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Deletion Analysis of the Subunit Genes of V-Type Na^+-ATPase from *Enterococcus hirae*

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The V_1V_o-ATPase from *Enterococcus hirae* catalyzes ATP hydrolysis coupled with sodium translocation. Mutants with deletions of each of 10 subunits (NtpA, B, C, D, E, F, G, H, I, and K) were constructed by insertion of a chloramphenicol acetyltransferase gene into the corresponding subunit gene in the genome. Measurements of cell growth rates, $^{22}Na^+$ efflux activities, and ATP hydrolysis activities of the membranes of the deletion mutants indicated that V-ATPase requires nine of the subunits, the exception being the NtpH subunit. The results of Western blotting and V_1-ATPase dissociation analysis suggested that the A, B, C, D, E, F, and G subunits constitute the V_1 moiety, whereas the V_0 moiety comprises the I and K subunits.

Key words: ATPase activity, deletion mutant, Na^+ efflux activity, subunit composition, V-type ATPase.

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DCCD, *N*,*N*′-dicyclohexylcarbodiimide; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; KLH, keyhole limpet hemocyanin.

Vacuolar-type ATPase (V-ATPase), which resembles ATP synthase (F-ATPase), functions as a proton pump in the acidic organelles and plasma membranes of eukaryotic cells and bacteria (*1*, *2*). This acidic environment is essential for such processes as receptor-mediated endocytosis, intercellular targeting of lysosomal enzymes, protein processing and degradation, and so on. V-ATPase is composed of two structural domains, the hydrophilic V_1 moiety, and the membrane-embedded V_o domain. A cytoplasmic, membrane associated part V_1 is responsible for ATP hydrolysis, and an integral membrane V_o domain is responsible for the translocation of H^+. Its function is based on a "rotation catalysis" mechanism (*3*). Eukaryotic V-ATPases contain more than 13 different subunits (*4*), subunits A–H for the V_1 moiety, with a probable stoichiometry of 3:3:1:1:1:1:2:1 (*5*, *6*), and subunits a, c, c′, c″, and d for the V_o moiety, with a probable stoichiometry of 1:5–6:5–6:1:1 (*5*). The V_1 moiety contains nucleotide binding sites, with catalytic sites located primarily on the A subunits and the noncatalytic sites on the B subunits. The central stalk is mainly comprised of D, F and d subunits (*5*). Subunits C, E, G and H might constitute the peripheral stalk of the V-ATPase (*5*).

V-ATPase is also found in bacteria such as *Enterococcus hirae* and *Thermus thermophilus* (*7–9*). *E. hirae* V-ATPase transports Na^+ rather than H^+ under physiological conditions. The Na^+-ATPase from *E. hirae* is encoded by the *ntp* operon, which consists of 11 *ntp* genes (*ntpFIKECGABDHJ*) (*10*). We have reported the large scale purification of V_1V_o-ATPase using a construct of the full size V-ATPase expression vector of *E. hirae* (*11*). Purified V_1V_o-ATPase contains nine subunits (NtpFIKECGABD) without the NtpH (7 kDa) and NtpJ (49 kDa) proteins (*11*). We found that the *ntpJ* gene, a cistron located at the distal end of the *ntp* operon, encodes a component of the KtrII K^+ transport system (*12*). We proved that NtpJ is a membranous component of the K^+ uptake system that functions under alkaline conditions, but is not a subunit of the V-type Na^+-ATPase complex (*12*). Therefore, the *ntp* operon contains at least two functional protein components (Na^+-ATPase and K^+ transporter). No protein homologous to NtpH has been found in any other species.

For this report, we constructed ten *ntp* gene deletion mutants other than *ntpJ* in *ntp* operon, and examined their phenotypes. All the subunits except NtpH were found to be necessary for the ATPase activity and $^{22}Na^+$ transport activity of the V-ATPase, suggesting that *E. hirae* V-ATPase is composed of nine subunits (A–G, I and K) as a minimum functional complex.

MATERIALS AND METHODS

Construction of pBSSE—Synthesized DNA with additional multi-cloning sites to those of pBluescript KS(+) (Stratagene, La Jolla, CA) with *Bss*HII sites at both ends was prepared as the following sequence: 5′-GCG CGC AAT TAA CCC TCA CTA AAG GGA ACA AAA GCT GGG TAC TAC TAG TGA GCT CGG TAC CGA TAT CCA ATT GCC CGG GTC TAG AGC ATG CCC ATG GAA GCT TGA ATT CTA GCT CCA ATT CGC CCT ATA GTG AGT CGT ATT AC GCG CGC-3′. This DNA fragment was digested with *Bss*HII nuclease and ligated into the *Bss*HII site in plasmid pBluescript KS(+), resulting in pBSSE with unique *Spe*I, *Sac*I, *Kpn*I,

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*Eco*RV, *Mun*I, *Sma*I, *Xba*I, *Sph*I, *Nco*I, *Hin*dIII, and *Eco*RI nuclease sites.

