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Respiration-dependent primary Na^+ pump in halophilic marine bacterium, *Alcaligenes* strain 201

Kazuhiro Kogure and Hajime Tokuda*

Ocean Research Institute, University of Tokyo, Minamidai, Nakano, Tokyo 164 and **Institute of Applied Microbiology, University of Tokyo, Bunkyo, Tokyo 113, Japan*

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The inverted membrane vesicles of marine bacterium *Alcaligenes* strain 201 generated inside positive membrane potential and accumulated Na^+ on the energization with NADH. Both the generation of membrane potential and the accumulation of Na^+ were resistant to a proton conductor, carbonyl cyanide *m*-chlorophenylhydrazone. Collapse of the membrane potential by valinomycin resulted in the stimulation of Na^+ accumulation. It was concluded that *Alcaligenes* strain 201 possesses a respiratory Na^+ pump which is analogous to that of *Vibrio alginolyticus*.

Inverted vesicle; Na^+ pump; Marine bacteria; Respiratory chain

1. INTRODUCTION

According to P. Mitchell's chemiosmotic theory [1], the formation of an electrochemical gradient of proton across the energy-transducing membrane is coupled to the ATP synthesis and other cellular activities. In 1981, Tokuda and Unemoto [2] first reported the presence of a sodium pump which is directly driven by a respiratory chain in halophilic bacterium *Vibrio alginolyticus*. Later, they clarified that the sodium extrusion occurs in NADH:quinone oxidoreductase segment, and is specifically inhibited by HQNO [3]. These findings led to a new concept of Na^+ cycle instead of a proton cycle in marine halophilic bacteria [4]. Since then, the presence of a similar sodium pump was also found in other bacterial groups, including *V. costicola* [5], *V. parahaemolyticus* [6], and bacterial strain Ba_1 [7,8]. Recently, we clarified that nine strains out of ten marine bacteria isolated from Indian Ocean possess a similar respiratory chain [9]. These included genera, *Alcaligenes*, *Alteromonas*, and *Vibrio*. This suggests that this type of respiratory chain is quite widely distributed among indigenous halophilic marine bacteria.

The objective of this study was to ascertain the presence of a respiration-dependent sodium pump in the strain 201, which is one of the ten strains reported in the previous paper [9]. The formation of membrane potential and the accumulation of sodium by the inverted membrane vesicles were investigated in the presence of ionophores or metabolic inhibitors.

Correspondence address: K. Kogure, Ocean Research Institute of Applied Microbiology, University of Tokyo, Minamidai, Nakano, Tokyo 164, Japan

Abbrevations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; HQNO, 2-heptyl-4-hydroxyquinoline *N*-oxide; 4ψ, membrane potential

2. EXPERIMENTAL

2.1. *Bacterial strain*

Marine bacterium *Alcaligenes* sp. 201 was obtained from the Indian Ocean during the KH-76-5 cruise of R/V Hakuho-Maru, Ocean Research Institute, University of Tokyo (ORIUT), and was a generous gift from Dr U. Simidu (ORIUT). The strain was tentatively identified as *Alcaligenes* sp. according to the scheme by Simidu [10]. This strain is a Gram negative rod with peritricous flagella, and requires sodium for growth. The occurrence of Na^+-dependent NADH:quinone oxidoreductase in this strain was reported in the previous paper [9].

2.2. *Generation of $\Delta\psi$ and Na^+ accumulation by inverted membrane vesicles of strain 201*

Inverted membrane vesicles were prepared as described

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elsewhere [11]. $\Delta\psi$ (inside positive) and ΔpNa ($[Na^+]_{in} > [Na^+]_{out}$) generated by inverted membrane vesicles were observed by flow dialysis, using $KS^{14}CN$ (58 μCi/mM, 65 μM) and $^{22}NaCl$ (1.2 μCi, carrier-free), respectively. Energization with NADH was performed by the addition of 5 mM NAD in the presence of a NADH-generating system containing 20 U alcohol dehydrogenase (EC 1.1.1.1) and 1% (v/v) ethanol as was reported previously [5,11].

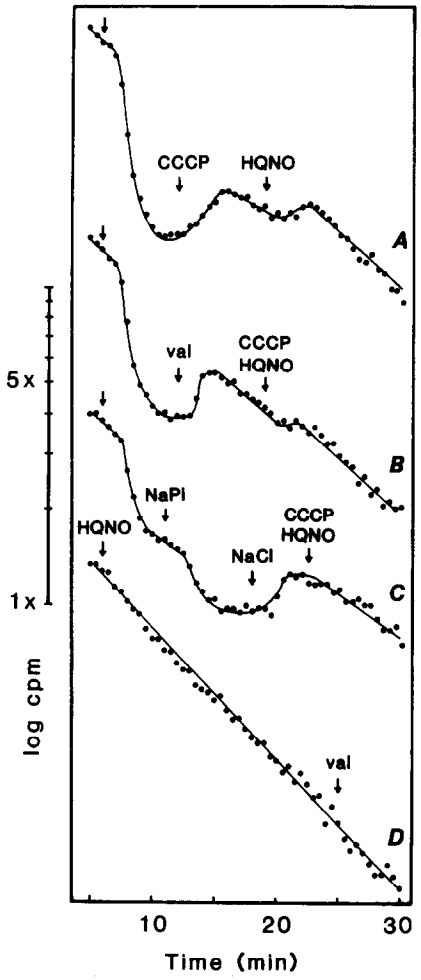

Fig.1. Generation of $\Delta\psi$ by inverted membrane vesicles. Inside positive $\Delta\psi$ by inverted membrane vesicles was observed using flow dialysis as described in section 2. The assay mixture contains 1% (v/v) ethanol, 20 U alcohol dehydrogenase (ADH), 5 mM NAD, 5 mM sodium phosphate buffer, and inverted membrane vesicles (10 mg protein) in 0.4 ml of 0.2 M potassium phosphate buffer (pH 8.5). The first arrow in each assay indicates the addition of ADH and NAD. The assay was started by the addition of $KS^{14}CN$ in the upper chamber at zero time. The final concentrations of the chemicals are as follows: CCCP, 5 μM; HQNO, 50 μM; valinomycin (val), 5 μM; NaCl, 50 mM.

