

Correlation between Possession of a Respiration-Dependent Na⁺ Pump and Na⁺ Requirement for Growth of Marine Bacteria

SUWAN OH,† KAZUHIRO KOGURE,* KOUICHI OHWADA, AND USIO SIMIDU

Ocean Research Institute, University of Tokyo, Minamidai, Nakano, Tokyo 164, Japan

Received 12 December 1990/Accepted 18 March 1991

The possession of a respiration-dependent primary sodium pump and the requirement of Na⁺ for growth were investigated in bacterial isolates from marine environments. The bacteria in which NADH oxidase specifically required Na⁺ for maximum activity were believed to possess a primary sodium pump. All bacteria that failed to grow without the addition of NaCl possessed a primary Na⁺ pump. All bacteria that had no primary Na⁺ pump grew without additional NaCl. The primary Na⁺ pump seems to be involved in the Na⁺ requirement of marine bacteria, and this can be regarded as a criterion for the definition of marine bacteria.

According to Mitchell's chemiosmotic theory (4), living organisms acquire energy or synthesize ATP by establishing a proton gradient across the energy-transducing membrane. This is accomplished by a proton pump, which is coupled to the respiratory chain and H⁺-ATP synthetase. However, Tokuda and Unemoto (8) clarified that the marine bacterium *Vibrio alginolyticus* possesses a primary Na⁺ pump that is directly coupled to the respiratory chain. They also showed that NADH oxidase of *V. alginolyticus* requires Na⁺ for maximum activity, whereas the oxidase of a mutant that lacks the Na⁺ pump is not specifically stimulated by Na⁺ anymore (9). The NADH oxidase of a revertant strain again showed the Na⁺ requirement for maximum NADH oxidase activity. Since then, other marine bacteria have been found to have a similar Na⁺ pump (1, 2, 7, 10, 11). So far, all of these bacteria share common characteristics, i.e., Na⁺ extrusion at the NADH:quinone oxidoreductase segment in the respiratory chain, Na⁺ requirement for maximum activity of NADH oxidase, and inhibition of NADH oxidase activity by 2-heptyl-4-hydroxyquinoline *N*-oxide. From these observations, it can be concluded that bacteria that possess a primary Na⁺ pump have NADH oxidase that requires sodium for the maximum activity. We used this criterion to investigate the distribution of this particular respiratory chain among bacteria from the marine environment.

Marine bacteria are generally defined as those living in seawater and requiring sodium for their growth (3). It is usually explained that the ion is necessary for membrane stability, enzyme activity, active transport, and so on. However, the physiological roles of sodium are not yet clear, and the bioenergetic approach has been almost completely ignored. This situation causes ambiguous characterization and definition of marine bacteria. The purpose of this work was to examine the relationship between a sodium requirement for growth and the possession of a respiration-dependent primary Na⁺ pump in marine bacteria.

Thirty-seven bacterial strains were isolated from the littoral zone of Aburatsubo Inlet, Kanagawa Prefecture, Japan, on 24 April and 6 June 1987. Samples were also taken from Sagami Bay, Japan, on 8 August 1988 during the

KT-88-15 cruise on board the R/V *Tansei-maru* (Ocean Research Institute, University of Tokyo). Colonies that appeared on ZoBell 2216E agar (1/10 dilution, referred to as 1/10 ZoBell 2216 E agar) plates were isolated after incubation for 2 weeks at 20°C. The isolates were tentatively identified as described by Simidu (5). Besides the isolates, 12 type strains were also investigated. Each bacterial strain was cultivated in 250 ml of 1/10 ZoBell 2216E culture medium in a reciprocal shaker at 25°C. Cells in the late logarithmic phase were harvested and washed, and the membrane fractions were obtained by osmotic lysis as reported elsewhere (6). The NADH oxidase activity in the membrane fraction was assayed as described by Tokuda and Unemoto (9). In brief, the activity was determined from the decrease in A₃₄₀ at 30°C. The assay was started by adding NADH (0.2 mM) to the assay mixture (final volume, 1 ml), which contained 20 mM Tris-HCl (pH 7.5), ca. 20 µg of membrane protein, and Na⁺ or K⁺ ion. The sodium requirement for growth was checked by using 1/10 ZoBell 2216E agar plates. The composition of the synthetic seawater medium was as follows (in 1 liter of distilled water): NaCl, 23.5 g; MgCl₂ · 6H₂O, 5 g; CaCl₂, 1.1 g; KCl, 0.66 g; KBr, 96 mg; H₃BO₃, 26 mg; SrCl₂,

