

pH dependence of the function of sodium ion extrusion systems in *Escherichia coli*

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Abstract

Escherichia coli has three systems for sodium ion extrusion, NhaA, NhaB and ChaA. In this study, we examined the effect of pH on the function of these transporters using mutants having one of them, and found that (1) a mutant having NhaB excreted sodium ions at pH 7.5 but not at pH 8.5, (2) the efflux of sodium ions from mutant cells having ChaA was observed at both pH 7.5 and 8.5, but the activity was lower at pH 7.5, and (3) sodium ions were excreted from mutant cells having NhaA at pH 6.5 to 8.5. The extrusion activity of cells having NhaA was higher than that of cells having NhaB or ChaA. These results indicate that NhaB functions at a pH below 8, and ChaA extrudes sodium ions mainly at an alkaline pH above 8. It was also suggested that the activity of NhaB and ChaA is not enough to maintain a low level of internal sodium ions when the external concentration of sodium ions is high, and NhaA is induced within a wide range of medium pH under such conditions. © 1998 Elsevier Science B.V.

Keywords: Antiporter, Na^+/H^+ ; pH dependence; *nhaA*; *nhaB*; *chaA*; (*Escherichia coli*)

1. Introduction

It has been shown that *Escherichia coli* has multiple systems for sodium ion extrusion. Three Na^+/H^+ antiporters, NhaA, NhaB and ChaA, have been found in this bacterium [1–4]. ChaA was first reported to be a $\text{Ca}^{2+}/\text{H}^+$ antiporter by Ivey et al. [4]. They have argued that sodium ion extrusion by ChaA is prevented under physiological conditions, and it extrudes sodium ions only when *chaA* is cloned with a multi-

copy vector, because the activity of ChaA to extrude sodium ions was low in a strain they used [4]. However, Ohyama et al. [5] have clearly shown that ChaA functions as a transporter for sodium ion extrusion even if it is encoded by chromosomal gene in the wild type strain, and argued that the *E. coli* strain Ivey et al. [4] used has a mutation which reduces the ChaA activity. The fourth system for sodium ion extrusion was found in *E. coli* [6], but it arose from a mutation of some gene. It has been suggested that a K^+/H^+ antiporter is able to extrude sodium ions, since the influx of protons induced by K^+ was prevented by preexisting Na^+ in membrane vesicles [7]. No sodium ion extrusion was observed in a mutant deficient in *nhaA*, *nhaB* and *chaA*, and the growth of the mutant was sensitive to sodium ions [5], suggesting that the wild type of *E. coli* uses

Abbreviations: Tricine, tris(hydroxymethyl)methylglycine; MES, 2-(*N*-morpholino)-ethanesulfonic acid monohydrate; CCCP, carbonyl cyanide *m*-chlorophenylhydrazine

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NhaA, NhaB and ChaA for sodium ion extrusion under physiological conditions. However, in disagreement with these results, Verkhovskaya et al. [8,9] have found a respiratory-driven Na^+ transport system in both the wild type and a mutant deficient in *nhaA* and *nhaB*.

The following physiological functions of a Na^+/H^+ exchanger have been proposed [1–3,10]. (1) Maintenance of a low level of internal sodium ions, (2) regulation of cytoplasmic pH, (3) control of cell volume, and (4) energy reservation. However, Ohyama et al. [5] have shown that an *E. coli* mutant deficient in all systems for sodium ion extrusion is able to grow at any pH value tested when growth medium contains a low level of sodium ions, suggesting that the major role of sodium ion extrusion is to keep a low level of internal sodium ions. If this is true, why are multiple transporters required for maintaining the cytoplasmic level of sodium ions? It has been proposed that NhaB functions within a wide range of medium pH. In contrast, the expression of NhaA was shown to be induced by Na^+ and Li^+ ions at high pH [11], and ChaA has been reported to function at high pH [4,5]. Thus, one of the explanations would be that *E. coli* uses a different transporter at a different pH value. Even so, it is still puzzling that three transporters function at alkaline pH.

