Distribution of Na⁺-dependent respiration and a respiration-driven electrogenic Na⁺ pump in moderately halophilic bacteria

TSUTOMU UNEMOTO,* AKIKO AKAGAWA, MEGUMI MIZUGAKI and MAKI HAYASHI

Laboratory of Membrane Biochemistry, Faculty of Pharmaceutical Sciences, Chiba University, Yayoi-cho, Inage-ku, Chiba 263, Japan

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Respiratory activities of moderately halophilic bacteria from diverse origins were studied with respect to the requirement for Na⁺, the site of Na⁺-dependent reactions in the respiratory chain and the presence of a redox-driven Na⁺ pump. All the moderately halophilic bacteria examined required 1.0–2.0 M-NaCl for optimum growth. The NADH oxidase activity of the respiratory chain was influenced by the method of membrane preparation. When the cells were disrupted by a French press, the presence of 0.2 M-Na₂SO₄ (or 0.4 M-NaCl) was required to protect against loss of Na⁺-dependent NADH oxidase activity. Membranes prepared by osmotic lysis retained high Na⁺-dependent NADH oxidase activity. Of eight moderate halophiles investigated, the six Gram-negative bacteria possessed Na⁺-dependent NADH oxidase activity. The site of Na⁺-dependent activation in the respiratory chain was located on the NADH : quinone reductase segment in all these halophiles. Other activities, such as succinate oxidase and the terminal oxidase, showed no specific requirement for Na⁺. Using inverted membrane vesicles prepared from these halophiles, it was found that membrane potential generation linked to NADH oxidation was not completely dissipated by addition of the protonophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), but was entirely sensitive to CCCP plus the Na⁺-conducting ionophore monensin. A Na⁺-dependent NADH oxidase was not detected in membranes from the two Gram-positive halophiles, *Marinococcus halophilus* and *Micrococcus varians* subsp. *halophilus*, that we investigated. Since the membrane potential generated by oxidation of NADH was completely dissipated by CCCP, these halophiles did not have any respiration-driven Na⁺ pumps.

Introduction

Since the discovery of a respiration-driven Na⁺ pump in a marine strain of *Vibrio alginolyticus* (Tokuda & Unemoto, 1981, 1982), respiration-driven Na⁺ pumps have been identified in a marine *V. parahaemolyticus* (Tsuchiya & Shinoda, 1985), a moderately halophilic *V. costicola* (Udagawa *et al.*, 1986), the halotolerant bacterium Ba₁ (Ken-Dror *et al.*, 1986*a, b*) and in several other marine bacteria (Tokuda & Kogure, 1989). Detailed studies on the Na⁺ pump of *V. alginolyticus* revealed that the extrusion of Na⁺ was directly coupled to the Na⁺-dependent NADH:quinone reductase segment in the respiratory chain (Tokuda & Unemoto, 1984), which is

composed of three subunits, α , β and γ (Hayashi & Unemoto, 1986, 1987). All of the above-described bacteria require Na⁺ for optimum growth and their respiratory activities (NADH oxidase) are specifically activated by Na⁺, the site of Na⁺ activation being Na⁺-dependent NADH:quinone reductase. At first, it appeared that the Na⁺-transport NADH:quinone reductase is commonly distributed only in marine and halophilic bacteria. However, Dimroth & Thomer (1989) demonstrated the presence of Na⁺-transport NADH:quinone reductase in the anaerobic bacterium *Klebsiella pneumoniae*, which is known to have an additional decarboxylase-driven Na⁺ pump (Dimroth, 1987), and Semeykina *et al.* (1989) reported the presence of Na⁺-motive terminal oxidase in the respiratory chain of the halotolerant *Bacillus* sp. strain FTU grown on succinate in the presence of 0.5 M-NaCl at pH 8.6. Later, this bacterium was shown to have H⁺-motive and Na⁺-motive NADH:quinone reductases and terminal-oxidases (Kostyrko *et al.*, 1991). The Na⁺-motive respiration of this bacterium was induced only if grown at high

* Author for correspondence. Tel. 43 251 1111 ext. 2738; fax 43 255 1574.

