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HQNO-sensitive NADH:Quinone Oxidoreductase of *Bacillus cereus* KCTC 3674

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The enzymatic properties of NADH:quinone oxidoreductase were examined in Triton X-100 extracts of *Bacillus cereus* membranes by using the artificial electron acceptors ubiquinone-1 and menadione. Membranes were prepared from *B. cereus* KCTC 3674 grown aerobically on a complex medium and oxidized with NADH exclusively, whereas deamino-NADH was determined to be poorly oxidized. The NADH oxidase activity was lost completely by solubilization of the membranes with Triton X-100. However, by using the artificial electron acceptors ubiquinone-1 and menadione, NADH oxidation could be observed. The activities of NADH:ubiquinone-1 and NADH:menadione oxidoreductase were enhanced approximately 8-fold and 4-fold, respectively, from the Triton X-100 extracted membranes. The maximum activity of FAD-dependent NADH:ubiquinone-1 oxidoreductase was obtained at about pH 6.0 in the presence of 0.1 M NaCl, while the maximum activity of FAD-dependent NADH:menadione oxidoreductase was obtained at about pH 8.0 in the presence of 0.1 M NaCl. The activities of the NADH: ubiquinone-1 and NADH:menadione oxidoreductase were very resistant to such respiratory chain inhibitors as rotenone, capsaicin, and $AgNO_3$, whereas these activities were sensitive to 2-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO). Based on these results, we suggest that the aerobic respiratory chain-linked NADH oxidase system of *B. cereus* KCTC 3674 possesses an HQNO-sensitive NADH:quinone oxidoreductase that lacks an energy coupling site containing FAD as a cofactor.

Keywords: *Bacillus cereus* KCTC 3674, NADH:menadione oxidoreductase, NADH:ubiquinone-1 oxidoreductase, Respiratory chain inhibitors

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Introduction

Although the NADH dehydrogenases (NDH) in the bacterial respiratory chains are referred to as NADH:ubiquinone oxidoreductase, many bacteria are known to possess quinones other than ubiquinones (Collins and Jones, 1981). Thus, the name NADH:quinone oxidoreductase in bacteria would seem more appropriate than NADH:ubiquinone oxidoreductase.

Three types of NADH:quinone oxidoreductase in the respiratory chain of bacteria have been reported (Yagi *et al.*, 1998). They are the H^+-translocating NADH:quinone oxidoreductase (designated NDH-1), the Na^+-translocating NADH:quinone oxidoreductase (designated Na^+-NDH), and the NADH:quinone oxidoreductase that lacks an energy coupling site (designated NDH-2). In general, NDH-1 or Na^+-NDH react with deamino-NADH as well as with NADH, show high affinities for NADH, and possess an energy coupling site (Matsushita *et al.*, 1987; Kim *et al.*, 1991). In contrast, NDH-2 reacts poorly with deamino-NADH, but with NADH without any energy coupling site, and with low affinity for NADH (Matsushita *et al.*, 1987; Kim *et al.*, 1991; Kim *et al.*, 1995). *Escherichia coli* (Matsushita *et al.*, 1987), *Vibrio alginolyticus* (Tokuda, 1983; Tokuda and Unemoto 1984), and *Thermus thermophilus* HB-8 (Yagi *et al.*, 1988) are known to possess two different types of NADH:quinone oxidoreductase whereas *Zymomonas mobilis* is known to possess only the NADH:quinone oxidoreductase that lacks an energy coupling site (Kim *et al.*, 1995).

Respiratory chain inhibitors have proven to be useful tools for probing the mechanisms of electron transfer and proton or sodium translocation in the respiratory chain. Generally, NDH-1 is inhibited by the respiratory inhibitors rotenone and capsaicin, whereas NDH-2 is only slightly inhibited by these inhibitors (Yagi, 1990; Yagi *et al.*, 1998). Interestingly, Na^+-NDH is known to be very resistant to rotenone and capsaicin (Yagi *et al.*, 1998), but highly sensitive to 2-heptyl-4-hydroxy-quinoline-*N*-oxide (HQNO) (Tokuda and Unemoto, 1984).

