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Primary structure of the α-subunit of vacuolar-type Na^+-ATPase in *Enterococcus hirae*

Amplification of a 1000-bp fragment by polymerase chain reaction

Yoshimi Kakinuma[1], Kazuei Igarashi[1], Kiyoshi Konishi[2] and Ichiro Yamato[3]

[1]*Faculty of Pharmaceutical Sciences, Chiba University, 1-33 Yayoi, Chiba 260,* [2]*Department of Biochemistry, Faculty of Medicine, Toyama Medical and Pharmaceutical University, Sugitani, Toyama 930-01, Japan* and [3]*Department of Biological Science and Technology, Science University of Tokyo, 2641 Yamazaki, Noda-shi, Chiba 278, Japan*

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A 1000-bp fragment of *Enterococcus hirae* genomic DNA was amplified by the polymerase chain reaction method, using the oligonucleotide primers designed from amino acid sequences of both amino-terminal and a tryptic fragment of the Na^+-ATPase α-subunit in this organism. DNA sequencing of this product revealed that the amino acid sequence of Na^+-ATPase α-subunit is highly homologous to the corresponding sequences of large (α) subunits of vacuolar (archaebacterial) type H^+-ATPases, supporting our proposal [Kakinuma, Y. and Igarashi, K. (1990) FEBS Lett. 271, 97–101] that the Na^+-ATPase of this organism belongs to the vacuolar-type ATPase.

Na^+-ATPase; Vacuolar ATPase; Polymerase chain reaction; *Enterococcus hirae*

1. INTRODUCTION

The fermentative bacterium *Enterococcus hirae* ATCC 9790 (formerly called as *Streptococcus faecalis (faecium)* ATCC 9790) contains a Na^+-translocating ATPase [1]. The ATPase activity is stimulated by Na^+ and Li^+, but not significantly by other ions. It is amplified by an increase in cytoplasmic [Na^+] as a signal, and plays a central role in Na^+ circulation of this organism at alkaline pH [2]. It is distinct from an electrogenic Na^+-translocating ATPase from *Propionigenium modestum* [3,4] as judged (i) by its resistance to dicyclohexylcarbodiimide and (ii) by the possibility that this enzyme exchanges Na^+ for K^+ ions [5].

Recently, we found (i) that a large multi-subunit peripheral membrane protein, which mainly consists of the polypeptides of 73 kDa (α) and 52 kDa (β), is the catalytic headpiece of the Na^+-ATPase, and (ii) that the Na^+-ATPase activity in the membranes is highly sensitive to nitrate and *N*-ethylmaleimide, inhibitors of the vacuolar-type H^+-ATPases [6,7], suggesting that the structure of Na^+-ATPase of this organism, at least that of its catalytic headpiece, resembles that of the vacuolar-type H^+-ATPase [7]. As the catalytic headpiece of this enzyme has been recently purified from EDTA extracts of the membranes [8], it is now possible to determine the primary structure of this ATPase and compare it with those of other ATPases.

In this communication we report the PCR amplification of a DNA fragment from *E. hirae* genomic DNA using primers designed from amino acid sequences of parts of the 73-kDa (α) subunit of this enzyme. The amino acid sequence deduced from DNA sequence of this amplified product showed that it retains the commonly conserved amino acid sequences of vacuolar (archaebacterial) ATPases.

Abbreviation: PCR, polymerase chain reaction

Correspondence address: Y. Kakinuma, Faculty of Pharmaceutical Sciences, Chiba University, 1-33 Yayoi-cho, Chiba 260, Japan.

2. MATERIALS AND METHODS

2.1. *Amino acid sequencing of the Na^+-ATPase α-subunit*

The catalytic portion of Na^+-ATPase from *E. hirae* ATCC 9790, purified as reported elsewhere [8], was electrophoresed in a denaturing gel system (SDS-PAGE) according to Laemmli [9], and was then electroblotted onto PVDF membranes (Immobilon Transfer, Millipore). After staining with Coomassie blue, the band corresponding to the α- (73 kDa) subunit was cut out and the N-terminal amino acid residues were then analyzed with a peptide sequenator (Applied Biosystem 470A). In order to determine the internal sequences of this subunit, the 73-kDa protein band was electrophoretically extracted from the gel, and dialyzed thoroughly against 1 mM Tris-HCl (pH 7.5) for three days. The sample was chemically reduced, carboxymethylated and then cleaved with trypsin (enzyme/substrate ratio of 1:100) at 37°C for 16 h. The digested solution was applied to a Cosmosil 5C18-300 column (NacaraiTesque, Japan), fractionated by high-performance liquid chromatography, and isolated peak fractions were analyzed with a peptide sequenator.

