Potassium/Proton Antiport System Is Dispensable for Growth of Enterococcus hirae at Low pH

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An energy-dependent $K^+/H^+$ antiport system is found in Enterococcus hirae ATCC 9790 cultured in a standard complex medium (Y. Kakinuma, and K. Igarashi, J. Biol. Chem. 263:14166-14170, 1988). We have now found that the activity of this antiport system was totally missing in cells cultured in a defined medium. In this defined medium, E. hirae did not grow well at pH near 9, but grew normally at pH below 7.5. This antiport system is important at high pH but dispensable at lower pH for ion homeostasis of this bacterium.

Key words: $K^+/H^+$ antiporter; ion homeostasis; Enterococcus hirae

Potassium is a major cytoplasmic cation in growing bacterial cells and is important for cell physiology. Cellular $K^+$ activates several cytoplasmic enzymes and is required for protein synthesis.1) $K^+$ movement across the cell membrane is involved in the maintenance of turgor pressure and in the regulation of cytoplasmic pH (pHin).3) $K^+$ is circulated across the membrane by a variety of transport systems in bacteria;8) characterization of $K^+$ transport systems at the molecular level is most advanced in Escherichia coli.4,5) In the Gram-positive bacterium Enterococcus hirae, two $K^+$ uptake systems, KtrI and KtrII, and one $K^+$ extrusion system, here designated Kep ($K^+$ extrusion pump), were reported.9) KtrI is the major $K^+$ uptake system and likely to be constitutive. Its features appear similar to the E. coli Trk system;6) both generation of proton motive force and ATP (or related high energy compound) are required for the reaction. KtrII is an inducible $K^+$ uptake system independent of proton motive force.7) Its activity is regulated, as well as the Na$^+$-ATPase activity, in response to medium Na$^+$ concentration.8) Recent molecular biological studies on a vacuolar-type Na$^+$-translocating ATPase of this bacterium indicated that one component of the KtrII system is encoded by the ntpJ gene, a tailed cistron of the Na$^+$-responsive Na$^+$-ATPase (ntp) operon.9) We assume that the reaction of the KtrII system is linked in some manner with the sodium electrochemical gradient generated by the action of the Na$^+$-ATPase, although the details are still obscure. Kep is a unique $K^+$ extrusion system because it extrudes $K^+$ uphill against a $K^+$ concentration gradient. $K^+$ extrusion by this system requires the generation of ATP (or a related metabolite) and occurs when the pHin is alkaline such as 9. Concomitantly with $K^+$ extrusion, the pHin falls from 9 to 8, at which point K$^+$ extrusion ceases. The Kep system is thus proposed to be the primary transport system that expels K$^+$ by exchange for H$^+$. We think that the Kep transport system is important for homeostasis of $K^+$ and H$^+$, especially for cytoplasmic pH regulation, in E. hirae in alkaline pH conditions.1,10,11)

In this paper we found that this Kep $K^+/H^+$ antiport activity was missing in this organism when cultured in a defined medium. In this defined medium, E. hirae did not grow at pH above 9.0, but grew normally at pH below 7.5. The results suggest the significance of the Kep system in E. hirae at high external pH. This system is dispensable for the physiology of this bacterium at acidic pH range.

Materials and Methods

E. hirae ATCC 9790, which was kindly supplied by F. M. Harold, Colorado State University, Fort Collins, was used. Cells were grown at 37°C in a standard complex medium: KTY (10 g of tryptone (Difco Laboratories), 5 g of yeast extract (Difco), 10 g of K$_2$HPO$_4$, and 10 g of glucose, per liter) or KPi medium, which is a modification of TrisM defined medium described previously;12) the concentration of K$_2$HPO$_4$ was increased from 10 to 50 mM. When necessary, the pH of these media were adjusted with HCl, KHCO$_3$ or K$_2$CO$_3$, but in all cases the final concentration of $K^+$ was adjusted to the same value by addition of KCl. The concentrations of $K^+$ and Na$^+$ of KTY complex medium were 120 mM and 20 mM, respectively, and those of $K^+$ and Na$^+$ of KPi defined medium were 120 mM and less than 1 mM, respectively. The growth was monitored by measuring the optical density at 600 nm with a Perkin-Elmer spectrophotometer (model 35). The growth rates were measured between the optical density of 0.1 and 0.2. There was no significant change in the medium pH during this period, remaining at the initial pH. For measurement of the $K^+/H^+$ antiport activity,10) cells were harvested at late logarithmic phase, washed twice with, suspended, and incubated in 2 mM MgSO$_4$ for 30 min on ice. By this treatment, the energy was depleted;14) the ATP level was decreased to less than 10% of the growing cells. For measurement of the uphill $K^+$ extrusion, cells were resuspended in 50 mM 2-[N-cyclohexylamino]ethanesulfonic acid (CHES)-KOH (pH 9.2) containing 0.2 M KCl and 50 mM diethanolamine-HCl, at a cell density of 1 mg/ml, and incubated at 25°C. At intervals, the cell samples (0.1 ml) were collect-