In this study, we investigated the pH dependence of the function of each system for sodium ion extrusion using mutants having one of three transporters, and found that (1) NhaB functions at a pH below 8, but not above 8, and (2) ChaA works mainly at alkaline pH. A mutant having only NhaA had a high activity to excrete sodium ions within a wide range of medium pH from 6.5 to 8.5. No significant efflux of

sodium ions was again observed in a mutant deficient in both *nhaA*, *nhaB* and *chaA* under our experimental conditions, contradicting the previous report by Verkhovskaya et al. [8].

2. Materials and methods

2.1. Bacterial strains and growth media

The *E. coli* strains used are listed in Table 1. *E. coli* was grown at 37°C in minimal medium containing 5 mM K_2HPO_4 , 20 mM NH_4Cl , 1 mM MgSO_4 and 0.1 mM CaCl_2 . Glucose (1%) or lactic acid (1%) was used as an energy source. The lactic acid solution was neutralized by KOH. Sixty mM MES and tricine were added to the medium as buffers at pH values below and above 7, respectively, and the medium pH was adjusted by the addition of KOH. Growth was monitored by measuring the absorbance of the medium at 600 nm (light path length was 1 cm).

2.2. Measurement of sodium ion efflux

The efflux of sodium ions from cells was measured as described previously [12], except that laurylbromide [13] was used instead of the mixture of silicone oil and mineral oil. Cells were suspended in the growth medium without glucose at 1 to 2 mg protein per ml. After the addition of 0.15 M NaCl, the cell suspension was kept on ice for 2 h. At zero time, 1 ml of the cell suspension was mixed with 4 ml of the same growth medium preheated at 37°C without NaCl. When the chilled cells were diluted with growth medium containing 0.28 M sucrose,

Table 1
E. coli strains used in this study

Strains	Relevant genotype	Sources or references
W3110	Wild type	Y. Anraku
TO101	W3110 <i>recD</i> ::Tn10 <i>nhaB</i> ::Em ^r	[5]
TO110	W3110 <i>chaA</i> ::Cm ^r	[5]
TO112	W3110 <i>nhaA</i> ::Km ^r <i>chaA</i> ::Cm ^r	[5]
TO113	W3110 <i>nhaA</i> ::Km ^r <i>nhaB</i> ::Em ^r	[5]
TO114	W3110 <i>nhaA</i> ::Km ^r <i>nhaB</i> ::Em ^r <i>chaA</i> ::Cm ^r	[5]
TO116	W3110 <i>nhaB</i> ::Em ^r <i>chaA</i> ::Cm ^r	TO110 × P1(TO101) (this study)

Cm, Chloramphenicol; Km, kanamycin; Em, erythromycin.

1/20 volume of 1,10-dibromodecane was added to laurylbromide. The temperature of the resulting mixture was 30°C, and the efflux was measured at this temperature. The amount of internal sodium ions at zero time was measured by the same procedure except that the cell suspension was mixed with growth medium chilled on ice, and the mixture was immediately centrifuged at 0°C. The content of extracellular sodium ions coprecipitated with cells was measured as follows. The cell suspension was chilled on ice over 30 min. After addition of 30 mM NaCl, the chilled cells were immediately centrifuged through laurylbromide. The sodium ion content in the pellet was $0.11 \pm 0.02 \mu\text{mol/mg}$ cellular protein, which was identical to the value of 0.10 reported previously [12]. The internal level of sodium ions was calculated after this value was subtracted.

2.3. Other methods and chemicals

Protein determination [14] and P1 transduction [15] were carried out as described before. Reagents used were of analytical grade.