2.2. *DNA sources*

Genomic DNA from *E. hirae* ATCC 9790 was prepared as described elsewhere [10]. Oligonucleotide primers used for PCR and

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DNA sequencing were synthesized using a DNA synthesizer (Applied Biosystem 381A).

2.3. *Polymerase chain reaction*

The reaction mixture (100 μl) for PCR contained 200 ng of genomic DNA (template), 1 μM of each primer, 10 mM Tris-HCl (pH 8.9), 80 mM KCl, 1.5 mM $MgCl_2$, 500 μg/ml BSA, 0.1% sodium cholate, 0.1% Triton X-100, 0.25 mM of each nucleotide (dATP, dCTP, dTTP, dGTP) and 4 U of Tth DNA polymerase (*Thermus thermophilus* HB8; TOYOBO, Japan). The amplification was performed in a Zymoreactor (ATTO Bioinstrument, Japan) using the program set to preheat at 72°C for 1 min, denature at 94°C for 1 min, anneal at 52°C for 2 min, and extend at 72°C for 2 min for a total of 30 cycles. The final step at 72°C was extended to 7 min.

2.4. *DNA sequencing*

The reaction mixture of PCR was washed by repeating filtration (Ultrafree C3HK, Millipore) to remove salts etc., and direct double-stranded sequencing of the product was performed with Sequenase (TOYOBO, Japan) using [α-^{35}S]thio-dATP (Amersham); sequencing was performed in both directions such that the final sequence was the result of 4 independent sequencing reactions.

3. RESULTS AND DISCUSSION

The sequences of both the twenty-amino-acid residues of the N-terminal of the 73 kDa α-subunit and the fifteen-amino-acid residues in one of its tryptic fragments were determined by the peptide sequenator as described in section 2. These amino acid sequences were aligned with the corresponding sequences of the α-subunits of archaebacterial H^+-ATPases [11,12], the large subunits of vacuolar H^+-ATPases [13,14] and β-subunit of F_1-ATPase from *E. coli* [15] (Fig. 1A). The N-terminal sequence of *E. hirae* subunit retained the sequence (G-X-X-X-X-V-X-G-P-X-V-X-A) common to the N-terminal region of large (α) subunits of V (A) type ATPases, and the sequence (E-M-P-X-X-X-G-X-P-X-Y-L) commonly conserved in the middle part of amino-acid sequences of these subunits is also observed in the tryptic fragment of the *E. hirae* subunit (Fig. 1A, open and closed circles). Thus, we can expect a structural analogy of the *E. hirae* subunit to the large (α) subunits of V (A) type ATPases but not so much to the EF1 β-subunit.

To determine the amino acid sequence between the N-terminal and this tryptic fragment of the *E. hirae* α-subunit, PCR amplification of genomic DNA using primers designed from these amino-acid sequences (Fig. 1A, underlined) was performed. The sequences of oligonucleotides used as PCR primers are shown in Fig. 1B. In order to design the oligonucleotide primer, the degeneracy of the genetic code must be taken into account: the bases underlined in Fig. 1B were chosen as the most probable ones by the alignment of base sequences of the corresponding regions of large (α) subunits from these V (A) type ATPases (Fig. 1A). Fig. 2 shows the result of PCR under the conditions described in section 2. When both LEFT and RIGHT primers were used to amplify *E. hirae* DNA, the product of about 1 kbp was exclusively amplified. This product is actually amplified only in the presence of both primers, since no product