3. RESULTS

3.1. *Generation of $\Delta\psi$ by the inverted membrane vesicles of strain 201*

Fig.1 shows the accumulation of $KS^{14}CN$ to see the generation of $\Delta\psi$ (inside positive) by the energized inverted membrane vesicles. The membrane potential was not completely abolished by CCCP whereas the subsequent addition of HQNO collapsed it (A). The potential was also still partly maintained with valinomycin, a potassium specific ionophore (B). Although sodium ion stimulates $\Delta\psi$, Cl^- collapsed it, probably due to the permeability of this ion through membrane (C). HQNO-treated membrane vesicles did not generate membrane potential (D).

3.2. *Accumulation of Na^+ by the inverted membrane vesicles of strain 201*

Fig.2 shows the accumulation of ^{22}Na. After

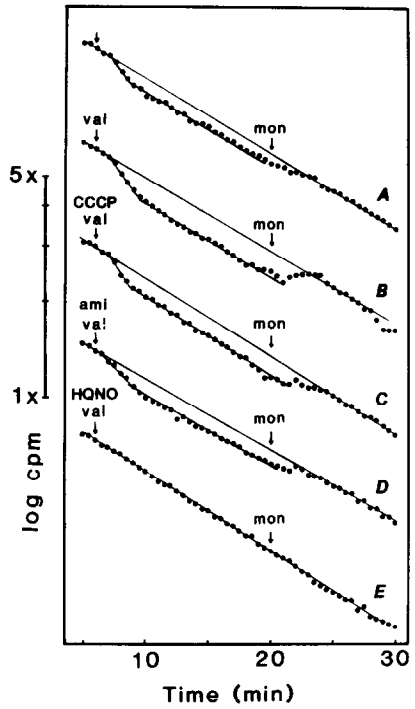

Fig.2. Na^+ accumulation by inverted membrane vesicles. Na^+ accumulation by inverted membrane vesicles was observed using flow dialysis as described in section 2. Unless otherwise stated, the conditions are the same as in fig.1. The final concentrations of monensin (mon) and amiloride (ami) were 20 μM and 3 mM, respectively. The concentrations of other chemicals are the same as in fig.1.

energization, the vesicles accumulated sodium (A). Neither valinomycin nor CCCP suppressed this process (B,C). The former clearly stimulated sodium influx, indicating that sodium influx leads to the generation of inside positive membrane potential. In the presence of amiloride, which is known as a specific inhibitor of Na^+/H^+ antiporter [12], accumulation of sodium still took place (D). On the other hand, HQNO completely inhibited it (E). Monensin caused the sodium gradient to collapse by exchanging sodium ions with a proton or another monovalent cation.

4. DISCUSSION

The preliminary study indicated that the growth of *Alcaligenes* strain 201 is insensitive to CCCP at alkaline pH and the cells formed inside acidic ΔpH in the presence of CCCP (data not shown). These characteristics are similar to those of *V. alginolyticus* [13], which is known to possess a respiration-dependent Na^+ pump. The previous report [9] further clarified that NADH oxidase of *Alcaligenes* strain 201 requires sodium for its maximum activity, and the step of semiquinone reduction seemed to be the site of Na^+ extrusion. The present investigation using inverted membrane vesicles with specific ionophores confirms the presence of respiration-dependent primary Na^+ pump in this strain.

Although the inverted membrane vesicles proved to accumulate sodium and form Δp*Na*, no detectable ΔpH was established under the same condition (data not shown). Sodium accumulation was inhibited by HQNO, which had been proved to specifically block the sodium extrusion site in the NADH:quinone oxidoreductase of *V. alginolyticus* [3,11], *V. costicola* [5], and the bacterial strain Ba_1 [8].

The bacteria of genus *Alcaligenes* are commonly distributed in the marine aquatic environment. The present investigation indicates that the respiration-dependent primary sodium pump is not restricted to a certain group of halophilic bacteria, especially *Vibrio*, but is quite widespread among diverse groups of marine bacteria. We presume that all nine bacterial strains investigated in the previous paper [9] have also the same type of sodium pump in their respiratory chains. Although they belong to at least three different generic groups, *Alcaligenes, Alteromonas*, and *Vibrio*, all strains share the common characteristics of an N^+-dependent NADH:quinone oxidoreductase. Considering the evolution of marine bacteria and the possible mechanisms of gene transfer among them, this seems quite interesting. Recently, it was suggested that the respiration-dependent sodium pump in *V. alginolyticus* is coded by plasmid DNA [14], although it remains to be elucidated for other bacteria.

In conclusion, it was confirmed that the marine bacterium *Alcaligenes* strain 201 possesses a respiration-dependent primary sodium pump, which is analogous to *V. alginolyticus* or *V. costicola*. This type of sodium pump is quite widespread among halophilic marine bacteria of diverse taxonomical groups. The results indicate the importance of the sodium cycle, instead of the proton cycle, in the energetics of halophilic marine bacteria.

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