3. Results and discussion

3.1. Effect of sodium ions on the growth of various mutants

The growth rate of both the wild type (W3110) as well as *chaA*⁺ (TO113) decreased with an increase in medium concentration of sodium ions at pH 8.5, but whereas the rate of the mutant was only slightly lower than that of the wild type below 0.2 M, the difference increased with further addition of sodium ions (Fig. 1A). Neither *nhaB*⁺ (TO112) nor a strain deficient in all systems (TO114) grew in medium containing 0.1 M NaCl at pH 8.5 (Fig. 1A), suggesting that NhaB does not support growth in the medium containing a high concentration of sodium ions at pH 8.5. These results suggest that although to a limited extent, ChaA supports growth in medium containing a high level of sodium ions at pH 8.5. *nhaA*⁺ (TO116) grew as well as wild type at any concentration of sodium ions tested at pH 8.5 (Fig. 1A). Since the growth rate of *chaA*⁺ in the presence of 0.3 M NaCl was lower than that of *nhaA*⁺, NhaA seems to

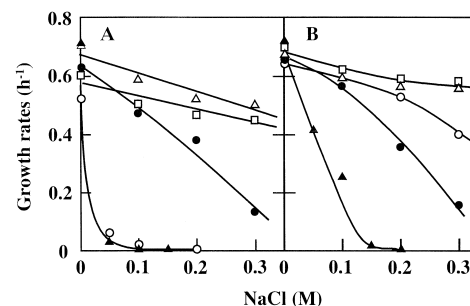


Fig. 1. Effect of sodium ions on the growth of mutants having only one transporter in the medium containing glucose. The growth rates were measured in the presence of various amounts of NaCl at pH 8.5 (A) and 7.2 (B) as described in Section 2. Symbols: (○), *nhaB*⁺; (●), *chaA*⁺; (□), *nhaA*⁺; (▲), mutant deficient in *nhaA*, *nhaB* and *chaA*; (Δ), wild type.

be more important than ChaA for growth at a high concentration of sodium ions.

chaA⁺ grew at pH 7.2 in medium containing 0.1 M NaCl, but the growth rate decreased as the concentration of NaCl increased above 0.1 M at pH 7.2. *nhaB*⁺ grew at pH 7.2 in the presence of 0.2 M NaCl and the decrease in growth rate was observed at a concentration of NaCl above 0.2 M (Fig. 1B). Thus, both ChaA and NhaB are suggested to function at a pH around 7.2, but the activity of both transporters does not seem to be enough to support growth in the presence of sodium ions above 0.2 M. The growth rate of *nhaA*⁺ was as good as that of the wild type at pH 7.2 (Fig. 1B), suggesting that NhaA is essential to growth at a high concentration of sodium ions at pH 7.2 as well as at 8.5. When lactic acid was used as an energy source, similar results were obtained, except that growth rates in the glucose medium were higher than those in the lactic acid medium.

Since all mutants grew as well as wild type in medium containing sucrose at pH 7.2 and 8.5, it can be argued that growth of mutants in the presence of sodium ions depends on the ability to extrude the ions. Similar to that of the wild type, the growth rates of all mutants decreased to one-fifth with the addition of 0.3 M NaCl at pH 6.0. A similar decrease in growth rate was observed when the medium osmolarity was increased by sucrose, suggesting that *E. coli* is more susceptible to hyperosmotic stress at low pH. Therefore, we could not examine the growth resistance of our mutants to sodium ions at an acidic pH.

Growth of the wild type was insensitive to a low concentration of sodium ions in growth medium we used (Fig. 1), contradicting the data by Neidhardt et al. [16] that NaCl significantly increases the growth rate of *E. coli* B and B/r up to 25 mM. Since *E. coli* has many Na⁺-dependent transport systems, sodium ions are thought to be essential for growth. The growth medium we used contained 1 to 3 mM of contaminating sodium ions, which may be enough for growth of the strain we used.

