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NOTE

The Influence of NaCl and Carbonylcyanide-*m*-Chlorophenylhydrazone on the Production of Extracellular Proteases in a Marine *Vibrio* Strain

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In general, the salinity of the ocean is close to 3.5% and marine *vibrios* possess the respiratory chain-linked Na^+ pump. The influence of sodium chloride and the proton conductor carbonylcyanide *m*-chlorophenylhydrazone (CCCP) on the production of extracellular proteases in a marine *Vibrio* strain was examined. At the concentration of 0.5 M, sodium chloride minimally inhibited the activity of extracellular proteases by approximately 16%, whereas at the same concentration, the producton of extracellular proteases was severely inhibited. On the other hand, the production of extracellular proteases was completely inhibited by the addition of 2 μM CCCP at pH 8.5, where the respiratory chain-linked Na^+ pump functions.

Key words: a marine bacterium, *Vibrio* sp. strain 60, extracellular protease producton, NaCl, CCCP

Halophilic marine bacteria can be defined as microorganisms living in sea water that absolutely require Na^+ for growth. These microorganisms always live in Na^+-rich habitats and little is known about the mechanism of Na^+ circulation between their cytoplasm and the surrounding medium. In halophilic marine bacteria, the Na^+ concentration of the cytoplasm is less than that of the environment. Thus, energy is required to maintain the internal Na^+ concentration at a lower level against its concentration gradient. It has been reported that the respiratory chain-linked NADH oxidase system, a primary Na^+ pump, plays a central role in the energetics of halophilic marine bacteria including *Vibrio alginolyticus* (Tokuda and Unemoto, 1982; Tokuda and Unemoto, 1984; Kim *et al.*, 1991), *Vibrio costicola* (Udagawa *et al.*, 1986), *Vibrio parahaemolyticus* (Tsuchiya and Shinoda, 1985), *Alcaligenes* strain 201 (Kogure and Tokuda, 1989), and the halotolerant bacterium Ba_1 found in the Dead Sea (Ken-Dror *et al.*, 1986). The extrusion of Na^+ was revealed to be specifically coupled to the NADH:quinone oxidoreductase segment of the NADH oxidase system in *V. alginolyticus*. The Na^+ electrochemical gradient generated by the respiratory Na^+ pump is very resistant to the proton conductor carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). This CCCP-resistant Na^+ electrochemical gradient can be used by the cell to drive energy consuming reactions, such as the active transport of sucrose and amino acids, flagella motility, cell growth, and protein translocation across the cytoplasmic membrane.

Extracellular proteins are synthesized initially as preproteins on the cytoplasmic membrane bound-ribosomes and then are excreted to the exterior of the cell along the secretion pathway. The process concerning the synthesis and secretion of bacterial enzyme proteins excreted into the growth medium has long been of interest to scientists who wish to produce enzyme proteins of commercial use. However, not enough is known about how the extracellular proteins are excreted into the external milieu or what factors affect their efficient production.

Gram-negative *Vibrio* sp. strain 60, which is a typical marine bacterium, excretes several extracellular proteins including protease, amylase, DNase, and hemagglutinin (Oishi *et al.*, 1979; Ichige *et al.*, 1988). In order to understand what factors affect the efficient production of extracellular proteases in *Vibrio* sp. strain 60, the influence that sodium chloride (NaCl) and the proton conductor carbonylcyanide *m*-chlorophenylhydrazone (CCCP) have on the production of extracellular proteases was investigated.

The bacterial strain used in this study was *Vibrio* sp. strain 60, which is isolated from sea water (Oishi *et al.*, 1979; Ichige *et al.*, 1988). It was grown aerobically at 37°C in a liquid medium containing 0.5% polypeptone, 0.5% yeast extract, and 1% (or 3%) NaCl in a 50 mM

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phosphate buffer (pH 7.5). Cell growth was monitored by measuring turbidity at 600 nm with a Varian Cary 3 spectrophotometer.