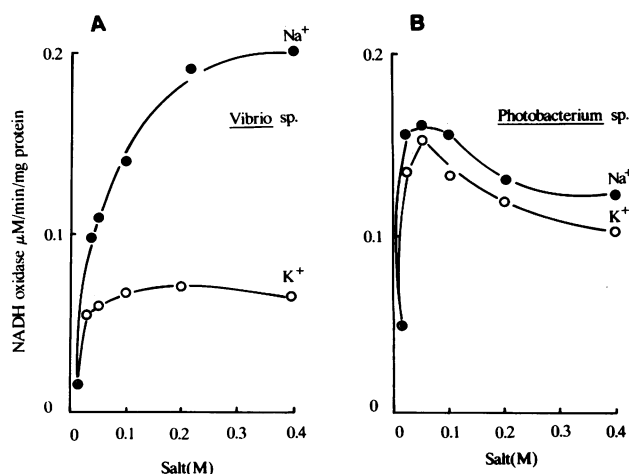


FIG. 1. Effect of salt on NADH oxidase activity of membrane fractions from bacteria with (A) and without (B) a Na⁺ pump.

* Corresponding author.

† Present address: Sagami Chemical Research Center, Sagami-hara, Kanagawa 229, Japan.

TABLE 1. Minimum NaCl concentration required for bacterial growth

Bacterial isolate(s)	No. of bacteria growing at the following NaCl concn (%):				
	0	0.2	0.5	1.0	2.0
With Na⁺ pump					
Marine isolates					
<i>Alteromonas</i> species	1	1	1		
<i>Moraxella</i> species		1			
<i>Pseudomonas</i> species	1	1	1		
<i>Vibrio</i> species	2	4	4	2	1
Type strains					
<i>Marinomonas vaga</i> IAM 12923			1		
<i>Photobacterium augustum</i> IAM				1	
<i>Photobacterium leiognathi</i> ATCC 25521				1	
<i>Pseudomonas marina</i> IAM 12928	1				
<i>Vibrio alginolyticus</i> NCMB 1903				1	
<i>Vibrio campbellii</i> ATCC 25920			1		
<i>Vibrio fischeri</i> ATCC 7744					1
<i>Vibrio harveyi</i> ATCC 14126			1		
<i>Vibrio natriegens</i> CCM 2575				1	
<i>Vibrio parahaemolyticus</i> ATCC 17802				1	
Without Na⁺ pump					
Marine isolates					
<i>Alcaligenes</i> species	9				
<i>Alteromonas</i> species	2				
<i>Bacillus</i> species	1				
Members of the family <i>Enterobacteriaceae</i>	1				
<i>Flavobacterium</i> species	2				
<i>Micrococcus</i> species	1				
<i>Pseudomonas</i> species	3				
<i>Vibrio</i> species	4				
Type strains					
<i>Alteromonas putrefaciens</i> IAM 12079	1				
<i>Pseudomonas aeruginosa</i> IAM 1514	1				

24 mg. Media with various concentrations of NaCl were obtained by substituting KCl for NaCl. After incubation at 20°C, visible colonies were transferred to a new plate with the same NaCl concentration. The second incubation was used to confirm growth: if no colony appeared after 2 weeks of incubation, the strain was judged unable to grow on the plate.

A typical relationship between NADH oxidase activity and Na⁺ or K⁺ concentration of a strain with a primary Na⁺ pump is shown in Fig. 1A. It required sodium for maximum activity; K⁺ did not serve as a substitute for NaCl. Other monovalent cations, including Rb⁺, Li⁺, and Cs⁺, also failed to induce maximum activity (data not shown). In contrast, Na⁺ had no specific effect on the NADH oxidase activity of isolates that lack a Na⁺ pump (Fig. 1B), and monovalent cations stimulated the activity only at low concentration. In the present study, when Na⁺ induced at least two times more activity than K⁺ did, the strain was judged to possess a primary Na⁺ pump coupled to the respiratory chain.

Among 27 strains possessing Na⁺ pumps, as indicated by the Na⁺ requirement for maximum NADH oxidase activity, 22 required sodium for growth (Table 1). Most of them failed

to grow on media with NaCl concentrations below 0.5%. Five strains, however, were able to make visible colonies without additional NaCl. On the other hand, all bacteria that did not have a Na⁺-stimulated NADH oxidase could grow on plates without NaCl. In other words, all strains that required NaCl for their growth proved to have a primary Na⁺ pump.

