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A respiratory-driven and an artificially driven ATP synthesis in mutants of *Vibrio parahaemolyticus* lacking H^+-translocating ATPase

Yuki Sakai, Chie Moritani, Masaaki Tsuda and Tomofusa Tsuchiya

Department of Microbiology, Faculty of Pharmaceutical Sciences, Okayama University, Tsushima, Okayama (Japan)

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Mutants of *Vibrio parahaemolyticus* lacking the H^+-translocating ATPase were isolated to evaluate both the role of this enzyme and the possibility of the involvement of other cation-translocating ATPase in the energy transduction in this organism. Dicyclohexylcarbodiimide-sensitive ATPase activity which represents the H^+-translocating ATPase was not detected either in the membrane vesicles or in the cytosol of the mutants. Three major subunits, α, β and γ, of the H^+-translocating ATPase were missing in the membranes of the mutants. Although ATP was synthesized in wild type cells when an artificial H^+ gradient was imposed, little ATP was synthesized in the mutants. However, we observed a large ATP synthesis driven by the respiration not only in the wild type but also in the mutants. The respiratory-driven ATP synthesis in wild type was inhibited by an H^+ conductor, carbonylcyanide *m*-chlorophenylhydrazone, by about 50%. On the other hand, the ATP synthesis in the mutants was not affected by the H^+ conductor. Since this organism possesses a respiratory Na^+ pump, Na^+-coupled ATP synthesis might take place. In fact, we observed some ATP synthesis driven by an artificially imposed Na^+ gradient both in the wild type and the mutant.

Introduction

Vibrio parahaemolyticus, a slightly halophilic marine bacterium, possesses a respiratory Na^+ pump [1]. Extrusion of Na^+ by this pump takes place under alkaline conditions. An electrochemical potential of Na^+ across the cytoplasmic membrane established by this pump is utilized to drive transport of amino acids [1], nucleosides [2], certain nucleotide [3] and perhaps other nutrients.

The respiratory Na^+-pump was first reported in *V. alginolyticus* [4]. It has been reported that the electrochemical potential of Na^+ is also utilized to drive ATP synthesis [5] and flagella rotation [6] in this organism. The ATP synthesis driven by an artificial Na^+ gradient has been reported in the cells grown on glucose under anaerobic conditions [5]. If Na^+-translocating ATPase is present in *V. alginolyticus* and if it is operative only under anaerobic conditions, it would not be involved in oxidative phosphorylation. So far, it is not clear whether Na^+-coupled ATP synthesis takes place in the cells of *V. alginolyticus* or *V. parahaemolyticus* grown under aerobic conditions where the oxidative phosphorylation proceeds.

Generally, *Vibrio* prefers slightly alkaline conditions for its growth. It has been reported that *V. alginolyticus* could grow even in the presence of an H^+ conductor under alkaline conditions [7]. We confirmed this growth property with *V. parahaemolyticus.* Furthermore, *V. parahaemolyticus* grew on lactate in the presence of H^+ conductors such as CCCP or TCS at pH 8.5 under aerobic conditions (unpublished results). These results suggest the existance of an ATP-synthesizing system coupled to ion(s) other than H^+. Thus, the Na^+-coupled oxidative phosphorylation in addition to the H^+-coupled oxidative phosphorylation seems possible in *Vibrio.* Since *V. parahaemolyticus* seemed to possess H^+-translocating ATPase [1], it would be rather difficult to

Abbreviations: CCCP, carbonylcyanide *m*-chlorophenylhydrazone; TCS, tetrachlorosalicylanilide; F_1, extrinsic and catalytic portion of the H^+-translocating ATPase; Tricine, *n*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; Mops, 4-morpholinepropanesulfonic acid; Ches, 2-(cyclohexylamino)ethanesulfonic acid; Tes, *N*-tris(hydroxymethyl)-methyl-2-aminomethanesulfonic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DCCD, dicyclohexylcarbodiimide.