A preculture, grown overnight, was used to inoculate the main culture (100 ml), and provided a turbidity of approximately 0.05. Aliquots (1 ml) were withdrawn from the main culture to measure protease activity, and then centrifuged at 15,000 rpm for 20 min at 4°C in a microcentrifuge. The supernatant was saved for a thorough analysis. A measurement of extracellular protease activity followed the procedure of Prestige *et al.* (Prestidge *et al.*, 1971) with minor modifications.

Hydrolysis of 0.25 ml of azocasein (2% in water) was carried out in a mixture containing 0.15 ml of the enzyme sample, 50 µl of 1 M Tris-HCl (pH 7.5), and 50 µl of distilled water. After an incubation of 30 min at 45°C, the reaction was stopped by the addition of 1 ml of 7% perchloric acid. The mixture was centrifuged at 15000 rpm for 10 min at 4°C. After the addition of 0.15 ml of 10 N NaOH to 1 ml of the supernatant, the absorbance was measured at 436 nm.

Vibrio sp. strain 60 is a marine bacterium and it excretes proteases of large quantities into the growth medium. In general, the salinity of the ocean is close to 3.5%. In order to investigate whether high concentrations of NaCl affect the production of extracellular proteases, the mentioned experiments were performed. The production of extracellular proteases occurred during the logarithmic growth phase and was the greatest when the cultures reached the stationary growth phase (data not shown). At the concentration of 0.5 M (about 3%), sodium chloride minimally inhibited the activity of extracellular proteases by approximately 16%, when compared to 0 M NaCl (Fig. 1). On the other hand, the growth of *Vibrio* sp. strain 60 in the presence of 3% NaCl was only slightly inhibited when compared to its growth in the presence of 1% NaCl (Fig. 2A). In contrast, the production of extracellular proteases in the presence of 3% NaCl was severely inhibited when compared to the production of extracellular proteases in the presence of 1% NaCl (Fig. 2B). In order to minimize

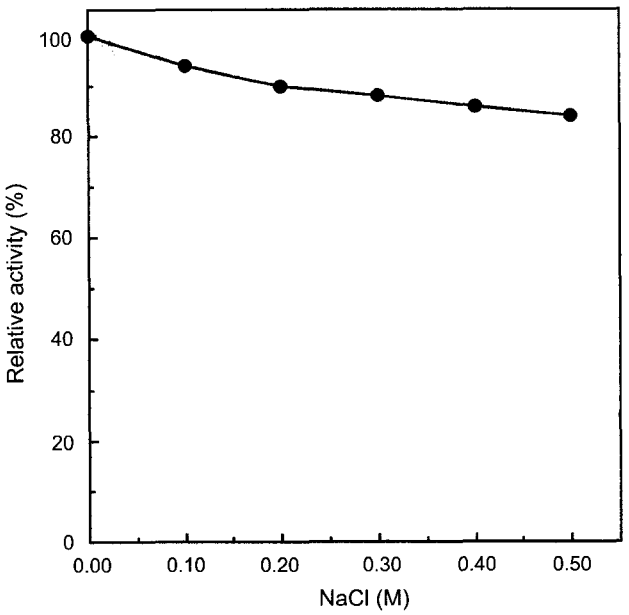

Fig. 1. Effect of NaCl on the activity of extracellular proteases in *Vibrio* sp. strain 60. The proteolytic activity in the culture supernatants was assayed with different concentrations of NaCl. Hydrolysis of 0.25 ml of azocasein (2% in water) was carried out in a mixture containing 0.15 ml of culture supernatant, 100 mM Tris-HCl (pH 7.5), and a given concentration of NaCl.