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; Q-1, ubiquinone-1; TMPD, *N,N,N',N'*-tetramethylphenylene diamine; oxonol-VI, bis(3-propyl-5-oxoisoxazole-4-yl)pentamethine-oxonol.

pH and high NaCl concentrations. The Na⁺-motive terminal oxidase was also reported in a non-halophilic and strictly aerobic bacterium, *Vitreoscilla* sp., where cytochrome *o* terminal oxidase was shown to be a redox-driven Na⁺ pump (Efiok & Webster, 1990). Although the site of Na⁺ transport in the respiratory chain has not been characterized, *Escherichia coli* grown on succinate at alkaline pH in the presence of NaCl was reported to possess a respiration-driven Na⁺ pump (Avetisyan *et al.*, 1989). These growth conditions were selected so as to lower the proton-motive force, such that the cells were expected to be forced to perform an Na⁺-motive oxidative phosphorylation (Skulachev, 1989). In methanogenic bacteria, the presence of at least two redox-driven Na⁺ pumps has been reported (Müller *et al.*, 1988; Kaesler & Schonheit, 1989). Thus redox-driven Na⁺ pumps are not restricted to marine and halophilic bacteria and are widely distributed in various species of bacteria.

During our studies on the Na⁺-dependent reactions in the respiratory chain of marine bacteria, only the NADH:quinone reductase segment was found to require Na⁺ for activity; none of the other respiratory segments was Na⁺-dependent. Furthermore, the Na⁺-translocating NADH:quinone reductase of marine bacteria was not affected by growth conditions such as medium pH and NaCl concentration, suggesting constitutive formation of this Na⁺ pump. Since several different types of redox-driven Na⁺ pumps have been reported in other bacteria, it is of interest to search further for the existence of respiration-driven Na⁺ pumps in halophilic bacteria. Marine bacteria are slightly halophilic bacteria: this group requires 0.5 M-NaCl for optimal growth but is unable to grow above 2 M-NaCl. On the other hand, moderately halophilic bacteria are able to grow in a wide range of NaCl concentrations (0.5–4.0 M) and are highly adapted to salt environments.

Except for a few examples, such as *V. costicola* (Udagawa *et al.*, 1986) and halotolerant Ba₁ (Ken-Dror *et al.*, 1986a, b), moderately halophilic bacteria have not been studied in detail from the bioenergetic point of view. We therefore examined the distribution of Na⁺-dependent respiration and respiration-driven Na⁺ pumps in several species of moderately halophilic bacteria from diverse origins.

Methods

Bacterial species and culture conditions. The moderately halophilic bacteria used in this study are listed in Table 1, together with the isolation sources. *Pseudomonas halosaccharolytica* was grown in a medium composed of 1% (w/v) polypeptone, 0.1% yeast extract, 23.5 mM-NaNO₃, 16 mM-MgSO₄ and 0.4–0 M-NaCl (Ohno *et al.*, 1979). *Vibrio costicola* (NCMB 701^T), *Pseudomonas beijerinckii* and the unidentified halophile (NRCC 41227) were grown in a medium composed of 0.5% polypeptone, 0.5% yeast extract, 0.5% Tryptone, 5.7 mM-K₂HPO₄, 10 mM-MgSO₄ and 0.4–0 M-NaCl. *Halovibrio variabilis* was grown in a medium composed of 1% (w/v) polypeptone, 0.5% yeast extract, 54 mM-glycerol, 5.7 mM-K₂HPO₄, 20 mM-MgSO₄ and 0.4–0 M-NaCl. *Deleya halophila* was grown in a medium composed of 1% (w/v) polypeptone, 1% (w/v) yeast extract, 0.1% glucose, 5.7 mM-K₂HPO₄ and 0.4–0 M-NaCl. *Marinococcus halophilus* and *Micrococcus varians* subsp. *halophilus* were grown in a medium composed of 1% (w/v) polypeptone, 1% (w/v) yeast extract, 5.7 mM-K₂HPO₄ and 0.4–0 M-NaCl. The medium pH was adjusted to 7.4 with 1 M-NaOH.