Our results demonstrated that sodium ion extrusion systems were essential to growth of *E. coli* at a high sodium ion concentration, but this bacterium was able to grow without operation of the transporters when the external surroundings contained a low level of sodium ions. It has been proposed that a Na⁺/H⁺ antiporter is involved in the regulation of cytoplasmic pH, which is required for growth at high pH [1–3]. Shimamoto et al. [17] have reported that a mutant deficient in *nhaB* is unable to grow at alkaline pH. However, it has been suggested that their mutant Hit-1 has a second mutation which affects on growth in an alkaline medium, because its inability to grow at high pH was not complemented by a plasmid encoding *nhaB* [3]. We have obtained the same result (data not shown). There have been reports to show that *E. coli* is able to grow in the presence of CCCP at alkaline pH [12,18]. These results may imply that *E. coli* is able to grow without the regulation of cytoplasmic pH at alkaline pH, because pH homeostasis is not maintained under such conditions. Our present results rather support the latter hypothesis.

3.2. Efflux of sodium ions from cells grown at various values of medium pH

When the wild type was loaded with 0.15 M NaCl, cells contained approximately 0.5 $\mu\text{mol Na}^+$ per mg cellular protein at pH 8.5 (Fig. 2A). The internal cell volume was 2.9 μl per mg cellular protein [12]. From these data, the intracellular level of sodium ions at zero time was calculated to be 0.17 M, suggesting that the equilibrium was attained for 2 h incubation on ice. It should be noted that the level of intracellular sodium ions was 1.5-fold higher than the medium level in the presence of 100 μM CCCP, mainly because of the Donnan potential [12]. The wild type cells excreted sodium ions and the intra-

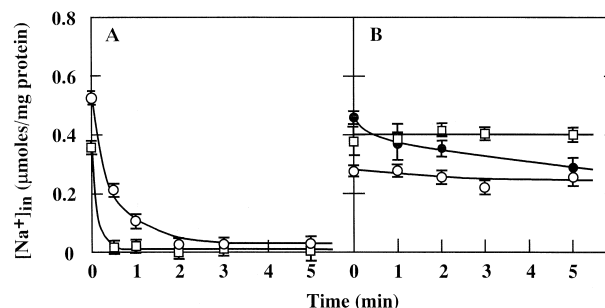


Fig. 2. Efflux of sodium ions in the wild type and mutant deficient in all three transporters grown at various pH values. The wild type (A) and the mutant (B) were grown at various pH values, and the efflux of sodium ions was measured in the same growth medium as described in Section 2. When cells were cultured, glucose was added as an energy source. Each point represents mean \pm standard deviation of two samples prepared separately. Symbols: (\circ), pH 8.5; (\square), pH 7.5; (\bullet), pH 6.5.

cellular content was lowered to near zero. In contrast to the wild type, no significant efflux of sodium ions was observed in the strain deficient in *nhaA*, *nhaB* and *chaA* at pH 7.5 and 8.5, but a slow efflux took place at pH 6.5 (Fig. 2B).

Sodium ions were excreted from *chaA*⁺ cells at both pH 7.5 and 8.5, but the activity was lower at pH 7.5 (Fig. 3A). In contrast to *chaA*⁺, *nhaB*⁺ excreted sodium ions at pH 7.5 but not at pH 8.5 (Fig. 3B). *nhaB*⁺ grown at pH 7.5 was still unable to excrete sodium ions at pH 8.5 (Fig. 3B), suggesting that NhaB had a very low activity at pH 8.5. Thus, it has been suggested that ChaA functions mainly at alka-

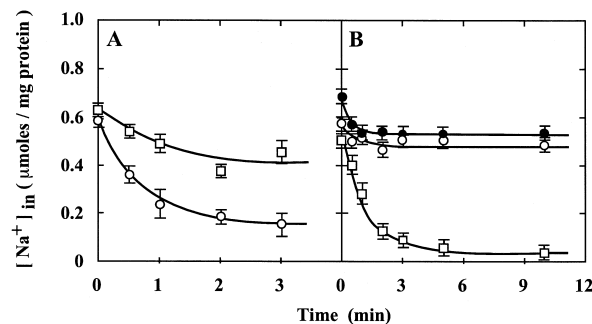


Fig. 3. Efflux of sodium ions in *chaA*⁺ and *nhaB*⁺ grown at various pH values. *chaA*⁺ (A) and *nhaB*⁺ (B) were grown at various pH values, and the efflux of sodium ions was measured in the same growth medium as described in the legend of Fig. 2. Symbols: (\circ), pH 8.5; (\square), pH 7.5; (\bullet), *nhaB*⁺ was grown at pH 7.5 and the efflux of sodium ions was measured at pH 8.5.