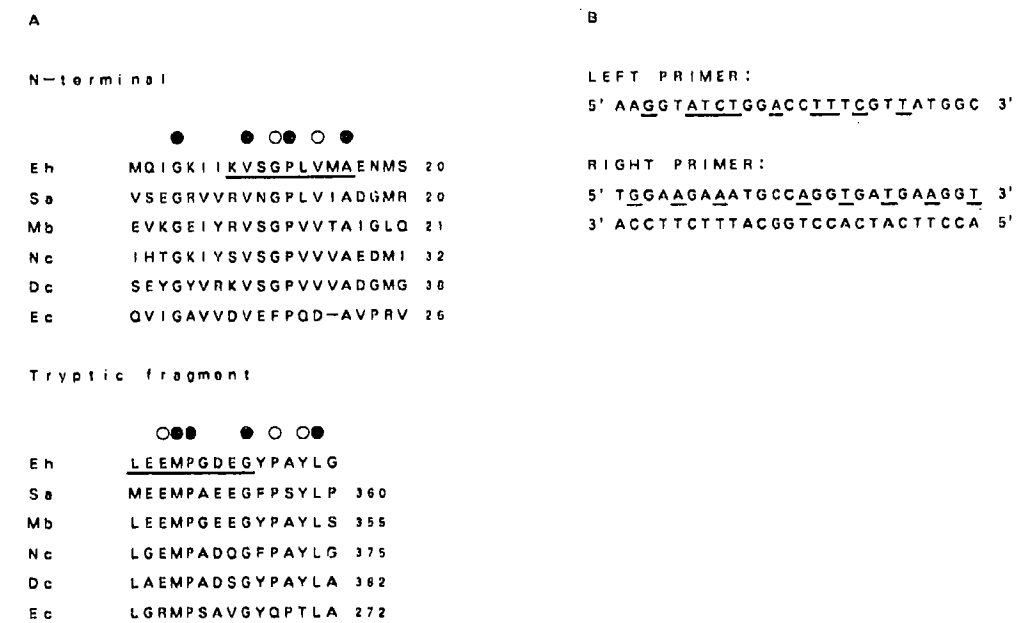

Fig. 1. Partial amino-acid sequences of the Na^+-ATPase α-subunit (A) and primers used for PCR (B). A. Amino-acid sequences of both the N-terminal and a tryptic fragment of the purified α-subunit were determined with an amino-acid sequencer, and aligned to those of the α-subunits of *S. acidicaldarius* ATPase (Sa) [11] and *M. barkeri* ATPase (Mb) [12], the large subunits of *N. crassa* (Nc) [13] and carrot (Dc) [14] vacuolar ATPases, and the EF_1 β subunit (Ec) [15]. Positions with identical amino-acid residues in all ATPases (closed circles) or in V- and A-type ATPases (open circles) were indicated. The sequence used for primers was underlined. B. Primers used for PCR amplification. The underlined bases were chosen as the most probable ones by the alignment of base sequences of the corresponding regions of large (α) subunits from these V (A) type ATPases.

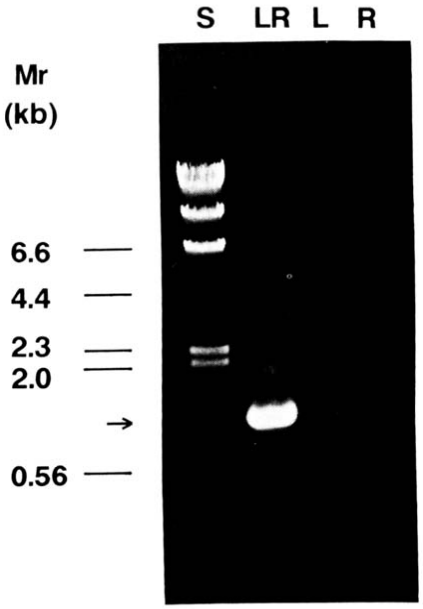

Fig. 2. PCR amplification of *E. hirae* genomic DNA. The gel of 1% agarose was stained with ethidium bromide. S, molecular mass marker (*λ-Hin*dIII digest); LR, amplification using LEFT and RIGHT primers; L, same as LR without RIGHT primer; R, same as LR without LEFT primer. The arrow indicates the amplified product.

was observed when PCR was performed using only one primer (LEFT or RIGHT) (Fig. 2). The amino acid sequence used to design the RIGHT primer is aligned to the middle portion of amino acid sequences in large (α) subunits of V (A) type ATPases (Fig. 1A). It is noteworthy that the size of this amplified product (about 1 kb: namely 333 amino-acid residues) just fits the primary structure of this subunit which is expected to be homologous to those of V (A) type ATPases.

