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Relationship of K^+-Uptaking System with H^+-Translocating ATPase in *Enterococcus hirae*, Grown at a High or Low Alkaline pH

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Abstract. Potassium ion pool was studied in glycolyzing *Enterococcus hirae*, grown at high or low alkaline pH (pH 9.5 and 8.0, respectively). Energy-dependent increase of K^+ pool was lower for the wild-type cells, grown at pH 9.5, than that for the cells grown at pH 8.0. It was inhibited by N,N′-dicyclohexylcarbodiimide (DCCD). The stoichiometry of DCCD-inhibited K^+ influx to DCCD-inhibited H^+ efflux for the wild-type cells, grown at pH 9.5 or 8.0, was fixed for different K^+ external activity. DCCD-inhibited ATPase activity of membrane vesicles was significantly stimulated by K^+ for the wild-type cells grown at pH 9.5, and required K^+ for the wild-type cells grown at pH 8.0, while the levels of α and β subunits of the F_1 and b subunit of the F_0 were lower for the cells grown at pH 9.5 than that for the cells grown at pH 8.0. Such an ATPase activity was residual in membrane vesicles from the *atpD* mutant with a nonfunctional F_0F_1. ATPase activity of membrane vesicles from the mutant with defect in Na^+-ATPase was higher for the cells grown at pH 9.5 than that for the cells grown at pH 8.0, and was inhibited by DCCD. An energy-dependent increase of K^+ pool in this bacterium, grown at a high or low alkaline pH, is assumed to occur through a K^+ uptaking system, most probably the *Trk*. The latter functions in a closed relationship with the H^+-translocating ATPase F_0F_1.

Anaerobic neutrophile *Enterococcus hirae* grows well at a high or low alkaline pH [7, 8, 11], but a regulation of cytoplasmic pH is unclear. In such a regulation K^+ transport systems have a role: pH was raised by H^+ extrusion through the F_0F_1 and K^+-influx, when cells were growing at an acidic pH [2, 5]. A model for the regulatory mechanism of cytoplasmic pH has been proposed recently: the pH is regulated by changes in amount and activity of the F_0F_1, which are dependent on the cytoplasmic pH [8].

E. hirae is known to have multiple systems for K^+ uptake [4]; however, operation of these systems is still not clear. In cells grown at an acidic or low alkaline pH, the main low-affinity K^+-uptaking system, the Trk or Ktr1, is assumed to operate as a secondary carrier; using transmembrane gradient of electrochemical potential for H^+ ($\Delta\mu_H{}^+$) to drive K^+-uptake, ATP regulates its function [2]. It means that the K^+ gradient between the cytoplasm and the medium could be higher than the membrane potential ($\Delta\Psi$), but never exceeds $\Delta\mu_H{}^+$. However, there were data that K^+ uptake in *E. hirae* was inhibited by DCCD [5, 10] and correlated with the F_0F_1 activity [1]. These results could be explained alternatively by a direct interaction of K^+ uptaking system with the F_0F_1 with formation of a supercomplex, functioning as a pump [10]. The ''pump'' model suggests that the K^+ gradient can be greater than $\Delta\mu_H{}^+$ and serve as a buffer to stabilize it [3]. However, there are no data on K^+ accumulation in *E. hirae* grown at a high alkaline pH. If this ion is needed for cells, is K^+ uptake carried out through the Trk?

Data represented in this paper show that *E. hirae*, grown at high or low alkaline pH, accumulates K^+ most probably by the Trk. Results suggest the operation of the Trk under a closed relationship with the F_0F_1.

Materials and Methods

Bacteria and growth media. Wild-type of *E. hirae ATCC9790* and its mutant *7683* (defective in Na^+ extrusion) were generously supplied by F.M. Harold (Colorado State University, Denver, CO, USA). The *atpD* mutant *E. hirae MS116* (defective in the β subunit of F_1) was isolated by H. Kobayashi and coworkers (unpublished data).