Cultures (10 ml) were incubated aerobically at 37 °C in a 25 ml volume L-tube, shaken at 52 strokes min⁻¹. The culture growth was monitored by measuring the optical density (OD) at 600 nm in a Perkin-Elmer model 35 spectrophotometer. Pre-cultures grown in the presence of 1 M-NaCl were inoculated at an OD₆₀₀ of 0.02 into fresh medium containing various concentrations of NaCl. The doubling time was calculated from the linear part of the exponential curve and the growth rate was expressed as the number of doublings h⁻¹.

Preparation of a membrane fraction. Membranes were prepared by disrupting the cells with an osmotic lysis or with a French pressure cell (Aminco). Cells were grown in the presence of 1 M-NaCl, harvested

Table 1. Moderately halophilic bacteria used in this study

Bacterial species	Isolation source	Reference
<i>Pseudomonas halosaccharolytica</i> (ATCC 29423)	Crude salt	Ohno <i>et al.</i> (1979)
<i>Pseudomonas beijerinckii</i> (ATCC 19372, NCIB 9041)	Salted beans	Breed <i>et al.</i> (1957)
Unidentified halophile (NRCC 41227)	Laboratory contaminant on salt-agar plate	(Bergey's Manual, 7th edn) Matheson <i>et al.</i> (1976)
<i>Vibrio costicola</i> (NCMB 701 ^T , NRCC 37001)	Bacon curing brines	Flannery <i>et al.</i> (1952)
<i>Halovibrio variabilis</i> (DSM 3051 ^T)	Great Salt Lake, Utah, USA	Fendrich (1988)
<i>Deleya halophila</i> (CCM 3662 ^T)	Saline soils near Alicante, Spain	Quesada <i>et al.</i> (1984)
<i>Marinococcus halophilus</i> (CCM 2706 ^T , IAM 12844)	Salted mackerel	Hao <i>et al.</i> (1984)
<i>Micrococcus varians</i> subsp. <i>halophilus</i> (ATCC 21971)	Soy sauce mash	Kamekura & Onishi (1974)

during the late exponential phase of growth and then washed twice by centrifugation with a medium containing 1.0 M-NaCl and 20 mM-HEPES/Tris, pH 7.5. For osmotic lysis, washed cells were suspended in a lysis medium containing 10 mM-NaCl, 5 mM-EDTA and 20 mM-HEPES/Tris, pH 7.5, at a cell density of 40 mg wet wt cells ml⁻¹. After 5 min at 25 °C, the suspension was centrifuged at 20000 g for 20 min. The precipitate was resuspended in 10 mM-NaCl, 0.5 mM-EDTA and 20 mM-HEPES/Tris, pH 7.5, and then DNAase (5 µg ml⁻¹) and MgSO₄ (2 mM) were added. After 10 min at 25 °C, EDTA was added at 5 mM, and the suspension was incubated for 5 min, before being centrifuged at 20000 g for 20 min. The precipitate was suspended in 10% (w/v) glycerol, 10 mM-NaCl and 10 mM-HEPES/Tris, pH 7.5, and stored at -75 °C.

For disruption of cells by using a French press, washed cells were suspended in a medium containing 5 mM-MgSO₄, 1 mM-dithiothreitol, 10 µg DNAase ml⁻¹, 10% (w/v) glycerol, 10 mM-HEPES/Tris, pH 7.5, and 5 mM- or 0.2 M-Na₂SO₄ at a cell density of 250 mg wet wt cells ml⁻¹. The suspension was passed through the French pressure cell at 8000 lbf in⁻² (55200 kPa) or 14000 lbf in⁻² (96600 kPa). Unbroken cells were removed by centrifugation at 25000 g for 10 min and the supernatant was centrifuged at 250000 g for 90 min. The precipitate was washed with and then suspended in 10% (w/v) glycerol, 10 mM-MgSO₄, 10 mM-HEPES/Tris, pH 7.5, and 5 mM- or 0.2 M-Na₂SO₄, at about 10 mg protein ml⁻¹, and stored at -75 °C.