Disruption of ntp *Genes*—Each *ntp* gene of the *E. hirae* chromosome was disrupted by the insertion of a chloramphenicol acetyltransferase gene. DNA fragments of about 1,000 bp containing the respective *ntp* genes, promoter-ntpI (pPI), ntpF-ntpK-ntpE (pFKE), ntpI-ntpE-ntpC (pIEC), ntpI-ntpK-ntpC-ntpG (pIKCG), ntpK-ntpE-ntpG-ntpA (pKEGA), ntpE-ntpC-ntpA (pECA), ntpC-ntpG-ntpB (pCGB), ntpG-ntpA-ntpD (pGAD), ntpB-ntpH-ntpJ (pBHJ), and ntpB-ntpD-ntpJ (pBDJ), were subcloned from pKAZ191 (Fig. 1A) onto a pBSSE vector (Fig. 1B) (*10*). A fragment of pBEST4C (*13*) containing a chloramphenicol resistance gene (Cm^r) was cut out using restriction sites (*Sma*I). This chloramphenicol acetyltransferase gene was inserted into each respective *ntp* gene using the *Sma*I restriction sites of pPI (for *ntpF*), pFKE (for *ntpI*), pIEC (for *ntpK*), pIKCG (for *ntpE*), pKEGA (for *ntpC*), pECA (for *ntpG*), pCGB (for *ntpA*), pGAD (for *ntpB*), pBHJ (for *ntpD*), and pBDJ (for *ntpH*). We obtained the respective plasmids, prPcatI (NtpF mutant), pFcatKE (NtpI mutant), pIcatEC (NtpK mutant), pIKcatCG (NtpE mutant), pKEcatGA (NtpC mutant), pECcatA (NtpG mutant), pCGcatB (NtpA mutant), pGAcatD (NtpB mutant), pBcatHJ (NtpD mutant), and pBDcatJ (NtpH mutant) (Fig. 1C). Finally, the mutated plasmids were linearized by *Bss*HII digestion and introduced into strain ATCC9790 by electroporation. The preparation of *E. hirae* cells competent for electrotransformation, and the transformation process itself, were performed as described elsewhere (*12*). Transformants resistant to chloramphenicol were selected. Chromosomal DNA was isolated from the transformants as described previously (*10*), and PCR was performed using the appropriate primers in the neighboring genes. PCR fragments were compared with those obtained from a wild-type strain. We obtained deletion mutants for the respective *ntp* genes, namely NAD (NtpA mutant), NBD (NtpB mutant), NCD (NtpC mutant), NDD (NtpD mutant), NED (NtpE mutant), NFD (NtpF mutant), NGD (NtpG mutant),