Correspondence: T. Tsuchiya, Department of Microbiology, Faculty of Pharmaceutical Sciences, Okayama University, Tsushima, Okayama 700, Japan.

analyze Na^+-translocating ATPase, even if it is present. Therefore we tried to isolate mutants defective in the H^+-translocating ATPase. Such mutants would be suitable to investigate the role of the H^+-translocating ATPase in the oxidative phosphorylation and Na^+-coupled ATP synthesis, if present.

Here we report the isolation of mutants of *V. parahaemolyticus* lacking the H^+-translocating ATPase and their properties in ATP synthesis.

Materials and Methods

V. parahaemolyticus AQ3334 [1] was used as a wild type strain. Unless otherwise stated, cells were grown in medium S (pH 7.8) [8] supplemented with 0.5% polypeptone (Daigo Eiyo Co.) and 20 mM lactate (K^+ salt) under aerobic conditions at 37°C. Solid media used included a polypeptone plate (medium S containing 0.5% polypeptone), a neomycin plate (polypeptone plate containing 100 μg neomycin sulfate per ml) and a succinate plate (medium S containing 40 mM succinate (Na^+ salt)), each containing 1.5% agar.

Mutants were isolated as follows. A portion (0.05 ml) of fully grown cells (in medium S supplemented with 0.5% polypeptone) was added to a 5 ml of fresh medium S containing 100 μg/ml neomycin supplemented with 0.5% polypeptone and 40 mM glycerol. After 37 h (the absorbance at 650 nm was about 0.5) an aliquot was diluted 10^6-fold and spread on the neomycin plate. Ten colonies were picked up from each plate (total 40 colonies), and replica-plated on the polypeptone and succinate plate. Mutants which grew on polypeptone (although poorly) and did not grow on succinate (after 4 days) were isolated. Activity of the H^+-translocating ATPase of those mutants was assayed.

To prepare the membrane vesicles, cells were harvested at the late exponential phase of growth. Everted membrane vesicles were prepared using a French press cell as described previously [8] with slight modification. Modified points are as follows: (1) Pressure of the French press was 1000 kg/cm^2; (2) the buffer used for membrane washing consisted of 10 mM Mops-Tris (pH 7.5)/0.3 M choline chloride/25 mM $MgSO_4$/1 mM phenylmethylsulfonyl fluoride. Membrane vesicles were suspended in this buffer.

F_1 portion of the H^+-translocating ATPase was stripped off by washing the membrane vesicles with a buffer containing 3 mM Tricine-Tris (pH 8.0), 0.5 mM EDTA (2 K^+) and 1 mM 2-mercaptoethanol.

SDS-PAGE was performed as described by Laemmli [9].

ATPase activity was determined as follows. The standard assay mixture (0.6 ml) consisted of 20 mM Ches-Tris (pH 9.0)/4 mM $MgSO_4$/0.5 M Na_2SO_4/4 mM ATP (2 Na^+) and about 3 μg (wild type) or 12 μg (mutants) protein of the membrane vesicles. Reaction mixtures were incubated at 37°C for 15 min, and the inorganic phosphate released was determined [10]. 1 unit of activity is defined as the release of 1 μmol inorganic phosphate per min.

Formation of an H^+ gradient in the everted membrane vesicles was estimated by the quenching of quinacrine [11]. The vesicles were suspended in 2 ml of 10 mM Tricine-choline (pH 8.0)/0.3 M choline-chloride/25 mM $MgSO_4$/0.5 μM quinacrine and 0.2 mg protein of the membrane vesicles. Fluorescence was monitored with an excitation wavelength at 420 nm and emission at 500 nm.