Enzyme assays. NADH oxidase activity was assayed at 30 °C in a reaction mixture containing 0.2 mM-NADH, 25 mM-Tris/HCl, pH 7.5, an appropriate amount of NaCl and membranes in a total volume of 1.0 ml. The reaction was started by addition of membranes and the decrease in absorbance at 340 nm was measured with a Hitachi 200 spectrophotometer. The rate of NADH oxidation was calculated using an absorption coefficient of 6.22 mM⁻¹ cm⁻¹.

NADH:quinone reductase activity was assayed at 30 °C by following the formation of reduced quinone as previously described (Unemoto & Hayashi, 1989). The reaction mixture contained 0.1 mM-NADH, 5 µM-ubiquinone-1 (Q-1), 25 mM-Tris/HCl, pH 7.5, 10 mM-KCN, salt and membranes in a total volume of 2.0 ml. The membranes were incubated with KCN for 2 min, and then the reaction was started by addition of NADH. Changes in absorbance difference at the wavelength pair, 242–270.5 nm, were recorded with a Hitachi 557 dual-wavelength spectrophotometer. The rate of Q-1 reduction was calculated using an absorption coefficient of 9.6 mM⁻¹ cm⁻¹ (Unemoto & Hayashi, 1989).

Succinate oxidase and tetramethylphenylene diamine (TMPD) oxidase activities were measured at 30 °C by using an oxygen electrode (Yellow Springs Instruments, model 5331). Succinate oxidase was assayed in a reaction mixture containing 6 mM-succinate, 25 mM-Tris/HCl, pH 7.5, NaCl and membranes in a total volume of 3.0 ml. TMPD oxidase was assayed in a reaction mixture containing 1 mM-ascorbate, 0.5 mM-TMPD, 25 mM-Tris/HCl, pH 7.5, NaCl and membranes in a total volume of 3.0 ml.

One unit of activity was defined as the amount of enzyme catalysing the oxidation of 1 µmol NADH, or the reduction of 1 µmol Q-1 or 1 µmol oxygen atoms, in 1 min.

Measurement of the membrane potential. Generation of a membrane potential, positive inside, in membrane vesicles was monitored by following the absorbance band shift of oxonol-VI at 625–587 nm with the dual-wavelength spectrophotometer. Inverted membrane vesicles were prepared by using the French press at a shear force of 8000 lbf in⁻² in the presence of 0.2 M-Na₂SO₄. The reaction mixture contained 0.1 mM-NADH, 10 mM-MgSO₄, 0.2 M-Na₂SO₄, 3 µM-oxonol VI, membrane vesicles (0.1 mg protein ml⁻¹) and 50 mM-HEPES/Tris, pH 7.5, in a total volume of 2.0 ml. The reaction was started by the addition of NADH at 20 °C.

Results and Discussion

Effect of NaCl on the growth of moderately halophilic bacteria

Since *H. variabilis*, isolated from the Great Salt Lake (Fendrich, 1988), showed optimum growth at 30 °C rather than at 37 °C, the results for this bacterium were obtained at 30 °C; all other cultures were grown at 37 °C. As shown in Fig. 1, the growth rate of the marine bacterium *V. alginolyticus* was high, with the optimum between 0.3 and 0.8 M-NaCl, which is typical of slightly halophilic bacteria. Conversely, *V. costicola*, *D. halophila*, *H. variabilis*, *Ps. halosaccharolytica*, *Ps. beijeirneckii* and NRCC 41 227 were able to grow at a wide range of NaCl concentrations, with the optimum between 1.0 and 2.0 M-NaCl. The NaCl dependence of growth rate observed here is typical of moderately halophilic bacteria.