Yagi (1990) showed that the respiratory inhibitor capsaicin inhibited H^+-translocating NADH:quinone oxidoreductase but

did not inhibit the NADH oxidase and NADH:quinone oxidoreductase of *Bacillus subtilis*. *Bacillus cereus* KCTC 3674, which is a gram-positive facultative anaerobic spore-forming rod-shaped bacterium, is known to possess a menaquinone with seven isoprene units as the respiratory quinone (Kim *et al*., 1998). Until the present, very little attention has been applied to the enzymatic properties of the aerobic respiratory chain-linked NADH oxidase system in the genus *Bacillus*. In the present paper, we have identified and describe the enzymatic properties of NADH:quinone oxidoreductase by using the artificial electron acceptors ubiquinone-1 and menadione in a study of the aerobic respiratory chain linked-NADH oxidase system of *B. cereus* KCTC 3674. We suggest the possibility that the NADH:quinone oxidoreductase of *B. cereus* KCTC 3674 is an enzyme that lacks an energy coupling site containing FAD as a cofactor.

Materials and Methods

Bacterial strain and experimental conditions. The bacterial strain used in this study was *B. cereus* KCTC 3674 (Kim *et al*., 1998). The bacterium was grown aerobically at 37°C in a liquid medium which contained 0.5% polypeptone and 0.5% yeast extract in 50 mM Tris-HCl buffer (pH 7.5). A preculture grown overnight was used to inoculate the main culture yielding a turbidity of approximately 0.03.

Preparation of membranes for the determination of respiratory activities. The protoplast formation for the preparation of membranes from *B. cereus* KCTC 3674 was carried out at 37°C. Cells harvested in the logarithmic growth phase were suspended in 50 mM potassium phosphate (pH 7.5) containing 5 mM EDTA at a concentration of 1 g (wet weight) per 80 ml; lysozyme was added at a concentration of 300 μg/ml (freshly prepared). The mixture was incubated for 30 min, after which the protoplasts were harvested by centrifugation at 14,000 × *g* for 30 min. Protoplasts were washed once in 50 mM potassium phosphate (pH 7.5) containing 5 mM EDTA, and centrifuged at 14,000 × *g* for 30 min at 4°C. Washed protoplasts were resuspended in 50 mM potassium phosphate (pH 7.5) containing 5 mM EDTA to give a concentration of 20 ml/g and protoplast suspensions were passed through a French pressure cell twice at 25,000 psi. Unbroken cells and cell debris were removed by centrifugation at 10,000 × *g* for 10 min at 4°C, and the supernatant was centrifuged at 120,000 × *g* for 2 h at 4°C to sediment the membrane fractions. A membrane pellet was washed in 50 mM potassium phosphate (pH 7.5) containing 5 mM EDTA. After sedimentation at 120,000 × *g* for 2 h at 4°C, membranes obtained were rewashed in 50 mM potassium phosphate (pH 7.5) containing 10% glycerol. Membranes were resuspened in 50 mM potassium phosphate (pH 7.5) containing 10% glycerol at a protein concentration of about 25 mg/ml, then was rapidly frozen in liquid nitrogen and stored at –80°C.

Preparation of the Triton X-100 extracts. To prepare the Triton X-100 extracts, membranes containing 27 mg protein were solubilized in 10 ml of 50 mM Tris-HCl (pH 8.0) containing 0.1% Triton X-100. The mixture was incubated for 30 min with gentle stirring on ice, and then centrifuged at 120,000 × *g* for 2 h at 4°C. If not immediately used, the supernatant containing 20% glycerol was rapidly frozen in liquid nitrogen and stored at –80°C.

Measurement of enzyme activities. The activities of NADH oxidase, NADH:ubiquinone-1 oxidoreductase, and NADH:menadione oxidoreductase were measured at 37°C from a decrease in A_{340} by using a Varian Cary 3E spectrophotometer. The assay mixture of NADH oxidase contained 125 μM NADH or deamino-NADH in 2 ml of 50 mM Tris-HCl (pH 8.5) containing 0.1 M NaCl. The assay was started by adding 200 μg of *B. cereus* membrane protein, and the activity calculated by using a millimolar extinction coefficient of 6.22. The assay mixture for NADH: ubiquinone-1 oxidoreductase contained a Triton X-100 extract of *B. cereus* membranes (10 μg protein), 30 mM KCN, 50 μM Q-1, and 50 μM FAD in 2 ml of 50 mM MES-KOH (pH 6.0) containing 0.1 M NaCl. The assay mixture for NADH:menadione oxidoreductase contained a Triton X-100 extract of *B. cereus* membranes (30 μg protein), 30 mM KCN, 150 μM menadione, and 50 μM FAD in 2 ml of 50 mM Tris-HCl (pH 8.0) containing 0.1 M NaCl. Activity was calculated by using millimolar extinction coefficients of 6.81 and 6.22, respectively, for Q-1 and menadione. All reactions for oxidoreductases were started by the addition of 125 μM NADH.

