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Escherichia coli Is Able To Grow with Negligible Sodium Ion Extrusion Activity at Alkaline pH

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The *Escherichia coli* mutant NM81, which is deficient in the *nhaA* gene for the sodium/proton antiporter, still has a sodium ion extrusion activity because of a second antiporter encoded by *nhaB* (E. Padan, N. Maisler, D. Taglicht, R. Karpel, and S. Schuldiner, J. Biol. Chem. 264:20297–20302, 1989). By chance, we have found that *E. coli* pop6810 already contains a mutation affecting the sodium ion circulation, probably in or near *nhaB*, and that its Δ*nhaA* mutant, designated RS1, has no sodium ion extrusion activity at alkaline pH. The growth of RS1 was inhibited completely by 0.1 M sodium, whereas growth inhibition of NM81 was observed only at sodium concentrations greater than 0.2 M. RS1 grew at a normal rate in an alkaline medium containing a low sodium concentration. Furthermore, RS1 grew with a negligible proton motive force in the alkaline medium containing carbonyl cyanide *m*-chlorophenylhydrazone. The transport activities for proline and serine were not impaired in RS1, suggesting that these transport systems could be driven by the proton motive force at alkaline pH. These findings led us to conclude that the operation of the sodium/proton antiporter is not essential for growth at alkaline pH but that the antiporter is required for maintaining a low internal sodium concentration when the growth medium contains a high concentration of these ions.

Escherichia coli possesses sodium/proton antiporters (2, 3, 6, 23, 30, 35), whose physiological roles have been reported as regulation of internal pH in the alkaline range (1, 4, 24, 25, 37, 38), generation of an electrochemical sodium gradient across the membrane (6), and energy buffering (5). The sodium gradient drives the transport systems of glutamate, proline, serine, and melibiose in *E. coli* (for a recent review, see reference 8).

The *nhaA* gene, which encodes the sodium/proton antiporter, has been cloned, and its nucleotide sequence has been determined (10, 15, 32). Padan et al. (23) isolated a mutant deficient in NhaA, designated NM81, by inserting a gene encoding kanamycin resistance into the *nhaA* gene. They found that the mutant grew at alkaline pH and that the growth was sensitive to sodium concentration at high pH; they also found that melibiose, whose transport was driven by sodium circulation, supported the growth of NM81 and that the sodium/proton antiporter activity was detected in membrane vesicles of NM81. From these findings, they suggested the presence of a second system, NhaB, for extrusion of sodium ions. Pinner et al. (26) recently cloned the *nhaB* gene, which maps at 25.5 min, and its nucleotide sequence has been determined.

Since the sodium/proton antiporters were reported to be driven by the proton motive force, it could be expected that either the transport system driven by the sodium circulation or the regulatory system for the internal pH did not function without this force. However, it was shown that *E. coli* could grow with a negligible proton motive force in the presence of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) at alkaline pH (22) as well as at neutral pH (9, 16).

To clarify this discrepancy, we constructed Δ*nhaA* mutants by transduction with P1*kc* grown on NM81. By chance, we found that *E. coli* pop6810 had a mutation in the gene affecting the sodium ion circulation functioning at alkaline pH, which was different from *nhaA*, and hence its Δ*nhaA* transductant, designated RS1, was unable to extrude sodium ions at alkaline pH. Our present data obtained with RS1 have suggested that *E. coli* can grow with an apparent lack of activity of the sodium/proton antiporter in alkaline media but that the antiporter is required for maintaining a low internal sodium concentration when cells are surrounded by a high concentration of sodium.

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MATERIALS AND METHODS

Bacterial strains. *E. coli* NM81 (TA15 *nhaA*::Km^r [23]) and plasmid pGM36 (10) containing the *nhaA* gene were generously supplied by E. Padan, Hebrew University, Jerusalem, Israel. W3110 and pop6810 [Δ(*lac-pro*) *ara malB*Δ*107 gyrA*] were generously donated by Y. Anraku, Tokyo University, Tokyo, Japan. HIT-1 (14) and its parent strain, W3133-2, were gratefully received from T. Tsuchiya, Okayama University, Okayama, Japan. RS1 (pop6810 *nhaA*::Km^r) was obtained by P1 transduction with pop6810 and NM81 as recipient and donor, respectively. HIT-1 Δ*nhaA* was obtained with P1*kc* grown on NM81. Strains containing transposon Tn*10* used for mapping were supplied by A. Nishimura, Genetic Stocks Research Center, National Institute of Genetics, Mishima, Japan.

Culture media. *E. coli* was grown at 37°C in L-broth (18), rich medium RM688, or the following minimal media. Minimal medium M488 contained 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). Minimal medium M570 was the same as medium M488 except that 60 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.0) was used instead of Tricine. Medium RM688 contained 60 mM Tricine (pH 8.8), 1% tryptone, and 0.5% yeast extract. The pH values of the media were adjusted by the addition of KOH. The minimal media contained 1 to 3 mM contaminating sodium. For the growth of proline auxotrophs, 0.5 mM proline was added to the growth medium. When NM81 was cultured, 0.5 mM threonine was added. Kanamycin (50 μg/ml) was added when kanamycin-resistant strains were