For the measurement of ATP synthesis, energy starved cells were prepared by a published procedure [5] with minor modification. Cells were harvested, washed with a buffer consisting of 50 mM diethanolamine-HCl (pH 8.5)/0.4 M KCl/25 mM $MgSO_4$, suspended in the same buffer, and shaken at 25°C for 10 min. Cells were collected by centrifugation, suspended in the same buffer and shaken at 25°C for another 10 min. Cells were washed twice with a buffer consisting of 25 mM Tris-H_2SO_4 (pH 8.5)/0.4 M KCl/25 mM $MgSO_4$, and resuspended in the same buffer at about 10 mg protein/ml. In respiration-driven type experiments, 50 mM NaCl was added to the assay mixture. Nitrogen gas was introduced into the assay vessel to keep anaerobic conditions, and then oxygen gas was introduced in place of nitrogen to start respiration. The H^+-pulse type experiments and the Na^+-pulse type experiments were performed under anaerobic conditions in the presence of 10 mM KCN to inhibit respiration. Eleven μl of 2 M HCl was added to the cell suspension to impose an H^+ gradient. Thus the pH of the assay mixture was lowered by 5 pH unit. For the Na^+-pulse type experiments, the assay mixture containing cells was diluted 2-fold with a similar buffer containing 0.5 M NaCl instead of 0.4 M KCl to impose the Na^+ gradient. Samples were taken at intervals, and ATP was extracted by heating them in 9 vol. of 10 mM Tes-NaOH (pH 7.0) containing 40 mM $MgSO_4$ for 6 min. Then the supernatant was obtained by centrifugation, and ATP contents were determined by using Lumicounter ATP-237 (Advantec Toyo Co.). Intracellular ATP level was calculated on being the intracellular water space estimated to be 2.9 μl/mg cell protein [2].

Oxygen consumption was measured as described previously [1].

Protein was determined by the method of Lowry et al. [12] with bovine serum albumin as a standard.

Results

Isolation of the mutants lacking H^+-translocating ATPase

It has been reported in *Escherichia coli* that the H^+-translocating ATPase mutants could be easily isolated from neomycin-resistant mutants [13]. We first

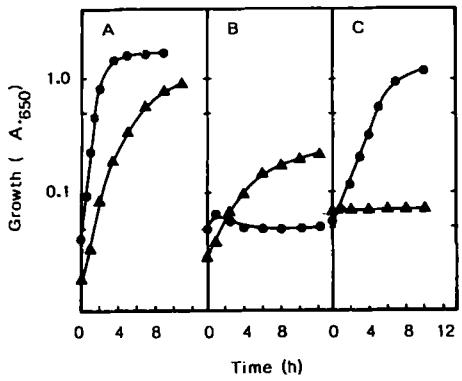

Fig. 1. Growth properties of the wild type and the mutant YS-1. Cells were shaken at 37°C in the medium S containing 0.5% polypeptone/40 mM glycerol (A), 0.5% polypeptone/40 mM glycerol/100 μg per ml of neomycin (B), or 40 mM succinate (K^+) (C). Growth was monitored turbidimetrically at 650 nm. Symbols: ●, wild type; ▲, YS-1.

isolated a neomycin-resistant mutant of *V. parahaemolyticus*, then mutants which did not grow on succinate (at pH 7.5) as a sole source of carbon were selected. We isolated many mutants, and three of them (YS-1, YS-2 and YS-3) were used for further analyses. These three mutants were obtained independently.

Growth properties of one of the mutants, YS-1, were shown in Fig. 1. The mutant grew on glycerol plus polypeptone (amino acid mixture) although at a slower rate than the parent. The mutant YS-1 grew in the presence of 100 μg/ml of neomycin, whereas the parent did not. The mutant could not grow on succinate, whereas the parent could. Thus, YS-1 seemed to lack the ability of oxidative phosphorylation. We checked whether the respiratory chain of the mutant is normal or not. The O_2 consumption with NADH as a respiratory substrate in everted membrane vesicles of the mutant was normal, and was stimulated by Na^+ (data not shown) as reported in the wild type [1]. These results suggest that the mutant YS-1 lacks the H^+-translocating ATPase activity. Very similar growth properties and respiratory activity were observed both in YS-2 and YS-3 (data not shown).

