The *ntpJ* Gene in the Enterococcus hirae ntp Operon Encodes a Component of KtrII Potassium Transport System Functionally Independent of Vacuolar Na\(^+\)-ATPase*

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The *ntpJ* gene, the tail end in the vacuolar type Na\(^+\)-ATPase (ntp) operon of Enterococcus hirae, encodes a putative 49-kDa hydrophobic protein resembling K\(^+\) transporter protein in Saccharomyces cerevisiae (Takase, K., Kakinuma, S., Yamato, I., Konishi, K., Igarashi, K., and Kakinuma, Y. (1994) J. Biol. Chem. 269, 11037–11044). Northern blotting experiment revealed that the *ntpJ* gene was transcribed as a cistron in the ntp operon. We constructed an Enterococcus strain in which the *ntpJ* gene was disrupted by cassette mutagenesis with erythromycin resistance gene. The growth of *ntpJ* mutant was normal at low pH. However, the mutant did not grow at high pH in *K* \(^+\)-limited medium (less than 1 mM), while the wild type strain grew well; the internal K\(^+\) concentration of this mutant was as low as 7% of that of the wild type strain, suggesting that the K\(^+\) accumulation at high pH was inactivated by disruption of the *ntpJ* gene. Potassium uptake activity via the KtrII system, which had been proposed as the proton potential-independent, Na\(^+\)-ATPase-coupled system working at high pH (Kakinuma, Y., and Harold, F. M. (1985) J. Biol. Chem. 260, 2086–2091), was missing in this mutant strain. However, this mutant retained as high activities of Na\(^+\)-ATPase and Na\(^+\) pumping as the wild type strain. From these results, we conclude that the NtpJ is a membrane component of the KtrII K\(^+\) uptake system but not a functional subunit of vacuolar Na\(^+\)-ATPase complex; the interplay between the KtrII system and the Na\(^+\)-ATPase was discussed.

All living cells show Na\(^+\) circulation across the cell membrane. This circulation is driven by active transport systems, which extrude sodium ions and maintain the Na\(^+\) concentration gradient directed inward (1–3). In animal cells, the familiar Na\(^+\),K\(^+\)-ATPase expels sodium ions, in which K\(^+\) uptake is tightly coupled. Bacteria have evolved diverse mechanisms for active sodium extrusion. Secondary Na\(^+\)/H\(^+\) antiporters are widely distributed (4), and some bacteria have been found to produce primary sodium pumps coupled with chemical reactions such as decarboxylation (5), electron transport (6), and ATP hydrolysis (7). Na\(^+\) reenters the cells via the Na\(^+\) gradient consumer, such as Na\(^+\)-coupled secondary co-transport systems, as the widespread route (8). Furthermore, Na\(^+\)-motive flagellar motor and the Na\(^+\) potential-driven ATP synthase are known for their physiological importance in some bacteria (9, 10).

The Gram-positive bacterium Enterococcus hirae lacks the respiratory chain; the proton motive force is generated by proton expulsion via the F0F1, H\(^+\)-translocating ATPase (11). This bacterium has two sodium extrusion systems: Na\(^+\)/H\(^+\) antiporter (12, 13) and an ATP-driven primary pump, Na\(^+\)-translocating ATPase (7). There has been no clear observation that suggests the Na\(^+\) gradient-consuming systems in this organism. The physiological role of sodium extrusion systems may be the elimination of sodium ions from cytoplasm and making room for K\(^+\) accumulation (14, 15).

Our recent biochemical and molecular biological work on E. hirae Na\(^+\)-ATPase (16–23) has suggested that this enzyme is the vacuolar type ATPase distributed in various eukaryotic endomembrane systems and archaeabacteria (24–26). This enzyme is encoded by a gene cluster (ntp operon) consisting of 11 ntp genes: ntpF, -I, -K, -E, -C, -G, -A, -B, -D, -H, and -J (22). In addition to the homologous counterparts of eukaryotic V-ATPases, A, B, and K (16-kDa proteolipid) subunits, we found that six other Ntp proteins (F, I, E, C, G, and D) were similar counterparts of V-ATPase subunits in eukaryotes (22, 27–29). Thus, the expected molecular structure of E. hirae Na\(^+\)-ATPase resembles those of the eukaryotic vacuolar type H\(^+\)-ATPase complex.

