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Physiological Role of the *chaA* Gene in Sodium and Calcium Circulations at a High pH in *Escherichia coli*

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Ohyama et al. previously isolated *Escherichia coli* mutant RS1, which had a negligible activity for sodium ion extrusion at alkaline pH (T. Ohyama, R. Imaizumi, K. Igarashi, and H. Kobayashi, J. Bacteriol. 174:7743–7749, 1992). Our present study showed that the mutation of RS1 was compensated for by a cloned *chaA* gene. It has been proposed that sodium ion extrusion by ChaA is prevented under physiological conditions (D. M. Ivey, A. A. Guffanti, J. Zemsky, E. Pinner, R. Karpel, E. Padan, S. Schuldiner, and T. A. Krulwich, J. Biol. Chem. 268:11296–11303, 1993). In order to clarify the physiological role of *chaA* in sodium ion circulation at alkaline pH, we constructed a Δ*chaA* mutant. The resultant mutant, TO112, deficient in both *nhaA* and *chaA*, was unable to grow at pH 8.5 in medium containing 0.1 M sodium chloride and had negligible sodium ion extrusion activity. However, TO112 grew at pH 7.0 in medium containing 0.4 M sodium chloride. Sodium ions were extruded from TO112 cells at neutral pH. The extrusion activity at pH 7.5 was greatly reduced by the deletion of *nhaB*. These data demonstrate that the activity of *nhaB* is low at high pH and that ChaA extrudes sodium ions at alkaline pH. The uptake of calcium ions by everted membrane vesicles prepared from the Δ*chaA* mutant TO110 was 60% of the activity observed in the vesicles of the wild-type strain at pH 8.5, but the activity at neutral pH was not reduced by the deletion of *chaA*. Therefore, it was also suggested that ChaA plays a role in calcium ion circulation at alkaline pH.

It has been shown that bacteria have multiple systems for sodium ion extrusion. This multiplicity was first discovered with *Enterococcus hirae* (formerly *Streptococcus faecalis*); the accumulation of sodium ions by everted membrane vesicles of this bacterium was inhibited by uncouplers at neutral pH but was uncoupler resistant in an alkaline buffer (5). The accumulation at high pH was proposed to be mediated by a sodium-translocating ATPase (4, 5), and the ATPase level was regulated by the internal sodium ion level (10). It was found that *E. hirae* had a sodium/proton antiporter that functioned at neutral pH (7). Both genes governing the sodium ion extrusion system have recently been cloned (25, 28). In *E. hirae*, the proton motive force was generated by the proton-translocating ATPase, which functions at a low pH in order to regulate the internal pH, and hence this force was not available at an alkaline pH (11–13). This is why this bacterium has two systems for sodium ion extrusion; one system is driven by the proton motive force at a low pH, and the other is the ATP-driven system that functions in the absence of this force at an alkaline pH.

Three genes encoding the sodium ion extrusion system, *nhaA*, *nhaB*, and *chaA*, have been identified and cloned in *Escherichia coli*, and all of them are driven by the proton motive force (3, 6, 9, 19, 21, 22, 24). In contrast to *E. hirae*, *E. coli* can generate the proton motive force at pHs from 6 to 8.5 (1). NhaB was proposed to extrude sodium ions within a wide range of medium pH, from 6.5 to 8.5 (19, 21, 26), while NhaA functions at an alkaline pH (8, 24). Ivey et al. (6) have argued that ChaA functions as a calcium/proton antiporter and that sodium ion extrusion by this antiporter is prevented under physiological conditions. However, our present data showed that the cloned *chaA* gene compensated for the mutation in RS1, isolated previously (18), which had negligible sodium ion extrusion activity at alkaline pH. In order to clarify the physiological significance of this *chaA* gene, we constructed a *chaA* deletion mutant. Our data for this mutant suggest that ChaA functions in both sodium and calcium circulation at an alkaline pH and that the activity of NhaB is not sufficient for net extrusion of sodium ions in an alkaline medium. The extrusion of calcium ions at pH 7.0 was not affected by the deletion of *chaA*, supporting the idea that *E. coli* has multiple transporters for calcium ion extrusion.