ATPase activity in the mutants

Everted membrane vesicles were prepared from a parent and mutants, and their ATPase activity was measured. Since there is a strong activity of ATP hydrolysis due to the 5′-nucleotidase which requires Cl^- in the membrane vesicles of *V. parahaemolyticus* [8], Cl^- was omitted from the assay mixture to avoid the involvement of the 5′-nucleotidase. Membrane vesicles of the parental cells showed a strong activity of ATPase. On the other hand, the ATPase activity of the membrane vesicles prepared from the mutant YS-1 was very low, about 1% compared to the parent (Table I). When the vesicles were washed with a buffer containing EDTA, the ATPase activity in the vesicles of the parent was greatly reduced (data not shown). This observation is consistent with the view that F_1 portion of the H^+-translocating ATPase was removed from the membrane vesicles by such washing, similar to the case of *E. coli* [14]. The washed membrane vesicles of the mutant showed slightly higher specific activity than the unwashed vesicles. This suggests that the ATPase activity observed in the membrane vesicles of the mutant, although it is very low under our experimental conditions, is due to the other ATPase than the H^+-translocating ATPase. The increase in the specific activity would be due to the removal of the weakly bound membrane proteins by washing. The ATPase activity of the vesicles of the parent was strongly inhibited by DCCD, an inhibitor of the H^+-translocating ATPase of *E. coli* (Table I). Therefore most of the ATPase activity observed here seems to be due to the H^+-translocating ATPase. The weak ATPase activity observed in the vesicles of the mutant was not affected by DCCD at all. This result also supports the idea that the weak ATPase activity observed in the membrane vesicles of the mutant is due to other ATPase. ATPase activity in the cytosol fraction was very low in either the parent or the mutant (Table I). Very similar results were obtained with other mutants, YS-2 and YS-3.

The absence of the functional H^+-translocating ATPase, an ATPase capable of translocating H^+ with expenditure of ATP, in the mutants was confirmed by measuring fluorescence quenching of quinacrine. As shown in Fig. 2, an addition of ATP to the everted membrane vesicles of the wild type elicited fluorescence quenching of quinacrine, indicating a formation of pH gradient as a result of inwardly directed H^+ translocation. DCCD inhibited the fluorescence quenching (data not shown). An addition of NaCl caused a significant reversal of the fluorescence quenching due to Na^+/H^+ antiport [11]. The addition of an H^+ conductor TCS, caused further reversal of the quenching, because of dissipation of the pH gradient. It should be pointed out that the addition of ATP alone caused some extent of decrease in basal level of the fluorescence intensity. Therefore, the fluorescence level did not return to the

TABLE I

ATPase activity in the wild type and the mutant

ATPase activity of the membrane fraction and the cytosol fraction in the wild-type and the mutant YS-1 was compared. DCCD was added at 5 μM.

Strain	ATPase activity (unit/mg protein)			
	Membrane		Cytosol	
	control	+DCCD	control	+DCCD
Wild type	1.72	0.19	0.10	0.06
YS-1	0.02	0.02	0.05	0.05

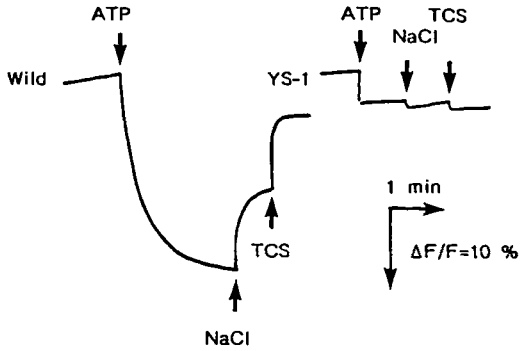

Fig. 2. Quenching of quinacrine fluorescence in the everted membrane vesicles of the wild type and YS-1. Assays were performed at 25° C in 10 mM Tricine-choline (pH 8.0)/0.3 M choline chloride/5 mM $MgSO_4$/0.5 μM quinacrine-HCl/0.1 mg protein per ml of the membrane vesicles. At the time points indicated by arrows, 0.25 mM ATP (2 K^+), 5 mM NaCl or 5 μM TCS was added to the assay mixtures.