As Gram-positive halophilic bacteria, *Mar. halophilus* and *Mic. varians* subsp. *halophilus* were selected for use in this study. The former was first isolated from salted mackerel as *Planococcus halophilus* and later reclassified as a marine coccus (Hao *et al.*, 1984). To examine the NaCl growth response of this bacterium, the concentrations of NaCl in the pre-culture were varied from 0.5 to 4.0 M-NaCl and then these pre-cultures were used for measurements of growth rate. The length of lag phase was greatly influenced by the concentration of NaCl in the pre-culture. For example, when a pre-culture grown at 4.0 M-NaCl was inoculated into fresh medium containing 4.0 M-NaCl, there was a 10 h lag phase, which was prolonged to 48 h with a pre-culture grown at 0.5 M-NaCl. However, the exponential growth rate at the various NaCl concentrations was unaffected by the pre-culture (Fig. 2). *Mar. halophilus* apparently required 1.0–2.0 M-NaCl for optimum growth.

Mic. varians subsp. *halophilus* is able to grow in complex media containing 1 M-NaCl, -KCl, -RbCl or other salts (Kamekura & Onishi, 1982). Since this bacterium has been reported to grow even in the absence of salts after a 5 d lag phase (Kamekura & Onishi, 1974), it did not have any specific requirement for NaCl for growth. However, similar to the results of Kamekura & Onishi (1974), this bacterium showed optimum growth between 1.0 and 3.0 M-NaCl (data not shown), exhibiting the NaCl growth response typical of moderately halophilic bacteria.

From these results, the bacterial species used in this study should be classified as moderately halophilic bacteria.

Fig. 1

Fig. 1. Effect of NaCl on growth of halophilic bacteria. The growth rate of each bacterium in the presence of various NaCl concentrations was measured at 37 °C as described in Methods (except for the growth rate of *H. variabilis*, which was measured at 30 °C). Values are means of two or three separate experiments. ×, *V. alginolyticus*; ●, *V. costicola*; ■, *D. halophila*; ▲, *H. variabilis*; ○, NRCC41227; △, *Ps. beijerinckii*; □, *Ps. halosaccharolytica*.

Fig. 2

Fig. 2. Effect of pre-culture on the subsequent NaCl growth response of *Mar. halophilus*. *Mar. halophilus* was pre-cultured in the presence of 0.5 (○), 1.0 (●), 2.0 (□) and 4.0 M-NaCl (■), and then grown on at the NaCl concentrations indicated.

Cation dependence of NADH oxidase in membrane fractions

In the marine *V. alginolyticus*, membranes prepared by osmotic lysis exhibited high rates of oxidation of exogenously added NADH (Unemoto *et al.*, 1977). This membrane preparation, however, cannot be used for measurements of the membrane potential due to its leakiness (Unemoto *et al.*, 1977). It was necessary, therefore, to prepare inverted membrane vesicles by disrupting the cells with a French press. To determine suitable conditions for membrane preparation, cells were disrupted under low or high pressure in the presence or absence of 0.2 M- Na_2SO_4 as described in Methods. The cation dependence of NADH oxidase in the membranes prepared by different methods from *D. halophila* was measured. In all preparations, the NADH oxidase was greatly activated by NaCl, but not by KCl. The Na^+ -dependent NADH oxidase activity was unaffected by the pressure of the French press, but addition of 0.2 M- Na_2SO_4 or 0.4 M-NaCl was required to maintain high activity, which is comparable to results obtained with membranes prepared by osmotic lysis. Similar results were obtained with *V. costicola* and *H. variabilis*.

Fig. 3 shows the effect of NaCl and KCl on the NADH oxidase of membranes prepared from NRCC 41227. The membranes prepared by the French press always showed high NADH oxidase activity in the absence of salts and only a slight activation was observed with Na^+ . Although not shown here, with the membranes prepared by the French press in the absence of 0.2 M- Na_2SO_4 , it was difficult to detect Na^+ -dependent activation of NADH oxidase. However, with membranes prepared by osmotic lysis, activity in the absence of salts decreased

Fig. 3. Effect of method of membrane preparation on NADH oxidase activity of NRCC 41227. NADH oxidase activity was measured in the presence of NaCl (filled symbols) or KCl (open symbols). The membranes were prepared under the following conditions: French press at 8000 lbf in⁻² in the presence of 0.2 M- Na_2SO_4 (■, □); osmotic lysis (●, ○). Results presented are typical of those obtained in three separate experiments.

and Na^+ -dependent activation became apparent (Fig. 3). Similar results were obtained with *Ps. halosaccharolytica* and *Ps. beijerinckii*.