On the other hand, one decade ago, Kakinuma and Harold (30) reported a peculiar feature of E. hirae Na\(^+\)-ATPase. They examined the proton potential-independent K\(^+\) transport activity (KtrII system) in this bacterium (31) and found that this activity depended on the activity of Na\(^+\)-ATPase. An apparent equimolar exchange of the internal Na\(^+\) for the external K\(^+\) was observed in the absence of the proton potential. They proposed the mechanism of the KtrII system as the direct Na\(^+\)/K\(^+\) exchange by the Na\(^+\)-ATPase. Since the molecular mechanism of this enzyme had not been elucidated, it was the simplest explanation (32). In this connection, we paid attention to the function of the *ntpJ* gene of the ntp gene cluster. This gene encoded a putative 49-kDa hydrophobic protein, which resembles those of K\(^+\) transport systems of Saccharomyces cerevisiae (Trk1 and Trk2) and of Escherichia coli (Trk) (22) and has not been assigned so far to other V-ATPase subunits. Thus, we thought that the NtpJ protein was the K\(^+\) transporting component for the KtrII activity in the Na\(^+\)-ATPase.

*The abbreviations used are: V-ATPase, vacuolar type ATPase; kb, kilobase(s); DCCD, N,N'-dicyclohexylcarbodiimide; CHES, 2-(cyclohexylethylamino)ethanesulfonic acid.*

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In this work we disrupted the ntpJ gene by cassette mutagenesis and examined the properties of this mutant. Although the Na⁺-ATPase was alive in this mutant, the K₅⁺ uptake activity was deficient, suggesting that the NtpJ protein is a memranous component of this K⁺ uptake system but not the essential one of the Na⁺-ATPase complex. The K₅⁺ transport system is important for this organism in K⁺-limited medium at high pH.

EXPERIMENTAL PROCEDURES

Strains and Growth Conditions—E. hirae strains used were ATCC 9790 (wild type strain), obtained from the American Type Culture Collection with authentic cultures designated 9790 (lane 1) and mutant 9790 (lane 2). Strain NA121 is a derivative of wild-type E. hirae and mutant JEM2 (one of the ErmR transformants) (Fig. 2A). First, the 2.4-kb HindIII-HindIII fragment covering the entire chromosomal locus of the ntpJ gene was isolated from strain 9790, mutant JEM2 (one of the ErmR transformants) (Fig. 2A). The HindIII fragment was liberated from pJEM2 by HindIII digestion and electrophoresed in a 1% agarose gel. The 2.4-kb HindIII-HindIII genomic fragments from strain 9790 and mutant JEM2 were subcloned into pUC119 (pKAZ132). The genes are represented by open boxes with italic type, and the portion of the vector is represented with thick lines. The shaded box represents the erythromycin resistance gene that was introduced into the SphI site of the ntpJ gene of pKAZ132 (pJEM2). The details of the plasmid manipulation and the disruption of the chromosomal gene are described under “Experimental Procedures.” Restriction enzyme sites are as follows: HindIII (H) and SphI (S). B, Southern hybridization. The pJEM2 (lane 3) genome DNAs isolated from 9790 (lane 2), and mutant J EM2 (lane 1) were digested with HindIII, and hybridization was performed with pJ EM2 as the probe as described under “Experimental Procedures.”