original level after the dissipation of the pH gradient by adding TCS. The addition of ATP to the vesicles of YS-1 just caused the decrease of the base line of fluorescence, which was not reversed by the addition of NaCl or TCS (Fig. 2). This result indicates that no detectable H^+ translocation take place in the membrane vesicles of the mutant YS-1. Similar results were obtained with the vesicles of YS-2 and YS-3 (data not shown). Thus, we conclude that there is no detectable H^+-translocating ATPase in the membranes of the mutants.

The presence or absence of the major subunits of the H^+-translocating ATPase in the mutants was checked by SDS-PAGE. As in *E. coli* [14], F_1 portion of the H^+-translocating ATPase of *V. parahaemolyticus* was released from the membranes by washing through a buffer containing EDTA. Protein bands corresponding to α, β and γ subunits of F_1 were detected in the supernatant fraction of the wild type (Fig. 3). The apparent molecular masses of the three subunits were 56, 53 and 31 kDa, respectively. These values are slightly larger than those of α, β and γ of *E. coli* [14]. The bands corresponding to α, β and γ were detected neither in the supernatant fraction (EDTA-extract) nor in the residual membrane fractions of YS-1, YS-2 and YS-3 (Fig. 3). Thus, the mutants not only lost the activity of the H^+-translocating ATPase but also at least three major subunits. Another polypeptide (about 150 kDa) was also missing in the EDTA-extract of the mutants (Fig. 3). It is not clear whether this polypeptide is a subunit of F_1 of *V. parahaemolyticus* or not.

ATP synthesis

Synthesis of ATP driven by an artificially imposed H^+ gradient across the cell membranes has been reported in the energy-starved cells of *E. coli* [15]. We tested ATP synthesis in the energy-starved cells of the wild type and the mutant YS-1 in a similar way. When the H^+ gradient was imposed across the cell membrane