These results indicated that the membrane-bound Na^+ -dependent NADH oxidase of moderate halophiles was influenced by the method of membrane preparation. Addition of 0.2 M- Na_2SO_4 or 0.4 M-NaCl protected against loss of Na^+ -dependent activity, especially when membranes were prepared by the French press.

In contrast to the Gram-negative halophiles, the Gram-positive halophiles were stable in a hypotonic medium and membranes could not be prepared by osmotic lysis. Therefore, cells of *Mar. halophilus* and *Mic. varians* subsp. *halophilus* were disrupted using the French

Fig. 4. Cation dependence of the NADH:quinone reductase segment of the respiratory chain. Membranes were prepared by the French press at 8000 lbf in⁻² (55 200 kPa) in the presence of 0.2 M-Na₂SO₄ as described in Methods, and the NADH:quinone reductase activity was assayed in the presence of NaCl (filled symbols) or KCl (open symbols). (a) *D. halophila* (●, ○); *Mic. varians* subsp. *halophilus* (■, □). (b) *Ps. halosaccharolytica*. Results presented are typical of those obtained in three separate experiments.

press. The membrane-bound NADH oxidase from these bacteria was slightly stimulated by addition of either NaCl or KCl, and the Na⁺-dependent activity could not be detected in any membrane preparations, implying the absence of Na⁺-dependent NADH oxidase.

Site of the Na⁺-dependent reaction in the respiratory chain

The cation dependence of the partial reaction in the respiratory chain was examined in order to locate the site of the Na⁺-dependent reaction. As shown in Fig. 4(a), the NADH:quinone reductase of membranes from *D. halophila* required Na⁺ for activity and K⁺ was practically ineffective. The NADH:quinone reductase of membranes from *Ps. halosaccharolytica* also required Na⁺ for activity (Fig. 4b). In contrast, the NADH:quinone reductase from *Mic. varians* subsp. *halophilus* did not require salt for activity and the activity was not affected by NaCl and KCl (Fig. 4a). *Mar. halophilus* also gave the same results (data not shown).

Table 2 shows the effects of NaCl and KCl on respiratory activities. In the case of *D. halophila* and *H. variabilis*, the presence of Na⁺-dependent NADH oxidase was easily detected due to the low activity of NADH oxidase in the absence of salts. Similar results have been shown in *V. costicola* (Unemoto *et al.*, 1977). On the other hand, in the case of *Ps. halosaccharolytica*, *Ps. beijeirinkii* and NRCC 41227, Na⁺-dependent NADH oxidase was difficult to detect because of the presence of high activities in the absence of salts. However, when the activity of the NADH:quinone reductase segment was measured, a Na⁺-dependent activation was clearly observed with these bacteria. These results imply that in the latter case the Na⁺-dependent reaction was not a limiting step in the respiratory chain.

In all of the Gram-negative halophiles examined, succinate oxidase, which bypasses the NADH:quinone reductase segment, and the terminal oxidase measured as oxidation of TMPD (TMPD oxidase) showed no specific requirement for Na⁺. Thus, the site of the Na⁺-

Table 2. Effects of NaCl and KCl on respiratory chain activities

Membranes prepared by the French press at 8000 lbf in⁻² (55 200 kPa) in the presence of 0.2 M-Na₂SO₄ were used for assays. Numbers in parentheses denote salt concentrations used for assays. Results presented are typical of those obtained in three separate experiments.