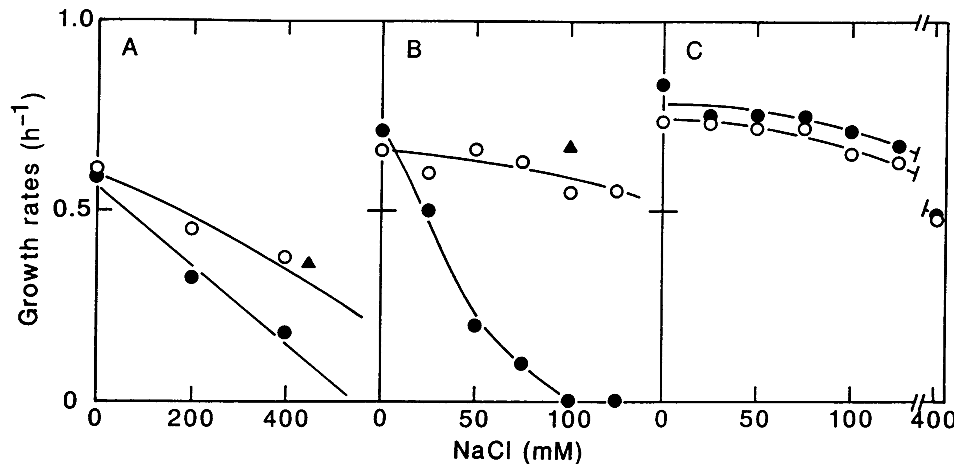

FIG. 1. Effect of sodium concentration on the growth of Δ*nhaA* mutants. (A) NM81 (●) and NM81/pGM36 (○) were grown in medium M488. (B) RS1 (●) and RS1/pGM36 (○) were grown in medium M488. (C) RS1 (●) and RS1/pGM36 (○) were grown in medium M570. KCl was used instead of NaCl when either NM81 or RS1 was cultured (▲). When the growth rate was measured, the medium pH values were 8.5, 8.5, and 7.0 in panel A, B, and C, respectively.

cultured. Ampicillin (50 μg/ml) was added when cells harboring pGM36 were grown.

Measurement of intracellular sodium ion levels. Medium (1.5 ml) was overlaid on a mixture of 0.30 ml of silicone oil (Toray SH-550) and 0.05 ml of mineral oil (Sigma light white oil) in a 2.0-ml microcentrifuge tube, and the cells were immediately centrifuged through the oil at 10,000 × *g* for 5 min. After the supernatant and oil were removed, the pellets were suspended in 1 ml of 5% trichloroacetic acid. The resulting suspension was heated at 90°C for 5 min and was then centrifuged at 6,000 × *g* for 5 min. The sodium content in the supernatant was measured with an atomic absorption spectrophotometer (Z-7000; Hitachi).

The sodium content from the medium that coprecipitated with cells was measured as follows. The culture was chilled on ice for over 30 min. After the addition of 0.1 M NaCl, the chilled cells were immediately centrifuged as described above. The sodium content of the chilled cells was subtracted from the content of growing cells to provide the intracellular sodium content. The internal cell volume used for calculating the internal concentration was 2.9 μl/mg of protein (22).

Measurement of the efflux of sodium ions. Cells grown in medium M488 were washed with the same medium containing no lactate and suspended in the same medium (minus lactate) at 1 to 2 mg of 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 suspension was added to 4 ml of medium M488 preheated at 37°C. After the mixture was incubated at 37°C for various periods, 1.5 ml of the mixture was overlaid on the oil mixture and the intracellular sodium content was measured as described above. The sodium concentration from the medium that coprecipitated with cells was calculated as described above except that 0.03 M NaCl was added instead of 0.1 M.

Measurement of transport activities of proline and serine. Cells grown in minimal medium M488 were harvested at the mid-logarithmic phase of growth and were washed twice with the same growth medium containing no carbon source. Washed cells were suspended in the same medium without carbon source at 0.1 to 0.2 mg of protein per ml. After chloramphenicol (100 μg/ml) and 1% lactate had been added, the cell suspension was preincubated for 3 min at 30°C. The reaction was started by the addition of either 10 μM [^{3}H]proline or 10 μM [^{3}H]serine (0.25 to 1.0 μCi/ml), and 500-μl aliquots were filtered through a nitrocellulose filter (pore size, 0.45 μm; Advantec) at certain intervals. The filter was washed twice with 5 ml of the same medium at room temperature, and the radioactivity trapped on the filter was counted in a liquid scintillation counter with a Bray solution.

Other procedures. *E. coli* was transformed by the standard calcium-heat shock method (29). Transduction of *E. coli* by P1*kc* was carried out as described previously (18). Protein was determined as described previously (19). The growth was monitored by measuring the A_{540} of the culture medium (the light path length was 1 cm).

Chemicals. [^{3}H]proline and [^{3}H]serine were purchased from American Radiolabeled Chemicals Inc. Other reagents used were of analytical grade.

RESULTS

Growth inhibition by sodium ions at alkaline pH. Both NM81 (23) and our Δ*nhaA* mutant RS1 (Fig. 1) grew at alkaline pH when no NaCl was added to the medium. Under our growth conditions, the growth rate of NM81 at pH 8.5 was slightly decreased by the addition of a relatively high concentration of NaCl (Fig. 1). In contrast, the growth of RS1 was greatly inhibited by a low sodium concentration (Fig. 1). When KCl was used instead of NaCl, no growth inhibition was observed in either RS1 or NM81 (Fig. 1), suggesting that sodium inhibits the growth but chloride has no effect. RS1 harboring pGM36 was resistant to sodium ions. The growth inhibition by sodium ions was not observed at pH 7.0 in RS1 (Fig. 1) or in NM81 (data not shown).