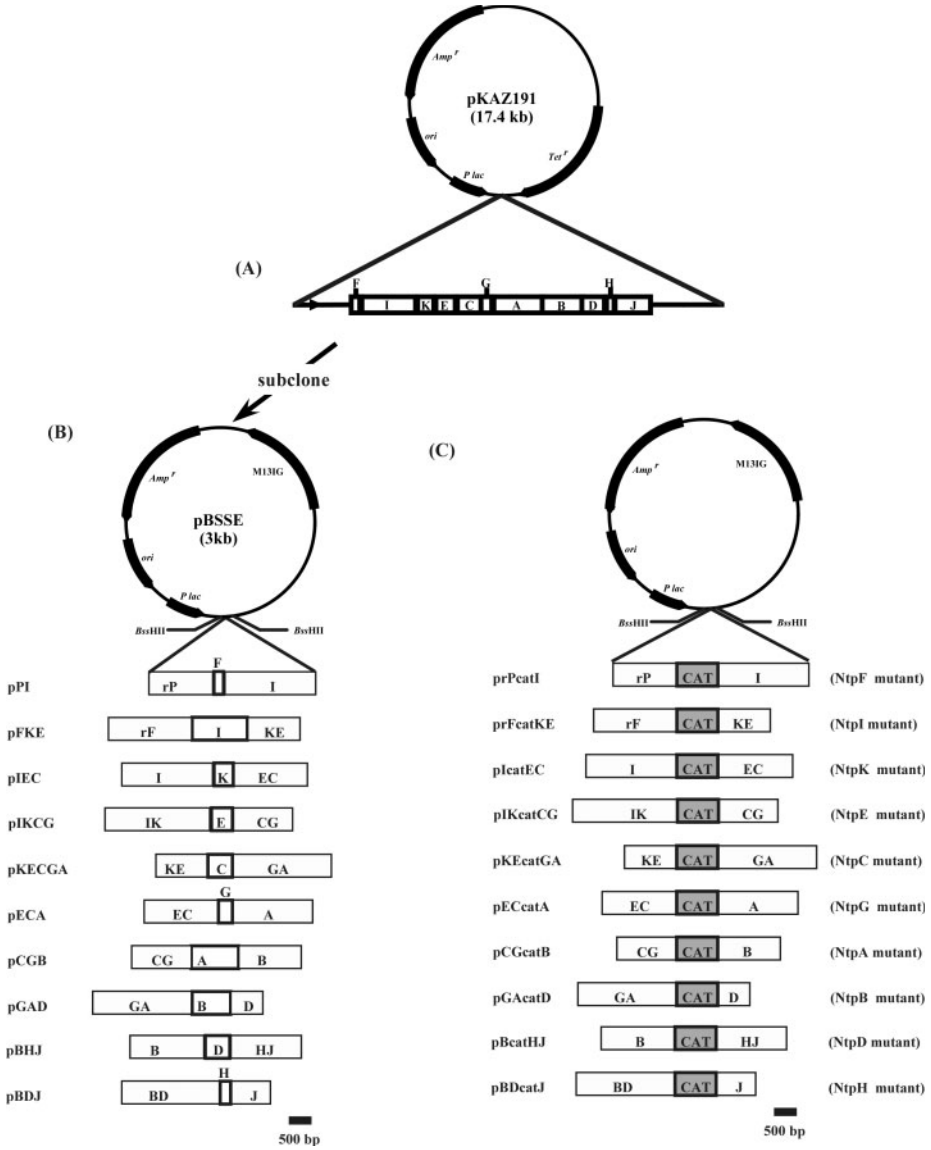

Fig. 1. **Schematic models of the *ntp* operon and disruption of each *ntp* gene**. (A) Open boxes indicate the open reading frames for *ntp* genes in pKAZ191 (*10*), and arrows indicate the direction of transcription. The length of the operon is about 13 kb. (B) Each fragment from the *ntp* gene cluster was subcloned into pBSSE. Genes are represented by open boxes. (C) Shaded boxes represent the chloramphenicol acetyltransferase gene from pBEST4C (*12*) that was introduced into the appropriate restriction enzyme site on each *ntp* gene. The details of plasmid manipulation and disruption of the chromosomal gene are described under "MATERIALS AND METHODS."

NHD (NtpH mutant), NID (NtpI mutant), and NKD (NtpK mutant).

Media and Growth Conditions—Cells were grown in the following standard medium: NaTY (Tryptone 10 g/liter, yeast extract 5 g/liter, Na_2HPO_4 10 g/liter, 1% glucose) (*14*). For culture of mutant strains, erythromycin (10 μg/ml) and/or chloramphenicol (10 μg/ml) was added to the medium. Cell growth was monitored as turbidity at a wavelength of 600 nm. For the preparation of cell membranes, cells were grown in medium containing 0.5 M NaCl. When required, Na_2CO_3 was added to alkalinize the medium.

Construction of Expression Vectors for V-ATPase Subunits—DNA fragments containing each respective *ntp* gene were amplified by PCR using pKAZ1 (*10*) as a template. Suitable primers with appropriate restriction enzyme sites were used. The PCR fragments were subcloned into pHEex (*15*), which has the *ntp* promoter, using the restriction sites *Xba*I and *Eco*RI. The plasmids pHEexA (for *ntpA*), pHEexB (for *ntpB*), pHEexC (for *ntpC*), pHEexD (for *ntpD*), pHEexE (for *ntpE*), pHEexF (for *ntpF*), pHEexG (for *ntpG*), pHEexI (for *ntpI*), and pHEexK (for *ntpK*) were transformed into NAD, NBD, NCD, NDD, NED, NFD, NGD, NID, and NKD, respectively by electroporation, as described previously (*16*). The erythromycin-resistant transformants were selected. We obtained transformants except in the case of NHD/pHEexH.

Growth of Wild-Type Strain ATCC9790 and the Disrupted Mutants at Different pHs—To elucidate the physiological roles of the respective Ntp proteins we examined the growth phenotypes of the respective *ntp* gene–disrupted mutants. Each mutant strain was cultured in NaTY medium (pH 7.5) at 37°C. Two hours after inoculation (1/100 dilution) the medium pH was shifted to 9.5 by the addition of Na_2CO_3 to a final concentration of 50 mM.

Measurement of $^{22}Na^+$ Efflux from Intact Cells—Cells were grown in 50 ml NaTY medium with appropriate antibiotics at 30°C overnight. The cells were washed once with A buffer (100 mM HEPES-KOH (pH 7.5), 50 mM maleate-KOH (pH 7.5), and 2 mM $MgSO_4$), and resuspended in the same buffer at a density of 4 mg protein/ml. $^{22}NaCl$ (30 kBq/ml, 20 mM) was added and the suspension was incubated at room temperature for 30 min. After further incubation of 2 ml cell suspension with 40 μl of 10 mM DCCD (*N,N′*-dicyclohexylcarbodiimide) (final concentration 0.2 mM), 2 μl of 5 mM CCCP (carbonyl cyanide *m*-chlorophenyl-hydrazone) (final concentration 5 μM) and 2 μl of 5 mM valinomycin (final concentration 5 μM) at room temperature for 10 min, the reaction was started by the addition of 10 μl of 40% glucose or 10 μl of distilled water as a control. At intervals, 200 μl of the reaction mixture was filtered on a nitrocellulose filter (0.45 μm pore size, AdvantecToyo, Tokyo) with suction, and washed quickly twice with 2.5 ml of A buffer. The radioactivity trapped on the filter was measured with a liquid scintillation counter.