Fig. 3 shows the DNA sequence and the deduced amino-acid sequence of this 1-kb product. The GC content of this sequence is 44% reflecting a low GC content of *Streptococcal* DNA [16]. As a control for possible Tth DNA polymerase errors in PCR [17], 380 bp from the end of the sequence were confirmed by repeating the experiment using an independently amplified sample of *E. hirae* genomic DNA. Both sequences were identical. Thus, under the conditions used, the error rate of the Tth polymerase is less than 0.3%.

The deduced amino-acid sequence for this product was again aligned with the corresponding regions of α-subunits of archaebacterial ATPases, large subunits of vacuolar ATPases, and the EF_1 β subunit (Fig. 4). The amino-acid sequence of this product is highly similar to those of V (A) type ATPases; 52, 56, 47 and 50% of the amino-acid residues of the product were identical with those of *S. acidocaldarius*, *M. barkeri*, *N. crassa* and Carrot, respectively. However, 30% of the amino-acid residues of the product was identical with that of EF_1 β subunit. Furthermore, it is noteworthy that 87% (127 residues) of the 146 amino acid residues conserved in these portions of V (A) type ATPases (Fig. 4, open and closed circles) are conserved in the sequence of this product. Only 13% (19 residues) of the common amino acids to all of other V (A) type ATPases are not seen in the sequence of this Na^+-ATPase (Fig. 4, open stars); most of these amino acids are changing to those with the

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AAGGTATCTGGACCTTTCGTTATGGC          A GAA AAT ATG TCA GAA GCA AGT ATT CAA GAC ATG TGT TTA GTG GGA GAT TTA GGT    55
LEFT PRIMER                           Glu Asn Met Ser Glu Ala Ser Ile Gln Asp Met Cys Leu Val Gly Asp Leu Gly    18

GTC ATC GGC GAA ATC ATT GAG ATG CGT CAA GAT GTG GCG TCT ATT CAA GTA TAT GAA GAA ACT TCA GGA ATT GGT CCC GGA   136
Val Ile Gly Glu Ile Ile Glu Met Arg Gln Asp Val Ala Ser Ile Gln Val Tyr Glu Glu Thr Ser Gly Ile Gly Pro Gly    45

GAA CCT GTT CGT TCC ACT GGG GAA GCA CTA TCT GTT GAG CTA GGA CCA GGA ATC ATT TCA CAA ATG TTT GAC GGG ATT CAA   217
Glu Pro Val Arg Ser Thr Gly Glu Ala Leu Ser Val Glu Leu Gly Pro Gly Ile Ile Ser Gln Met Phe Asp Gly Ile Gln    72

AGA CCA CTG GAT ACA TTT ATG GAA GTG ACT CAA AGT AAC TTC GTA GGA CGT GGG GTT CAA TTA CCA GCT TTA GAT CAT GAG   290
Arg Pro Leu Asp Thr Phe Met Glu Val Thr Gln Ser Asn Phe Val Gly Arg Gly Val Gln Leu Pro Ala Leu Asp His Glu    99

AAA CCA TGG TGG TTT GAA GGC ACA ATC GAA GGA GGA ACC GAA GGA AGT TCT GGA AAC CTC ATT GGG TAC CTG GAT GAA ACC   379
Lys Pro Trp Trp Phe Glu Gly Thr Ile Glu Gly Gly Thr Glu Gly Ser Ser Gly Asn Leu Ile Gly Tyr Leu Asp Glu Thr   126

AAG GTC ATT CAG CCC AAA ATT ATG GTC CCT AAT GGT ATC AAA GGA ACT GTA CAA AAA ATT GAA TCT GGA TCA TTT ACG ATC   460
Lys Val Ile Gln Pro Lys Ile Met Val Pro Asn Gly Ile Lys Gly Thr Val Gln Lys Ile Glu Ser Gly Ser Phe Thr Ile   153

GAT GAT CCG ATT TGT GTG ATC GAA ACG GAA CAA GGC TTA AAA GAG CTG ACG ATG ATG CAA AAA TGG CCA GTA CGT CGT GGT   541
Asp Asp Pro Ile Cys Val Ile Glu Thr Glu Gln Gly Leu Lys Glu Leu Thr Met Met Gln Lys Trp Pro Val Arg Arg Gly   180

CGA CCA ATC AAA CAA AAA TTA AAT CCA GAT GTA CCG ATG ATC ACC GGT CAA AGG GTC ATT GAC ACG TTT TTC CCA GTA ACT   622
Arg Pro Ile Lys Gln Lys Leu Asn Pro Asp Val Pro Met Ile Thr Gly Gln Arg Val Ile Asp Thr Phe Phe Pro Val Thr   207