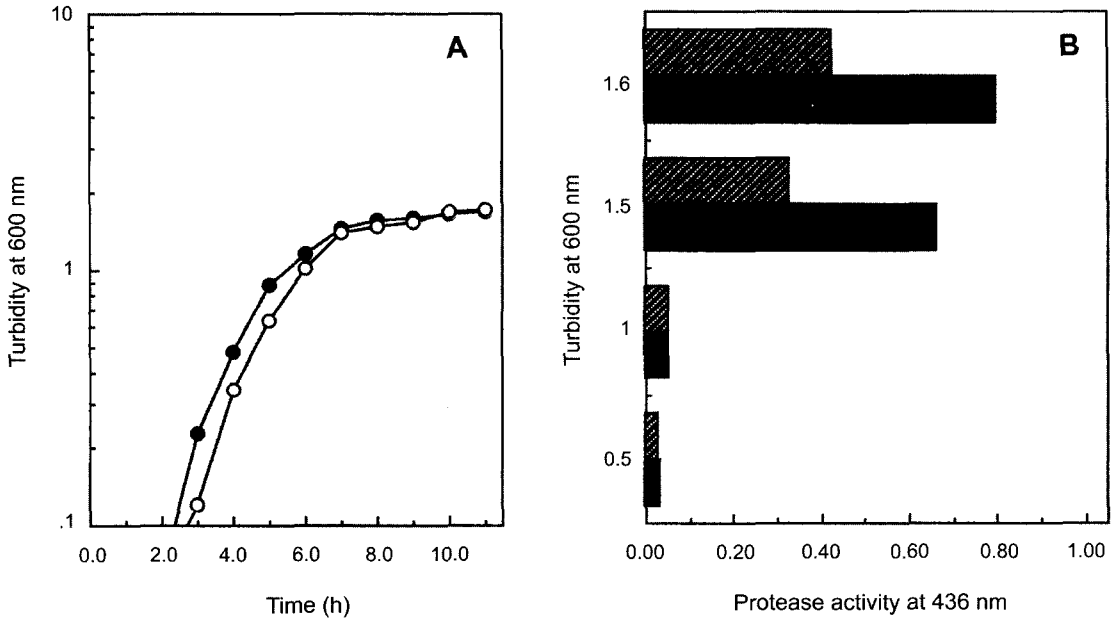

Fig. 2. Effect of NaCl on the growth (A) and production of extracellular proteases (B) of *Vibrio* sp. strain 60. 1% NaCl (●, ■); 3% NaCl (○, ▨).

the inhibition of cell growth caused by NaCl, concentrated stationary growth phase cultures were used. That is, cells in 300 ml of the medium were grown having been shaken at 37°C for 6 h. The cells were collected by centrifugation at 7000×g at 37°C for 20 min, and suspended in 200 ml of fresh medium. As shown in Fig. 3, the production of extracellular proteases in the presence of 3% NaCl was inhibited by approximately 56% at 6 h when compared to its production in the presence of 1% NaCl. From these results, it is assumed that the production of extracellular proteases in *Vibrio* sp. strain 60 is affected by the concentration of NaCl.

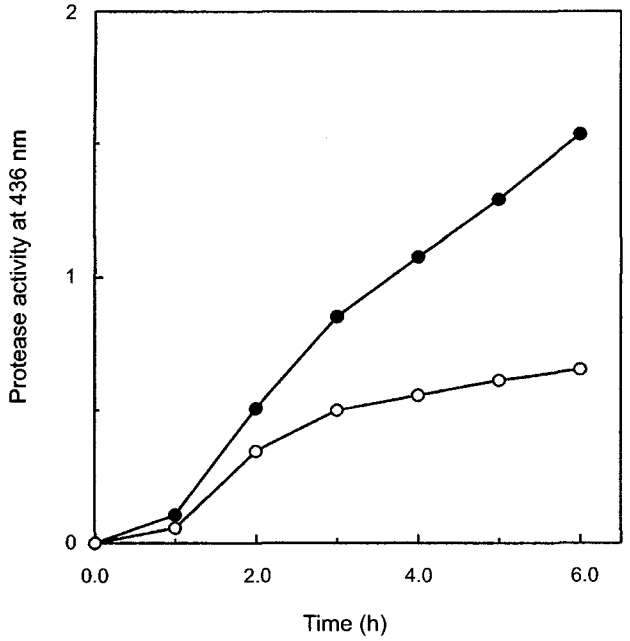

Fig. 3. Effect of NaCl on the production of extracellular proteases in concentrated cells from *Vibrio* sp. strain 60.

