Respiration-Driven Na\(^{+}\) Pump of the Marine Vibrio Is Encoded by Chromosomal DNA

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A plasmid-cured strain of the marine Vibrio alginolyticus 138-2 retains a respiration-driven Na\(^{+}\) pump. Examinations of several strains of V. alginolyticus and V. parahaemolyticus revealed that these marine Vibrio always possessed the respiration-driven Na\(^{+}\) pump irrespective of the presence or absence of plasmids. These results strongly suggested that the genes for the Na\(^{+}\) pump were encoded by chromosomal DNA.

Keywords: respiration-driven Na\(^{+}\) pump; NADH-quinone reductase; plasmid; Na\(^{+}\) pump; marine bacterium; marine Vibrio

The marine Vibrio alginolyticus has a respiration-driven Na\(^{+}\) pump, which is coupled to the Na\(^{+}\)-dependent NADH-quinone reductase segment of the respiratory chain.\(^{1,2}\) Several mutants, defective in Na\(^{+}\) pump activity, were isolated from V. alginolyticus 138-2.\(^{2}\) Among them, a mutant designated Nap-1, which was devoid of all three subunits of Na\(^{+}\)-dependent NADH-quinone reductase, was found to recover the Na\(^{+}\) pump activity by conjugation with wild type cells at a frequency of 10\(^{-4}\).\(^{3}\) The wild type cells contained two plasmids identified by agarose gel electrophoresis. Since the respiration-driven Na\(^{+}\) pump is widely distributed in marine bacteria,\(^{4}\) it has been suggested that the Na\(^{+}\) pump might be encoded by a plasmid.\(^{3}\) We observed that the plasmids in the wild type cells were spontaneously deleted from the cells during storage, yet these plasmid-cured cells still retained Na\(^{+}\) pump activity. In the past we surveyed the distribution of plasmids in naturally occurring V. parahaemolyticus and about one half of the strains isolated from stools of diarrhea patients were found to contain plasmids.\(^{5}\) Although most of the plasmids detected were cryptic, a new drug-resistant plasmid, pSA55, was isolated from a strain of V. parahaemolyticus.\(^{6}\) If the Na\(^{+}\) pump were encoded by a plasmid, strains of marine Vibrio without plasmid would be expected to be devoid of Na\(^{+}\) pump activity. To elucidate the relationship between the Na\(^{+}\) pump and plasmids, we examined the Na\(^{+}\) pump activity of several strains of marine Vibrio with special reference to the presence or absence of plasmids.

Materials and Methods

Bacterial Strains Four strains of V. alginolyticus ST202, ST204, ST212 and ST224 were employed. These strains were confirmed to have no plasmid by a \([^{3}H]\)thymidine labeling technique described below. A multiple drug-resistant V. parahaemolyticus ST350\(^{7}\) and a drug-susceptible strain ST559\(^{8}\) have been described previously. The former has a drug-resistant plasmid pSA55. V. alginolyticus ST224, containing pSA55 (ST224/pSA55), was prepared by conjugation with Escherichia coli bearing pSA55 as previously described.\(^{9}\) A plasmid-cured strain of V. alginolyticus 138-2, designated Vaw-1, was obtained from a stock culture of the strain 138-2.

Detection of Plasmid DNA Plasmids were detected by agarose gel electrophoresis as previously described.\(^{9}\) For the separation of plasmid DNA, \([^{3}H]\)thymidine was incorporated into DNA. Then, the cleared cell lysate was mixed with ethidium bromide–CsCl and then subjected to density gradient centrifugation.\(^{10}\)

Preparation of Membranes The membrane fraction of marine Vibrio was prepared by osmotic lysis of cells as previously described.\(^{9}\) Cells were aerobically grown at 37°C in a complex medium containing 0.5% polypeptone, 0.5% yeast extract, 0.4% K-HPO\(_4\) and 0.5 mM NaCl, and then harvested during the late exponential phase of growth.

