Potassium/Proton Antiport System of Growing
Enterococcus hirae at High pH

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The cytoplasmic pH (pHin) of *Enterococcus hirae* growing at pH 9.2 was maintained at about 8.1. Membrane-permeating amines such as ammonia alkalinized the pHin from 8.1 to 9.0 at a high concentration and induced K^+ extrusion. The pHin alkalinization was transient; the pHin fell from 9.0 to the original value of pH 8.1, at which point K^+ extrusion ceased, and remained constant. Cells accumulated ammonium ion to an extent stoichiometrically equivalent to the K^+ loss. This bacterium continued to grow well under this condition. These results suggest that the pHin-responsive primary K^+/H^+ antiport system (Y. Kakinuma, and K. Igarashi, J. Biol. Chem. 263:14166–14170, 1988) works for the pHin regulation of this organism growing at a high pH.

It is generally accepted that bacterial cytoplasmic pH (pHin) is regulated by various cation transport systems (3). In aerobic bacteria growing at a low pH, the pHin is alkalinized by a combination of proton expulsion via the respiratory chain and an electrogenic potassium influx. At a high pH, it is assumed that the pHin is acidified by the flux of protons via secondary Na^+/H^+ and K^+/H^+ antiporters (3, 15). The fermentative bacterium *Enterococcus hirae*, which lacks the respiratory chain, survives in a broad range of environmental pHs of 5 to 10 (5). The pHin is maintained within a relatively narrow range of 7.5 to 8.0 (9, 13). In this bacterium growing at a low pH, the pHin is alkalinized by a combination of proton expulsion via a proton-translocating F_0F_1-ATPase and an electrogenic potassium influx (14). At a high pH, on the other hand, the mechanism of H^+-coupled secondary systems cannot be fit for acidification of the pHin of *E. hirae*. First, the generation of a proton potential at a high pH was too small to drive the Na^+/H^+ antiporter (8, 17) since the activity of the H^+-ATPase was negligible at the high pH (14). Second, this bacterium grows well in high-K^+ medium at a high pH, when the outwardly directed potassium gradient needed to drive H^+ influx via the assumed K^+/H^+ antiporter is negligible (9). Although *E. hirae* Na^+/H^+ antiporter (NapA) and *Escherichia coli* KefC have similar sequences throughout their hydrophobic domains (16), KefC activity (*N*-ethylmaleimide-induced K^+ efflux) has not been observed in this bacterium (4). Instead of the secondary system, we found primary K^+/H^+ antiport activity in resting *E. hirae* (10); this activity is expressed at alkaline pHin and turned off, at which point the pHin is acidified to about 8.0. We believe that it plays an important role for pHin regulation in this bacterium. In this study, we examined the effect of pHin alkalinization by membrane-permeating amines such as ammonia on the growth of *E. hirae* at a high pH. The pHin alkalinized by ammonia was acidified to the original pH value with K^+-ammonium exchange, and the cells continued to grow well.

E. hirae (formerly called *Streptococcus faecalis* or *S. faecium*) ATCC 9790, which was kindly supplied by F. M. Harold, Colorado State University, Fort Collins, was cultured in alkaline complex medium (9); the K^+ and Na^+ concentrations were

about 150 and 10 mM, respectively. Cell growth was determined by monitoring the A_{600}, and growth rates were determined as described previously (9). The pH gradient (interior acid) and membrane potential (interior negative) across the cell membrane were determined by use of [^{14}C]benzylamine (10 μM, 185 MBq/mmol) and [^{3}H]tetraphenylphosphonium ion (10 μM, 185 MBq/mmol), respectively, as described previously (9). To measure cellular cation contents, cells (10-ml culture with an A_{600} of 0.2) were collected by filtration with a filter (pore size, 0.45 μm; Millipore Inc.,), washed once with 2 mM $MgSO_4$, and extracted with hot 5% trichloroacetic acid. Aliquots were then analyzed for K^+ and Na^+ by flame photometry. Intracellular ammonium was extracted as follows: the cell culture was centrifuged through a layer of silicone oil into 0.5 ml of an extraction mixture containing 1% phenol, 1% toluene, 10% Tween 80, and 5% KCl in water. Ammonium was determined as reported previously (11). To measure [^{14}C]methylamine (0.37 MBq/mmol) accumulation, cells were collected on filters and counted by liquid scintillation counter. The A_{600} was measured before filtering the cell culture, and the dry weight of cells was calculated from the relationship of A_{600} to cell dry weight. The cytoplasmic water space was taken to be 1.75 μl/mg of cells (1, 9).