MATERIALS AND METHODS

Bacterial strains and culture media. The *E. coli* strains used are listed in Table 1. Strains containing transposon Tn*10* used for mapping were generously supplied by A. Nishimura (Genetic Stocks Research Center, National Institute of Genetics, Mishima, Japan).

E. coli was grown at 37°C in minimal medium M488, containing 5 mM K_2HPO_4, 20 mM NH_4Cl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 1% lactate, and 60 mM Tricine (pH 8.8), or in minimal medium M570, which was the same as medium M488 except that 60 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.0) was used instead of Tricine. The pH of the media was adjusted by the addition of KOH. The minimal media contained 1 to 3 mM contaminating sodium ions. Threonine (0.5 mM) was added when strain NM81 was cultured. Kanamycin (30 μg/ml), erythromycin (160 μg/ml), and chloramphenicol (30 μg/ml) were added as indicated. Growth was monitored by measuring the absorbance of the medium at 540 nm (light path length, 1 cm).

Plasmids. Phage clones 13H6(248) and 11G8(244) were received from Y. Kohara (National Institute of Genetics). Plasmid pVA838 (16) was generously donated by D. B. Clewell (University of Michigan). Plasmid pVA838 was first restricted with *Ava*I, both ends were filled in, and the *Hin*dIII-*Ava*I fragment containing the erythromycin resistance gene was

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TABLE 1. *E. coli* strains used in this study

Strain	Relevant genotype[a]	Origin or reference
W3110	Wild type	Y. Anraku
NM81	*melB* Lid *ant*$^+$ *lacZY nhaA*::Km^r *thr*	E. Padan (19)
pop6810[b]	Δ(*lac-pro*) *ara malB*Δ107 *gyrA chaA*	Y. Anraku
RS1[b]	pop6810 *nhaA*::Km^r *chaA*	pop6810 × P1 (NM81) (18)
TO100	W3110 *recD*::Tn*10* *chaA*::Cm^r	This study
TO101	W3110 *recD*::Tn*10* *nhaB*::Em^r	This study
TO110	W3110 *chaA*::Cm^r	W3110 × P1 (TO100) (this study)
TO111	W3110 *nhaA*::Km^r	W3110 × P1 (NM81) (this study)
TO112	W3110 *nhaA*::Km^r *chaA*::Cm^r	TO111 × P1 (TO110) (this study)
TO113	W3110 *nhaA*::Km^r *nhaB*::Em^r	TO111 × P1 (TO101) (this study)
TO114	W3110 *nhaA*::Km^r *nhaB*::Em^r *chaA*::Cm^r	TO112 × P1 (TO113) (this study)

[a] Km, kanamycin; Cm, chloramphenicol; Em, erythromycin.
[b] It has been suggested in the present study that *chaA* is mutated in RS1, and pop6810 probably has the same mutation.

excised by *Hin*dIII digestion. The resulting fragment was ligated to pUC118 cut with *Hin*dIII and *Hin*cII, yielding plasmid pFT1. Plasmid pNB2 containing *nhaB* was constructed by ligation of the *Eco*RV-*Bam*HI fragment of phage clone 11G8 to pACYC184 cut with *Eco*RV and *Bam*HI (Fig. 1). Plasmid p13H6 containing *chaA* was constructed by insertion of the *Hin*dIII-*Hin*dIII fragment of phage clone 13H6 into the *Hin*dIII site of pACYC184 (Fig. 2). Plasmids p13H6-3B and p13H6-3F were obtained by deletion of the *Bam*HI-*Bam*HI or *Eco*RV-*Eco*RV fragment, respectively, from p13H6.

Disruption of *nhaB*. The disruption of *nhaB* is shown in Fig. 1. The *Hin*dIII site located on the vector of pNB2 was first deleted; this plasmid was cut with *Cla*I and *Eco*RV, both ends were filled in, and it was ligated. The *Hin*dIII-*Kpn*I fragment of the resulting plasmid, pNBΔ1, was removed, and the *Hin*dIII-*Kpn*I fragment containing the erythromycin resistance gene prepared from pFT1 was inserted. The resulting plasmid containing the disrupted *nhaB* gene was designated pNBΔEM. The inactivated *nhaB* gene was transferred to the chromosome by transformation of strain W3110 *recD*::Tn*10* with pNBΔEM cut with *Bam*HI and *Eco*RI (*Eco*RI site was located on the vector). The Δ*nhaB* gene was transferred to TO111 by P1 transduction, and strain TO113 (W3110 Δ*nhaA* Δ*nhaB*) was obtained.