Disruption of the ntpJ Gene in E. hirae Na⁺-ATPase Operon

Disruption of the ntpJ Gene—The chromosomal locus of the ntpJ gene was disrupted by insertion of an erythromycin resistance gene as shown in Fig. 2A. First, the 2.4-kb HindIII-HindIII fragment covering the entire chromosomal locus of the ntpJ gene was subcloned from strain 9790 into pUC119 using the DNA fragment liberated from it. Strain NA121 is a derivative of wild-type E. hirae and mutant JEM2 (one of the ErmR transformants) (Fig. 2A). The HindIII fragment was liberated from pJEM2 by HindIII cleavage and electrophoresed in 1% agarose gel. The 2.4-kb HindIII-HindIII genomic fragments from strain 9790 and mutant JEM2 were subcloned into pUC119 (pKAZ132). The genes are represented by open boxes with italic type, and the portion of the vector is represented with thick lines. The shaded box represents the erythromycin resistance gene that was introduced into the SphI site of the ntpJ gene of pKAZ132 (pJEM2). The details of the plasmid manipulation and the disruption of the chromosomal gene are described under “Experimental Procedures.” Restriction enzyme sites are as follows: HindIII (H) and SphI (S). B, Southern hybridization. The pJEM2 (lane 3) genome DNAs isolated from 9790 (lane 2), and mutant JEM2 (lane 1) were digested with HindIII, and hybridization was performed with pJEM2 as the probe as described under “Experimental Procedures.”

Measurement of the Internal Na⁺ and K⁺—The cellular contents of K⁺ and Na⁺ in growing cells were determined by flame photometry (37). Samples of cell suspension (10 ml at OD₆₀₀ = 0.2) were spun at 12,000 × g for 10 min and resuspended in 200 ml of 0.1 M phosphate buffer (pH 7.0). Part of the supernatant was removed, and the remainder was carefully removed by suction; the tip of the centrifuge tube, containing the pellet, was cut off and extracted with hot 5% trichloroacetic acid. Aliquots were analyzed for K⁺ and Na⁺ by a flame photometer. The cytoplasmic water space was taken to be 1.75 μl/mg of cells (37).

Transport Assays—To measure the K₁⁺ activity (30), the cells were loaded with Na⁺ as described by Bakker and Harold (37) and suspended in 50 mM Na⁺-CHEES buffer, pH 9.0, at a density of 1 mg (dry weight)/ml. After incubation with 10 mM glucose and 20 μM tetrachlorosalicylanilide for 10 min, the reaction was initiated by the addition of 1 mM KCl. Cell samples were collected by filtration on membrane filters (pore size, 0.4 μm; nuclepore polycarbonate, Costar Scientific Co., Cambridge, MA) and washed with 2 ml MgSO₄. Sodium and potassium contents were determined by flame photometry after extraction of the cells with hot 5% trichloroacetic acid. Sodium extrusion was monitored with ²²Na⁺ as described previously (12). Cells harvested in the late log phase were used directly as K⁺-loaded cells. Washed cells were suspended at 4 mg (dry weight)/ml in 50 mM K⁺-HEPES buffer (pH 7.0) containing 100 mM maleate-KOH with 20 mM ²²NaCl (2.315 MBq/mmol) and incubated at 25 °C for 60 min. At intervals, samples (0.2 ml) were filtered through membrane filters (pore size, 0.45 μm; Toyo Roshi Co., Tokyo) and washed with the same buffer, and the radioactivity was measured with a liquid scintillation counter.

Miscellaneous Methods—Western blotting was performed as described elsewhere (33) and visualized by using goat-anti-rabbit IgG conjugated to alkaline phosphatase. The cell membranes were prepared by the standard procedure as described previously (17) and, if necessary, stored frozen at −80 °C. The Na⁺-stimulated ATPase activity of the membranes was determined at pH 8.5 in the presence or absence of 25 mM NaCl as described previously (30). Denatured polyacrylamide gel electrophoresis was carried out using the system of Laemmli with 10% polyacrylamide (38). Protein was determined by the method of Lowry et al. (39) with bovine serum albumin as a standard. The membrane potential of intact cells was calculated on the basis of the distribution of [³H]tetraphenylphosphonium ion as described previously (30).