Effect of amine on the pHin of growing *E. hirae*. Membrane-permeating amines are useful for manipulation of the pHin of growing cells. Amines traverse the cell membrane in their unprotonated form by passive diffusion, bind intracellular protons, and accumulate in their protonated forms because of the relative acidity of the cell interior (12). Amine accumulation at a low concentration can be used as a measure of a pH gradient (interior acid), but at high concentrations, amines collapse the pH gradient. The effects of various membrane-permeating amines on the pHin of cells growing at pH 9.2 were examined (Table 1). The pHin of *E. hirae* growing in complex medium (pH 9.2) was acidified to 8.1 (9). At a concentration of 5 mM, ammonium (pK_a, 9.3), ethanolamine (pK_a, 9.5), or methylamine (pK_a, 10.6) affected the pHin little. At 50 mM, however, all of these amines alkalinized the pHin from 8.1 to 9.0 to 9.1. On the other hand, 50 mM triethanolamine (pK_a, 7.8) did not affect the pHin. These results reflect the pK_a values of these membrane-permeating amines; the amines with high pK_a values collapsed the pH gradient of *E. hirae* growing at a high pH. When 5 mM [^{14}C]methylamine was used to monitor the entry of the amine, the calculated intracellular methylamine concentration was about 50 mM.

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TABLE 1. Effects of various amines on the pHin of growing *E. hirae*[a]

Addition[b]	Concn (mM)	pHin
None		8.1
Triethanolamine (7.8)	50	8.3
Ammonium (9.3)	5	8.2
	50	9.0
Ethanolamine (9.5)	5	8.2
	50	9.1
Methylamine (10.6)	5	8.2
	50	9.0

[a] Cells were cultured in high-K^{+} complex medium (pH 9.2), and at an A_{600} of 0.2, various concentrations of amines were added. The pHin at 30 s after amine addition was determined by equilibrium distribution of [^{14}C]benzylamine as described in Materials and Methods.
[b] The values in parentheses are pK_a values at 25°C.

Effect of ammonium on cell growth. Elegant work by Harold and Van Brunt (7) has shown that circulation of H^{+} and K^{+} is not obligatory for the growth of *E. hirae*, provided that the cells are cultured in a rich medium with a high K^{+} concentration and a slightly high pH of 7.5 to 8.0. Cell growth in high-K^{+} medium was not inhibited by nigericin, the K^{+}/H^{+} antiport ionophore, and gramicidin D, the channel ionophore for monovalent cations, at pH 7.5 (9). However, nigericin collapsed the pH gradient (interior acid) of cells growing at pH 9.2 and completely stopped cell growth (Fig. 1). When the medium pH was shifted from 9.2 to 7.5, growth in the presence of the ionophore was restored (data not shown; 9), suggesting
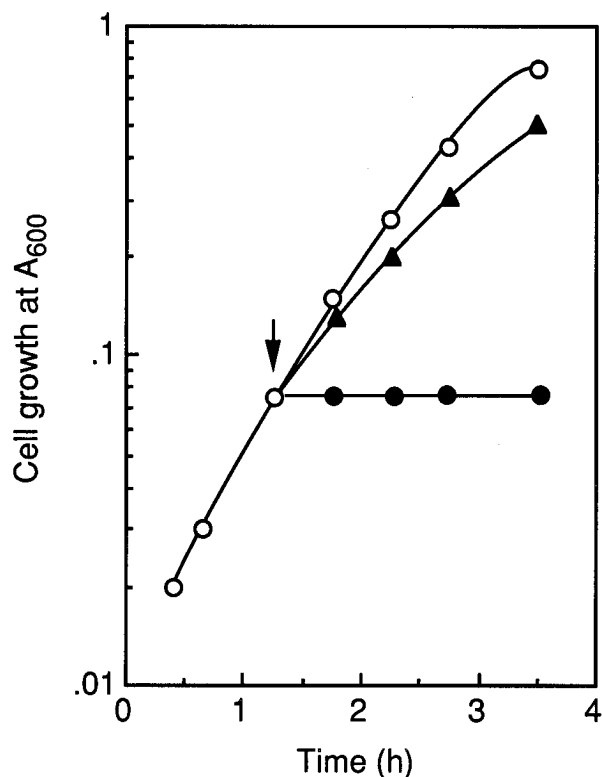

FIG. 1. Effect of ammonium chloride on the growth of *E. hirae* at pH 9.2. A cell culture was started in high-K^{+} complex medium at pH 9.2. The arrow indicates the addition of nigericin or ammonium chloride at a final concentration of 5 μg/ml or 50 mM, respectively. Symbols: ○, no addition; ●, nigericin; ▲, ammonium chloride.
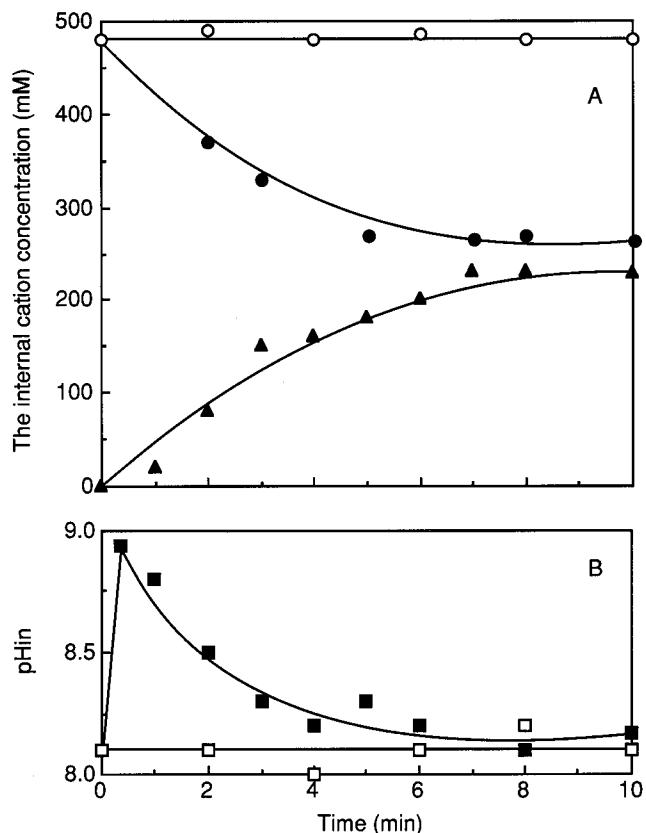