All transductants of pop6810 isolated independently had the same sensitivity to NaCl at alkaline pH as did RS1. We next constructed Δ*nhaA* transductants of W3110. All these transductants grew on the medium RM688 plate containing 0.2 M NaCl, suggesting that only transductants of pop6810 were very sensitive to sodium ion at alkaline pH.

Sodium ion extrusion in RS1. The cytoplasmic sodium concentration increased up to 0.4 M when 0.1 M NaCl was added to the growing cells of RS1 in an alkaline medium (Fig.

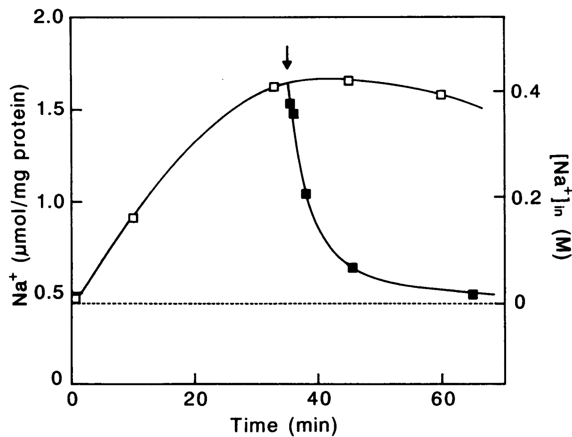

FIG. 2. Change in the internal sodium concentration. RS1 was cultured in medium M488, and 0.1 M NaCl was added to cells growing at the logarithmic growth phase (A_{540} = 0.5) at zero time. A 1.5-ml sample of the medium was centrifuged through oil at certain intervals, and the total sodium concentration in the pellet (□) was measured as described in Materials and Methods. At the arrow, 60 mM HEPES (■) was added to decrease the medium pH from 8.30 to 7.60. The left and right axes represent the total sodium concentration in the pellet and the internal sodium concentration, respectively. The concentration was calculated after the value obtained with chilled cells (dotted line) was subtracted.

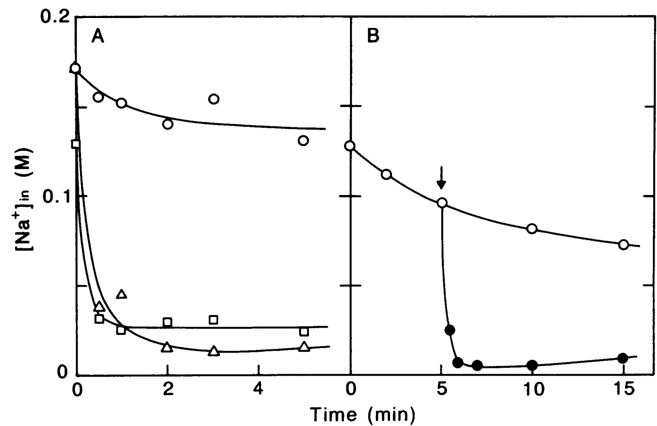

FIG. 4. Efflux of sodium ions from cells preloaded with sodium. RS1 (○), pop6810 (△), and NM81 (□) were grown in medium M488, and the efflux of sodium ions from preloaded cells was measured as described in Materials and Methods. HEPES (60 mM) (●) was added at the arrow to decrease the medium pH from 8.50 to 7.65. The internal sodium concentration was calculated as described in Materials and Methods, and the value subtracted was 0.1 μmol per mg of protein.

2). When the medium pH was decreased from 8.3 to 7.6, the accumulated ions were extruded (Fig. 2). In the next experiment, sodium ions accumulated for 30 min in the alkaline medium containing 0.1 M NaCl and then CCCP was added. The internal sodium concentration decreased from 0.34 M to 0.15 M after 30 min (Fig. 3A). The internal concentration in the presence of CCCP was higher than the medium concentration, mainly because of the Donnan potential, which causes accumulation of sodium ions in the absence of the proton motive force. These results suggested that RS1 had no sodium ion extrusion activity at alkaline pH but that sodium was extruded at a neutral pH. In contrast, the sodium ion content stayed at a low level in both pop6810 and RS1/pGM36 under the same conditions (Fig. 3A).

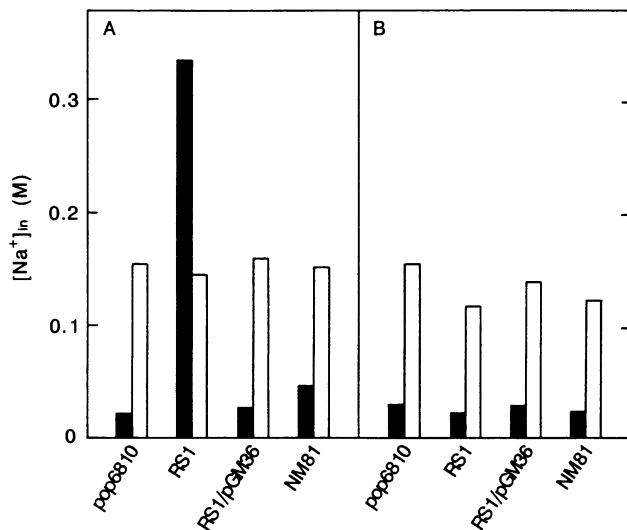

FIG. 3. Internal sodium concentration. Cells were grown in medium M488 (A) and medium M570 (B). NaCl (0.1 M) was added to cells at the logarithmic growth phase, and they were allowed to grow for 30 min. The medium was divided into two parts. One was used for measuring the internal sodium concentration as described in Materials and Methods (solid bars), and the other was allowed to grow for another 30 min in the presence of 100 μM CCCP and then used for measurement of the internal sodium concentration (open bars). The internal sodium concentration was calculated as described in the legend to Fig. 2.