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ed by filtration through Millipore filter (0.45 μm pore size), washed twice with 2 mM MgSO₄, and extracted with hot 5% trichloroacetic acid. Portions were then analyzed for K⁺ by flame photometry. Background due to the binding of buffer K⁺ to the filters was subtracted from the data. To measure the uptake of [¹⁴C]methylamine (0.37 μBq/mm), cell samples collected on filters were counted in a liquid scintillation counter. The cytoplasmic water space was taken to be 2 μl/mg of cells. The pH gradient and the membrane potential across the cytoplasmic membrane, inside acid and negative, were measured with [¹²C]benzylamine (10 μM, 148 MBq/mol) and [³H]tetraphenylphosphonium ion (TPP⁺) (10 μM, 185 MBq/mol), respectively. The procedures have been described previously.¹¹

Results and Discussion

Streptococcus (Enterococcus) is an obligate heterotroph, requiring a variety of nutrients such as amino acids, vitamins, and nucleotide bases for growth. The defined medium KPi used here for *E. hirae* contains twenty amino acids, eight vitamins, and three bases. Strain 9790 grew well in this KPi medium at pH 7.5, with the doubling time of about 35 min, equivalent to the doubling time of about 30 min in the KTY standard complex medium at pH 7.5. No notable difference in morphology has been reported between *E. hirae* cells cultured in the complex medium and those cultured in the defined medium. Figure 1 shows movements of K⁺ and H⁺ (pHin) by the cells grown in different media: KTY and KPi media at pH 7.5. The K⁺ concentration of the buffer used for the experiment was about 250 mM. Since the internal K⁺ concentrations in cell grown on KTY (KTY cells) and KPi (KPi cells) media were calculated to be 350 and 300 mM, respectively, the K⁺ gradients [K⁺]i/[K⁺]o of these cells were insignificant under the experimental conditions. The internal pH of KTY and KPi cells suspended in the buffer containing 50 mM diethanolamine were estimated to be 8.9 and 8.9, respectively. Addition of glucose induced K⁺ extrusion in the KTY cells. Concomitantly, the internal pH was acidified from 8.9 to 8.1, and at which point K⁺ extrusion ceased (Fig. 1A). On the other hand, addition of glucose did not induce K⁺ extrusion and cytoplasmic acidification by the KPi cells (Fig. 1B); K⁺/H⁺ antiport activity was missing in the KPi cells. There are several explanations for the lack of the K⁺/H⁺ activity in the KPi cells. A possible explanation is likely that alteration of the permeabilities for K⁺ and H⁺ across the cell membrane and defect in energy production by glucose metabolism occurred when grown in the defined medium. The passive K⁺ efflux rates of these KTY and KPi cells were examined with K⁺-loaded cells suspended in K⁺-free buffer containing 200 mM Na⁺ at pH 9.2. Proton permeabilities of the KTY and KPi cells were also measured by monitoring the change of the medium pH after a proton pulse as described elsewhere. There was no significant difference in flux rates of K⁺ and H⁺ between the KTY and KPi cells (data not shown). The membrane potential (inside negative) of the KTY and KPi cells were −40 and −45 mV, respectively, under the assay conditions in Fig. 1. Glycolytic activities of these cells at pH 9.2 were examined by measuring glucose-induced acid production, but no great difference between these cells was observed. These results suggest that there was no alteration of K⁺ and H⁺ permeabilities and no defect in glucose metabolism in KPi cells. Instead, the activity of the Kepp K⁺/H⁺ antiport system is deficient in the KPi cells. We also examined activities of several other energy-dependent cation transport systems in the KPi cells. K⁺ uptake via the Ktl system, dependent on ATP and proton motive force, and sodium extrusion via the Na⁺/H⁺ antiporter did not show any significant difference between the KTY and KPi cells (data not shown). The activity of the F₀F₁, H⁺-ATPase of inverted membrane vesicles was normal. Furthermore, we also observed the normal activities of the Na⁺-ATPase and Ktll K⁺ uptake system by the cells cultured in KPi medium supplemented with 100 mM NaCl; these systems are induced in high Na⁺ medium.⁸

The effects of medium pH on the growth rates of *E. hirae* in KTY and KPi media were examined (Fig. 2). *E. hirae* grew well in KTY medium over a broad range of medium pH from 6 to 10. In KPi medium this bacterium grew as well at pH below 7.5, but, at pH above pH 8.0, the growth severely decreased. At pH above 9, *E. hirae* nearly stopped growing in this defined medium. The Kep activity as shown in Fig. 1 has been also observed in cells cultured in KTY medium at pH 6.0 and 9.0, respectively. However, the cells cultured in KPi medium at

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**Fig. 1.** Simultaneous Measurements of K⁺ Extrusion and pHin by *E. hirae* ATCC 9790 grown in different media.

Cells were grown in KTY medium (A and C) or KPi medium (B and D) at pH 7.5, washed and suspended in 50 mM CHES-KOH buffer (pH 9.2) containing 200 mM KCl and 50 mM diethanolamine-HCl. At intervals, samples were collected, and cellular K⁺ (circles) contents and the pHin (interior acid) were measured as described in text. At the arrow, 10 mM glucose was added. Open symbols represent no addition of glucose. The internal water space was taken as 2 μl per dry weight. Symbols; circles, K⁺ content; squares, pHin value.