line pH, while NhaB functions at a pH below 8. It has been proposed that ChaA is a $\text{Ca}^{2+}/\text{H}^{+}$ antiporter with low affinity to sodium ions [4], but the activity of ChaA in the efflux of sodium ions was similar to that of NhaB (Fig. 3). Furthermore, *chaA*⁺ was resistant to 0.2 M NaCl at pH 8.5 (Fig. 1A). It is hence apparent that ChaA has a significant capacity to excrete sodium ions.

Previous reports [19,20] have shown that the activity of NhaB is only slightly higher at pH 7.0 than that at pH 8.5. Thelen et al. [21] have reported that the activity is equal within a wide range of pH from 7.0 to 8.5. The disagreement between these previous reports and our results may be due to a difference in the method to measure the transport activity; the activity was measured with indirect method in the previous studies, while we measured the movement of sodium ions from cells directly. In the first two reports [19,20], the $\text{Na}^{+}/\text{H}^{+}$ antiport activity was measured by the dequenching of a fluorescence probe induced by proton efflux coupled with the influx of sodium ions via a $\text{Na}^{+}/\text{H}^{+}$ antiporter using membrane vesicles. The dequenching of a fluorescence probe can be observed only when the efflux of protons by a $\text{Na}^{+}/\text{H}^{+}$ antiporter exceeds its influx via the respiratory chain. Since the respiratory activity decreases at high pH [22], it is likely that the activity of NhaB was overestimated at high pH in the previous study [19,20]. Thelen et al. [21] have calculated the NhaB activity from the external concentrations of sodium ions measured at equilibrium state before and after addition of melibiose.

In the present study, we measured a net movement of sodium ions using intact cells, and no significant efflux of sodium ions was observed at pH 8.5 in *nhaB*⁺ (Fig. 3B). However, our results do not suggest that the activity of NhaB is zero at pH 8.5. As shown previously [12], sodium ions were accumulated by cells devoid of all sodium extrusion systems. Since the rate was slow, it can be argued that the accumulation is caused by the passive movement driven by the membrane potential, as first proposed by Heefner and Harold [23]. Thus, our data suggest that the activity of NhaB to expel sodium ions is too low to exceed the passive influx at pH 8.5.

nhaA⁺ excreted sodium ions within a wide range of medium pH from 6.5 to 8.5, and the activity was increased when cells were grown in the presence of

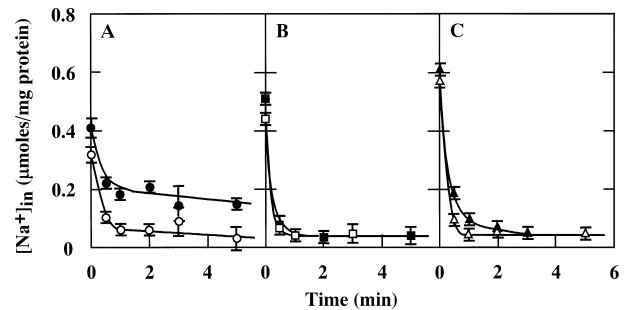


Fig. 4. Efflux of sodium ions in *nhaA*⁺ grown at various pH values. *nhaA*⁺ was grown in the presence (open symbols) and absence (closed symbols) of 0.1 M NaCl at pH 6.5 (A), 7.5 (B) and 8.5 (C), and the efflux of sodium ions was measured in the same growth medium without NaCl as described in the legend of Fig. 2.