It has been proposed that *E. coli* has at least two sodium/proton antiporters, NhaA and NhaB, and NM81 is deficient in only NhaA (23, 32). This was supported by our result that NM81 maintained a low cytoplasmic sodium concentration but that this level was higher than that in the wild-type strain (Fig. 3A). As described above, growth of NM81 at alkaline pH was more resistant to sodium than was growth of RS1.

No accumulation of sodium ion was observed at pH 7.0 in any of the strains we used (Fig. 3B), suggesting that at a neutral pH, RS1 has an extrusion system for sodium ions that functions similarly to that of other strains.

We next observed the downward movement of sodium ions from cells preloaded with these ions. Both NM81 and pop6810 cells expelled sodium ions quickly in an alkaline medium (Fig. 4A). In contrast, extrusion was very slow in RS1 cells at alkaline pH (Fig. 4) but rapid at neutral pH (Fig. 4B). These results again support the above suggestion that RS1 has no sodium ion extrusion activity at alkaline pH.

Is the mutated gene of RS1 *nhaB*? Our results suggest that, in addition to Δ*nhaA*, RS1 has a second mutation which affects sodium ion extrusion. Is the second gene *nhaB*? Recently, Pinner et al. (26) mapped *nhaB* at 25.5 min. We isolated tetracycline-resistant transductants of RS1 with P1*kc* grown on *tet*-marked strains. By looking at the growth of the transductants on the medium RM688 plate containing 0.2 M NaCl, we found that the gene compensating the mutation of RS1 was cotransduced with a marker at 25.1 min (frequency = 0.22, *n* [total number tested] = 94) and at 26.3 min (frequency = 0.02, *n* = 52) but not with a marker at 24.3 min (*n* = 89). These results suggested that *nhaB* was altered

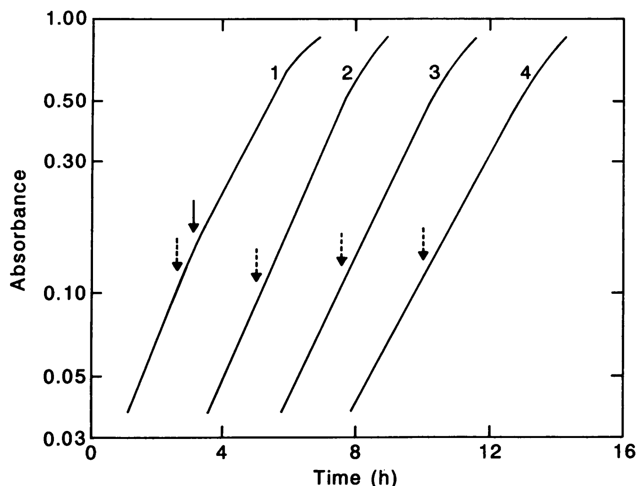

FIG. 5. Growth of RS1 in the presence of CCCP. RS1 (curve 1) was grown in medium M488 containing 1% glucose instead of lactate, and 100 μM CCCP was added at the solid arrow. Strains pop6810 (curve 2), RS1 (curve 3) and RS1/pGM36 (curve 4) were grown in medium M488 containing 1% glucose instead of lactate in the presence of 100 μM CCCP. The medium pH values were measured at the dotted arrows and were 8.7, 8.6, 8.6, and 8.5 for curves 1, 2, 3, and 4, respectively.

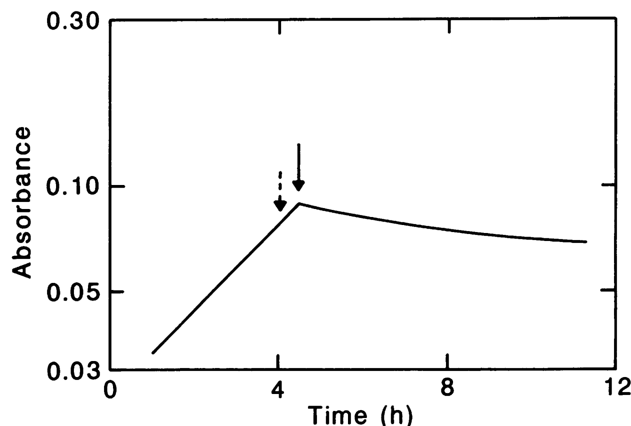

FIG. 6. Effect of CCCP on the growth of RS1. RS1 was grown in medium M488 containing 0.5% sodium succinate instead of lactate, and 100 μM CCCP was added at the solid arrow. The medium pH measured at the dotted arrow was 8.8.

in pop6810 and hence that its Δ*nhaA* transductant RS1 had no sodium ion extrusion activity at alkaline pH.

