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

TAKAO OHYAMA, KAZUEI IGARASHI, AND HIROSHI KOBAYASHI*

Faculty of Pharmaceutical Sciences, Chiba University, Chiba 263, Japan

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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. Guiffanti, 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 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 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 firstly 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 function 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 Tn10 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 K₂HPO₄, 20 mM NH₄Cl, 1 mM MgCl₂, 0.1 mM CaCl₂, 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-hydroxyethylpipеразин-N'-2-этиленацетоновокислая, 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 AvaI, both ends were filled in, and the HindIII-AvaI fragment containing the erythromycin resistance gene was

* Corresponding author. Mailing address: Faculty of Pharmaceutical Sciences, Chiba University, 1-33, Yayoi-cho, Inage-ku, Chiba, 263, Japan. Phone: 81-43-290-2898. Fax: 81-43-290-2900 or 81-43-255-1574.
TABLE 1. E. coli strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Origin or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>W3110</td>
<td>Wild type</td>
<td>Y. Anraku</td>
</tr>
<tr>
<td>NM81</td>
<td>melB Lid anti* lacZY nhaA::Km' thr</td>
<td>E. Padan (19)</td>
</tr>
<tr>
<td>pop6810t</td>
<td></td>
<td>Y. Anraku</td>
</tr>
<tr>
<td>RS1b</td>
<td></td>
<td>pop6810 x P1 (NM81)</td>
</tr>
<tr>
<td>TO100</td>
<td>W3110 recD::Tn10 chaA::Em'</td>
<td>This study</td>
</tr>
<tr>
<td>TO101</td>
<td>W3110 chaA::Cm'</td>
<td>TO110 x P1 (TO100)</td>
</tr>
<tr>
<td>TO110</td>
<td>W3110 chaA::Cm'</td>
<td>TO110 x P1 (NM81)</td>
</tr>
<tr>
<td>TO111</td>
<td>W3110 nhaA::Km'</td>
<td>TO111 x P1 (TO110)</td>
</tr>
<tr>
<td>TO112</td>
<td>W3110 nhaA::Km' chaA::Cm'</td>
<td>TO111 x P1 (TO110)</td>
</tr>
<tr>
<td>TO113</td>
<td>W3110 nhaA::Km' nhaB::Em'</td>
<td>TO112 x P1 (TO113)</td>
</tr>
<tr>
<td>TO114</td>
<td>W3110 nhaA::Km' nhaB::Em' chaA::Cm'</td>
<td>TO112 x P1 (TO113)</td>
</tr>
</tbody>
</table>

* Km, kanamycin; Cm, chloramphenicol; Em, erythromycin.

with p13H6CM cut with BglI (BglI site was located on the vector). The ΔchaA 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.

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 P1kc (14), and protein determination (15) were carried out as described before.

Chemicals. 45CaCl₂ 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 ΔnhaA mutant, RS1, isolated in our laboratory by transduction of strain pop6810 with P1kc 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.
In order to identify the gene encoding the sodium ion extrusion system which functions at alkaline pH in ΔchaA 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 BamHI-HindIII 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 EcoRV-HindIII fragment of 13H6 could not compensate for the mutation of RS1, this suggested that the gene located between the BamHI and EcoRV 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 ΔchaA mutant at an alkaline pH is mediated mainly by ChaA.

For further characterization of the chaA product, we constructed a ΔchaA mutant. The sensitivity of the growth of TO110 (W3110 ΔchaA) 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 ΔnhaA; 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 NM8191AB, a derivative of NM81 which became resistant to sodium ions again (6). Therefore, a possible explanation for this discrepancy might be that the ChaA of NM81 has a low activity because of some unknown mutation and that this mutation reverted in the NM8191AB 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', chloramphenicol resistance gene.
Calcium ion movement in a ΔchaA 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 CaCl₂ 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 Ca²⁺ 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 to the calcium ion concentration of the medium have been mapped to 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 RSI, suggesting that NhaB activity is low at alkaline pH. In order to confirm this, we constructed a ΔnhaB mutant. Mutant TO113 (W3110 ΔnhaA ΔnhaB) 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 ΔnhaA ΔnhaB ΔchaA) 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 ΔnhaB); the sensitivity of

FIG. 5. Calcium ion uptake by everted membrane vesicles of the ΔchaA 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 ΔnhaA ΔchaA 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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REFERENCES