Protein determination. Protein concentration was measured by the Bio-Rad protein assay, based on the method of Bradford, by using bovine serum albumin as a standard.

Results

Properties of membrane-bound NADH oxidase activity. The effects of salts and pH on the activity of NADH oxidase were examined with membranes prepared from *B. cereus* KCTC 3674. The membranes oxidized NADH, but very little deamino-NADH as substrates (data not shown). The maximum activity of NADH oxidase was obtained at pH 8.5 in the presence of 0.1 M KCl or NaCl (data not shown). This NADH oxidase activity was abolished on solubilization of the membranes with Triton X-100. However, by using the artificial electron acceptors ubiquinone-1 and menadione, NADH oxidation could be observed. To examine the enzymatic properties of NADH: quinone oxidoreductase, membranes were solubilized with 0.1% Triton X-100. Under these conditions, approximately 18% of the membrane proteins were solubilized (Table 1 and Fig. 1) and about an 8-fold and a 4-fold purification was achieved from the extracts of membranes by Triton X-100 on the activities of NADH:ubiquinone-1 and NADH: menadione oxidoreductase, respectively (Table 1).

Effect of the flavins FMN and FAD on the activity of NADH:ubiquinone-1 oxidoreductase. The effect of flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) as a cofactor on the activity of NADH:ubiquinone-1 oxidoreductase was examined. The activity of NADH: ubiquinone-1 oxidoreductase was increased about 1.3-fold by 50 μM FAD at pH 8.0 (Fig. 2B), whereas the activity was not

Table 1. Activities of NADH:ubiquinone-1 and NADH:menadione oxidoreductase following solubilization of *B. cereus* KCTC 3674 membranes with 0.1% Triton X-100

Enzymes	Total protein (mg)	Specific activity (μmol/min/mg protein)	Purification (fold)
NADH:ubiquinone-1 oxidoreductase from membranes	27	0.85	
Membranes incubated with 0.1% Triton X-100	27	0.9	
0.1% Triton X-100 supernatant	5	7.12	8
NADH:menadione oxidoreductase from membranes	27	0.53	
Membranes incubated with 0.1% Triton X-100	27	0.9	
0.1% Triton X-100 supernatant	5	3.6	4

Each enzyme activity was measured as described in the Materials and Methods.

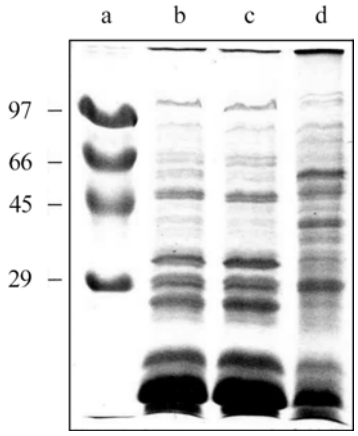

Fig. 1. SDS-PAGE (12.5% polyacrylamide) analysis of *B. cereus* KCTC 3674 membranes. All samples were heated at 100°C for 3 min. The protein bands were stained with Coomassie brilliant blue R-250. Molecular mass markers (lane a) are phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), and carbonic anhydrase (29 kDa). Lane b: *B. cereus* membranes, lane c: Membranes solubilized by 0.1% Triton X-100, lane d: a Triton X-100 extract of *B. cereus* membranes.