Preparation of Membrane Vesicles—Cells were grown in 2 liters of NaTY plus 0.5 M NaCl medium, with appropriate antibiotics, at 30°C (A_{600} = 0.6). Cell membranes were prepared by a standard procedure, as described previously (*17*).

Dissociation from Cell Membranes and Purification of V_1-ATPase—V_1-ATPase was dissociated from the V_o moiety by dialysis of wild-type cell membranes against 3 liters of C buffer [1 mM Tris-HCl (pH 7.5), 2 mM ethylenediaminetetraacetic acid (EDTA), 1 mM DTT, 20 μM nafamostat mesilate (protease inhibitor), and 10% glycerol] at 4°C overnight, and separated from the V_1 stripped membranes (V_o-membranes) by centrifugation at 150,000 × *g* for 60 min (*17*). The released V_1 moiety was concentrated by ultrafiltration (YM10 filter, Amicon, Beverly, MA) at 4°C. The V_o-membranes were suspended in the same C buffer, incubated for 50 min at 25°C, and centrifuged again. The membranes were finally suspended in 10 ml of 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM DTT, 10% glycerol, and 10 mM KCl.

The supernatant containing V_1-ATPase was loaded onto an anion-exchange column (Bio-Scale Q20, Bio-Rad, Hercules, CA) equilibrated at room temperature with D buffer [20 mM Tris-HCl (pH 7.5), 10% glycerol, 5 mM $MgCl_2$, 1 mM DTT, and 100 mM KCl]. After the column had been washed with 40 ml of D buffer, the enzyme was eluted with a linear gradient of 10 to 700 mM KCl in the same buffer at a flow rate of 0.5 ml/min. The V_1-ATPase samples were pooled and concentrated to 2 ml by ultrafiltration (YM10 filter, Amicon, Beverly, MA) at 4°C. The sample containing V_1-ATPase was loaded onto an anion-exchange column (Bio-Scale Q20, Bio-Rad) equilibrated at room temperature with E buffer [20 mM MES-Tris (pH 6.0), 10% glycerol, 5 mM $MgCl_2$, 1 mM DTT, and 100 mM KCl]. After the column had been washed with 40 ml of E buffer, the enzyme was eluted with a linear gradient of 0 to 700 mM KCl in the same buffer at a flow rate of 0.5 ml/min. The V_1-ATPase samples were pooled and concentrated to 1 ml by ultrafiltration (YM10 filter, Amicon) at 4°C. One milliliter of the concentrated ATPase fraction was loaded onto a gel filtration column (Superose 6HR, Amersham Biosciences, Piscataway, NJ) equilibrated with 20 mM Tris-HCl (pH 7.5), 10% glycerol, 5 mM $MgCl_2$, 1 mM DTT, and 50 mM KCl and eluted with the same buffer at a flow rate of 0.3 ml/min.

Other Methods—Western blotting was performed as described elsewhere (*12*). 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 (*18*). Protein concentration was determined by the method of Lowry *et al*. (*19*) using bovine serum albumin as a standard.

Materials—Enzymes for recombinant DNA techniques were purchased from Takara Shuzo Co. (Kyoto, Japan). $^{22}NaCl$ was from Daiichi Pure Chemical Co. (Tokyo, Japan). All reagents used were commercial products of analytical grade. Anti-sera against V_1, NtpI, E, F, and K were obtained as described previously (*11*). Anti-serum against a peptide of NtpG (GTLGIGLEEIQNSV) was obtained from rabbits that had received several subcutaneous injections of the synthesized peptide conjugated to KLH (keyhole limpet hemocyanin) by Takara Shuzo, Co. Kyoto.

RESULTS

Complementation Test—Growth of the respective *ntp* gene–disrupted mutants, with the exception of NHD (*ntpH* mutant),was inhibited at alkaline pH (Fig. 2). Cells transformed with pHEex plasmids containing the respective wild-type *ntp* genes restored growth rates to