0.1 M NaCl (Fig. 4). When *nhaA*⁺ cells preloaded with sodium ions were diluted with medium containing 0.28 M sucrose to avoid hypoosmotic stress, the same rate of sodium ion efflux was observed at any pH value tested (data not shown), ruling out the possibility that the efflux was caused by hypoosmotic stress. It has been generally accepted that the cytoplasm is maintained at pH around 7.6 in cells growing in an acidic medium. The low level of sodium ions at zero time at pH 6.5 (Fig. 4A) may be due to the presence of the pH gradient (internal alkaline), which prevents the loading of sodium ions via a $\text{Na}^{+}/\text{H}^{+}$ antiporter. The activity of NhaA to expel sodium ions (Fig. 4) was higher than that of NhaB or ChaA (Fig. 3), and was close to that of the wild type (Fig. 2). *nhaA*⁺ was more resistant to sodium ions than any other mutant (Fig. 1). These results suggest that NhaA is the most powerful transporter in *E. coli*.

NhaA was reported to have the optimum activity at an alkaline pH [24,25]. Karpel et al. [11] have shown that alkalization of medium markedly increases the sensitivity of expression of *nhaA* to external sodium ions. However, they used an *E. coli* strain having NhaB, which can keep a low level of internal sodium ions at a pH below 8 as shown above. A plasmid encoding *nhaA* conferred the halotolerance of a mutant deficient in both NhaA and NhaB at pH 7.5 [25], and *nhaA*⁺ was resistant to NaCl at pH 7.2 (Fig. 1). Dover et al. [26] have shown that the expression of *nhaA* at pH 6.5 is higher than that at pH 8.4 in cells devoid of $\text{Na}^{+}/\text{H}^{+}$ antiport activity, while the ex-

pression is very low at pH 6.5 in cells having NhaB. It has been proposed that the expression of *nhaA* is regulated by the intracellular level of sodium ions [3]. Therefore, it can be argued that the expression of *nhaA* is suppressed after an enough amount of NhaA to maintain a low level of internal sodium ions is induced; the amount of NhaA is large when its molecular activity is low. It should be noted that such the suppression does not take place when a fusion gene is used, because no active NhaA is produced. Thus, our results that *nhaA*⁺ has an activity to extrude sodium ions at any pH value rather support the previous proposal on the regulation of expression of *nhaA*.

The efflux rate of sodium ions from *nhaA*⁺ was approximately 1200 nmol min⁻¹ mg⁻¹ protein at pH 8.5 (Fig. 4), although the rate was too high to measure precisely in the present study. The movement of sodium ions across the cytoplasmic membrane has been measured by filtration of the assay medium through a membrane filter [6,27], but the rate measured by this method was again too high [6]. Thus, the rate of sodium ion extrusion by NhaA seems to be higher than that of potassium ion accumulation by Trk system, which was reported to be 200 to 600 nmol min⁻¹ mg⁻¹ cell weight at 30°C [28].

Verkhovskaya et al. [8,9] have found a respiratory-dependent Na⁺ transporter in both the wild type of *E. coli* and mutant EP432 deficient in *nhaA* and *nhaB*. In disagreement with their data, our mutant deficient in *nhaA*, *nhaB* and *chaA* had no significant capacity to excrete sodium ions (Fig. 2B). It should be noted that our assay medium contained approximately 60 mM potassium ions. The reason for this discrepancy remains to be clarified. One possibility is that the disagreement is due to a difference in the assay method; the activity of the respiratory-dependent Na⁺ transporter is not measurable under our assay conditions. As described above, a net efflux can be observed only when the extrusion activity exceeds the passive influx driven by the membrane potential under our conditions. A K⁺/H⁺ exchanger of the strain they used might have an elevated affinity for sodium ions. The possibility that our mutant has a mutation which reduces the activity of the respiratory-dependent transporter can't be ruled out. The growth of another mutant deficient in *nhaA*, *nhaB* and *chaA* derived from a strain having a different

genetic background was sensitive to sodium ions (data not shown). However, if the mutated gene is located near *nhaA*, *nhaB* or *chaA*, the mutation would be transferred with the deletion of the antiporter by P1 transduction. Finally, it might be possible that the respiratory-dependent Na⁺ efflux is mediated by ChaA. As discussed previously [5], *E. coli* mutant NM81 has a mutation which reduces the activity of ChaA. Since EP432 has a similar background genetically to that of NM81 [20], it is possible that EP432 has a residual activity of ChaA, while the activity is null in our mutant with disrupted *chaA* by insertion of a drug resistant gene. The identification of the gene for the respiratory-dependent Na⁺ transporter would be required for clarifying these points.

