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Patch clamp analysis of the respiratory chain in *Bacillus subtilis*

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ABSTRACT

Bacillus subtilis is a representative Gram-positive bacterium. In aerobic conditions, this bacterium can generate an electrochemical potential across the membrane with aerobic respiration. Here, we developed the patch clamp method to analyze the respiratory chain in *B. subtilis*. First, we prepared giant protoplasts (GPs) from *B. subtilis* cells. Electron micrographs and fluorescent micrographs revealed that GPs of *B. subtilis* had a vacuole-like structure and that the intravacuolar area was completely separated from the cytoplasmic area. Acidification of the interior of the isolated and purified vacuole-like structure, due to H^+ translocation after the addition of NADH, revealed that they consisted of everted cytoplasmic membranes. We called these giant provacuoles (GVs) and again applied the patch clamp technique. When NADH was added as an electron donor for the respiratory system, a significant NADH-induced current was observed. Inhibition of KCN and 2-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO) demonstrated that this current is certainly due to aerobic respiration in *B. subtilis*. This is the first step for more detailed analyses of respiratory chain in *B. subtilis*, especially H^+ translocation mechanism.

1. Introduction

The respiratory chain plays an essential role in oxidative phosphorylation. The H^+ or Na^+ electrochemical gradient across the cellular membrane is generated via a respiratory chain in aerobically grown bacteria. These electrochemical gradients are accordingly utilized as the driving force for the synthesis of ATP or the uptake of nutrient molecules (e.g., amino acids or sugars) [1,2]. On the other hand, the potential is also used as a driving force for the efflux of antibiotics or heavy metal cations [3,4]. So, the respiratory chain has been investigated in bacteria, chloroplasts, and mitochondria.

Bacillus subtilis is a genus of Gram-positive rod-shaped bacteria and one of the best-understood prokaryotes, in terms of molecular biology and cell biology. Because of easier handling, *B. subtilis* has been used as a model organism for many biological functions. The respiratory chain in *B. subtilis* has been studied since the early twentieth century, and several components have been investigated in great detail.

The respiratory chain in *B. subtilis* consists of NADH dehydrogenases, quinol/cyt *c* oxidoreductase, and terminal oxidases. The reaction in the respiratory chain goes through dehydrogenation of NADH, as a first step. *Bacillus* NADH dehydrogenases are likely to be encoded by *yjlD*, *yumB*, or *yutJ*; their activity has been investigated with oxygen consumption in partially purified particles [5]. Electrons are transferred from NADH to menaquinol, which serves as an electron carrier, and then, the pathway goes through two main branches. The first is one in which electrons go through the cytochrome *c* and cytochrome oxidase, in turn. The second contains only quinol oxidase. In each branch, electrons are finally accepted by oxygen molecules. As terminal oxidases, one cytochrome *c* oxidase and four quinol oxidases are known [6–9].

In many reports, the activities of these enzymes have been elucidated with spectrometric studies or by oxygen consumption [6,10–13]. However, H^+ translocation coupled to electron transfer is also an important parameter. One of the most useful methods for the measurement of cellular ion transport is the patch clamp method [14,15]. With this method, a lot of important analyses, such as those of Na^+/K^+ ATPase in guinea pig myocardial cells or rat hippocampal synaptic currents, have been achieved [16,17]. Even in bacterial cells, Martinac et al. prepared spheroplasts (whose diameter is approximately 6 μm) from *E. coli* cells and analyzed the current by

Abbreviations: GP, giant protoplast; GV, giant provacuole; EtBr, ethidium bromide; HQNO, 2-heptyl-4-hydroxyquinoline-*N*-oxide

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mechanosensitive channels with the patch clamp technique [18]. It was possible to apply the patch clamp technique for enlarged bacterial cells. Furthermore, we applied whole-cell mode patch clamp for giant provacuoles (GVs), which were observed in giant protoplasts (GPs), and detected the current by respiratory chain or F_OF_1-ATPase [19]. We aimed to apply this method to *B. subtilis* cells.

In this study, we succeeded in the enlargement of representative Gram-positive bacteria *B. subtilis*. In addition, we applied whole-cell (whole-vacuole) mode patch clamp for a GV (whose diameter was at least 10 μm) and analyzed exact H^+ translocation across the membrane as the current.

2. Materials and methods

2.1. Preparation of GPs from B. subtilis cells

GPs were prepared by using a modification of Kusaka's method [20] and spheroplast incubation method [19]. Cells of *B. subtilis* JH642 and 168 were grown in PYG medium (0.5% polypeptone, 0.25% yeast extract, 1% glucose, 0.05% K_2HPO_4, 0.03% KH_2PO_4, 0.01% NaCl, 0.013% $CaCl_2 \cdot 2H_2O$, 0.03% $MgSO_4 \cdot 7H_2O$, 0.0018% $MnSO_4 \cdot 4H_2O$, 0.0006% $FeSO_4 \cdot 7H_2O$; pH was adjusted to 6.8 by NaOH). The cells were harvested in the late exponential phase of growth and suspended in PS buffer (0.1 M NaPi, 0.6 M sucrose; pH 6.8). Lysozyme (300 μg/ml) was added to the cell suspension, and the suspension was shaken at 30 °C for 25 min at 100 rpm. After this treatment, harvested cells were resuspended in PYN medium (0.5% polypeptone, 0.25% yeast extract, 0.05% K_2HPO_4, 0.03% KH_2PO_4, 4.1% NaCl, 0.013% $CaCl_2 \cdot 2H_2O$, 0.03% $MgSO_4 \cdot 7H_2O$, 0.0018% $MnSO_4 \cdot 4H_2O$, 0.0006% $FeSO_4 \cdot 7H_2O$; pH was adjusted to 6.8 by NaOH) supplemented with 60 μg/ml ampicillin. A 2.5-μl aliquot of the suspension was added to 2 ml of PYN medium supplemented with 60 μg/ml ampicillin. This suspension was shaken at 23 °C for 48–72 h at 45 rpm.