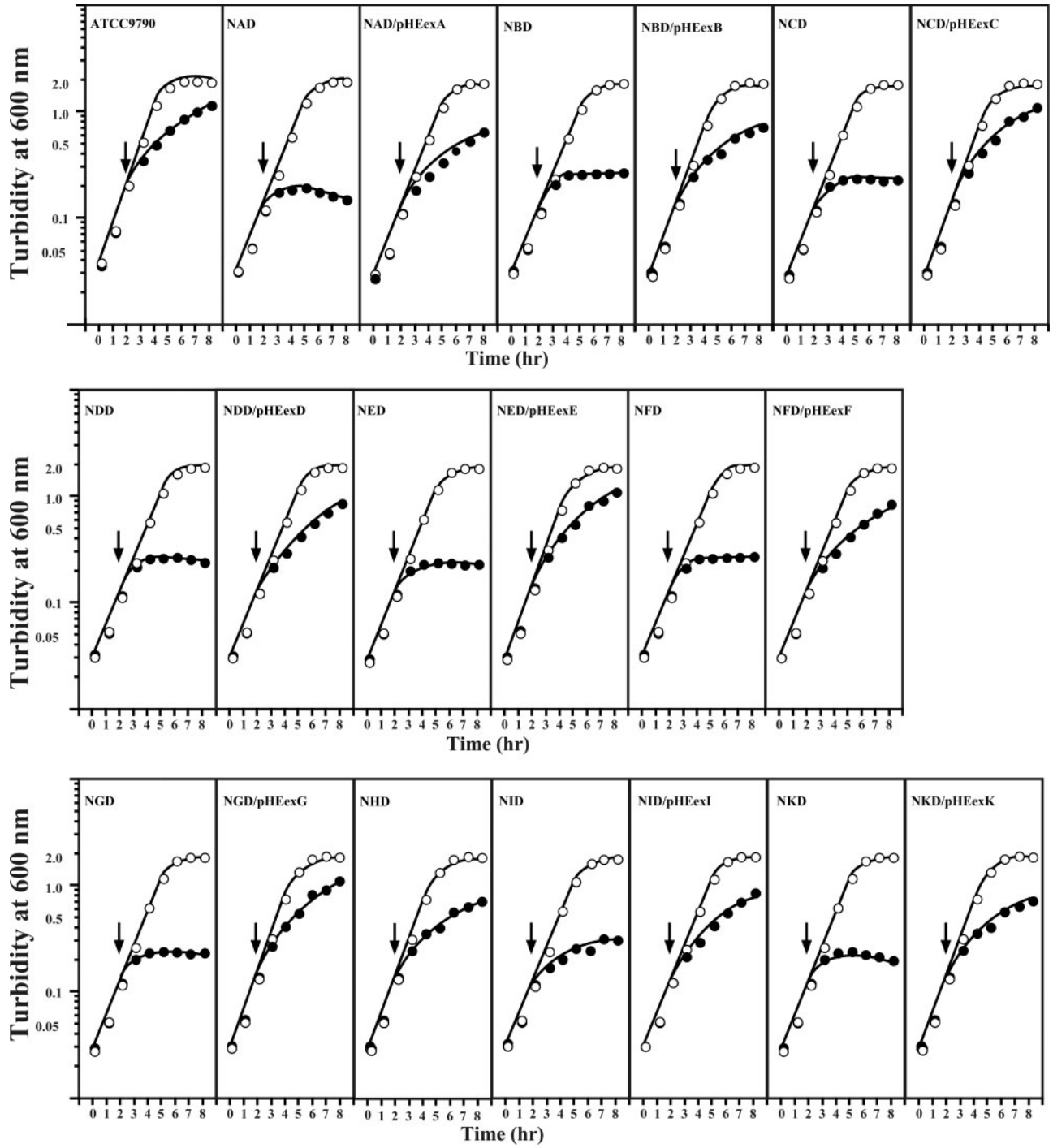

Fig. 2. **Effect of medium pH on the growth of *E. hirae* strains.** Cells were grown in NaTY medium (pH 7.5) (open circles) at 37°C. At the times indicated by the arrow, the medium pH was shifted to 9.5 by the addition of 50 mM Na_2CO_3 (closed circles). Cell growth was monitored by measuring turbidity at a wavelength of 600 nm. NAD, NBD, NCD, NDD, NED, NFD, NGD, NHD, NID, and NKD indicate the NtpA mutant, NtpB mutant, NtpC mutant, NtpD mutant, NtpE mutant, NtpF mutant, NtpG mutant, NtpH mutant, NtpI mutant, and NtpK mutant, respectively. NAD/pHEexA, NBD/pHEexB, NCD/pHEexC, NDD/pHEexD, NED/pHEexE, NFD/pHEexF, NGD/pHEexG, NID/pHEexI, and NKD/pHEexK indicate strains having pHEex plasmids with the respective wild-type genes.

those seen in the wild type. These results indicate that 9 of the 10 *ntp* genes (the exception being *ntpH*) are necessary for growth at alkaline pH.

Measurement of $^{22}Na^+$ Efflux from Intact Cells—Figure 3 shows the $^{22}Na^+$ efflux activities of the intact mutant cells. All the mutants except for NHD (H subunit mutant) showed the induction of defects in $^{22}Na^+$ efflux activities in response to the addition of glucose. Cells transformed with pHEex plasmids showed efflux activities similar to those of the wild type.