3.3. Effect of sodium ions on the expression of ChaA

The rate of sodium ion excretion in *chaA*⁺ grown at pH 8.5 was higher than that of cells grown at pH 7.5 (Fig. 5). The activity increased when cells were grown in the presence of 0.2 M NaCl at pH 8.5, while no significant increase was observed at pH 7.5 (Fig. 5). Thus, the expression of ChaA may be regulated by both pH and sodium ion concentration of medium. *chaA*⁺ grown in medium without the addition of NaCl had the activity to excrete the ions (Fig. 5). The growth medium we used contained 1 to 3 mM of contaminating sodium ions, which may be enough for induction of ChaA. Another possibility is that *E. coli* has a basal level of ChaA and sodium

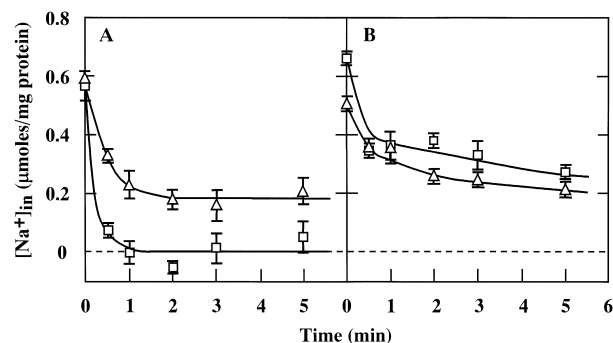


Fig. 5. Effect of NaCl on the expression of ChaA. *chaA*⁺ was grown at pH 8.5 (A) and 7.5 (B), and the efflux of sodium ions was measured in the growth medium without NaCl at pH 8.5 as described in the legend of Fig. 2. Symbols: (Δ), no addition; (□), 0.2 M NaCl was added to the growth medium.

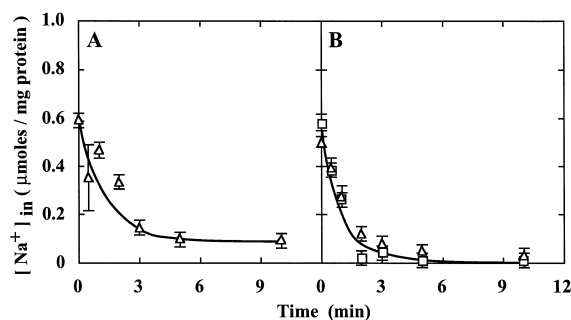


Fig. 6. Effect of NaCl on the expression of NhaB. *nhaB*⁺ was grown at pH 8.5 (A) and 7.5 (B), and the efflux of sodium ions was measured in the growth medium without NaCl at pH 7.5 as described in the legend of Fig. 2. Symbols: (Δ), no addition; (□), 0.2 M NaCl was added to the growth medium.

ions stimulate its expression, resulting in an increase in the expression level. The efflux of sodium ions by NhaB was affected by neither pH nor sodium ion concentration of growth medium (Fig. 6), suggesting that NhaB is a constitutive antiporter.

On the basis of these findings, we concluded that (1) NhaB functions at a low pH below 8, (2) ChaA works mainly at a high pH above 8, and (3) when the external concentration of sodium ions is high, the activity of NhaB and ChaA is insufficient to keep a low level of internal sodium ions, and hence more powerful antiporter NhaA is induced within a wide range of medium pH from 6.5 to 8.5.

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