Note

Sodium ATPase and Sodium/proton Antiporter Are Not Obligatory for Sodium Homeostasis of Enterococcus hirae at Acid pH

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Enterococcus hirae grows in a broad pH range from 5 to 11. An E. hirae mutant 7683 lacking the activities of two sodium pumps, Na⁺-ATPase and Na⁺/H⁺ antiporter, does not grow in high Na⁺ medium at pH above 7.5. We found that 7683 grew normally in high Na⁺ medium at pH 5.5. Although an energy-dependent sodium extrusion at pH 5.5 was missing, the intracellular levels of Na⁺ and K⁺ were normal in this mutant. The Na⁺ influx rates of 7683 and two other strains at pH 5.5 were much slower than those at pH 7.5. These results suggest that Na⁺ elimination of this bacterium at acid pH is achieved by a decrease in Na⁺ entry and a normal K⁺ uptake.

Key words: Na⁺-ATPase; Na⁺/H⁺ antiporter; Na⁺ homeostasis; Enterococcus hirae

Sodium circulation is driven by active transport systems that extrude sodium ions and maintain the Na⁺ concentration gradient directed inward.¹,² Bacteria have evolved diverse mechanisms for active sodium extrusion. Secondary Na⁺/H⁺ antiporters are widely distributed,³ and some bacteria produce primary sodium pumps coupled with chemical reactions such as decarboxylation,⁴ electron transport⁵ and ATP hydrolysis.⁶ Na⁺ reenters the cells via transport systems using the energy of a Na⁺ gradient such as Na⁺-coupled secondary co-transporters.⁷ Moreover, the Na⁺ gradient is used for ATP synthesis and flagellar motion in some bacteria.⁴,⁸

The Gram-positive bacterium Enterococcus hirae grows in a broad pH range from 5 to 11.⁹ This bacterium has two sodium extrusion systems: a NapA Na⁺/H⁺ antiporter¹⁰,¹¹ and a vacuolar Na⁺-translocating ATPase.¹² E. hirae lacks the respiratory chain. The electrochemical concentration gradient of protons (proton potential) is generated by proton extrusion via the F₁F₀, H⁺-translocating ATPase.¹³ Since the activity of the H⁺-ATPase is optimal around pH 6.5, the proton potential is generated well at acid pH but is minimal at alkaline pH.⁹ Therefore, Na⁺/H⁺ antiporter operates for Na⁺ extrusion only at acid pH. The Na⁺-ATPase is important for Na⁺ extrusion under conditions where the proton potential is low, such as at alkaline pH.¹⁴ There is no clear evidence to suggest that there are Na⁺ gradient-consuming systems in this bacterium. It has been suggested that Na⁺ circulation is not always required for the growth of E. hirae in high K⁺ culture conditions.¹⁵ Therefore, it is speculated that the physiological role of sodium extrusion systems in this bacterium may be the elimination of Na⁺ from cytoplasm to make room for K⁺ accumulation.¹⁶,¹⁷

A mutant 7683 defective in both activities of Na⁺/H⁺ antiporter and Na⁺-ATPase has been isolated;¹⁸ no glucose-dependent sodium extrusion was observed by this mutant at pH 8.¹⁸,¹⁹ This double mutant did not grow at pH 8 in a complex medium NaTY, containing high Na⁺ and traces of K⁺,¹⁴,¹⁸ where the ratio of intracellular Na⁺ and K⁺ was nearly equivalent to that of external ones. In this study, we found that 7683 as well as the wild-type grew normally in NaTY medium at acid pH. The intracellular Na⁺ concentration of this mutant was maintained at a low level although sodium extrusion did not occur. The rate of Na⁺ influx decreased greatly at acid pH rather than alkaline pH, suggesting that Na⁺ elimination of this bacterium is achieved by a decrease in Na⁺ entry at acid pH.

All of the experiments were done with E. hirae ATCC 9790 (wild-type), 7683,¹⁸ which is deficient in both activities of Na⁺-ATPase and Na⁺/H⁺ antiporter, and WD4,¹¹ in which the Na⁺/H⁺ antiporter gene is disrupted, derived from 9790. Cells were cultured in a complex medium NaTY (10 g of Difco tryptone, 5 g of Difco yeast extract, 8.5 g of Na₂HPO₄ and 10 g of glucose per liter) and KTY (the same as above except for replacing Na₂HPO₄ with 10 g of K₂HPO₄).¹⁰ The Na⁺ and K⁺ concentrations of NaTY were 120 and 15 mM, respectively, and those of KTY were 20 and 130 mM, respectively. The medium pH was adjusted by adding HCl. Cell growth was monitored by measuring the optical density at 540 nm with a spectrophotometer and the growth rates were measured between the optical densities of 0.1 and 0.2; the stated values of medium pH represent the initial pH. Potassium and sodium contents of growing cells were measured by flame photometry.