Disruption of *chaA*. A gene for resistance to chloramphenicol was inserted into the *chaA* gene as shown in Fig. 3, yielding plasmid p13H6CM. The inactivated *chaA* gene was transferred to the chromosome by transformation of W3110 *recD*::Tn*10* with p13H6CM cut with *Bgl*I (*Bgl*I site was located on the vector). The Δ*chaA* gene was transferred to W3110 by P1 transduction, and TO110 was obtained. The deletion of *chaA* was confirmed by Southern blot analysis of TO110 chromosomal DNA.

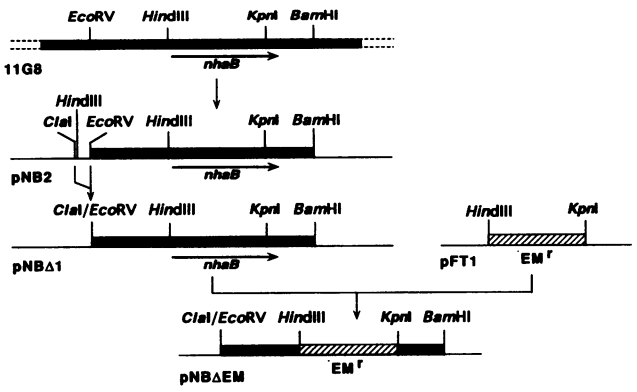

FIG. 1. Physical maps of plasmids containing *nhaB* and strategy for disruption of *nhaB*. Solid lines represent chromosomal DNA. EM^r, erythromycin resistance gene.

Measurement of calcium ion transport activity in everted membrane vesicles. Cells were grown in medium M488 or M570 and harvested at the logarithmic phase of growth. Everted membrane vesicles were prepared and calcium ion uptake was assayed as described previously (27) except that the pHs of all buffers used were adjusted to 8.5 and 7.0 for cells grown in medium M488 and M570, respectively.

Measurement of internal sodium ion content. For measurement of the intracellular amount of sodium ions, 1.5 ml of cells growing at 37°C was centrifuged through oil at certain intervals, and the total amount of sodium ions in the pellet was measured as described previously (18). The internal concentration of this ion was calculated after the value obtained with chilled cells was subtracted, as described previously (18).

Other procedures. Transformation of *E. coli* by the standard calcium-heat shock method (23), transduction of *E. coli* by P1*kc* (14), and protein determination (15) were carried out as described before.

Chemicals. $^{45}CaCl_2$ was purchased from DuPont/NEN Research Products. Other reagents used were of analytical grade.

RESULTS AND DISCUSSION

ChaA extrudes sodium ions at alkaline pH. *E. coli* mutant NM81, deficient in *nhaA*, had residual sodium ion extrusion activity at pH 8.5 and could grow at a sodium ion level as high as 0.4 M at the same pH (18, 19). It has been suggested that NhaB functions within a wide range of medium pHs, from 6.5 to 8.5 (19, 21, 26). Another Δ*nhaA* mutant, RS1, isolated in our laboratory by transduction of strain pop6810 with P1*kc* grown in NM81, had negligible sodium ion extrusion activity in alkaline medium, and its growth was inhibited completely by 0.1 M NaCl (18). It was reported earlier that the second gene for sodium ion extrusion, which is located in or near *nhaB*, was deficient in strain RS1 (18). These previous data suggested that the residual sodium ion extrusion activity in NM81 is mediated by NhaB. However, plasmid pNB2 containing *nhaB* (Fig. 1) could not compensate for the mutation in RS1, suggesting that the *nhaB* gene in RS1 was not mutated. Thus, the mapping in the previous study (18) would seem to be wrong; the position of the *tet* marker was probably incorrect in strains used previously.