Vibrio sp. strain 60 possesses an aerobic respiratory chain-linked NADH oxidase system that generates a CCCP-resistant Na^+ electrochemical potential (unpublished results). Thus, the influence that CCCP-resistant Na^+ electrochemical potential has on the production of extracellular proteases, was examined. The growth of *Vibrio* sp. strain 60 at pH 6.5 was completely stopped by the addition of 4 M CCCP, whereas its growth at pH 8.5 was only slightly inhibited (Fig. 4A). In order to minimize the inhibition of *Vibrio* sp. strain 60 growth caused by CCCP, concentrated stationary growth phase cultures were used. As shown in Fig. 4B, the production of extracellular proteases at pH 8.5 was almost completely inhibited by the addition of 2 μM CCCP. These results suggest that the production of extracellular proteases in *Vibrio* sp. strain 60 is not affected by the Na^+ electrochemical potential, but instead affected by the H^+ electrochemical potential.

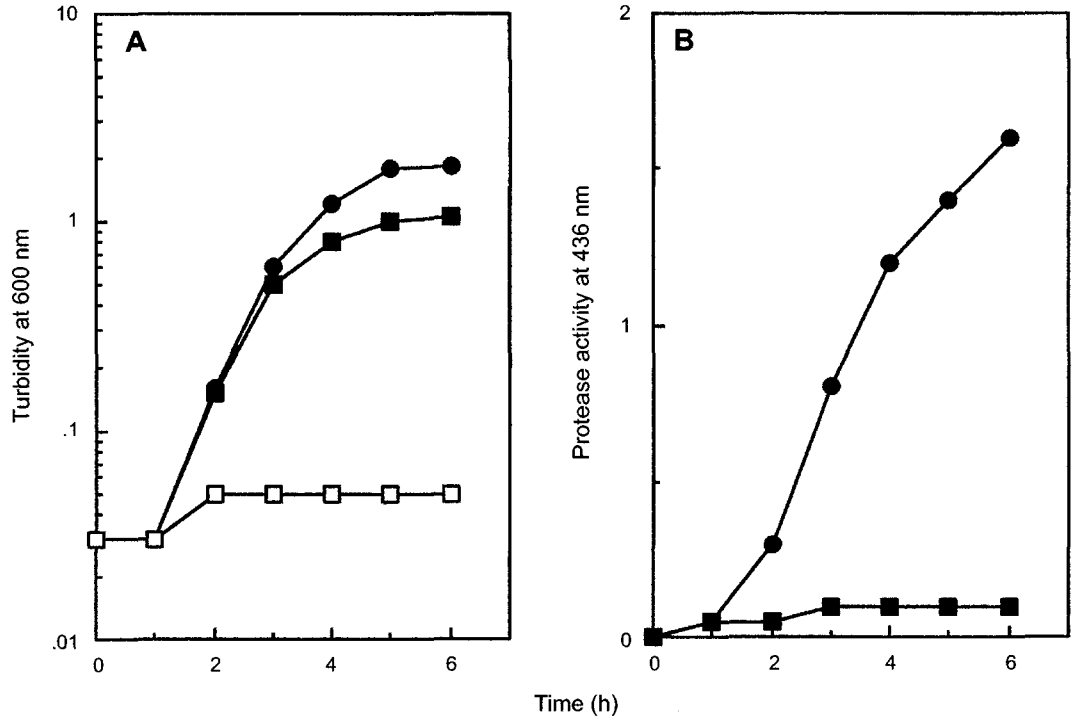

Fig. 4. Growth of *Vibrio* sp. strain 60 on media containing CCCP and the effect of CCCP on the production of its extracellular proteases. (A) The growth of *Vibrio* sp. strain 60 was followed by measuring the cell density at 600 nm. CCCP was added to give the final concentrations of 0 (●) and 2 μM (■, □) at pH 6.5 (open symbol) and 8.5 (closed symbol), respectively. (B) Extracellular proteolytic activities were measured in the absence of (●) and the presence of (■) 2 μM CCCP. In order to minimize the inhibition of cell growth caused by CCCP, concentrated stationary growth phase cultures were used as described under the text.

Acknowledgments

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