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Table 1. K^+-pool in *E. hirae*, grown anaerobically at high and low alkaline pH, in different growth phase and pH of the medium

Growth pH		Duration of growth (*h*)	Protein concentration (*mg/ml*)	K^+-pool (*μmol/mg protein*) pH of medium	
Start	End			7.5	9.0
9.5	9.4	3.0	0.03	2.66 ± 0.02	2.43 ± 0.13
	9.3	5.0	0.07	1.39 ± 0.07	1.58 ± 0.07
8.0	ND[a]	3.5	0.19	3.12 ± 0.01	2.65 ± 0.12
	7.3	5.0	0.27	1.64 ± 0.03	1.72 ± 0.08
	ND	14.0	0.31	0.73 ± 0.01	0.74 ± 0.06

Washed cells of the wild-type strain *E. hirae ATCC9790*, grown in KTY medium, were suspended in the buffer A, containing 1 mM KCl, at different pHs indicated. After 1 min K^+ was measured in aliquots as described under Materials and Methods. Average data from two to four experiments are shown with standard errors.
[a] ND, not determined

Bacteria were grown anaerobically at 37°C in flasks, filled completely with growth medium. Growth medium KTY contained 10 g of trypton, 5 g of yeast extract, 10 g of K_2HPO_4, and 10 g of glucose per liter. Medium NaTY contained Na_2HPO_4 instead of K_2HPO_4. When a bacterium was grown at pH 9.5, 50 mM Na_2CO_3 was added. The pH of the medium was adjusted by addition of KOH (for KTY) or NaOH (for NaTY) and HCl. Growth of bacteria at different pHs was monitored by measuring the optical density of the suspension at 600 nm with a colorimeter. Precultured cells were transferred into a fresh medium at the optical density of 0.05 to 0.07. The growth rate was expressed as the growth constant defined as 0.693/doubling time in minutes.

Preparation of membrane vesicles. *E. hirae* membrane vesicles were prepared as described previously [7], with lysozyme to destroy the cell wall. After removal of intact cells and unlysed protoplasts, the pellet obtained by centrifugation at 127,000 *g* for 30 min was used throughout, without a pass through a French pressure cell.

Measurement of intracellular K^+. Washed cells with distilled water by centrifugation were transferred in the prewarmed Buffer A (100 mM Tris-phosphate, pH indicated, containing 0.4 mM $MgSO_4$, 1 mM KCl, and 1 mM NaCl); glucose or other reagents were supplemented. When DCCD was used, cells were preincubated in the buffer with this reagent, 0.2 mM, for 10 min. The cell suspension was incubated at 37°C with magnetic stirring. Aliquots of 1.5 ml taken at times indicated were centrifuged at 8000 *g* for 5 min. The pellet was laid with 1.5 ml of 5% trichloroacetic acid (TCA), vortexed well (for approximately 30 s), boiled for 5 min, and centrifuged again. The supernatant was transferred into a plastic tube and analyzed for K^+ by the atomic absorption spectrophotometer (Hitachi Z-8000). The pellet was defined for protein.

Determination of cation fluxes. Washed cells were transferred in the prewarmed Buffer A; glucose or other reagents were supplemented. When DCCD was used, cells were preincubated in the buffer with this reagent, 0.2 mM, for 10 min. The cell suspension was incubated at 37°C with magnetic stirring. K^+ and H^+ fluxes were determined simultaneously with selective glass electrodes as described [10]. Changes in K^+ and H^+ activity were titrated with KCl for 0.05 mM and HCl for 0.2 mM, respectively. The kinetics was recorded with a fountain-potentiometer. The DCCD-inhibited fluxes were calculated from the differences between rates in the absence and presence of DCCD, 0.2 mM. The cell suspension was defined for bacterial count.