Thelen et al. (33) also mapped *nhaB* to 25.6 min by using HIT-1 (14). However, all tetracycline-resistant transductants of HIT-1 Δ*nhaA* with P1*kc* grown on the *tet*-marked strain at 25.1 min failed to grow on the medium RM688 plate containing 0.2 M NaCl (n = 60). It is therefore suggested that the mutated gene of HIT-1 is different from that of RS1 or that HIT-1 has another mutation.

Growth of RS1 in an alkaline medium containing CCCP. It has been proposed that the cytoplasmic pH is kept neutral by the sodium/proton antiporter in *E. coli* growing in alkaline media (37, 38). However, the above data obtained with RS1 suggested that the sodium circulation was not essential for growth at alkaline pH when the growth medium contained a low sodium concentration (less than 3 mM; see Materials and Methods). This notion was reinforced by the following observations.

RS1 grew without any lag time at alkaline pH after the addition of CCCP (Fig. 5, curve 1). pop6810, RS1, and RS1/pGM36 all grew in the presence of CCCP at the same growth rate (Fig. 5, curves 2 to 4). When succinate was used instead of glucose, no growth was observed after the addition of CCCP (Fig. 6), suggesting that ATP synthesis via oxidative phosphorylation was inhibited by this drug under our growth conditions. The active accumulations of proline and serine were inhibited by CCCP at alkaline pH (Fig. 7 and 8). CCCP completely inhibits the accumulations of tetraphenylphosphonium ion and methyl thiogalactoside in cells growing in alkaline media (22). These results clearly show that CCCP indeed functions as a protonophore under our growth conditions but that the growth is not inhibited by this agent. Even if RS1 has some residual activity of the sodium/proton antiporter, it would be inhibited by CCCP. It is therefore suggested that the operation of the sodium/proton antiporter is not required for growth at alkaline pH.

Since pop6810 and RS1 are proline auxotrophs, the uptake of proline is essential for growth. Our data showed that the active accumulation of proline was inhibited by CCCP (Fig. 7). However, the downward movement of proline mediated by its carrier is not inhibited by CCCP and may in fact be stimulated. The internal level of proline was equal to the external level in cells growing in the presence of CCCP (data not shown).

In previous studies (9, 16), growth stopped for a time after the addition of CCCP and then resumed. However, no cessation of growth was observed in the present study (Fig. 5, curve 1). Besides RS1, the wild-type strain W3110 also grew without any lag time after the addition of CCCP under the same conditions. Since there was no lag time at alkaline or at neutral pHs, growth cessation may not be due to the pH of the medium. Thus, the cessation of growth after the

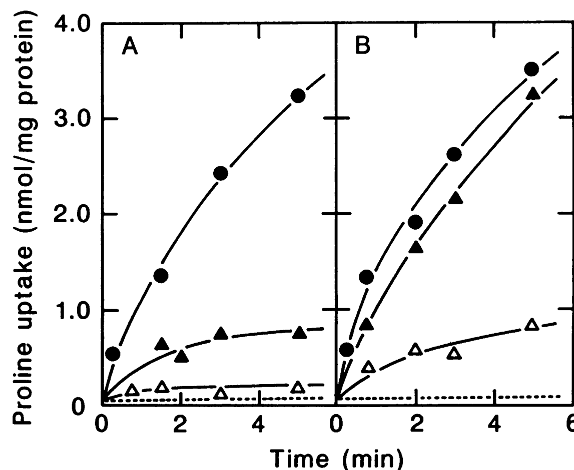

FIG. 7. Proline transport activity of RS1. RS1 (A) and RS1/pGM36 (B) were grown in medium M488, and the proline transport activity was measured as described in Materials and Methods. Symbols: △, grown in the presence of 20 mM NaCl and assayed in the presence of 20 mM NaCl; ▲, grown in the presence of 20 mM NaCl and assayed in the absence of 20 mM NaCl; ●, grown in the absence of 20 mM NaCl and assayed in the absence of 20 mM NaCl; – – –, assayed in the presence of 100 μM CCCP.

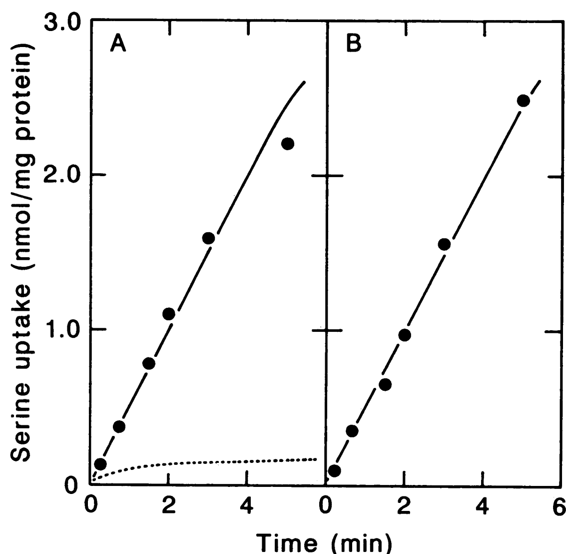

FIG. 8. Serine transport activity of RS1. RS1 (A) and RS1/pGM36 (B) were grown in medium M488, and their serine transport activity was measured as described in Materials and Methods. The dotted line represents the transport activity in the presence of 100 μM CCCP.

addition of CCCP may be restricted to the particular media used in those previous studies (9, 16).