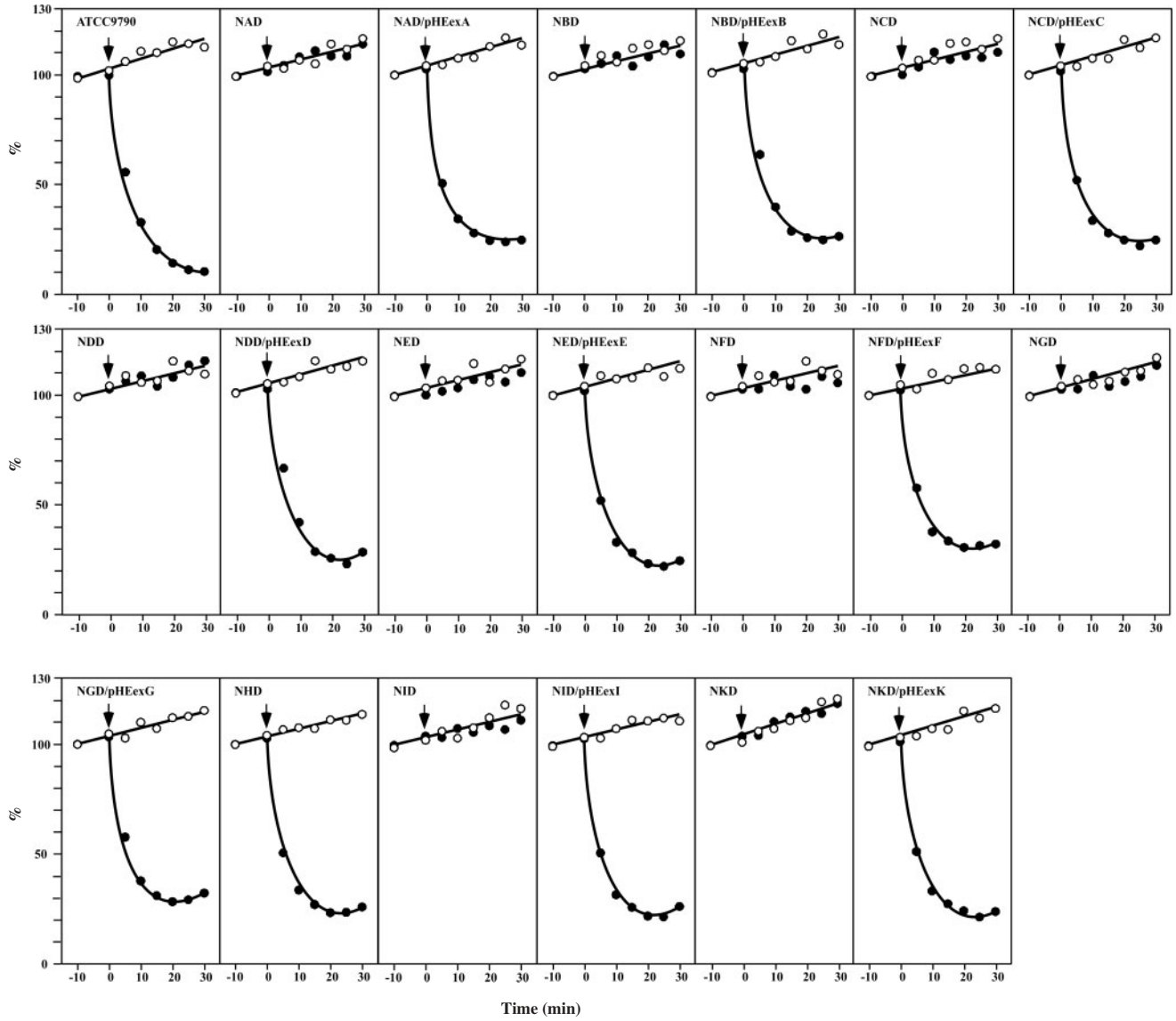

Fig. 3. **Sodium extrusion activity in *E. hirae* strains.** Cells were grown in NaTY medium at 30°C for 24 h. After harvest, the cells were suspended at 4 mg dry weight/ml in 50 mM K^+-HEPES buffer (pH 7.0) containing 400 mM K^+-maleate. $^{22}NaCl$ (20 μM; 30 kBq/ml) was added and the cell suspension was incubated at 25°C for 40 min. In the presence of 0.2 mM DCCD, 5 μM CCCP, and 5 μM valinomycin, $^{22}Na^+$ extrusion was initiated with 10 mM glucose, as indicated by the arrow. NAD, NBD, NCD, NDD, NED, NFD, NGD, NHD, NID, NKD, NAD/pHEexA, NBD/pHEexB, NCD/pHEexC, NDD/pHEexD, NED/pHEexE, NFD/pHEexF, NGD/pHEexG, NID/pHEexI, and NKD/pHEexK are described in the legend to Fig. 2.

Na^+-ATPase Activity of Cell Membranes—Table 1 shows the Na^+-ATPase activities of cell membranes (inverted membrane vesicles from various mutant cells). Membrane vesicles from all the mutant strains other than NHD (H subunit mutant) showed no Na^+-stimulated ATP hydrolysis activity. Membranes from cells transformed with pHEex plasmids recovered their Na^+-ATPase activities to the wild-type level. The NHD strain showed Na^+-ATPase activity similar to that of the wild type. These results indicate that 9 of the 10 *ntp* genes are necessary for Na^+-ATPase and Na^+ efflux activities.