The present data indicate a relationship between possession of a primary Na⁺ pump and Na⁺ requirement for growth. However, commercially available agar and other chemicals usually contain some sodium. Therefore, the failure in growth on agar plates without added NaCl does not always mean that the strain does not require sodium for growth. Furthermore, the growth on agar plates is not the same as that in liquid culture medium.

It was reported that *V. alginolyticus* has both a primary H⁺ pump and a Na⁺ pump (8). It seems that the H⁺ pump functions at lower pH, whereas the Na⁺ pump functions at an alkaline pH, such as in seawater. Although the evolution of the Na⁺ pump is still not clear, there may be a transient group of bacteria which have Na⁺ pumps but lack or lost the requirement of sodium for their growth. Conversely, previous work showed that there is at least one bacterial strain that requires sodium for growth but lacks a Na⁺ pump (7). It is possible that the gene coding for the Na⁺ pump was somehow transferred to the non-Na⁺-requiring bacterium.

Although further investigation is necessary, the present results indicate that the occurrence of a respiration-dependent primary sodium pump is closely related to the sodium requirement. This raises questions about the evolution and definition of marine bacteria. Since the respiratory chain or energy-transducing system is one of the most fundamental biological processes in living organisms, we propose that the possession of a respiration-dependent primary Na⁺ pump should be one of the criteria for the definition of marine bacteria.

We thank officers and crew of R/V *Tansei-maru* for their helpful assistance in getting samples.

This work was partly supported by the Ministry of Education, Japan.

REFERENCES

- Ken-Dror, S., J. K. Lanyi, B. Schobert, B. Silver, and Y. Avi-Dor. 1986. An NADH:quinone oxidoreductase of the halotolerant bacterium Ba₁ is specifically dependent on sodium ion. *Arch. Biochem. Biophys.* **244**:766-772.
- Kogure, K., and H. Tokuda. 1989. Respiration-dependent primary Na⁺ pump in halophilic marine bacterium, *Alcaligenes* strain 201. *FEBS Lett.* **256**:147-149.
- MacLeod, R. A. 1968. On the role of inorganic ions in the physiology of marine bacteria, p. 95-126. *In* M. R. Droop and E. J. F. Wood (ed.), *Advances in microbiology of the sea*, vol. 1. Academic Press, Inc. (London), Ltd., London.
- Mitchell, P. 1961. Coupling of phosphorylation to electron and hydrogen transfer by a chemiosmotic type of mechanism. *Nature (London)* **191**:144-148.
- Simidu, U. 1985. Identification of marine bacteria, p. 228-233. *In* H. Kadota and N. Taga (ed.), *Methods in marine microbiology*. Gakkai Shuppan Center, Tokyo.
- Tokuda, H. 1986. Sodium translocation by NADH oxidase of *Vibrio alginolyticus*: isolation and characterization of the sodium pump-defective mutants. *Methods Enzymol.* **125**:520-530.
- Tokuda, H., and K. Kogure. 1989. Generalized distribution and common properties of Na⁺-dependent NADH:quinone oxidoreductases in Gram-negative marine bacteria. *J. Gen. Micro-*

- biol. **135**:703–709.
8. Tokuda, H., and T. Unemoto. 1981. A respiration dependent primary sodium extrusion system functioning at alkaline pH in the marine bacterium *Vibrio alginolyticus*. *Biochem. Biophys. Res. Commun.* **102**:265–271.
 9. Tokuda, H., and T. Unemoto. 1984. Na⁺ is translocated at NADH:quinone oxidoreductase segment in the respiratory chain of *Vibrio alginolyticus*. *J. Biol. Chem.* **259**:7785–7790.
 10. Tsuchiya, T., and S. Shinoda. 1985. Respiration-driven Na⁺ pump and Na⁺ circulation in *Vibrio parahaemolyticus*. *J. Bacteriol.* **162**:794–798.
 11. Udagawa, T., T. Unemoto, and H. Tokuda. 1986. Generation of Na⁺ electrochemical potential by the Na⁺-motive NADH oxidase and Na⁺/H⁺ antiport system of a moderately halophilic *Vibrio costicola*. *J. Biol. Chem.* **261**:2616–2622.