Transport activities of proline and serine in RS1. It has been reported that NM81 can take up melibiose and that this process is driven by sodium circulation (23). Proline transport was also reported to be driven by sodium circulation (7, 21, 31, 36). We therefore examined the proline transport activity of RS1 and found it to be the same as that of RS1 harboring pGM36 (Fig. 7). As described above (Fig. 2 and 3), no sodium gradient (interior low) was generated in RS1, whereas RS1 harboring pGM36 maintained a low internal sodium concentration, resulting in the generation of the sodium gradient (interior low).

When RS1 was grown in the presence of 20 mM NaCl, the transport activity of proline was greatly reduced (Fig. 7). Since the high internal sodium concentration decreases the sodium ion motive force, it might be assumed that the decrease in the proline uptake shown in Fig. 7 is due to the decrease in the motive force. However, this is unlikely, inasmuch as the activity was partially recovered when measured without the addition of NaCl to further decrease the sodium ion motive force.

The proline transport activity in RS1 harboring pGM36 was reduced when cells were grown in the presence of sodium ions, but the activity was completely recovered when sodium ions were not added to the assay medium. These results suggest that the proline transport system is not driven by the sodium gradient across the cell membrane at alkaline pH in RS1 but is inhibited by the high internal sodium concentration.

It has been reported that serine transport is also driven by sodium circulation (11). Again, the serine transport activities of RS1 and RS1 harboring pGM36 were the same in the medium containing only contaminating sodium ions (Fig. 8).

DISCUSSION

In agreement with the report of Padan et al. (23), we found that NM81 had a sodium ion extrusion activity at alkaline pH, although the activity was low compared with that of the wild-type strain. Taglicht et al. (32) have proposed that *E. coli* has two systems, NhaA and NhaB, for sodium-proton exchange and that NM81 is deficient only in NhaA. In contrast to NM81, RS1 accumulated sodium ions in an alkaline medium, suggesting that RS1 is deficient in all systems for sodium ion extrusion functioning at alkaline pH.

The sodium/proton antiporter activity has been examined mainly by two methods: measurement of the change in the pH gradient across the membrane vesicles (23), and measurement of the change in the external sodium concentration (33). Our method was different from these; we measured the change in cytoplasmic sodium concentration directly. The sodium concentration that accumulated at alkaline pH was threefold the concentration of sodium from the medium that coprecipitated with cells. Our method was reproducible; the sodium concentrations that accumulated after 30 min were the same in duplicate experiments shown in Fig. 2 and 3. These results supported the reliability of our method for measuring the internal sodium ion concentration. In general, we believe that direct measurements are better than indirect methods. Therefore, we used a direct method to measure the movement of sodium ions in the present study.

What is the route for the sodium ion accumulation observed in RS1 at alkaline pH? Heefner and Harold (13) reported the passive movement of sodium ions driven by the membrane potential in streptococci and they called this movement "energized leak." Thus, it is possible to assume that *E. coli* generating the membrane potential can accumulate sodium ions via energized leak. The accumulation of sodium ions observed in RS1 at alkaline pH was much slower than its extrusion mediated by a specific transport system in a neutral medium (Fig. 2), supporting our assumption. It should be noted that such passive movement is difficult to detect in cells with a sodium ion extrusion activity.

It is still not clear which system is deficient in RS1. Since our data showed that the mutated gene of RS1 was located at 25 min, it is quite possible that RS1 has a mutation in *nhaB*. It has been reported that the extrusion system encoded by *nhaB* functions at the same activity level within the wide range of pHs from 6.5 to 8.5 (23, 33), and RS1 can extrude sodium ions at a neutral pH. These findings may therefore rule out this possibility. However, since it is quite unusual for the activity to be the same at any pH value, the activity of NhaB may be higher at neutral than at alkaline pH. Thus, one possible explanation is that the activity of NhaB is reduced by some mutation in RS1 and hence that mutated NhaB cannot function at alkaline pH. It is also possible that *E. coli* has a third gene affecting sodium circulation, for example a regulatory gene for the NhaB system.

Our data clearly showed that if RS1 had sodium ion extrusion activity, it was undetectable by our methods. Many previous reports have suggested that the sodium circulation has a central role in pH homeostasis in *E. coli*. We found here that RS1 grew in the virtual absence of sodium ion extrusion activity at alkaline pH. Furthermore, both RS1 and its parent strain grew in the presence of CCCP and glucose at high pH, above 8.5. Even if RS1 possesses the residual activity of the sodium/proton antiporter, the antiporter is unable to function in the presence of CCCP, that is, in the absence of the proton motive force. These data rather support the opposing view that there is no pH homeostasis in *E. coli* growing in alkaline media and that this bacterium can adapt itself to an alkaline cytoplasmic pH, probably by making changes in its metabolism (22).

Another important finding was that the transport activities for proline and serine are independent from the sodium gradient in an alkaline medium. There are two possible explanations. One is that they can be driven by either the proton motive force or the sodium circulation and that the proton motive force is a primary energy source at alkaline pH. There have been many reports to suggest that sodium ion transport systems can transport protons instead. For example, sodium/potassium ATPase can transport protons (12, 27). Bacterial sodium ion-translocating ATPase pumps protons at a low sodium concentration (17). Conversely, proton/potassium ATPase can transport sodium ions instead of protons (28). The bacterial melibiose carrier uses sodium ions and protons as the symporter cations (34). The discrimination between sodium ions and protons does not seem to be strict in all the biological transport systems.