Assay of ATPase activity. ATPase activity was measured by means of colorimetric determination of the P_i liberated by the method of Fiske and Subbarow with slight modification as described [6]. The reaction mixture of 0.60 ml, containing the buffer B (50 mM Tris-HCl buffer, pH 7.5, 2.5 mM $MgSO_4$), was used. The membrane vesicles of 20–80 μg of protein, 5 or 100 mM KCl, or 0.2 mM DCCD as indicated were supplemented. The mixture was prewarmed at 37°C for 10 min. The reaction was initiated by adding 5 mM ATP (Tris or disodium salt) and stopped after 20 min with 0.3 ml of 15% TCA. P_i was determined after 10 min at 18°C by measuring the optical density at 660 nm. Corrections were made for blanks without ATP or membrane vesicles. One unit of ATPase was defined as the amount of enzyme releasing 1 μmol of P_i in 1 min.

Western blotting and immunoassay of blotted F_0F_1. Immunoblots of *E. hirae* membrane vesicles were developed with antibodies against F_1 and b-subunit of F_0 obtained as described [8]. Purified F_0F_1 was supplied by A. Yamaguchi (Chiba University, Chiba, Japan). Western blotting and its immunoassay were carried out with anti-rabbit IgG-conjugated peroxidase as described [12].

Other tests and chemicals. Bacterial count was calculated from the growth of a diluted suspension on solid medium. Protein was determined by the method of Lowry et al. [9] with bovine serum albumin as a standard. Trypton, yeast extract (Difco, USA), ATP (Tris or disodium salt), DCCD, 4-chloro-1-naphthol (Sigma, St. Louis, MO, USA) and other reagents of analytical grade were used.

Results

Energy-dependent increase of K^+-pool. K^+-pool was determined in intact cells, harvested from the growth medium at the pH indicated, washed, and transferred into the buffer at different pHs, containing 1 mM K^+ as described under Materials and Methods. While the growth rate constant of *E. hirae ATCC9790*, wild-type strain, had the value of 71% for growth at pH 9.5 from that at pH 8.0, K^+-pool depended on the growth phase and decreased during the growth (Table 1).

An increase of K^+-pool in *E. hirae*, wild-type strain, upon addition of glucose was observed for the cells grown at pH 9.5 or 8.0 and transferred into the medium with pH 7.5 (Fig. 1). Such an increase was not so high (Table 2). It depended not only on the growth pH, but also on the pH of the assay medium. K^+-pool of the cells grown at pH 9.5 increased at pH 7.5 to the level of cells grown at pH 8.0 (the data of Table 1 suggest that the level of K^+-pool is higher at pH 7.5 than at pH 9.0). This pool of the cells, grown at pH 8.0, decreased at pH 9.0 to the level of cells grown at pH 9.5. An increase in K^+-pool was higher for the cells grown at pH 9.5 than that for the cells grown at pH 8.0, and, moreover, it was higher when the assay pH was lower. Increase of K^+-pool in *E. coli*, grown at pH 9.5 or 8.0, was inhibited by DCCD (not shown).

Stoichiometry of K^+ influx to H^+ efflux. Stoichiometry of K^+ influx through the K^+ uptaking system to H^+ efflux via the F_0F_1 is an important fact to suggest a mode for