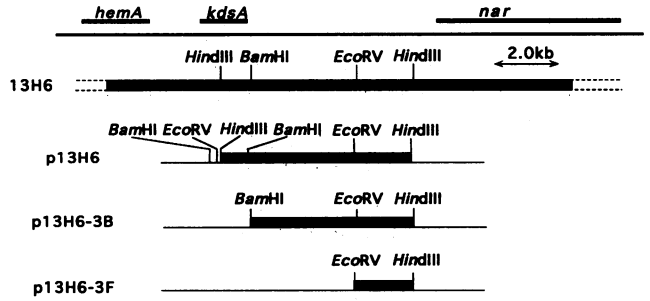

FIG. 2. Physical maps of plasmids containing *chaA*. Solid lines represent chromosomal DNA.

In order to identify the gene encoding the sodium ion extrusion system which functions at alkaline pH in Δ*nhaA* mutant, we again mapped the mutated gene in RS1 by using other *tet*-marked strains, and the frequencies of cotransduction were 27.1% (n = 46) and 13.8% (n = 65) with *tet* markers at 26.3 and 27.3 min, respectively. No cotransductant with a *tet* marker at 25.1 min was obtained. Our present mapping data show that the mutated gene of RS1 is located at 27 min, near the *nar* gene. Phage clone 13H6 of Kohara's library contained this region (Fig. 2). Plasmid p13H6-3B containing the *Bam*HI-*Hin*dIII fragment of 13H6 compensated for the mutation of RS1; RS1 containing this plasmid grew in medium containing 0.2 M NaCl at pH 8.5 (data not shown). Since plasmid p13H6-3F containing the *Eco*RV-*Hin*dIII fragment of 13H6 could not compensate for the mutation of RS1, this suggested that the gene located between the *Bam*HI and *Eco*RV sites was mutated in RS1. There was one open reading frame in this region, and its nucleotide sequence was identical to that of *chaA*, which was cloned previously by Ivey et al. (6). These data demonstrate that the sodium ion extrusion observed in the Δ*nhaA* mutant at an alkaline pH is mediated mainly by ChaA.

For further characterization of the *chaA* product, we constructed a Δ*chaA* mutant. The sensitivity of the growth of TO110 (W3110 Δ*chaA*) to sodium ions was higher than that of its parent strain W3110 at pH 8.5, but the sensitivity was less than that of TO111 (W3110 Δ*nhaA*; Fig. 4A and B). When both *nhaA* and *chaA* were deleted, the growth of the resulting mutant, TO112, was completely inhibited by 0.1 M NaCl (Fig. 4C). The sensitivity of TO112 to sodium ions was similar to that of RS1. The deletion of either *chaA* or *nhaA* had no effect on growth at a neutral pH, but the growth rate decreased somewhat when both genes were deleted (Fig. 5). TO112 could not extrude sodium ions but slowly accumulated them at pH 8.5 (Fig. 6), as observed previously in RS1 (18). These results clearly show that ChaA extrudes sodium ions at alkaline pH.

Ivey et al. (6) have argued that sodium ion extrusion by ChaA is prevented under physiological conditions but that it took place when this gene was cloned with a multicopy vector. In disagreement with this, our data suggested that ChaA had sodium ion extrusion activity even if it was encoded by the chromosomal gene. The *chaA* gene was cloned from NM8191ΔB, a derivative of NM81 which became resistant to sodium ions again (6). Therefore, a possible explanation for this discrepancy could be that the ChaA of NM81 has a low activity because of some unknown mutation and that this mutation reverted in the NM8191ΔB strain used for *chaA* cloning. In fact, the internal level of sodium ions in NM81 grown at pH 8.5 was higher than that in TO111 (data not shown), suggesting that the sodium ion extrusion activity of *chaA* is lower in NM81 than in TO111.