Respiratory Activity The NADH oxidase activity of the membrane fraction was assayed at 30°C in a reaction mixture containing 0.2 mM NADH, 25 mM Tris-HCl (pH 7.5) and a specified salt. The reaction was started by the addition of membranes and the activity was calculated from the decrease in absorbance at 340 nm. One unit of activity was defined as the amount of enzyme catalyzing the oxidation of 1 μmol NADH in 1 min.

Methyamine Uptake by the Na\(^{+}\)-Loaded Cells The Na\(^{+}\)-loaded cells were prepared by treatment with diethanolamine as previously described.\(^{9}\) The cell suspension (2 μl) corresponding to about 40 μg of cell protein was added to 100 μl of the reaction mixture containing 0.4 mM NaCl, 2 mM \([^{3}H]\)methyamine (8600 cpm) and 25 mM Tricine–NaOH (pH 8.9) at 25°C. At different times the reaction was terminated by the addition of 1.5 ml of 0.4 mM choline chloride containing 10 mM Tris-HCl (pH 7.2) and by immediate filtration through a Schleicher and Schull membrane filter (OE 67 (0.45 μm pore size). The filter was washed once with 1.5 ml of the above buffer and transferred into 15 μl of the above buffer. The radioactivity on the filter was determined. The internal concentration of methyamine and the internal pH were calculated as previously described.\(^{9}\)

Results and Discussion

The marine V. alginolyticus 138-2 contained two different sizes of plasmids as detected by agarose gel electrophoresis.\(^{3}\) We have previously observed that these plasmids were spontaneously deleted from the strain 138-2 during storage, but the plasmid-cured strain, designated Vaw-1, maintained a respiration-driven Na\(^{+}\) pump. To confirm the absence of plasmids in the strain Vaw-1, \([^{3}H]\)thymidine was incorporated into DNA, and then the labeled DNAs were analyzed by CsCl ultracentrifugation. As shown in Fig. 1, the strain Vaw-1 had no detectable satellite DNA. Under the same experimental conditions, the strain ST224, containing pSA55, had a clear satellite DNA, which corresponded to the plasmid DNA, pSA55. In the case of ST224/pSA55, the ratio of total radioactivity recovered in the main peak (1.67 × 10\(^{6}\) cpm) to that in the satellite peak (4.9 × 10\(^{4}\) cpm) corresponded to about 34. The size of pSA55 is known to be about 170 kbp.\(^{10}\) Therefore, assuming that the size of the chromosomal DNA is about 4000 kbp, the satellite peak observed in Fig. 1 was calculated to correspond to 0.7 plasmids/cell. In the case of Vaw-1, the radioactivity in the main peak and in the region of the satellite peak amounted to 1.74 × 10\(^{6}\) cpm and 3.7 × 10\(^{3}\) cpm, respectively. If the same size of plasmid as that in pSA55 was present in the Vaw-1 cells, the numbers of plasmids per cell was estimated to be 0.05. These results further suggest the absence of any plasmid in the strain

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Fig. 1. Separation of Plasmids by CsCl Density Gradient Centrifugation

V. alginolyticus Vaw-1 (□) and ST224/pSA55 (●) were labeled with [3H]-thymidine, and then the labeled DNAs were separated by CsCl ultracentrifugation. Each tube contained a fraction consisting of 3 drops of elute, and 10 μl of each fraction was used for the measurement of radioactivity.

Table I. Effect of NaCl and KCl on the NADH Oxidase Activity of Marine Vibrio

<table>
<thead>
<tr>
<th>Organism</th>
<th>NADH oxidase a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+ KCl</td>
</tr>
<tr>
<td>V. alginolyticus</td>
<td></td>
</tr>
<tr>
<td>Nap-1</td>
<td>0.70</td>
</tr>
<tr>
<td>Vaw-1</td>
<td>0.76</td>
</tr>
<tr>
<td>ST202</td>
<td>0.98</td>
</tr>
<tr>
<td>ST204</td>
<td>1.52</td>
</tr>
<tr>
<td>ST212</td>
<td>0.57</td>
</tr>
<tr>
<td>ST224</td>
<td>1.11</td>
</tr>
<tr>
<td>ST224/pSA55</td>
<td>1.38</td>
</tr>
<tr>
<td>V. parahaemolyticus</td>
<td></td>
</tr>
<tr>
<td>ST550</td>
<td>0.80</td>
</tr>
<tr>
<td>ST559</td>
<td>1.06</td>
</tr>
</tbody>
</table>

a) The activity of the membrane fraction was measured in the presence of 0.2 M KCl or 0.2 M NaCl, and was expressed in units per mg protein.