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cells (10 ml) at the middle-logarithmic phase were filtered through membrane filters (pore size, 0.4 mm; Nucleopore polycarbonate, Costar Scientific Co.), washed twice with 2 mM MgSO₄. The samples were obtained after extraction from the trapped cells with hot 5% trichloroacetic acid. Cytoplasmic water space was taken to be 2 μl/mg of protein. The sodium extrusion activity was assayed as described previously. 9) 9790 and 7683 were cultured in NaTY medium at pH 5.5 or 6.2. Cells were harvested at late logarithmic phase, washed twice with 2 mM MgSO₄, and suspended in 50 mM Tris-maleate (pH 6.0) containing 0.4 mM KCl, at a cell density of 1 mg/ml. After incubation with 20 mM ²²NaCl (2.3 MBq/mmol) at 25°C for 60 min, the reaction was initiated by addition of 10 mM glucose. At intervals, cell samples were collected on filters and the radioactivity on the filters was counted in a liquid scintillation counter. Na⁺ influx was examined as follows. Cells were cultured in NaTY medium, harvested at middle-logarithmic phase, and loaded with K⁺ using 2,4-dinitrophenol as described previously; 30) the ATP level was depleted in these cells. Cells were suspended in 50 mM Tris-maleate containing 0.4 mM KCl, at various pHs, and Na⁺ influx was started by addition of 200 mM NaCl.

The growth of the wild-type 9790 and mutant 7683 at various pHs was first examined (Figs. 1A and 1B). As reported elsewhere, 9) 9790 grew well at the pH range from 5.5 to 8.0 in NaTY (Na⁺-rich) medium as well as KTY (K⁺-rich) medium with the growth rates (h⁻¹) of around 1 (Fig. 1A). 7683 grew normally at these pHs in KTY medium with the growth rates of about 0.9 (Fig. 1B). This mutant did not grow in NaTY medium at pH above 7.8 (Fig. 1B) as reported previously. 18) Interestingly, 7683 grew in NaTY medium at acid pH; the growth rate in NaTY at pH 5.5 was equivalent with that in KTY medium at pH 5.5 (Fig. 1B). The intracellular concentrations of Na⁺ and K⁺ of 9790 and 7683 cells growing in NaTY medium at various pHs were estimated (Figs. 1C and 1D). The internal concentrations of K⁺ and Na⁺ of 9790 were 450–520 mM and 30–50 mM, respectively, at pH from 5.5 to 7.7 (Fig. 1C). As the sodium extrusion activity at pH 7.7 was impaired in 7683, 10) the internal concentrations of K⁺ and Na⁺ of 7683 at pH 7.7 were 50 and 500 mM, respectively. At acid pH range, however, the Na⁺ level of 7683 decreased in inverse proportion to an increase in the K⁺ level (Fig. 1D). The internal concentrations of K⁺ and Na⁺ of 7683 at pH 5.5 were 480 and 50 mM, respectively, equivalent with those of 9790 at pH 5.5 (Figs. 1C and 1D). In parallel with cell growth, the intracellular K⁺ and Na⁺ concentrations of 7683 were thus maintained at normal levels at acid pH.

Sodium extrusion by 7683 has not been examined at acid pH. 18) Since the genes for these two Na⁺ extrusion systems were not knocked out in 7683, it is possible that the activities may be somewhat active at acid pH. Figure 2 compares glucose-dependent ²²Na⁺ movement by 9790 and 7683 at pH 5.5 and 6.2. Cells were cultured in NaTY medium. Glucose-dependent ²²Na⁺ extrusion was observed by 9790 at pH 5.5 or 6.2, which was inhibited by addition of a protonophore: carbonyl cyanide m-chlorophenylhydrazone and valinomycin (Figs. 2A and 2C). Sodium extrusion is thus characterized by the activity of the Na⁺/H⁺ antiporter. Although the Na⁺-ATPase, which is optimal at pH 9.5, 12) was induced in this high Na⁺ medium, 14) its activity was negligible at acid pHs. Glucose-dependent sodium extrusion was not observed by 7683 at pH 5.5 and 6.2 (Figs. 2B and 2D). The results clearly indicate that both activities of the Na⁺-ATPase and Na⁺/H⁺ antiporter were inactive in this mutant, not participating in Na⁺ homeostasis at acid pH. It is possible that an unidentified sodium extrusion system, which is not linked with ATP and/or the proton potential and only active at acid pH, operates in this bacterium; its activity may not be observed under the assay condition as shown in Fig. 2. We are unable to clearly exclude this possibility. However, when sodium extrusion was
examined by the Na⁺-loaded cells in NaTY medium (pH 5.5), a decrease in the intracellular Na⁺ level was markedly observed in 9790 but not in 7683 (data not shown). Therefore, the contribution of an additional system, even if it were present, for sodium extrusion should be insignificant in vivo.