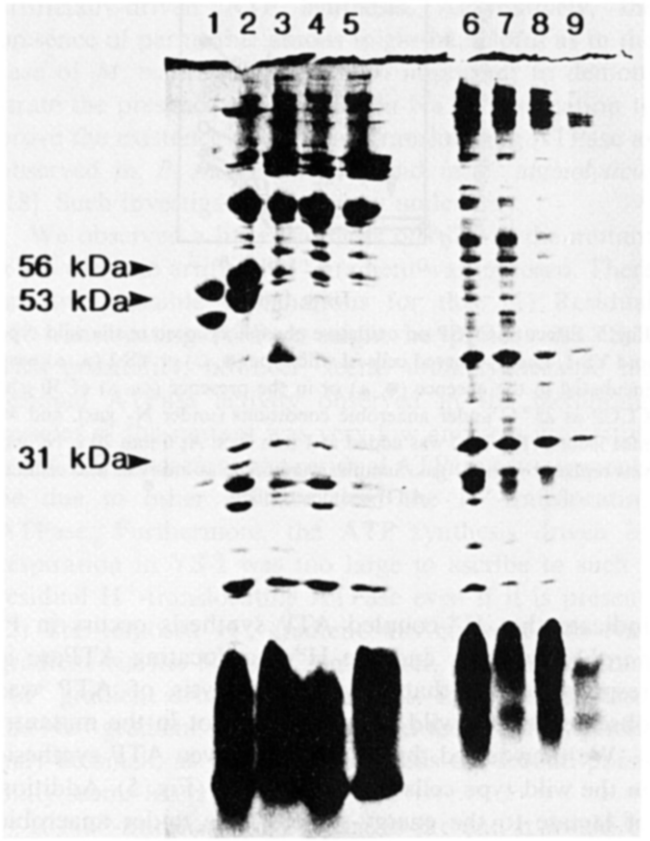

Fig. 3. Analysis of the membrane proteins of the wild type and the mutants by SDS-PAGE. Everted membrane vesicles were washed with a buffer containing EDTA as described in the text. The resultant EDTA-extract (lanes 2 to 5) and the residual membranes (lanes 6 to 9) were analyzed by SDS-PAGE and stained with Coomassie brilliant blue. Lane 1, F_1 of *E. coli*; lanes 2 and 6, wild type; lanes 3 and 7, YS-1; lanes 4 and 8, YS-2; lanes 5 and 9, YS-3.

by adding HCl to the medium, much ATP was synthesized rapidly in the wild type cells (Fig. 4). In contrast, only a little ATP synthesis was observed in the cells of YS-1 under similar conditions. These results

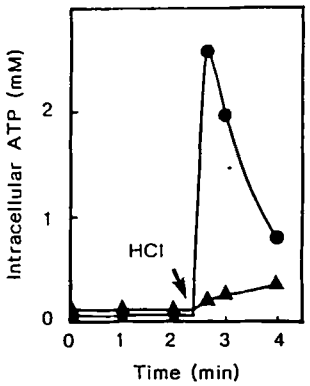

Fig. 4. ATP synthesis driven by an artificially imposed H^+ gradient. Energy-starved cells of the wild type (●) or YS-1 (▲) were suspended in a buffer and incubated at 25° C under anaerobic conditions. At the time point indicated by an arrow small amount of 2 M HCl was added to lower the pH as described in the text. Samples were taken at intervals and cellular ATP was determined.

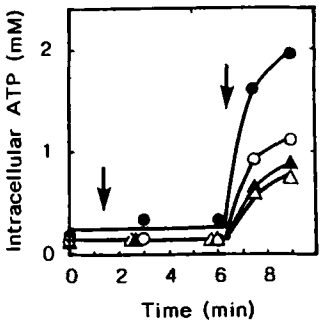

Fig. 5. Effect of CCCP on oxidative phosphorylation in the wild type and YS-1. Energy-starved cells of wild type (●, ○) or YS-1 (▲, △) were incubated in the absence (●, ▲) or in the presence (○, △) of 50 μM CCCP at 25 °C under anaerobic conditions (under N_2 gas), and 40 mM lactate (K^+ salt) was added at 1 min 20 s. At 6 min 20 s, N_2 gas was replaced with O_2 gas. Samples were taken at intervals and cellular ATP was determined.

indicate that H^+-coupled ATP synthesis occurs in *V. parahaemolyticus*, and the H^+-translocating ATPase is responsible for that. Rapid hydrolysis of ATP was observed in the wild type cells but not in the mutant.

We then tested the respiration-driven ATP synthesis in the wild type cells and the mutant (Fig. 5). Addition of lactate to the energy-starved cells under anaerobic conditions did not lead to the increase in intracellular ATP level. A few minutes later, oxygen gas was introduced into the assay vessels to initiate respiration. As expected, rapid synthesis of ATP was observed in the wild type cells. Unexpectedly, much ATP was also synthesized in the mutant YS-1, although at a slower rate than the wild type. Thus, although cells of YS-1 do not possess the H^+-translocating ATPase, they possess an ability of oxidative phosphorylation.

The effect of an H^+ conductor, CCCP, on the respiratory-driven ATP synthesis was tested. The ATP synthesis in wild type cells was inhibited by CCCP (50 μM) by about 50%, whereas that in mutant YS-1 was not affected (Fig. 5). The levels of the ATP synthesis in the presence of CCCP were similar in wild-type cells and in mutant cells.