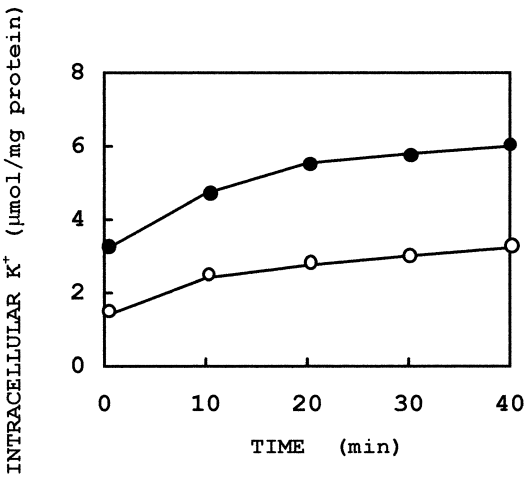

Fig. 1. K^+ accumulation by *E. hirae* cells, grown at high or low alkaline pH, when source of energy (glucose) was supplemented. Washed cells of the wild-type strain *E. hirae ATCC9790*, grown in the KTY medium until protein concentration of 0.03 mg/ml at pH 9.5, or 0.19 mg/ml at pH 8.0, were suspended in the buffer A; glucose (44 mM) was added at zero time. K^+ was measured in aliquots as described under Materials and Methods. Protein amounts were of 0.20 to 1.45 mg per sample of 1.5 ml. Symbols: ○, growth pH 9.5; ●, growth pH 8.0.

Table 2. Energy-dependent increase of K^+-pool in the *E. hirae* in the medium at different pHs

Growth medium	Change of K^+-pool (%) Growth at pH 8.0 Assay pH			Growth at pH 9.5 Assay pH		
	5.5	7.5	9.0	5.5	7.5	9.0
KTY	168	149	90	ND[a]	142	107
	147	126	ND	ND	163	126
NaTY	ND	130	72	190	109	ND

Washed cells of the wild-type cells *E. hirae ATCC9790*, grown on the KTY or NaTY medium and at a protein concentration of 0.19 to 0.25 mg/ml for pH 8.0, or 0.03 to 0.07 mg/ml for pH 9.5, were suspended in the buffer A with different pHs as indicated. K^+ was measured in aliquots as described under Materials and Methods. K^+-pool at 10 min after addition of glucose, 22 mM, was represented in comparison with that at zero time, which was 100%. Data from separate experiments are shown.

[a] Not determined.

operation of K^+ uptaking system. If the Trk is associated with the F_0F_1 to form a supercomplex, functioning as a single mechanism, accomplished cation exchange [8], the stoichiometry of fluxes must be independent of experimental conditions and, therefore, remains strictly fixed. If the Trk works separately [2], the stoichiometry would be variable. The stoichiometry of DCCD-sensitive cation fluxes in *E. hirae* wild-type strain grown at pH 9.5 or 8.0 was fixed with changes in K^+ external activity and equal to K^+:$2H^+$ (Table 3).

Table 3. Stoichiometry of DCCD-inhibited K^+ influx to DCCD-inhibited H^+-efflux in *E. hirae ATCC9790* wild-type strain, grown at high or low alkaline pH, at different K^+ external activity

Growth pH	K^+ activity (*mM*)	Cation fluxes Total H^+	(*mMmin. 10^{12} cells*) DCCD-inhibited K^+	DCCD-inhibited H^+	Stoichiometry of inhibited fluxes (K^+:H^+)
9.5	1.0	1.64	0.32	0.67	1:2.1
	1.9	2.38	0.51	1.22	1:2.4
	2.8	2.63	0.66	1.31	1:2.0
8.0	1.0	0.88	0.33	0.66	1:2.0
	1.9	1.22	0.44	0.88	1:2.0
	2.8	1.44	0.47	0.99	1:2.1

Washed cells, grown on the KTY medium, transferred into the buffer A, containing KCl of different concentration at pH 7.5; glucose was 22 mM. Initial fluxes were determined as described under Materials and Methods. Average data from two to four experiments are shown.

Stimulation of ATPase activity by K^+ and presence of the F_0F_1. ATPase activity in the membranes of *E. hirae* has been shown to be determined mainly by the F_0F_1, and its optimum pH was 6.5 [1]: the activity is low at pH above 8.0 [8]. The level of this ATPase in the bacterium is elevated when the cytoplasmic pH was shifted below 7.5, and the elevated level was attained by the increase in functional unit in the membranes, but not by activation of the enzyme. The amount of the F_0F_1 decreased to a basal level when the medium was alkalized again [8, 12].