AAA GGA GGA GCG GCA GCC GTT CCA GGT CCG TTT GGT GCA GGG AAG ACA GTT GTG CAA CAC CAG ATT GCT AAG TGG TCG GAC   703
Lys Gly Gly Ala Ala Ala Val Pro Gly Pro Phe Gly Ala Gly Lys Thr Val Val Gln His Gln Ile Ala Lys Trp Ser Asp   234

GTA GAT CTA GTG GTT TAC GTT GGT TGT GGG GAA CGA GGA AAT GAA ATG ACG GAT GTC GTC AAT GAA TTT CCT GAA CTG ATC   784
Val Asp Leu Val Val Tyr Val Gly Cys Gly Glu Arg Gly Asn Glu Met Thr Asp Val Val Asn Glu Phe Pro Glu Leu Ile   261

GAT CCA AAT ACA GGC GAG TCT TTG ATG GAA CGA ACT GTG TTG ATC GCT AAT ACA TCG AAC ATG CCA GTA GCT GCT CGA GAA   865
Asp Pro Asn Thr Gly Glu Ser Leu Met Glu Arg Thr Val Leu Ile Ala Asn Thr Ser Asn Met Pro Val Ala Ala Arg Glu   288

GCT TCT ATT TAT ACG GGA ATC ACG ATT GCC GAG TAC TTC CGT AAA ATG GGG AAT AAG TTG GCA ATC ATG GCA AAT TCC CCT   946
Ala Ser Ile Tyr Thr Gly Ile Thr Ile Ala Glu Tyr Phe Arg Lys Met Gly Asn Lys Leu Ala Ile Met Ala Asn Ser Pro   315

TTT TGG TGG GCC GAA GCC CCG CCT GAA AAT AGC CGA CGT T          TGGAAGAAATGCCAGGTGATGAAGGT                    986
Phe Trp Trp Ala Glu Ala Pro Pro Glu Asn Ser Arg Arg            RIGHT PRIMER                                  328
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Fig. 3. DNA sequence and the deduced amino-acid sequence of the PCR amplified DNA fragment. The incorporated LEFT primer and the complementary strand to the RIGHT primer are also shown.