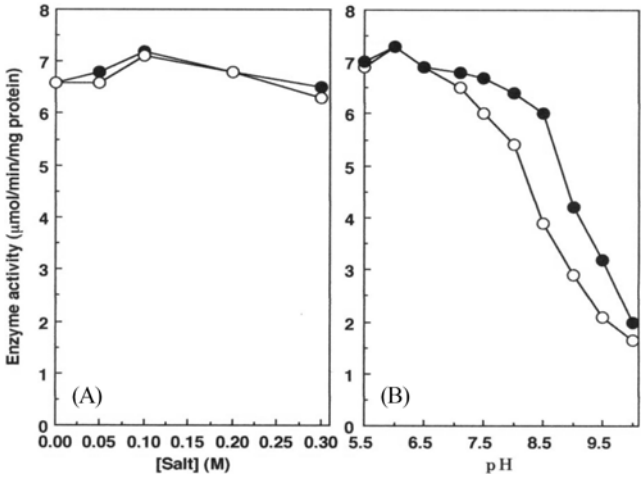

Fig. 2. Effect of salts and pH on the activity of NADH: ubiquinone-1 oxidoreductase. (A) The assay mixture of NADH: ubiquinone-1 oxidoreductase (2 ml) contained 50 mM MES-KOH (pH 6.0), a Triton X-100 extract of *B. cereus* membranes (10 μg protein), 30 mM KCN, 50 μM Q-1, and various concentrations of NaCl (●) or KCl (○). (B) The activity of NADH:ubiquinone-1 oxidoreductase was determined over a pH (5.5 to 10) containing 0.1 M NaCl in the presence of 50 μM FAD (●) or in the absence of FAD (○). Buffers used at 50 mM were MES-KOH (pH 6 to 6.5), Tris-HCl (pH 7.1 to 8.9), and CAPSO-HCl (pH 9 to 10). All assays were started by the addition of 125 μM NADH at 37°C.

affected by FMN for all pH ranges studied (data not shown).

The effect of salts, pH, and respiratory inhibitors on the activity of NADH:ubiquinone-1 oxidoreductase. The activity of NADH:ubiquinone-1 oxidoreductase was only slightly stimulated by Na^+ and K^+ at a concentration of 0.1 M (Fig. 2A). The optimal pH in the presence of 0.1 M NaCl was determined to be 6.0 (Fig. 2B). The respiratory inhibitor HQNO inhibited the NADH:ubiquinone-1 oxidoreductase activity by about 40% at a concentration of 20 μM (Fig. 3A). However, the activity of NADH:ubiquinone-1 oxidoreductase was resistant to such respiratory chain inhibitors as rotenone and capsaicin (Fig. 3B and C).

Effect of the flavins, FMN and FAD on the activity of NADH:menadione oxidoreductase. As shown in Fig. 4B, the activity of NADH:menadione oxidoreductase was increased nearly 2-fold by 50 μM FAD at pH 8.0, whereas the activity was not altered by FMN at the pH range studied (data not shown).

The effect of salts, pH, and respiratory inhibitors on the activity of NADH:menadione oxidoreductase. The activity of NADH:menadione oxidoreductase was only slightly stimulated by Na^+ and K^+ at a concentration of 0.1 M (Fig. 4A). The optimal pH was determined to be 8.0 (Fig. 4B). The respiratory inhibitor HQNO inhibited the activity of NADH: menadione oxidoreductase by about 45% at a concentration of 20 μM (Fig. 5A). However, the activity of NADH:menadione oxidoreductase was resistant to the respiratory chain inhibitors rotenone and capsaicin (Fig. 5B and C).