The intracellular ion level is balanced by uptake and extrusion of the ion. It is possible that the maintenance of the internal Na⁺ at a low level is due to reduced Na⁺ uptake at acid pH. Although the pathway of Na⁺ entry in E. hirae is still unknown, it has been reported that Na⁺ enters glycozyling E. hirae cells by a transport process of low affinity (Kᵢ > 20 mm) and high capacity (> 40 nmol/min/mg protein) at pH 7.2,⁵⁰ apparently in response to the membrane potential (Δψ). As shown in Figs. 2B and 2D, Na⁺ was taken up into 7683 cells when glucose was added, and it was inhibited by dissipation of Δψ. Glucose-dependent sodium uptake by 7683 at pH 5.5 (about 2 nmol/min/mg protein) was lower than that at pH 6.2 (about 8 nmol/min/mg protein) (Figs. 2B and 2D). Since Δψ generation at pH 5.5 was nearly equivalent with that at pH 6.2 under the experimental conditions of Figs. 2B and 2D,¹⁴,²² the activity of Na⁺ entry itself may be influenced by pH. Effects of external pH on energy-independent Na⁺ influx were examined (Fig. 3); Na⁺ uptake was initiated by addition of 200 mM NaCl in the absence of glucose. Na⁺ influx of 7683 was highly affected by external pH; the Na⁺ influx rates at pH 7.5, 6.0 and 5.5 were estimated as 3, 1 and 0.5 nmol/min/mg protein, respectively (Fig. 3A). pH-sensitive Na⁺ influx was also observed in 9790 (Fig. 3B) as well as WD4 defective in the NapA antiporter (Fig. 3C).¹¹ Although we cannot

Fig. 2. Sodium Movement by E. hirae at Acid pH.

9790 (A and C) 7683 (B and D) were cultured overnight in NaTY medium, washed twice with 2 mM MgSO₄ and suspended in buffer at pH 5.5 (A and B) or pH 6.2 (C and D). After incubation with 20 mM NaCl (2.3 MBq/µmol) for 60 min, the measurement of Na⁺ movement was started by addition of 10 mM glucose at 10 min. Symbols: open circles, without glucose; closed circles, with glucose; open triangles, with glucose (5 mM CCCP and 5 mM valinomycin were added at 5 min).

Fig. 3. Effects of pH on Na⁺ Uptake by E. hirae.

Cells were cultured overnight in NaTY medium, washed, and suspended in 2 mM MgSO₄ at 10 mg/ml of protein. The Na⁺ influx was initiated by suspending the cells into 50 mM Tris-maleate, at pH 5.5 (open circles). 6.0 (closed circles) or 7.5 (closed triangles), containing 400 msi KCl and 200 mM NaCl at 1 mg/ml of protein. The internal Na⁺ amounts were measured by flame photometry as described in the text. A, 7683; B, 9790; C, WD4.
decide whether the Na⁺ uptake reactions shown in Fig. 2 and in Fig. 3 are mediated by the same pathway, it is clear that Na⁺ influx of *E. hirae* greatly decreased at acid pH. This decreased Na⁺ uptake at acid pH is not an artifact, because it occurred under the physiological pH conditions (Figs. 1A and 1B). Instead, we think that it is the pH-sensitive manner of the Na⁺ entry pathway. Sodium extrusion is thus not important for Na⁺ homeostasis of *E. hirae* at acid pH. Therefore, it is likely that the intracellular balance of K⁺ and Na⁺ ions at acid pH (Fig. 1D) is maintained by the difference in the influx rates of these cations. In this bacterium, K⁺ uptake at acid pH is mediated by the KtrI K⁺ transport system, depending upon ATP and the proton potential;²⁰ K⁺ influx via the KtrI system was about 200 nmol/min/mg protein at pH 5.5,²⁰,²³ much higher than the Na⁺ influx as mentioned above. The pH-sensitive manner of Na⁺ entry is especially important for the K⁺/Na⁺ homeostasis of 7683 defective in sodium extrusion; the KtrI system was active in this mutant.³⁰ The Na⁺ elimination of this bacterium at acid pH is thus likely to be done by a decrease in Na⁺ entry.

Pathways for Na⁺ entry have been proposed in other microorganisms such as *E. coli*³⁰ and yeast.²³ Na⁺-coupled co-transporters likely operate for Na⁺ entry in *E. coli*. In yeast, Na⁺ influx is probably mediated by some transport systems with a broad substrate specificity such as Hxt3 (glucose transporter) and Hnm3 (choline transporter).²⁰ It is very important to characterize the Na⁺ entry pathway(s) at the molecular level for understanding the homeostasis of K⁺ and Na⁺ of *E. hirae*. However, the feature of Na⁺ entry has not been well characterized. The Na⁺/H⁺ antiporter does not participate in energy-independent Na⁺ influx (Fig. 3C). Recently we have found that the Na⁺ entry at alkaline pH is mediated by the KtrII K⁺ transport system³¹ (Kakinuma et al., unpublished results). This system, likely a Na⁺/K⁺ co-transporter, may be the Na⁺ entry pathway itself, but the details require further investigation.

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**References**