FIG. 2. Simultaneous measurement of pHin and exchange of K^{+} for ammonium. A cell culture was started in complex medium (pH 9.2), and at an A_{600} of 0.2, 50 mM ammonium chloride was added at time zero. At intervals, aliquots were collected and cellular K^{+} (circles) and ammonium (triangles) contents were determined as described in Materials and Methods. The pHin (interior acid) was determined as previously described (9). Open symbols represent no addition of ammonium, and closed symbols represent its addition. The internal water space was taken as 1.75 μl/mg of dry weight.

that acidification of the pHin is indeed required for the growth of *E. hirae* at a high pH. In contrast, cell growth at pH 9.2 was inhibited little by addition of 50 mM ammonium chloride when the pHin was shifted to 9.0 (Table 1 and Fig. 1). Even in the presence of 100 mM ammonium chloride, *E. hirae* still grew well at pH 9.2 with a growth rate of 1.2 h^{−1}. As pHin acidification at a high external pH is a prerequisite for cell growth, the effect of ammonia on pHin alkalinization should not be continuous. That is, a pHin alkalinized by a high concentration of ammonia may be restored to the original pH value.

K^{+}/H^{+} antiport induced by pHin alkalinization. Simultaneous measurements of the cellular cation contents and pHin of cells growing at pH 9.2 after addition of 50 mM ammonium chloride were made (Fig. 2). The intracellular concentrations of K^{+} and Na^{+} in this high-K^{+} medium were 480 and 5 mM, respectively, suggesting that the K^{+} gradient externally generated was only 3. The membrane potential (interior negative) of cells growing on this complex medium was negligible. Addition of 50 mM ammonium chloride at time zero induced K^{+} extrusion, as well as pHin alkalinization to 8.9 (Table 1 and Fig. 2A). Concurrently with K^{+} extrusion, the pHin was acidified to 8.1, where K^{+} extrusion leveled off (Fig. 2B). The cells accumulated ammonium to an extent stoichiometrically equivalent to the K^{+} loss (Fig. 2A). Thus, the pHin alkalinization by ammonium chloride was transient and it induced K^{+}-ammonium

exchange concomitant with acidification of the pHin to the original value. Apparent exchange of K^+ for ammonium, as well as pHin acidification, continued for at least 60 min after the addition of ammonium chloride (data not shown). It is noteworthy that replacement of 40% of the cellular K^+ with ammonium ion was not too toxic for the growth of *E. hirae* (Fig. 1 and 2A); glycolytic ATP formation was normally observed in the ammonium-loaded cells (6).

When the pHin of cells growing at pH 9.2 was alkalinized by addition of 50 mM ethanolamine or methylamine (Table 1), K^+ extrusion started and the pHin fell from 9.0 to 8.1, as shown in Fig. 2. [^{14}C]Methylamine accumulated in the cells for a charge balance with K^+ loss (data not shown). However, in contrast to ammonium (Fig. 1), 50 mM ethanolamine or methylamine severely inhibited cell growth; the growth rates after addition of these amines were 0.3 and 0.35 h^{-1}, respectively. Accumulation of methylamine or ethanolamine is toxic for this bacterium, although the action of these amines inside the cells is unknown.

These findings are interpreted by the primary K^+/H^+ antiport mechanism, which is active at alkaline pHin, found in resting cells (10). First, K^+-amine exchange occurred only in cells growing at a high pH, not in those growing at pH 7.5 (data not shown). Second, high concentrations of amines were required for the exchange, and ammonium or [^{14}C]methylamine uptake was not saturated, even at concentrations as high as 50 mM; ammonium transport systems reported in other bacteria show a high affinity for ammonium as a substrate (2). Third, most importantly, this K^+/H^+ antiport system is likely to be primary but not secondary, since (i) iodoacetic acid inhibited K^+/H^+ antiport activity, as well as cell growth (10); (ii) the antiport activity was also observed in an F_0F_1-ATPase-defective mutant (data not shown); and (iii) ammonium actively accumulated in the cells where the external K^+ gradient was negligible (Fig. 2A). Thus, the pHin-responsive primary K^+/H^+ antiport system works for pHin regulation of this bacterium growing at a high pH. For further investigation of the physiological role of this primary K^+/H^+ antiport system, isolation of mutants defective in its activity is in progress.

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