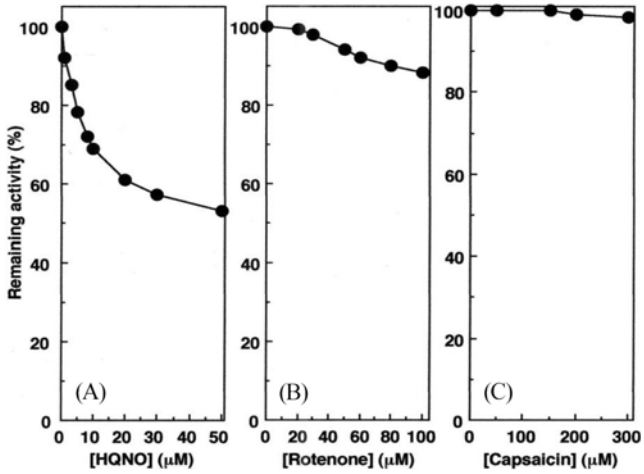

Fig. 3. Effect of respiratory chain inhibitors on the activity of NADH:ubiquinone-1 oxidoreductase. The activity of NADH: ubiquinone-1 oxidoreductase was measured with various concentrations of HQNO (A), rotenone (B), and capsaicin (C). The assay mixture of NADH:ubiquinone-1 oxidoreductase contained a Triton X-100 extract of *B. cereus* membranes (10 μg protein), 30 mM KCN, and 50 μM Q-1 in 2 ml of 50 mM MES-KOH (pH 6.0) containing 0.1 M NaCl. All assays were started by the addition of 125 μM NADH at 37°C.

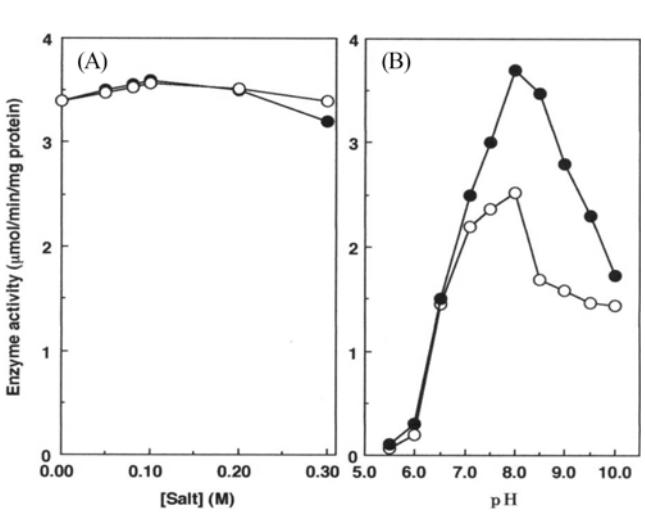

Fig. 4. Effect of salts and pH on the activity of NADH:menadione oxidoreductase. (A) The assay mixture of NADH:menadione oxidoreductase (2 ml) contained 50 mM Tris-HCl (pH 8.0), a Triton X-100 extract of *B. cereus* membranes (30 μg protein), 30 mM KCN, and 150 μM menadione, and various concentrations of NaCl (●) or KCl (○). (B) The activity of NADH:menadione oxidoreductase was determined over a pH (5.5 to 10) containing 0.1 M NaCl in the presence of 50 μM FAD (●) or in the absence of FAD (○). Buffers used at 50 mM were MES-KOH (pH 6 to 6.5), Tris-HCl (pH 7.1 to 8.9), and CAPSO-HCl (pH 9 to 10). All assays were started by the addition of 125 μM NADH at 37°C.

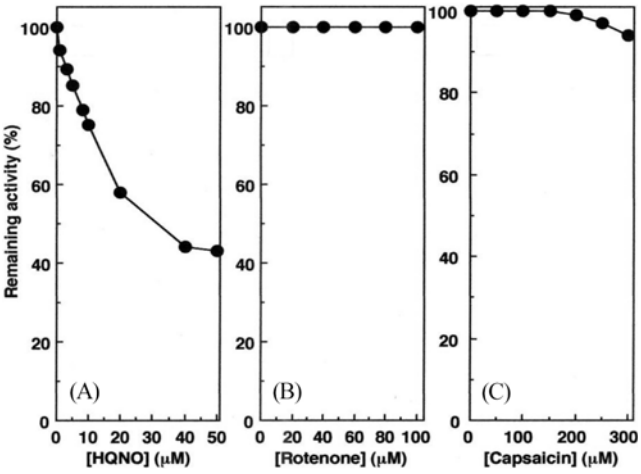

Fig. 5. Effect of respiratory chain inhibitors on NADH: menadione oxidoreductase. The activity of NADH:menadione oxidoreductase was measured with various concentrations of HQNO (A), rotenone (B), and capsaicin (C). The assay mixture of NADH:menadione oxidoreductase contained a Triton X-100 extract of *B. cereus* membranes (30 μg protein), 30 mM KCN, and 150 μM menadione in 2 ml of 50 mM Tris-HCl (pH 8.0) containing 0.1 M NaCl. All assays were started by the addition of 125 μM NADH at 37°C.