Bacterium	Respiratory chain activity [units (mg protein) ⁻¹]											
	NADH oxidase			Quinone reductase			Succinate oxidase			TMPD oxidase		
	None	NaCl	KCl	None	NaCl	KCl	None	NaCl	KCl	None	NaCl	KCl
<i>Deleya halophila</i>	0.10	1.54 (0.8 M)	0.34	0.01	1.28 (1.0 M)	0.17	0.79	0.72 (0.2 M)	0.84	0.70	0.62 (0.2 M)	0.31
<i>Halovibrio variabilis</i>	0.05	0.65 (0.3 M)	0.25	0.01	0.36 (0.4 M)	0.17	0.23	0.17 (0.3 M)	0.22	0.15	0.15 (0.3 M)	0.16
<i>Ps. halosaccharolytica</i>	0.51	0.69 (0.3 M)	0.36	0.13	0.34 (0.3 M)	0.12	0.15	0.16 (0.3 M)	0.18	0.10	0.13 (0.3 M)	0.12
<i>Ps. beijeirinkii</i>	0.56	0.71 (0.4 M)	0.40	0.10	0.27 (0.8 M)	0.07	0.10	0.15 (0.4 M)	0.16	0.10	0.11 (0.4 M)	0.13
NRCC 41227	0.48	0.57 (0.2 M)	0.38	0.07	0.21 (0.6 M)	0.10	0.31	0.24 (0.2 M)	0.29	0.28	0.25 (0.2 M)	0.22

Fig. 5. Generation of a membrane potential and its sensitivity to CCCP (3 μ M) and monensin (30 μ M) in inverted membrane vesicles prepared from Gram-negative halophiles.

dependent reaction in the respiratory chain was located solely on the NADH:quinone reductase segment. Furthermore, the Na⁺-dependent reaction was strongly inhibited by 5 μ M-2-*n*-heptyl-4-hydroxyquinoline *N*-oxide, which is a specific inhibitor of the Na⁺ pump. These results were very similar to those obtained with marine bacteria (Unemoto & Hayashi, 1989).

With respect to the Gram-positive halophiles, a Na⁺-dependent reaction site was not detected in any segment of the respiratory chain.

Generation of a CCCP-resistant membrane potential

The membrane potential generated by Na⁺-dependent oxidation of NADH is not completely dissipated by the proton conductor CCCP (Tokuda & Unemoto, 1982). Therefore, the sensitivity of the membrane potential generated by oxidation of NADH in inverted membrane vesicles was examined using the response of oxonol-VI as described in Methods. As shown in Fig. 5, the membrane potential generated in inverted membrane vesicles derived from Gram-negative halophiles was not completely dissipated by addition of 3 μ M-CCCP. By increasing the concentration of CCCP, the oxonol response diminished, but it still remained even at 25 μ M-CCCP. It was necessary to add the Na⁺-conducting ionophore monensin to completely dissipate the membrane potential. These results are in agreement with the functioning of a respiration-driven Na⁺ pump.

In contrast, with inverted membrane vesicles derived from Gram-positive halophiles, the membrane potential generated by oxidation of NADH was completely dissipated by addition of 3 μ M-CCCP (data not shown).

From these results, it was evident that among the moderately halophilic bacteria examined, the Gram-negative halophiles have a respiration-driven Na⁺ pump, whereas the Gram-positive halophiles have no respiration-driven Na⁺ pump. In all the Gram-negative halophiles, the site of the Na⁺-dependent reaction was located on the NADH:quinone reductase and other reactions in the respiratory chain showed no specific requirement for Na⁺. These properties are just the same as those of the marine bacterium *V. alginolyticus* (Unemoto & Hayashi, 1989). Thus, the moderately halophilic bacteria examined here are closely related to marine bacteria in that they possess a Na⁺ pump. On the other hand, the two species of Gram-positive moderate halophiles had no respiration-driven Na⁺ pump. Although the NaCl growth responses of these bacteria support their classification as moderate halophilic bacteria, they do not lyse in a hypotonic medium, which is in contrast to marine bacteria. Furthermore, *Mic. varians* subsp. *halophilus* can grow in complex medium in the absence of salts (Kamekura & Onishi, 1974). Thus, these halophiles differ from marine bacteria in many respects. It seems very important to determine the presence or absence of a respiration-driven Na⁺ pump when characterizing moderately halophilic bacteria.

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