus* species (Padan *et al.*, 1981; Booth, 1985; McMorro *et al.*, 1989). An *E. coli* mutant with a

defective Na⁺/H⁺ antiporter did not grow at alkaline pH, but it had K⁺/H⁺ antiporter and Ca²⁺/H⁺ antiporter activities that were normal (Ishikawa *et al.*, 1987). Everted membrane vesicles derived from the L-form have three distinct cation antiport systems: a K⁺/H⁺ antiport for K⁺, Rb⁺ and Na⁺; a Na⁺/H⁺ antiport for Na⁺ and Li⁺; and a Ca²⁺/H⁺ antiport for Ca²⁺ and Mn²⁺ (Onoda *et al.*, 1989). When the L-form was grown in alkaline NaPY and KPY media, growth was inhibited by amiloride, which blocks activity of the Na⁺/H⁺ antiporter, indicating that in NaPY and KPY media at alkaline pH, the activity of the Na⁺/H⁺ antiporter plays an important role in growth of the L-form, probably in pH_i regulation. We found, however, that in CaPY medium the L-form grew well at pH values below 7.8, but was unable to grow above pH 7.8, although the cells grew at pH values from 6.0 to 9.0 in NaPY and KPY media. Growth on CaPY medium was completely inhibited by addition of 10 µM-CCCP, indicating that the L-form requires a ΔμH⁺ for growth. Primary proton pumps such as the respiratory chain or H⁺-ATPase would be needed for the generation of ΔpH. In CaPY medium, oxygen uptake by intact cells and quenching of lactate-energized fluorescence of Ca-vesicles were repressed at alkaline pH. Roth *et al.* (1985) reported that osmotic stress, such as a hypertonic salt solution, inhibited the active transport of sugar by *E. coli*. They suggested that deformation of the membrane by osmotic stress resulted in the conversion of a membrane component of the transport system to a less functional conformation. With the L-form grown in CaPY medium, it was expected that deformation of membrane components by hypertonic Ca²⁺ may have inhibited electrogenic Na⁺/H⁺ antiporter activity. The generation of a ΔpH by primary proton pumps may have lead to cessation of cell division. Brey *et al.* (1980) reported that a more likely function of the K⁺/H⁺ antiporter would be as a regulator of pH_i. However, it is still uncertain whether this is so. The Ca²⁺/H⁺ antiporter is the other antiporter system found in many bacteria. However, the molecular mechanism of active transport of Ca²⁺ in bacterial membrane vesicles is unclear. Recently, we reported that the L-form requires an external Ca²⁺ concentration of 0.1 mM for optimal growth in KPY medium and 1.0 mM in NaPY medium (Onoda *et al.*, 1987; Onoda & Oshima, 1988). We have shown here that the L-form can grow at high calcium concentrations and requires an external Ca²⁺ concentration of about 0.2 M for optimal growth, but the physiological relationship of Ca²⁺ to carrier proteins remains unclear. Activities of cation antiporters and respiratory enzymes present on the cell membrane in hypertonic calcium solution may be regulated by pH_o.

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