We then tested a possibility of Na^+-coupled ATP synthesis in *V. parahaemolyticus*. An artificial Na^+ gradient was imposed across the cell membrane of the energy-starved cells of the wild type and the mutant YS-1, and the intracellular ATP was measured. ATP synthesis occurred after the addition of NaCl both in the wild type cells and in the mutant (Fig. 6). Similar pattern of increase in the intracellular ATP was observed both in the wild type and in the mutant. An addition of KCl instead of NaCl did not cause any increase in the intracellular ATP level. On the contrary, some decrease in ATP level was observed (data not shown).

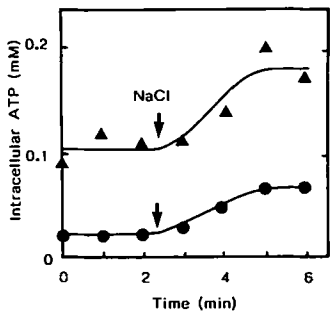

Fig. 6. ATP synthesis driven by an artificially imposed Na^+ gradient. Energy-starved cells of wild type (●) or YS-1 (▲) were incubated at 25 °C in the absence of added NaCl under anaerobic conditions in the presence of 10 mM KCN. At the time points indicated by arrows, equal volume of buffer containing 0.5 M NaCl was added as described in the text. Thus the final concentration of NaCl in the assay mixture was 0.25 M. Samples were taken at intervals and cellular ATP was determined.

Discussion

Discovery of the respiratory Na^+ pump in *V. alginolyticus* by Tokuda and Unemoto [4] raised a possibility that Na^+ may be utilized as a major coupling ion for many energy-linked processes in the membrane of this organism. The respiratory Na^+ pump [1] and Na^+-coupled cotransport systems [1–3] have been reported in *V. parahaemolyticus*. It was not clear whether H^+ circulation across the membrane plays a central role in the energy-linked membrane processes in this organism or not. One of the most important energy-linked membrane processes is the oxidative phosphorylation. Is H^+ the coupling ion for this process in *V. parahaemolyticus*? It was not clear whether the H^+-translocating ATPase is involved in the oxidative phosphorylation in this organism or not. More fundamentally, the presence of the H^+-translocating ATPase in this organism was not clear. Our results described in this paper answer these questions. The hydrolysis of ATP induced H^+ translocation or formation of the pH gradient in the everted membrane vesicles. Imposition of a pH gradient to the energy-starved cells elicited ATP synthesis. DCCD-sensitive ATPase activity was present in the membrane vesicles. This ATPase activity was lost when the membrane vesicles were washed with a buffer containing EDTA. Thus, it became clear that *V. parahaemolyticus* possesses the H^+-translocating ATPase in the membrane. Involvement of the H^+-translocating ATPase in these phenomena was proved by experiments using the mutants lacking the H^+-translocating ATPase.

Major subunits of F_1 portion of the H^+-translocating ATPase, α, β and γ, were not detected in the membrane fraction of the mutants. These mutants are spontaneous mutants, and the reversion frequencies were less than 10^{-10}. Thus these mutants do not seem to result from a single mutation. A mutant of the H^+-

translocating ATPase of *E. coli*, NR70, which was isolated for neomycin-resistance, lacks F_1 portion in the membrane [16]. This mutant has been revealed to harbor the deletion in the structural gene coding for γ subunit [17]. Perhaps the F_1 portion can not be assembled because of the defect in the γ subunit. Thus the functional H^+-translocating ATPase was lost in this mutant. Mutants YS-1, YS-2 and YS-3 may horbor similar defect. Functional F_1 was not detected also in the cytosol. It is not clear whether the subunits are present in the cytoplasm of YS-1, YS-2 and YS-3 or not.