Materials—Enzymes for recombinant DNA techniques were purchased from TOYOBO (Tokyo) and Takara Shuzo Co. (Kyoto). [α-³²P]dCTP (111 TBq/mmol) was purchased from Amersham (United Kingdom) and ²²NaCl was from Daiichi Pure Chemical Co. (Tokyo). All reagents used were commercial products of analytical grade.
DISRUPTION OF THE NTPJ GENE IN E. HIARE Na\textsuperscript{+}-ATPASE OPERON

The ntpj Gene Is a Cistron in the ntp Operon—Fig. 1 shows the structure of the ntp operon encoding the Na\textsuperscript{+}-ATPase and its neighboring genes; the ntpR and -X genes are in the opposite direction of the operon. Although nine ntp gene products, from F to D, in this operon are found to be the homologues of vacuolar type ATPase subunits (22, 27–29), we could not so far identify any vacuolar ATPase subunits corresponding to the ntpH and ntpj gene products. As there is no potential sequence for a ribosomal binding site preceding the start codon of ntpH, it is likely that the ntpH gene is not the reading frame. In addition, two sequences able to form stem-loop (or palindrome) structures lie in the sequence between the ntpD and ntpj genes, and a possible promoter sequence (−35 and −10 boxes) is observed in the region preceding the ntpj (22). It is, therefore, possible that the ntp operon is terminated at the end of ntpD and that the ntpj gene is in another operon. Indeed, the primary sequence of the ntpj gene product resembles K\textsuperscript{+} transporter (22) but not the V-ATPase subunits. Thus, we first examined the transcripts of the ntpj gene by northern hybridization. Fig. 3 shows the Northern blotting experiment with three different probes for ntpj genes (Fig. 1). E. hiare Na\textsuperscript{+}-ATPase is not constitutive; this enzyme is highly induced in its neighboring genes; the ntpR gene is in another operon. Indeed, the ntp operon is terminated at the end of ntpj gene (Fig. 1), was used, the RNA longer than 10-kb in the RNA extract of cells grown under high Na\textsuperscript{+} conditions (Fig. 3) (lane 1, 2.5-kb HindII-HindIII fragment; probe II, 0.8-kb PvuI-PvuII fragment; probe III, 1.2-kb PvuI-PvuII fragment) as the probes labeled with a random primer labeling kit using [\textsuperscript{32}P]dCTP.

RESULTS

The ntpj Gene Is a Cistron in the ntp Operon—Fig. 1 shows the structure of the ntp operon encoding the Na\textsuperscript{+}-ATPase and its neighboring genes; the ntpR and -X genes are in the opposite direction of the operon. Although nine ntp gene products, from F to D, in this operon are found to be the homologues of vacuolar type ATPase subunits (22, 27–29), we could not so far identify any vacuolar ATPase subunits corresponding to the ntpH and ntpj gene products. As there is no potential sequence for a ribosomal binding site preceding the start codon of ntpH, it is likely that the ntpH gene is not the reading frame. In addition, two sequences able to form stem-loop (or palindrome) structures lie in the sequence between the ntpD and ntpj genes, and a possible promoter sequence (−35 and −10 boxes) is observed in the region preceding the ntpj (22). It is, therefore, possible that the ntp operon is terminated at the end of ntpD and that the ntpj gene is in another operon. Indeed, the primary sequence of the ntpj gene product resembles K\textsuperscript{+} transporter (22) but not the V-ATPase subunits. Thus, we first examined the transcripts of the ntpj gene by northern hybridization. Fig. 3 shows the Northern blotting experiment with three different probes for ntpj genes (Fig. 1). E. hiare Na\textsuperscript{+}-ATPase is not constitutive; this enzyme is highly induced in its neighboring genes; the ntpR gene is in another operon. Indeed, the ntp operon is terminated at the end of ntpj gene (Fig. 1), was used, the RNA longer than 10-kb in the RNA extract of cells grown under high Na\textsuperscript{+} conditions (Fig. 3) (lane 1, 2.5-kb HindII-HindIII fragment; probe II, 0.8-kb PvuI-PvuII fragment; probe III, 1.2-kb PvuI-PvuII fragment) as the probes labeled with a random primer labeling kit using [\textsuperscript{32}P]dCTP.