2.2. Microscopy and electron microscopy

For ethidium bromide (EtBr) staining, the GPs were prepared from cells of *B. subtilis* JH642. GPs were harvested and suspended in PBS-GP (0.7 M NaCl, 0.1 M NaPi; pH 7.4). EtBr (final concentration, 1 μg/ml) was added to the suspension and incubated at room temperature for 1 h. After washing with PBS-GP, fluorescent micrographs of the protoplasts were taken with excitation at 548 nm (with emission at 615 nm).

Electron microscopic observation was performed on GPs from *B. subtilis* 168 strain.

2.3. Isolation of GVs from GPs of B. subtilis

GPs were harvested by centrifugation at 18,000 × *g*. The pellet was suspended with burst buffer (50 mM KPi, pH 7.5, 10 mM $MgCl_2$, 0.6 M sucrose), and 17.5 units/ml of DNase I. The suspension was shaken at 30 °C for 20–30 min (45 rpm). After centrifugation at 18,000 × *g*, the pellet was resuspended in a small volume of burst buffer containing 20% Percoll (Amersham Pharmacia Biotech). The suspension was placed in a centrifuge tube onto which an equal volume of burst buffer was overlaid. Percoll density gradient centrifugation was carried out at 400 × *g* for 60 min. GVs were included in the interface layer. The GVs were washed twice with the burst buffer and used for further analyses. Protein concentration was measured by a previously published procedure [21].

2.4. H^+ pumping activity in GVs

Measurement of H^+ pumping activity was carried out by the quinacrine fluorescence quenching method [22]. Provacuoles (100 μg of protein) were added to 2 ml of the assay mixture (20 mM Tricine–KOH, pH 8.0, 5 mM $MgSO_4$, 200 mM KCl) containing 1 μM quinacrine hydrochloride. After pre-incubation for 5 min at 25 °C, NADH was added. After fluorescence quenching had occurred, KCN was added as an inhibitor of the respiratory chain.

2.5. Electrical recording in GVs

GPs were harvested by centrifugation at 2000 × *g* and gently suspended in a small volume of the same medium as that used for cell growth. The GPs were put on a glass chamber and washed with burst buffer (50 mM HEPES–KOH, pH 7.4, 10 mM $MgCl_2$, 0.6 M sucrose). The chamber was carefully filled with burst buffer. The patch pipettes (Drummond Scientific Co.) were pulled to a diameter with a resistance of 12.5–25 MΩ (when measured in burst buffer) using a puller machine (model PC-10, Narishige) and then heat-polished (model MF-90, Narishige). The electrode was gently touched to a GV with mild suction (about 800 mm H_2O), producing an instantaneous seal of about 10 GΩ. Thereafter, the suction was stopped. A tiny hole was made in the membrane of the GV with a ZAP pulse (duration time 50 ms, amplitude 1.6 V). After that, the resistance was 0.5–1 GΩ. All substrates in the burst buffer were added through tandem six-way bulbs (GL Sciences Inc.). The patch amplifier used was CEZ-2400 (Nihon Kohden). A positive current represents positive charges moving from the exterior to the interior of the GV. All recordings were made using the standard patch clamp technique at 23 °C.

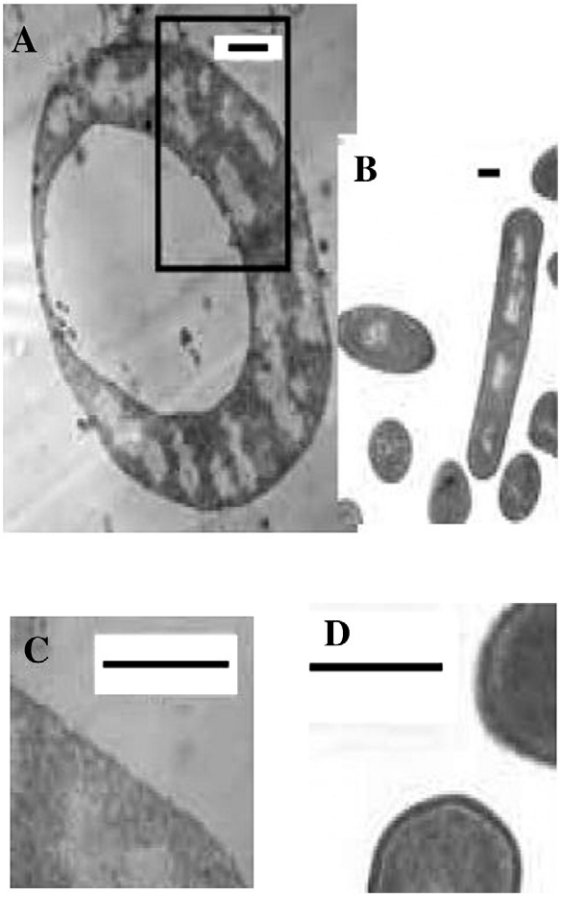

Fig. 1. Transmission electron micrographs of *Bacillus subtilis* GP. (A and C) GP. C is the magnified image of the section indicated by a square in A. GPs have no peptidoglycan on their surfaces, and this is very different to normal cells. (B and D) Normal *B. subtilis* cells. They have a thick peptidoglycan layer on their surfaces. The scale bars indicate 1 μm.