Western Blotting Analysis—Protein expression was examined by Western blotting using the respective membrane preparations. Western blotting using anti-V_1(A, B), E, F, I, and K sera revealed that the 69-, 65-, 52-, 24-, 15-, and 15-kDa polypeptides correspond to the A, I, B, E, F, and K subunits, respectively (Fig. 4). No polypeptide cross-reacting with antiserum against V_1 (A) was observed in the NAD and NED membrane fractions, and only very low amounts were observed in the NBD, NFD, and NID membranes as compared with the NCD, NDD, NGD, NHD ,and NKD membranes. Polypeptide cross-reacting with anti-V_1 (corresponding to the B subunit protein) serum was not observed in the NAD, NBD, NED, NFD, or NID membrane fractions, and very low amounts were found in the NKD membranes. Anti-I serum showed no cross-reactions with the NAD, NBD, NED, NFD, and NID membranes. These results suggest that the NtpA, B, E, F, and I subunits are important for assembly of the V-ATPase complex. Anti-E serum, which had low specificity and titer, showed no

Table 1. **Na^+-stimulated ATPase activities in membranes from mutant strains.**

Strain	25 mM NaCl	No addition	Na^+-stimulated
Wild-type	237.6	122.8	114.8
NAD	143.1	151.6	–
NAD/pHExA	261.3	159.3	102.0
NBD	144.2	148.7	–
NBD/pHExB	255.4	156.9	98.5
NCD	138.7	140.2	–
NCD/pHExC	227.4	137.8	89.6
NDD	184.6	188.1	–
NDD/pHExD	206.8	111.2	95.6
NED	225.9	227.5	–
NED/pHExE	338.5	258.1	80.4
NFD	135.2	139.0	–
NFD/pHExF	197.2	121.3	75.9
NGD	187.4	197.2	–
NGD/pHExG	225.5	152.9	72.6
NHD	259.6	162.4	97.2
NID	169.9	172.4	–
NID/pHExI	289.1	216.7	72.4
NKD	151.1	156.1	–
NKD/pHExK	257.3	158.6	98.7

ATPase activity (nmol min^{-1} mg^{-1} $protein^{-1}$) was measured as described in "MATERIALS AND METHODS."

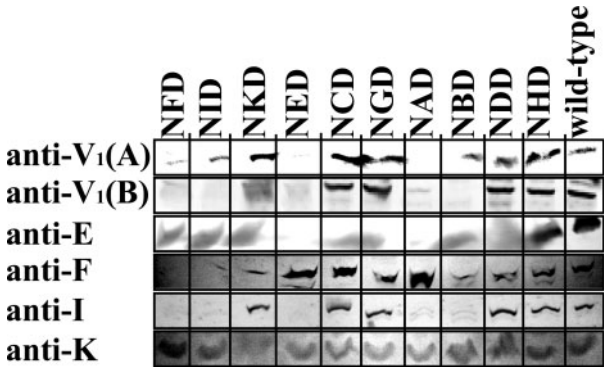

Fig. 4. **Western blotting analysis of cell membranes from *E. hirae* strains.** Cell membranes from mutant strains were subjected to 10% or 12.5% SDS-PAGE and Western blotting using anti-V_1, E, F, I, or K antisera. Anti-V_1 (A), anti-V_1 (B), anti-E, anti-F, anti-I, and anti-K represent anti-V_1, E, F, I, and K antisera, respectively, of V-ATPase subunits. NFD, NID, NKD, NED, NCD, NGD, NAD, NBD, NDD, and wild-type are described in the legend to Fig. 2.

cross-reactions with the NAD and NED membranes. Anti-F serum showed no cross-reactions with the NFD membranes and weak cross-reactions with the NBD, NDD, NID, and NKD membranes. Anti-K serum cross-reacted with most of the mutant membranes except for NKD. The NHD membranes showed cross-reactivities similar to those of the wild-type membranes. Because the antisera against the C and G subunits had very low specificity, we could not detect any cross-reaction in the wild-type.

From these results, we conclude that all nine subunits of the vacuolar Na^+-ATPase complex are encoded by genes *ntpA*, *ntpB*, *ntpC*, *ntpD*, *ntpE*, *ntpF*, *ntpG*, *ntpI*, and *ntpK* (*11*); we do not consider *ntpH* to be an open-reading frame. We speculate that NtpA, B, E, F, and I are important for the assembly of the V-ATPase complex, and that NtpA and NtpE form a stable pre-subcomplex of V-ATPase.

Dissociation of the V_1 and V_o Moieties and Purification of the V_1 Moiety—We purified V_1-ATPase by Q-column and gel filtration column chromatographies (Fig. 5, lanes 3, 4, 5). Using antiserum against the G polypeptides, we detected a G subunit in this V_1 fraction. Because the antiserum against the G subunit had low specificity, we analyzed the NH_2-terminal sequence of the 8-kDa (TYKIGVV) band and found that the polypeptide corresponded to the *ntpG* gene product of the Na^+-ATPase operon. We think that the G subunit belongs to the V_1 moiety, but the final assignment of the G subunit as part of V_1 or V_o needs further investigation. The purified V_1-ATPase consisted of NtpA, B, D, and G subunits (Fig. 5). The stoichiometries of the D and G subunits versus the A and B subunits were low, suggesting the instability of these subunits during purification.