Although functional H^+-translocating ATPase was lost in the mutant YS-1, we still observed a considerable respiratory-driven ATP synthesis in this mutant, which was resistant to CCCP. Also we observed a considerable respiratory-driven ATP synthesis in the presence of CCCP in wild-type cells. These results suggest the existence of another ATP synthetase which couples to the respiration. One possibility is the presence of Na^+-translocating ATPase. Since *V. parahaemolyticus* possesses Na^+-translocating respiratory chain, it seems likely that Na^+ circulation, in addition to H^+ circulation, plays a certain role in the oxidative phosphorylation in this organism. Since *V. parahaemolyticus* is able to grow on lactate under aerobic conditions in the presence of CCCP or TCS at pH 8.5 (unpublished results), the role of such Na^+ circulation would be very important at least under such conditions. Furthermore, the mutants grew on lactate as a sole source of carbon at alkaline pH although at a slower rate than the parent (Sakai, Y. and Tsuchiya, T., unpublished data). Recently, ATP synthesis driven by an artificially imposed Na^+ gradient has been reported with *V. alginolyticus* cells grown on glucose under anaerobic conditions [5]. Also ATP-driven Na^+ uptake by everted membrane vesicles has been shown in *V. alginolyticus* [18]. These suggest the presence of Na^+-translocating ATPase in *V. alginolyticus*. In *Propionigenium modestum* and *Methanococcus voltae* Na^+-coupled ATP synthesis [19, 20] and properties of the Na^+-translocating ATPase [21,22] have been reported.

If the Na^+-translocating ATPase is present in *V. parahaemolyticus*, the ATP synthesis driven by an electrochemical potential of Na^+, and Na^+ translocation driven by ATP hydrolysis would be observed. In fact, we observed the ATP synthesis driven by an artificially imposed Na^+ gradient in the energy-starved cells of the wild type and the mutant YS-1 which lacks the H^+-translocating ATPase. This supports the idea that the Na^+-translocating ATPase is present in *V. parahaemolyticus*. The level of ATP synthesized by an Na^+ pulse was not high. We think that an improvement of the experimental system would be necessary for the further investigation of the Na^+-driven ATP synthesis. Especially, imposition of a membrane potential together with an Na^+ gradient would be valuable to increase the artificially-driven ATP synthesis. Alternatively, the presence of permeable anions might be helpful as in the case of *M. voltae* [20]. It is also important to demonstrate the presence of ATP-driven Na^+ translocation to prove the existence of the Na^+-translocating ATPase as observed in *P. modestum* [19] and in *V. alginolyticus* [18]. Such investigations are now under way.

We observed a little synthesis of ATP in the mutant YS-1 when an artificial H^+ gradient was imposed. There are two possible explanations for this. (1) Residual H^+-translocating ATPase might be present in YS-1. This possibility, however, seems unlikely because the residual ATPase activity observed in the membrane vesicles of the mutant was very low and was insensitive to DCCD. Therefore, the residual ATPase activity would be due to other ATPase than the H^+-translocating ATPase. Furthermore, the ATP synthesis driven by respiration in YS-1 was too large to ascribe to such a residual H^+-translocating ATPase even if it is present. (2) The imposed H^+ gradient was converted to Na^+ gradient via the Na^+/H^+ antiporter, and the resultant Na^+ gradient drove ATP synthesis. If this is the case, the Na^+ gradient may be small and then ATP synthesis may be small, as was observed. Thus the second possibility seems likely.

If Na^+-translocating ATPase is present, it would be possible to detect the Na^+-stimulated ATPase activity in the everted membrane vesicles. We observed very weak ATPase activity in the membrane vesicles of the mutants. Such ATPase activity was insensitive to DCCD. We are now trying to find out the optimal conditions for such ATPase activity and to characterize it.

All of the mutants we isolated as neomycin-resistant ones were unable to grow on succinate as a sole source of carbon. It seemed possible that if Na^+-coupled ATP synthesis takes place then mutant cells lacking the H^+-translocating ATPase is still able to grow on succinate. When succinate was a respiratory substrate, we observed no oxygen consumption with starved cells of the mutants, whereas we observed normal oxygen consumption with everted membrane vesicles prepared from the mutant cells (unpublished results). These results indicate that succinate transport is also defective in those mutants. We are now investigating why all of the neomycin-resistant mutants were succinate transport-negative.

Acknowledgments

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