Membrane vesicles of *E. hirae* grown at pH 8.0 demonstrated a high ATPase activity, 0.21 units/mg protein. The DCCD-inhibited ATPase activity was stimulated by K^+ added (Fig. 2), but such a stimulation was not so high. Vesicles of the cells grown at pH 9.5 manifested a low ATPase activity; the DCCD-inhibited activity was absent (Fig. 2). At the same time, total and DCCD-inhibited ATPase activity was strongly stimulated by 100 mM K^+ so that the value of ATPase activity was similar to that in *E. hirae* grown at pH 8.0.

A high K^+-stimulated ATPase activity in *E. hirae* grown at a high alkaline pH is of interest, because the levels of α, β as well as of b subunits of the F_0F_1 visualized by immunoassay of Western blotted protein were lower in the cells grown at pH 9.5 than in the cells grown at pH 8.0 (not shown). These results are in good conformity with the data reported previously [8, 12].

ATPase activity in *E. hirae* mutants with defects in the F_0F_1 or in the Na^+-ATPase. If K^+-stimulated DCCD-inhibited ATPase activity is due to the F_0F_1, it should be absent in the *atpD* mutant with a nonfunctional F_0F_1. Actually, total ATPase activity of membrane vesicles from the mutant *E. hirae MS116*, grown at pH 9.5, was less, and K^+-dependent DCCD-inhibited ATPase activity

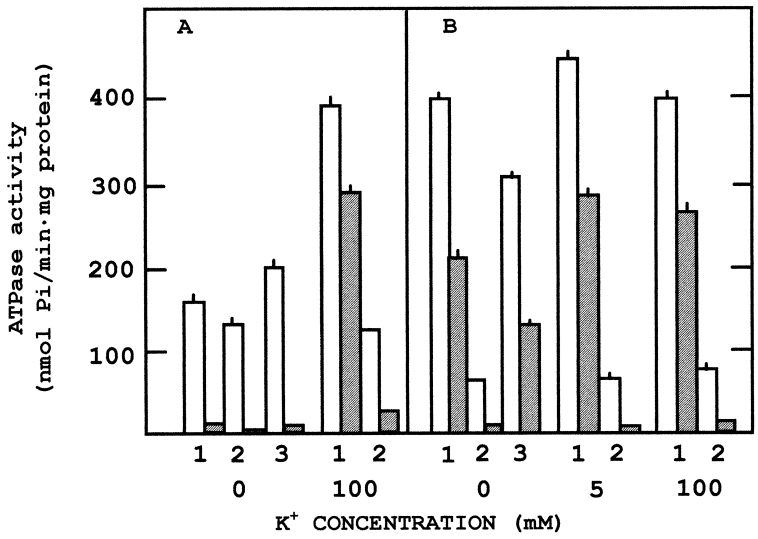

Fig. 2. ATPase activity of membrane vesicles from *E. hirae*, grown at high or low alkaline pH, at different K^+ concentration. Membrane vesicles, prepared as described under Materials and Methods, were suspended in the buffer B; KCl and DCCD, 0.2 mM, were added when indicated. Protein amounts were 18–33 µg per sample of 0.6 ml. ATPase activity was assayed as described under Materials and Methods. The activity represents µmol P_i liberated per min per mg protein. The DCCD-inhibited activity was calculated as the difference between the values in the absence and presence of reagent, 0.2 mM. The value of ATPase activity in the buffer without K^+ added was 100%. Average data are represented with standard errors. Symbols: A, growth pH 9.5; B, growth pH 8.0; lane 1, wild-type strain *ATCC9790*; lane 2, *atpD* mutant *MS116*; lane 3, Na^+-ATPase deficient mutant 7683; □, total activity; ▨, DCCD-sensitive activity.