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Fig. 2. Effects of Medium pH on the Growth Rates of E. hirae ATCC 9790 in Different Media.

Cells were grown at 37°C in KTY complex medium (open circles) or in KPi defined medium (closed circles), and the growth rates were measured as described in text.

Fig. 3. Effects of Difco Yeast Extract on the Potassium/Methyamine Exchange Activity.

Cells grown in KPi medium with 0.5% yeast extract at pH 7.5, were harvested at OD$_{600}$=0.4, and suspended in 50 mM CHES-KOH (pH 9.2) buffer containing 200 mM KCl and 10 mM glucose. At the arrow, the reaction was started by the addition of 50 mM $[^{14}C]$ methylamine chloride (0.37 nCi/mmol). Open symbols represent no addition of methylamine. Symbols: circles, $K^+$ content; triangles, methyamine content.

pH 6.0 or 9.0 did not show the $K^+/H^+$ antiport activity (data not shown). These results suggest that the activity of the Kep system is specifically missing in cells cultured in this defined medium. From these results, it is suggested that this ATP-linked $K^+/H^+$ antiporter system does not participate in homeostasis of $K^+$ and pH of E. hirae at acidic pH, which is consistent with the notion that the Kep system is active at alkaline pH. The importance of the Kep system in this bacterium is limited at alkaline external pH.

Figure 3 shows the $K^+/H^+$ antiport activity of the cells cultured in KPi medium at pH 7.5 with 0.5% yeast extract (Difco). Glucose-induced $K^+$ extrusion, not observed in the cells grown in KPi medium (Fig. 1B), was observed. $K^+$ extrusion occurred concomitantly with the equimolar accumulation of methylamine, and the internal pH was acidified from 8.9 to 8.0. The recovery of the $K^+/H^+$ antiport activity was also partially observed when KPi medium was supplemented with 1% Difco tryptone, but not by increasing the amounts of 20 amino acids (including related compounds), 8 vitamins, 3 bases and some minerals in media (data not shown). The Kep activity was observed if KPi cells were transferred in KPi medium supplemented with yeast extract. The activity was not induced when erythromycin was added to medium, suggesting that protein synthesis is related to expression of the Kep activity. It is likely that some minor ingredients contained in yeast extract or tryptone are required for the activity or gene expression of the Kep system of E. hirae at alkaline pH. SDS-PAGE of the membranes of KPi cells and KTY cells did not reveal any great difference between the membrane protein pattern (data not shown). In parallel with the recovery of the Kep activity, the cell growth in this defined medium at pH 9.5 was restored by addition of yeast extract (Fig. 4). The pHin of growing cells in KTY medium at pH 6–9 (Fig. 2) is maintained within the narrow range of 7.5 to 8.0. 5,11 In KPi medium, the pHin of E. hirae cells at the medium pH from 6 to 7.5 was maintained around 7.5, but the pHin of the cells grown at the external pH of 9.5 was 9.3 (Fig. 2). Acidification of the pHin at alkaline external pH was thus impaired in the cells cultured on KPi medium. On the other hand, the pHin of cells growing KPi medium with yeast extract at pH 9.5 (Fig. 4) was acidified to 8.2. Cell growth at pH 9.5 in these media as well as in KTY medium5,11 were inhibited by nigericin, $K^+/H^+$ antiporter ionophore (Fig. 4), suggesting that cytoplasmic acidification is important for the growth at alkaline pH. From these results we suppose that the key function of the Kep system is cytoplasmic acidification and is the prerequisite for the growth of this bacterium.
at alkaline pH. On the other hand, we know that the metabolism of E. hirae growing at alkaline pH is not always the same as those at neutral or acidic pH. Carbonate (CO$_3 ^-$) is required for the growth of E. hirae at alkaline pH;\textsuperscript{11} the glycolytic metabolism of Enterococci at alkaline pH is switched to a more effective system for ATP production in the presence of high concentrations of carbon dioxide.\textsuperscript{16,17} It is also reported that anaerobiosis compensates for the requirement for carbonate for the growth of enterococci at alkaline pH.\textsuperscript{18} Therefore, it is possible that other metabolic pathways are also impaired by the lack of minor nutrients in the defined medium. Although K$^+$ efflux and its relation to K$^+$ uptake are not yet fully understood in E. hirae, the cytoplasmic K$^+$ concentration should be controlled at a constant level by influx and efflux of K$^+$ ions. The size of the K$^+$ pool (400 mM) of growing KPi cells, in which the Kep system was inoperative, was not far from the value (450 mM) of growing KTY cells. Therefore, at least in these high K$^+$ media, the role of the Kep system in K$^+$ homeostasis is minimal. Dispensability of the Kep activity for the growth of E. hirae at low pH is very useful for isolating mutants. We are now going to isolate Kep mutants among alkaline pH-sensitive mutants for investigation of its functional roles.

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