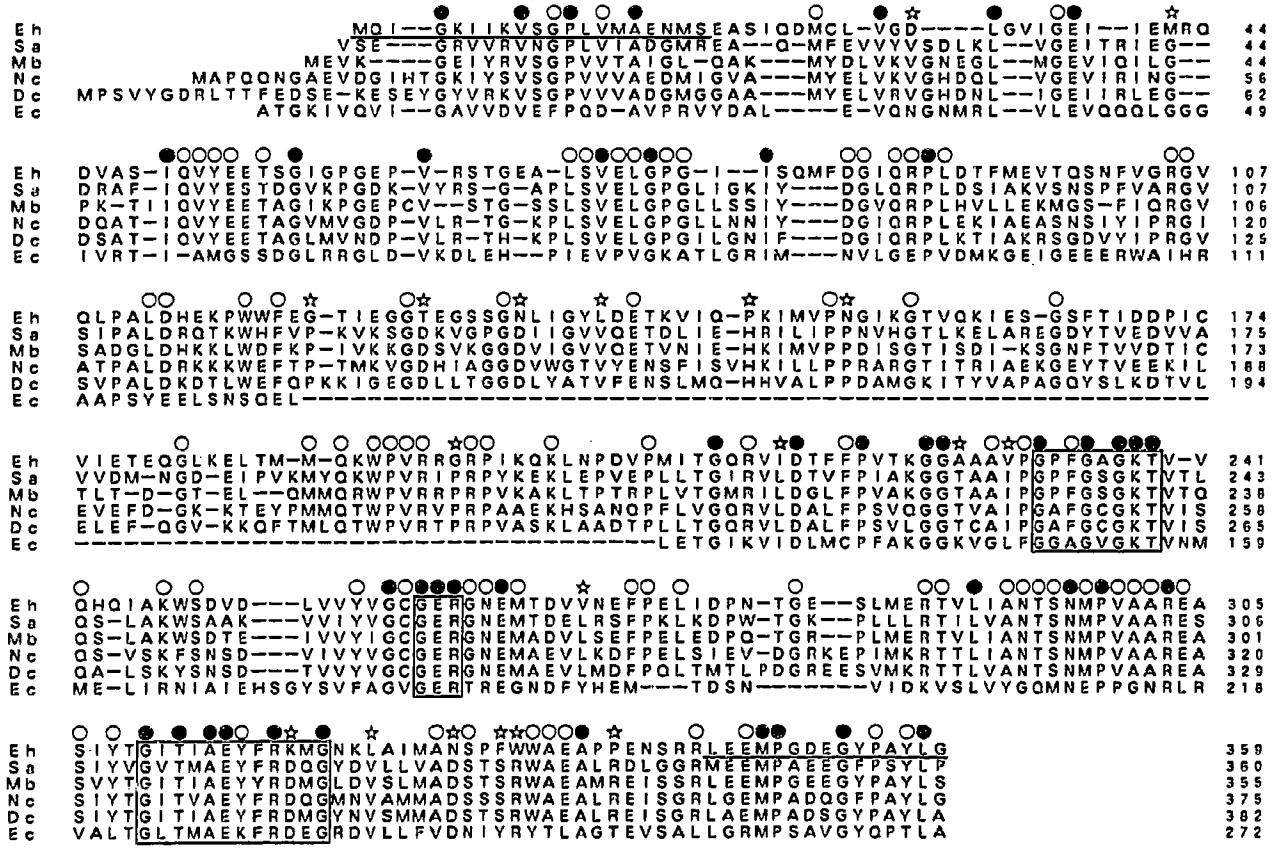

Fig. 4. Alignment of the deduced amino-acid sequence for the *E. hirae* α-subunit with those of other ATPases. Positions with identical amino-acid residues in all ATPases (closed circles), in V- and A-type ATPases (open circles) or positions different from those common to other ATPases (open stars) were indicated. The boxed amino-acid residues correspond to the regions homologous to other nucleotide binding proteins. The underlined sequences were determined with an amino acid sequenator. See also the legend of Fig. 1.

same side chain. The region between residues 122 and 208 of this product is not found in the EF_1 β subunit, as commonly observed in large (α) subunits of V (A) type ATPases [12].

A group of nucleotide-binding proteins have the conserved sequence G-X-X-X-X-G-K-T/S [18]. Similar to the large (α) subunits of other V (A) ATPases, this product has also a typically conserved sequence (residues 232–239) (Fig. 4, boxed). In addition, the sequence G-E-R [13] 21 residues downstream of the conserved sequence and the sequence (residues 310–321), which is quite similar to another conserved sequence (G-X-T-X-A-E-X-X-R-D-X-G) of nucleotide binding proteins [18], are also found in this sequence (Fig. 4, boxed).

In another tryptic fragment of this α subunit, we have also found the amino-acid sequence conserved to these V (A) type ATPases by chemical determination using a sequenator (data not shown).

Thus, the amino acid sequence deduced here for the α-subunit of *E. hirae* Na^+-ATPase is clearly homologous to those of large (α) subunits of V (A) type ATPases. In addition, the molecular weight of the purified catalytic portion of the Na^+-ATPase was approximately 400 kDa by gel filtration. It consists of polypeptides of 73 kDa (α), 53 kDa (β) and 29 kDa (δ): the stoichiometry of these subunits was probably 3:3:1 [8], supporting our proposal that the structure of Na^+-ATPase in this organism, at least its catalytic headpiece, belongs to the V (A) type but not F type as reported in the case of *P. modestum* Na^+-ATPase [7]. This is the first report indicating the existence of V(A) type ATPase in enterobacteria; here it is Na^+-transporting ATPase but not H^+-ATPase. It is well known that *E. hirae* retains F_0F_1 H^+-ATPase which extrudes protons and generates the proton potential [19]. Thus, two types of large multimeric ATPases are simultaneously expressed in this organism; F-type H^+-ATPase functioning at low pH and V-type Na^+-ATPase at high pH [2].

The structure of the membrane portion of *E. hirae* Na^+-ATPase is still unknown. We expect that it does not show simply the same structure as F_0, judging from the possibility that this enzyme does not uniport Na^+ ions but exchanges Na^+ for K^+ ions [5]. To clear up the structure and function of Na^+-ATPase, sequencing of the whole gene of this enzyme is now in progress.

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