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as did the wild type strain. Even when the medium osmolarity was increased by the addition of 0.5 M sorbitol, the cell growth of strain 9790 or JEM2 was not affected (data not shown). The defect of the ntpJ mutant may be specific for this bacterium's physiology at high pH.

Fig. 4C shows the growth of mutant JEM2 in NaTY medium where 10 mM KCl was also added. It is noteworthy that the mutant JEM2 grew well in this medium even after shifting the medium pH to 10.0 (Fig. 4C, closed circles). The internal concentrations of K⁺ and Na⁺ of JEM2 growing at pH 10.0 in this medium were 120 and 80 mM, respectively (Table I). It is important to point out here that the Na⁺-ATPase mutant, Nak1, did not grow in the same medium: NaTY medium supplemented with additional 10 mM KCl (33). These results suggest that E. hirae grows at high pH even where the internal K⁺ concentration was relatively moderate (120 mM; Table I) and that K⁺ is accumulated in the cells at high pH in an NtpJ-independent manner where the external K⁺ concentration is more than 12 mM. The expected mechanism for K⁺ transport in the ntpJ-disrupted strain is described under “Discussion.”

An ntpJ-disrupted Mutant Lacks the KtrII Activity—It is reported that the apparent Kᵢₐₐₚ value for K⁺ of the KtrII system was 0.5 mM (30, 31); this system should work for K⁺ accumulation under K⁺-limited growth conditions (32). Therefore, it is likely that the NtpJ protein is related with the activity of K⁺ uptake via the KtrII system. Fig. 5 shows the KtrII activities of strain 9790, the Na⁺-ATPase mutant, Nak1 (33), and mutant JEM2. In this experiment, cells were grown in NaTY medium, and the KtrII activity was assayed at pH 9.0, where the proton motive force was dissipated by addition of the protonophore tetrachlorosalicylanilide. The KtrII activity that exchanged nearly equivalently Na⁺ for K⁺ was observed in strain 9790 (Fig. 5A) but not in Nak1 (Fig. 5B). In contrast, in JEM2, K⁺ uptake activity was limited, although efflux of the internal Na⁺ was normal (Fig. 5C); Na⁺ efflux and K⁺ influx were not equimolar in this mutant. These results suggest that NtpJ protein is indeed essential for K⁺ uptake via the KtrII system. Furthermore, Na⁺ extrusion does not seem to be obligatorily coupled with K⁺ uptake.

The ntpJ-disrupted Mutant Has Normal Activity of Vacuolar Na⁺-ATPase—Glucose-dependent downhill Na⁺ efflux observed in the Na⁺-loaded cells, as shown in Fig. 5, has been ascribed to the activity of Na⁺-ATPase (30). Therefore, the result shown in Fig. 5C suggests that the Na⁺-ATPase activity is not damaged by disruption of the ntpJ gene. The activity of the Na⁺-ATPase was examined. Fig. 6 shows active sodium extrusion via the Na⁺-ATPase by the whole cells. In this experiment, cells were cultured in high Na⁺ medium (NaTY medium containing 0.5 mM NaCl) so as to induce the Na⁺-ATPase (16). The proton potential-independent active 22Na⁺ extrusion activity was observed by the parent strain (Fig. 6A); this activity was not observed by Nak1 (33). Active 22Na⁺ extrusion was also observed by JEM2 cells (Fig. 6B). Further-