Vaw-1.

The respiration-driven Na⁺ pump of the marine Vibrio is coupled to the Na⁺-dependent NADH-quinone reductase segment of the respiratory chain. Since the Na⁺-dependent NADH-quinone reductase is a limiting step in the respiratory chain of the marine Vibrio, the species of marine Vibrio having the respiration-driven Na⁺ pump exhibits Na⁺-dependent activation of the respiratory chain. As shown in Table I, the NADH oxidase activities of the membrane fractions from V. alginolyticus Vaw-1, ST202, ST204, ST212, ST224 and ST224/pSA55 were specifically activated by Na⁺. Except for the last strain, the others were confirmed to have no plasmid. As was expected, the NADH oxidase from the Na⁺ pump-defective mutant Nap-1 showed no specific activation by Na⁺. The Na⁺-dependent activation of NADH oxidase was also observed with V. parahaemolyticus ST550 and ST559. The strain ST550 possessed two different sizes of plasmids, whereas the strain ST559 possessed no detectable plasmids. The presence of a respiration-driven Na⁺ pump in V. parahaemolyticus has been reported by Tsuchiya and Shinoda. When the respiration-driven Na⁺ pump is functioning at alkaline pH, the membrane potential (∆Ψ) generated by respiration is not dissipated by the addition of the protonophore, carbonylcyanide m-chlorophenylhydrazone (CCCP). Moreover, due to the generation of CCCP-resistant ∆Ψ, a large pH gradient (∆pH), making the inside acidic, is produced in the presence of CCCP. Thus, the CCCP-induced acidification of the cell interior is reliable evidence for the presence of an electrogenic Na⁺ pump. Therefore, the generation of an internal acidic ∆pH was measured by the uptake of [14C]methylamine. When the Na⁺-loaded cells of V. alginolyticus Vaw-1 and Nap-1 were suspended in medium containing 0.4 M NaCl and 2 mM [14C]methylamine at pH 8.9, methylamine was taken up by both strains (Fig. 2). The internal concentration of methylamine was calculated to be 24 μM at equilibrium, which corresponded to an internal pH of 7.8. The internal acidic ∆pH was maintained by the regulation of cytoplasmic pH. Following the addition of 10 μM CCCP at 1 min, methylamine was further taken up by the strain Vaw-1, whereas it was released from the cells by the strain Nap-1 (Fig. 2). In the case of Vaw-1, the internal concentration of methylamine approached 136 μM, when the internal pH was calculated to be 7.1. Thus the CCCP-induced acidification of the cell interior was clearly observed with Vaw-1, but not with Nap-1. Since the addition of 50 μM 2-heptyl-4-hydroxyquinoline N-oxide, a specific inhibitor of the Na⁺ pump, prevented CCCP-induced methylamine uptake, it was apparent that the Na⁺ pump was functioning in Vaw-1.

In the results shown in Table I, the CCCP-induced acidification of the cell interior was observed with V. alginolyticus ST202, ST204, ST212, ST224 and ST224/pSA55 (data not shown). Thus, all the marine Vibrio examined possessed the respiration-driven Na⁺ pump, irrespective of the presence or absence of plasmids. These results strongly suggest that the gene for the Na⁺ pump was encoded by chromosomal DNA. Recently, we reported that Na⁺-dependent respiration and the respiration-driven Na⁺ pump are widely distributed among moderately halophilic gram-negative bacteria from diverse origins.
The presence of a respiration-driven Na\(^+\) pump is likely to be a common feature of gram-negative marine and halophilic bacteria.

References