was absent (Fig. 2). It could be owing to a Na^+-stimulated ATPase, shown previously for *E. hirae atp* mutant [7]. To test a Na^+-ATPase in *E. hirae*, ATPase activity of membrane vesicles from the mutant *7683* was studied. Vesicles from this mutant, grown at pH 9.5, had a high level of total ATPase activity (Fig. 2). But a stimulation of DCCD-inhibited ATPase activity by K^+ was significant for the cells grown at pH 9.5, but less than that of a wild-type strain (not shown). The nature of the mutation in this mutant is far from clear, but the results show that this bacterium, grown at pH 9.5, manifests the F_0F_1 and Na^+-stimulated ATPase.

Discussion

Results represented show that *E. hirae*, grown at a high or low alkaline pH, accumulates K^+ (Tables 1 and 2; Fig. 1). The important findings are as follows: (a) an increase of K^+-pool is inhibited by DCCD; (b) the stoichiometry of DCCD-inhibited K^+ influx to DCCD-inhibited H^+ efflux is determined by different experimental conditions and equal to K^+: $2H^+$ (Table 3); (c) DCCD-sensitive ATPase activity in membrane vesicles is high and stimulated by K^+ added (Fig. 2); such a stimulation is absent in *atpD* mutant with a nonfunctional F_0F_1 (Fig. 2).

A K^+ accumulation in *E. hirae*, grown at a high or low alkaline pH, can be explained by two models. The first one is that a K^+ uptake, possibly through the Trk system, is suggested to have a closed relationship with the F_0F_1; such a conclusion has been reported previously [1]. A relationship can be explained by an interaction of the K^+ uptake with the F_0F_1 with formation of a single mechanism, functioning as a K^+-H^+-pump. Such a model is similar to that proposed for glycolyzing *Escherichia coli*, grown anaerobically at a low alkaline pH [13, 14]. This model might be accepted for *E. hirae*: we have no direct proof for such a model, but each result represented above argues to support it. The second possible pathway for K^+ accumulation in *E. hirae*, grown at a high or low alkaline pH, can also be considered: K^+ uptake occurs through the putative system, which is an ATP-driven pump and inhibited by DCCD. If it is correct, the Trk does not function in the cells grown at a high pH, and the ATPase activity is not the F_0F_1-activity. It means that this bacterium at a high pH has a few F_0F_1 (see results; [8, 12]). However, *E. hirae* grown at a high alkaline pH had a high DCCD-inhibited ATPase activity, stimulated by K^+, and this activity was absent in the *atpD* mutant (Fig. 2). Moreover, K^+ was released from the latter mutant (data not shown). It is, therefore, assumed that the K^+ uptake system in *E. hirae*, grown at a high or low alkaline pH, operates under a closed relationship with the F_0F_1. The nature of this K^+ uptake system is not clear.

E. hirae survives in the broad range of environmental pH [7–8, 11] and grows at a lower rate at a high alkaline pH. It has been accepted that bacteria must generate a high level of H^+- or ion-motive force [3] and manifest the cytoplasmic pH within the range of 7.5 to 8.0 [7, 8] or the latter is not neutral at an alkaline pH above 8 [11]. The main generator of a $\Delta\mu_H{}^+$ in *E. hirae* is the F_0F_1. Its level has been shown previously to be elevated depending on pH [8, 12]. The levels of the α and β subunits of F_1 as well as of the b subunit of F_0 were lower in the cells grown at a high alkaline pH than at a low alkaline pH (not shown). Therefore, a lower level of $\Delta\mu_H{}^+$ in the cells grown at a high alkaline pH can be suggested, and the cytoplasmic pH cannot be acidified by Na^+/H^+- or K^+/H^+-antiporter, even if it is present,

because of the absence of $\Delta\mu_H^+$ at a high alkaline pH. Thus, it has a merit energetically that a K^+ uptake system interacts with the F_0F_1 to work an ATP-driven pump when the $\Delta\mu_H^+$ is low.

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