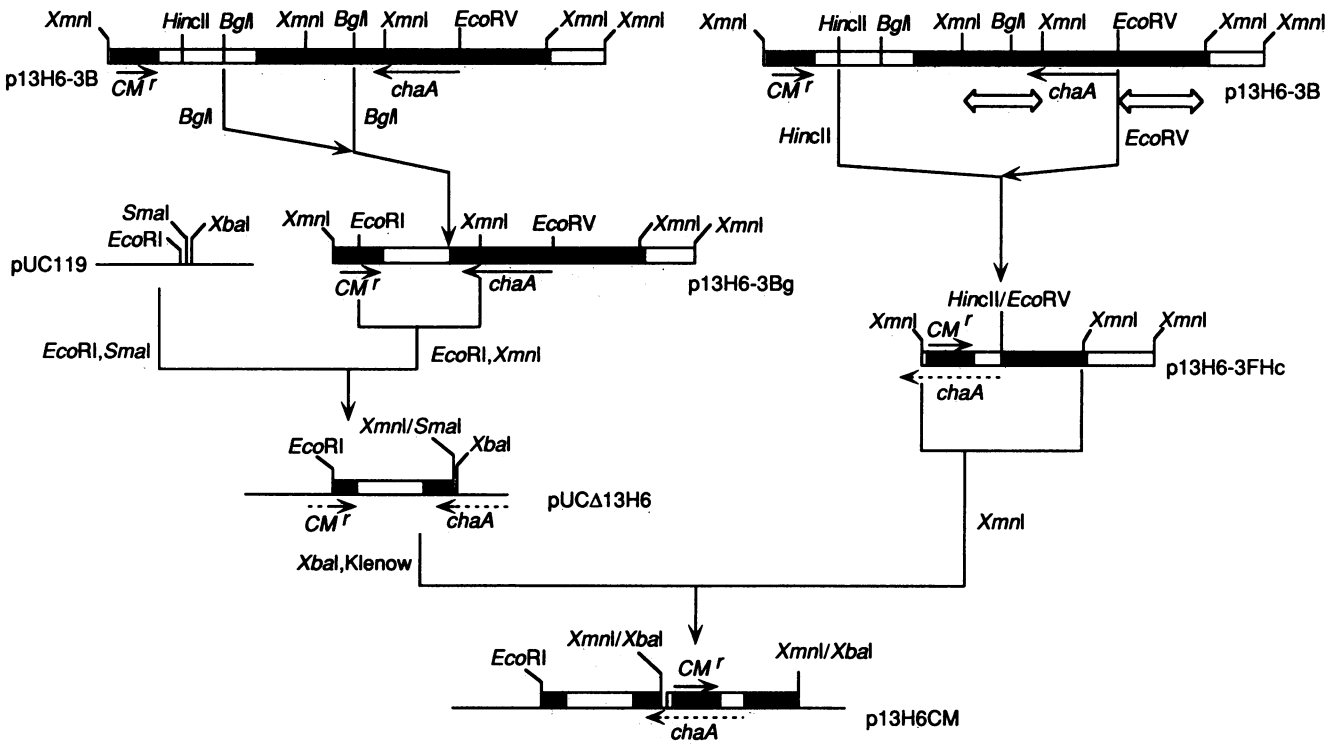

FIG. 3. Strategy for disruption of *chaA*. Solid lines represent chromosomal DNA. Probes used for Southern blot analysis are shown by open double-headed arrows. The solid and dotted arrows represent the intact and disrupted genes, respectively. CM^r, chloramphenicol resistance gene.

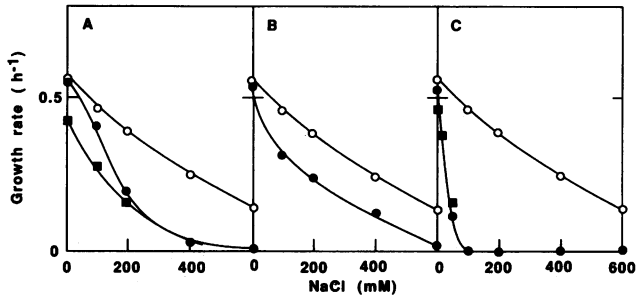

FIG. 4. Effect of sodium ion concentration on the growth of various mutants at pH 8.5. The growth rates of W3110 and its mutants in medium M488 containing various amounts of NaCl were measured as described in Materials and Methods. The pH of medium M488 was adjusted to 8.8 before use, but the pH decreased to 8.5 when cells reached the middle of the logarithmic growth phase. (A) ○, W3110; ●, TO111 growing in the presence of kanamycin; ■, TO113 growing in the presence of kanamycin and erythromycin. (B) ○, W3110; ●, TO110 growing in the presence of chloramphenicol. (C) ○, W3110; ●, TO112 growing in the presence of kanamycin and chloramphenicol; ■, TO114 growing in the presence of kanamycin, erythromycin, and chloramphenicol.