E. coli has multiple transport systems for proline. The major system is PutP, which is driven by the sodium motive force (21, 36). Therefore, the second possible explanation is that the proline transport system driven by the proton motive force generally functions instead of the PutP system at alkaline pH. The proline transport activity was low in the mutant with only PutP at alkaline pH (unpublished observation). Construction of RS1 with only PutP is required before this problem can be solved.

McMorrow et al. (20) have shown that *E. coli* was unable to grow at pH 8.5 when the sodium concentration in the medium was less than 100 μM. Our present data suggest that the maintenance of a low internal sodium concentration is essential for growth in media containing a high concentration of sodium. Thus, the maintenance of the intracellular sodium concentration at a certain low level may be important for the growth of *E. coli* at alkaline pH.

On the basis of these findings, we propose that the primary function of the sodium/proton antiporter is to maintain a low cytoplasmic sodium concentration at alkaline pH when *E. coli* is growing in alkaline media containing a high sodium concentration. It is then necessary to determine the physiological role of sodium circulation in neutral and acidic media. Sodium extrusion in the wild-type strain is inhibited by CCCP at neutral pH (2, 3, 6). CCCP equilibrated the internal sodium concentration at both neutral and alkaline pHs (Fig. 3). A high sodium concentration, above 0.2 M, inhibited the growth at pH 7.5 in the presence of CCCP (unpublished observation). These results suggest that the maintenance of a low internal sodium concentration is also required for growth at a neutral pH and that this may be mediated by NhaB. A Δ*nhaB* mutant would be useful for further studies of this problem. Thelen et al. (33) have mapped the gene affecting the sodium/proton antiporter activity at 25.6 min in their mutant HIT-1, and they proposed that HIT-1 is a mutant deficient in *nhaB*. We found that the transductant of W3133-2, the parent strain of HIT-1, with P1*kc* grown on NM81 had a low sodium ion extrusion activity in an alkaline medium and that therefore HIT-1 Δ*nhaA* obtained by P1 transduction with NM81 as the donor also had a low extrusion activity (data not shown). The gene for supporting the growth on the medium RM688 plate containing 0.2 M NaCl was not cotransduced with a *tet* marker at 25.1 min. Therefore, the mutated gene in HIT-1 may be different from *nhaB*; alternatively, HIT-1 may contain another mutation. Another mutant deficient in *nhaB* is required for further studies of this problem.

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REFERENCES

1. **Bakker, E. P.** 1990. The role of alkali-cation transport in energy coupling of neutrophilic and acidophilic bacteria: an assessment of methods and concepts. FEMS Microbiol. Rev. **75**:319–344.
2. **Bassilana, M., E. Damiano, and G. Leblanc.** 1984. Relationships between the Na^+-H^+ antiport activity and the components of the electrochemical proton gradient in *Escherichia coli* membrane vesicles. Biochemistry **23**:1015–1022.
3. **Beck, J. C., and B. P. Rosen.** 1979. Cation/proton antiport systems in *Escherichia coli*: properties of the sodium/proton antiporter. Arch. Biochem. Biophys. **194**:208–214.
4. **Booth, I. R.** 1985. Regulation of cytoplasmic pH in bacteria. Microbiol. Rev. **49**:359–378.
5. **Brown, I. I., M. Y. Galperin, A. N. Glagolev, and V. P. Skulachev.** 1983. Utilization of energy stored in the form of Na^+ and K^+ ion gradients by bacterial cells. Eur. J. Biochem. **134**:345–349.
6. **Castle, A. M., R. M. Macnab, and R. G. Shulman.** 1986. Measurement of intracellular sodium concentration and sodium transport in *Escherichia coli* by ^{23}Na nuclear magnetic resonance. J. Biol. Chem. **261**:3288–3294.
7. **Chen, C. C., T. Tsuchiya, Y. Yamane, J. M. Wood, and T. H. Wilson.** 1985. Sodium (lithium)-proline cotransport in *Escherichia coli*. J. Membr. Biol. **84**:157–164.
8. **Dibrov, P. A.** 1991. The role of sodium ion transport in *Escherichia coli* energetics. Biochim. Biophys. Acta **1056**:209–224.
9. **Fraimow, H. S., J. B. Greenman, I. M. Leviton, T. J. Dougherty, and M. H. Miller.** 1991. Tobramycin uptake in *Escherichia coli* is driven by either electrical potential or ATP. J. Bacteriol. **173**:2800–2808.
10. **Goldberg, E. B., T. Arbel, J. Chen, R. Karpel, G. A. Mackie, S. Schuldiner, and E. Padan.** 1987. Characterization of a Na^+/H^+ antiporter gene of *Escherichia coli*. Proc. Natl. Acad. Sci. USA **84**:2615–2619.
11. **Hama, H., T. Shimamoto, M. Tsuda, and T. Tsuchiya.** 1987. Properties of a Na^+-coupled serine-threonine transport system in *Escherichia coli*. Biochim. Biophys. Acta **905**:231–239.
12. **Hara, Y., and M. Nakao.** 1986. ATP-dependent proton uptake by proteoliposomes reconstituted with purified Na^+,K^+-ATPase. J. Biol. Chem. **261**:12655–12658.
13. **Heefner, D. L., and F. M. Harold.** 1980. ATP-linked sodium transport in *Streptococcus faecalis*. I. The sodium circulation. J. Biol. Chem. **255**:11396–11402.
14. **Ishikawa, T., H. Hama, M. Tsuda, and T. Tsuchiya.** 1987. Isolation and properties of a mutant of *Escherichia coli* possessing defective Na^+/H^+ antiporter. J. Biol. Chem. **262**:7443–7446.
15. **Karpel, R., Y. Olami, D. Taglicht, S. Schuldiner, and E. Padan.** 1988. Sequencing of the gene *ant* which affects the Na^+/H^+ antiporter activity in *Escherichia coli*. J. Biol. Chem. **263**:10408–10414.
16. **Kinoshita, N., T. Unemoto, and H. Kobayashi.** 1984. Proton motive force is not obligatory for growth of *Escherichia coli*. J. Bacteriol. **160**:1074–1077.
17. **Laubinger, W., and P. Dimroth.** 1989. The sodium ion translocating adenosinetriphosphatase of *Propionigenium modestum* pumps protons at low sodium ion concentrations. Biochemistry **28**:7194–7198.
18. **Lennox, E. S.** 1955. Transduction of linked genetic characters of the host by bacteriophage P1. Virology **1**:190–206.
19. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
20. **McMorrow, I., H. A. Shuman, D. Sze, D. M. Wilson, and T. H. Wilson.** 1989. Sodium/proton antiport is required for growth of