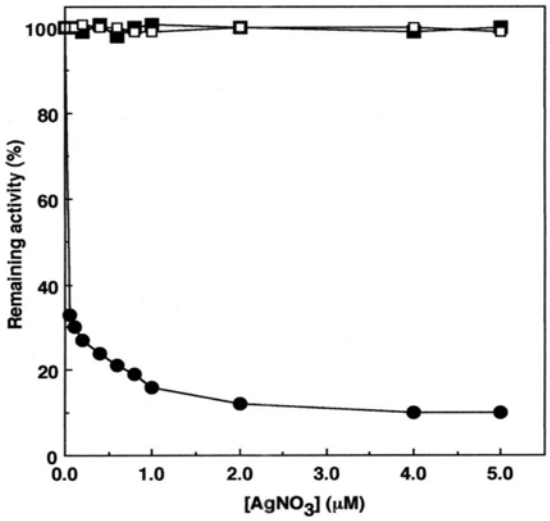

Fig. 6. Effects of $AgNO_3$ on the enzyme activities of the NADH oxidase system. NADH oxidase (●), NADH:ubiquinone-1 oxidoreductase (■) and NADH:menadione oxidoreductase (□). Each enzymatic activity was measured with various concentrations of $AgNO_3$.

Effects of Ag^+ on the enzyme activities of NADH oxidase, NADH:ubiquinone-1 oxidoreductase, and NADH:menadione oxidoreductase. $AgNO_3$ is known to inhibit Na^+-translocating NADH:quinone oxidoreductase (Asano *et al*., 1985). As shown in Fig. 6, the membrane-bound NADH oxidase activity was

highly sensitive to Ag^+ (●). In contrast, the activities of NADH:ubiquinone-1 and NADH:menadione oxidoreductase were not affected by Ag^+ at the concentrations used.

Discussion

The results of this study demonstrate that the aerobic respiratory chain-linked NADH oxidase system of *B. cereus* KCTC 3674 possesses an HQNO-sensitive NADH:quinone oxidoreductase that lacks an energy coupling site containing FAD as a cofactor (designated NDH-2). Membranes prepared from *B. cereus* KCTC 3674 oxidized NADH, but very little of the deamino-NADH substrate, and exhibited an apparent K_m value approximating 65 μM for NADH (data not shown). Generally, the NADH:quinone oxidoreductase that lacks an energy coupling site (NDH-2) oxidizes little deamino-NADH, and shows low affinity for NADH (Kim *et al*., 1991; Kim *et al*., 1995; Matsushita *et al*., 1987). The NADH:ubiquinone-1 oxidoreductase of *B. subtilis* that lacks an energy coupling site is little affected by capsaicin (Yagi, 1990). The NADH: ubiquinone-1 and NADH:menadione oxidoreductase of *B. cereus* KCTC 3674 in this study were little affected by capsaicin or rotenone, but their activities were affected by flavin adenine dinucleotide (FAD). $AgNO_3$ and HQNO are known to be the potent inhibitors of the Na^+-translocating NADH:quinone oxidoreductase (Asano *et al*., 1985; Tokuda and Unemoto, 1984). Interestingly, the NADH:ubiquinone-1 and NADH:menadione oxidoreductase of *B. cereus* KCTC 3674 were sensitive to HQNO, whereas they were not affected by Ag^+. The membrane-bound NADH oxidase was highly sensitive to Ag^+ and it is suggested that $AgNO_3$ does not inhibit the NADH:quinone oxidoreductase of *B. cereus* KCTC 3674, but rather inhibits the quinol oxidase. Alternatively, the NADH:ubiquinone-1 and NADH:menadione oxidoreductase of the NADH oxidase system were quite different in their pH optima.

As *B. cereus* KCTC 3674 vigorously excretes proteases into the extracellular environment (Kim *et al*., 2000; Kim *et al*., 2001) it is very difficult to prepare functional inverted membrane vesicles for an energy measurement experiment. As a result, the lack of a functional *in vitro* system limits the design and study of energetics investigations in the genus *Bacillus*. To examine whether the aerobic respiratory chain of *B. cereus* KCTC 3674 generates energy, we are in the process of developing an inverted membrane vesicular system for functional *in vitro* energy evaluation.

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