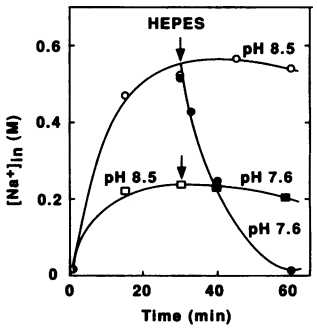

FIG. 6. Change in the internal level of sodium ions. Strains TO112 and TO114 were cultured in medium M488, and 0.1 M NaCl was added to cells in the logarithmic growth phase (absorbance, 0.5) at time zero. At the time marked by the arrows, 60 mM HEPES was added to decrease the medium pH from 8.5 to 7.6. The internal concentrations of sodium ions were measured as described in Materials and Methods. ○,●, TO112 growing in the presence of kanamycin and chloramphenicol; □,■, TO114 growing in the presence of kanamycin, erythromycin, and chloramphenicol. Solid symbols represent the internal level after HEPES was added.

Calcium ion movement in a Δ*chaA* mutant. The initial rate of calcium ion accumulation by everted membrane vesicles of TO110 was approximately 60% of the rate observed in membrane vesicles of its parent strain, W3110 (Fig. 7). The accumulation was the same as that of W3110 at pH 7.0 (Fig. 7). These results suggested that ChaA plays a role in calcium ion circulation at an alkaline pH. The growth of TO110 was not affected by the addition of 50 mM $CaCl_2$ at pH 8.5 (data not shown). At concentrations above 50 mM in an alkaline medium, the effect of calcium ions on growth could not be tested because of precipitation.

In agreement with the previous results (6), our data also suggest that ChaA extrudes calcium ions at an alkaline pH. Since Na^+ and Ca^{2+} are quite different in size and character, the molecular mechanism of ChaA activity is of particular interest. This protein may have two domains, one for sodium ions and the other for calcium ions. It is also possible that both ions are bound to the same site. Alternatively, yet another possibility is that *E. coli* has an extrusion system for calcium ions that is driven by the sodium ion gradient.

The mutant deficient in *chaA* still had calcium ion extrusion activity (Fig. 7), indicating the presence of another transporter(s) for this ion. Four genes affecting the sensitivity of *E. coli* growth to the calcium ion concentration of the medium have been mapped in different regions from *chaA* (2). Thus, it appears certain that *E. coli* has multiple transport systems for calcium ions. Cloning and characterization of the genes mapped by Brey and Rosen (2) would clarify the physiological roles of each of the calcium ion transport systems.

NhaB activity negligible at high pH. It was previously suggested that NhaB functions within a wide range of medium pHs, from 6.5 to 8.5 (19, 21, 26). However, as described above, pNB2 could not compensate for the mutation in RS1, suggesting that NhaB activity is low at alkaline pH. In order to confirm this, we constructed a Δ*nhaB* mutant. Mutant TO113 (W3110 Δ*nhaA* Δ*nhaB*) grew in an alkaline medium containing 0.2 M NaCl (Fig. 4A) and in medium containing 0.4 M NaCl at pH 7.5 (data not shown). No growth of TO114 (W3110 Δ*nhaA* Δ*nhaB* Δ*chaA*) was observed in medium containing 0.2 M NaCl at pH 7.0 to 8.5 (Fig. 4C and 5C). Pinner et al. (21) constructed mutant EP432 (NM81 Δ*nhaB*); the sensitivity of