Escherichia coli at alkaline pH. Biochim. Biophys. Acta **981:** 21–26.

21. **Mogi, T., H. Yamamoto, T. Nakao, I. Yamato, and Y. Anraku.** 1986. Genetic and physical characterization of *putP*, the proline carrier gene of *Escherichia coli* K12. Mol. Gen. Genet. **202:**35–41.
22. **Mugikura, S., M. Nishikawa, K. Igarashi, and H. Kobayashi.** 1990. Maintenance of a neutral cytoplasmic pH is not obligatory for growth of *Escherichia coli* and *Streptococcus faecalis* at an alkaline pH. J. Biochem. **108:**86–91.
23. **Padan, E., N. Maisler, D. Taglicht, R. Karpel, and S. Schuldiner.** 1989. Deletion of *ant* in *Escherichia coli* reveals its function in adaptation to high salinity and an alternative Na^+/H^+ antiporter system(s). J. Biol. Chem. **264:**20297–20302.
24. **Padan, E., and S. Schuldiner.** 1986. Intracellular pH regulation in bacterial cells. Methods Enzymol. **125:**337–352.
25. **Padan, E., D. Zilberstein, and S. Schuldiner.** 1981. pH homeostasis in bacteria. Biochim. Biophys. Acta **650:**151–166.
26. **Pinner, E., E. Padan, and S. Schuldiner.** 1992. Cloning, sequencing, and expression of the *nhaB* gene, encoding a Na^+/H^+ antiporter in *Escherichia coli*. J. Biol. Chem. **267:**11064–11068.
27. **Polvani, C., and R. Blostein.** 1988. Protons as substitutes for sodium and potassium in the sodium pump reaction. J. Biol. Chem. **263:**16757–16763.
28. **Polvani, C., G. Sachs, and R. Blostein.** 1989. Sodium ions as substitutes for protons in the gastric H,K-ATPase. J. Biol. Chem. **264:**17854–17859.
29. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
30. **Schuldiner, S., and H. Fishkes.** 1978. Sodium-proton antiport in isolated membrane vesicles of *Escherichia coli*. Biochemistry **17:**706–711.
31. **Stewart, L. M. D., and I. R. Booth.** 1983. Sodium involvement in proline transport in *Escherichia coli*. FEMS Microbiol. Lett. **19:**161–164.
32. **Taglicht, D., E. Padan, and S. Schuldiner.** 1991. Overproduction and purification of a functional Na^+/H^+ antiporter coded by *nhaA* (*ant*) from *Escherichia coli*. J. Biol. Chem. **266:**11289–11294.
33. **Thelen, P., T. Tsuchiya, and E. B. Goldberg.** 1991. Characterization and mapping of a major Na^+/H^+ antiporter gene of *Escherichia coli*. J. Bacteriol. **173:**6553–6557.
34. **Tsuchiya, T., and T. H. Wilson.** 1978. Cation-sugar cotransport in the melibiose transport system of *Escherichia coli*. Membr. Biochem. **2:**63–79.
35. **West, I. C., and P. Mitchell.** 1974. Proton/sodium ion antiport in *Escherichia coli*. Biochem. J. **144:**87–90.
36. **Yamato, I., M. Ohsawa, and Y. Anraku.** 1990. Defective cation-coupling mutants of *Escherichia coli* Na^+/proline symport carrier: characterization and localization of mutations. J. Biol. Chem. **265:**2450–2455.
37. **Zilberstein, D., V. Agmon, S. Schuldiner, and E. Padan.** 1982. The sodium/proton antiporter is part of the pH homeostasis mechanism in *Escherichia coli*. J. Biol. Chem. **257:**3687–3691.
38. **Zilberstein, D., V. Agmon, S. Schuldiner, and E. Padan.** 1984. *Escherichia coli* intracellular pH, membrane potential, and cell growth. J. Bacteriol. **158:**246–252.