Because the 16-kDa K subunit is a proteolipid (*20*) and the deduced amino acid sequence of the 76-kDa I subunit suggests that this protein has several membrane-spanning domains in its C-terminal half, both the K and I subunits were tentatively assigned to the V_o moiety. These results suggest that the A, B, C, D, E, F, and G subunits constitute the V_1 moiety, whereas the V_o moiety comprises the I and K subunits.

DISCUSSION

The growth experiment, $^{22}Na^+$ efflux, and ATP hydrolysis measurements for various mutants indicated that V-ATPase requires the NtpA, B, C, D, E, F, G, I, and K subunits, but not the NtpH subunit. There is no V-ATPase subunit homologous to NtpH in *Saccharomyces cerevisiae*. Furthermore, because there is no strong Shine-Dalgarno sequence upstream of the mini *ntpH* gene, we tentatively consider that *ntpH* is not an open reading frame.

The results of Western blotting of cell membranes suggest that NtpA, B, E, F, and I are important for the assembly of the V-ATPase complex (see Fig. 4). Although the process of assembly of V-ATPase has not been completely elucidated (*21*, *22*), we speculate from our results that NtpK alone can exist in membranes (*23*, *24*). NtpF may be stable by itself; it may attach to the membranes or NtpK or NtpC/NtpD/NtpG, and this may be why it showed cross-reactions with anti-F antiserum in all mutant membranes except NFD. NtpA and NtpE may form a partly stable transient complex and attach to the membranes or NtpC/NtpD/NtpK, and this may be the reason why they showed weak cross-reactions with anti-E antiserum in various mutant membranes other than NAD and NED. NtpI in membranes acts as an anchor for the assembly of hydrophilic components of the V_1 moiety (from Western blotting of NID), and then further assembly of NtpA, B, E, and F occurs, forming a stable NtpA/NtpB/NtpE/NtpF/NtpI complex (from Western-blotting of NAD, NBD, NED, NFD, and NID). Without the formation of a stable complex of NtpA/NtpB/NtpE/NtpF/NtpI, the NtpB and NtpI polypeptides seem unstable and to be digested or remain soluble, despite the hydrophobicity of NtpI. However, it is similarly possible that subunit proteins are simply

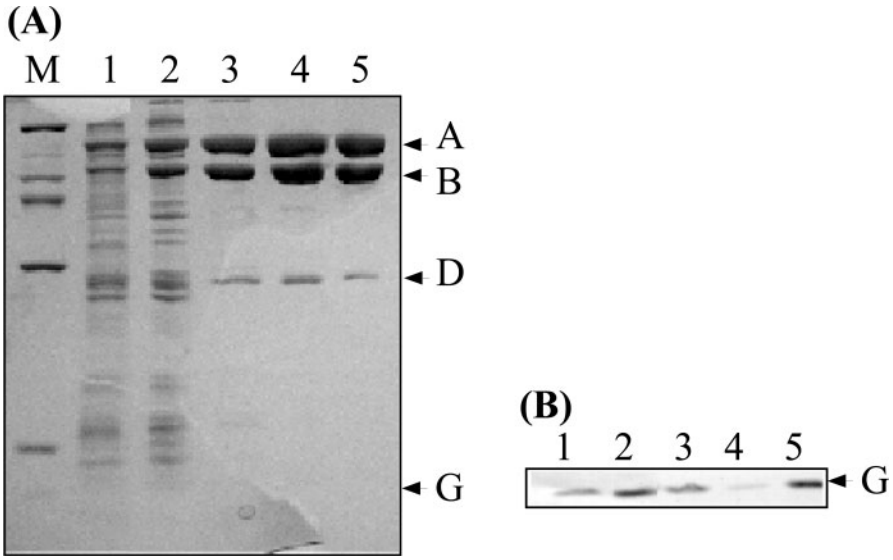

Fig. 5. **SDS-PAGE and Western blotting of various fractions during purification of V_1-ATPase**. (A) M, marker protein; lane 1, membranes; lane 2, EDTA extract (supernatant); lane 3, pool of Q column (pH 7.5); lane 4, pool from Q column (pH 6.0); lane 5, pool from Superose 6HR column. Symbols: A, subunit A; B, subunit B; D, subunit D; G, subunit G. (A), SDS-PAGE. (B) Western-blotting using anti-G antiserum. Numbers 1 to 5 as in (A).

precipitated on membranes as dead-end products without meaningful assembly. Further detailed analysis of such membrane-associated complexes is necessary to elucidate the assembly process of V-ATPase.

Rotation experiments of V-ATPase have shown that subunits D (corresponding to the D subunit of *E. hirae*) and F (corresponding to the G subunit of *E. hirae*) of *T. thermophilus* (*25*). Therefore, we speculate that the G subunit of *E. hirae* interacts with the D subunit, and also that these subunits, C (*26*), D, and G, constitute the rotor shaft part along with the K rotor subunit.

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