more, a Western blotting experiment using anti V₁ serum was performed. The molecular size of the purified V₁ moiety of Na⁺-ATPase was about 400 kDa, consisting of polypeptides of 69 kDa (A), 52 kDa (B), and 29 kDa (D) with a probable stoichiometry of 3:3:1 (23). Antiserum raised against this enzyme reacted intensely with the A subunit (Fig. 7, lane 1). Immunoblotting revealed that the amount of Na⁺-ATPase in the membranes of JEM2 was increased when grown under high Na⁺ and high pH conditions, as was the case for strain 9790 (Fig. 7). Western blotting of total cell lysates showed similar results (data not shown). The Na⁺-stimulated ATP hydrolytic activity of the cell membranes prepared from strain 9790 and JEM2 grown in the same high Na⁺ medium (Fig. 6) were 0.13 and 0.13 units/mg protein, respectively. These results suggest that the NtpJ-dependent K⁺ uptake, KtrII, is not directly coupled with the reaction of the Na⁺-ATPase and that the NtpJ protein is not essential for induction of the Na⁺-ATPase and its subunit assembly.

**DISCUSSION**

In bacteria, the transports of K⁺ and Na⁺ are mediated by separate transport systems usually linked to the chemiosmotic proton circulation (1–3). In E. hirae, in addition to two Na⁺ extrusion systems (Na⁺-ATPase and Na⁺/H⁺ antiporter), two distinct potassium uptake systems have been recognized. The major one, KtrI, is thought to be constitutive and resembles the Trk system of E. coli (37), dependent on the proton motive force and ATP. The second one, KtrII, which was internal Na⁺-dependent, but not dependent on the electrochemical potentials of either H⁺ or Na⁺, required ATP (30, 31). This system stoichiometrically exchanged Na⁺ for K⁺. The mechanism of the KtrII system has not been clearly understood. However, since (i) the mutant that lacked the Na⁺-ATPase also lacked KtrII and (ii)
the KtrII and the Na\(^{+}\)-ATPase were induced in parallel when cells were grown in media rich in Na\(^{+}\), particularly under the conditions that limit the generation of the proton motive force. Kakinuma and Harold proposed the simplest hypothesis, that the membrane potential of this Na\(^{+}\)-ATPase belongs to the vacuolar type ATPase, an electrogenic proton pump (24–26). This speculation should be now withdrawn and replaced by the following. First, the Na\(^{+}\)-ATPase transports Na\(^{+}\) electrogenically, not obligatorily linked with potassium ion transport (41); even in the absence of the potassium ion, the membrane potential was generated via the electrogenic Na\(^{+}\) transport (41); even in the absence of the potassium ion, the membrane potential of less than −70 mV across the cell membrane were generated. In a previous paper (30), the K\(^{+}\) gradient of at least 800 was generated by the Na\(^{+}\)-ATPase mutant under the same assay, where the size of the Na\(^{+}\) potential was negligible. These in vitro results are not consistent with the simple secondary co-transport mechanism and suggest the possibility of the primary pump mechanism; in this case, there should be gene(s) encoding the other component(s), such as the ATPase catalytic subunit(s) of KtrII, whose expression should also be regulated by the same signal for the ntpJ expression should also be regulated by the same signal for the ntpJ gene.

It is important to point out that the KtrII may not be only the route of K\(^{+}\) uptake at high pH. In growing JEM2, the K\(^{+}\) concentration gradient of about 10−17 was generated whether or not the medium was supplemented with additional 10 mM K\(^{+}\) (Table I). This NtpJ-independent K\(^{+}\) accumulation is consistent with the simple secondary co-transport mechanism as described above. We think that the membrane potential generated by the Na\(^{+}\)-ATPase is the driving force for the NtpJ-dependent K\(^{+}\) accumulation, since the membrane potential of about −70 mV was generated in JEM2; K\(^{+}\) accumulation by JEM2 was limited in the KtrII assay (Fig. 5C).

Thus, the physiological function of the E. hirae Na\(^{+}\)-ATPase is to extrude Na\(^{+}\) from cytoplasm and generate the sodium potential, which drives active K\(^{+}\) transport systems at high pH where the proton motive force is minimal. It is notable that two important genes for the cation homeostasis of E. hirae at high pH form an operon.

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