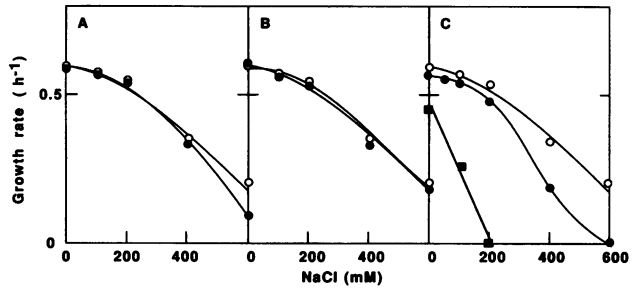

FIG. 5. Effect of sodium ion concentration on the growth of various mutants at pH 7.0. Experiments were carried out as described in the legend to Fig. 4 except that medium M570 was used.

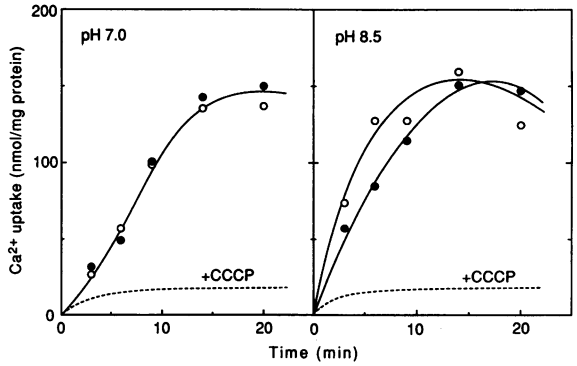

FIG. 7. Calcium ion uptake by everted membrane vesicles of the Δ*chaA* mutant. Everted membrane vesicles were prepared from strains W3110 and TO110 grown in medium M570 or medium M488, and calcium ion uptake was measured as described in Materials and Methods. Chloramphenicol was added to the growth medium for TO110. The assays were carried out at pH 7.0 and 8.5 for cells grown in medium M570 and M488, respectively. ○, W3110; ●, TO110. Dotted lines represent the uptake activity of TO110 in the presence of 0.1 mM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP).

this strain to sodium ions was similar to that of TO114 but higher than that of TO113. These data again suggest that the sodium ion extrusion activity of ChaA is low in EP432 and its parent strain, NM81.

Deletion of *nhaB* increased the sensitivity of growth to sodium ions at pH 7.0 in the Δ*nhaA* Δ*chaA* mutant TO112 (Fig. 5C). The sodium ion extrusion activity of TO112 was still greatly reduced by the deletion at pH 7.6 (Fig. 6), suggesting that NhaB of TO112 was active in this pH range. However, the growth rates of TO111 and TO112 as a function of the level of sodium ions were not affected by the deletion of *nhaB* at pH 8.5 (Fig. 4A and C). Net extrusion of sodium ions was not observed in TO112 at pH 8.5 (Fig. 6). Thus, our data demonstrated that NhaB functioned at a neutral pH and that its activity is not sufficient for net extrusion of sodium ions at an alkaline pH, although the activity can be detected by the dequenching of a fluorescent probe at a high pH, as shown previously (19, 21, 26). Measurement with a fluorescent probe is less quantitative, and the dequenching may be affected by proton influx via the respiratory chain, the activity of which decreases at alkaline pH (20).

The reason for the presence of multiple systems for sodium ion extrusion in *E. coli* still needs to be clarified. We have proposed that the cytoplasm is not kept neutral in *E. coli* growing in an alkaline medium; the cytoplasmic pH is increased by the alkalinization of the external surroundings (17, 18). If this is true, it may be reasonable to assume that the transport system functioning at neutral pH has a low activity in an alkaline medium, and hence a compensatory system would be required at alkaline pH. In fact, our present data show that the activity of NhaB is low at high pH. We suggest that two transport systems for sodium ions, NhaA and ChaA, function at high pH. A possible explanation for this is that the main function of ChaA is to extrude calcium ions, as proposed previously (6), and that sodium ions are extruded mainly by NhaA. Another possibility, one that we prefer, is that one of the two systems functions at a low sodium ion concentration and the other begins to function when the level of this ion is higher. Additional studies on the regulation of expression of these genes would clarify these points.

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