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Comprehensive metabolomic analyses of anode-respiring *Geobacter sulfurreducens* cells: The impact of anode-respiration activity on intracellular metabolite levels

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ABSTRACT

Comprehensive metabolomic analysis of anode-respiring *Geobacter sulfurreducens* cells revealed that intracellular levels of metabolites related to the tricarboxylic acid (TCA) cycle, gluconeogenesis, consumptions of nicotinamide adenine dinucleotide (NADH) and nicotinamide adenine dinucleotide phosphate (NADPH), and adenosine triphosphate (ATP) generation correlated well with the activity of microbial anode-respiration detected as microbial electric current. Use of a glassy carbon electrode as the anode material in a three-electrode system resulted in a higher microbial current at +0.2 V (vs. Ag/AgCl) than at −0.2 V. A larger current flow resulted in higher concentrations of TCA cycle metabolites and lower concentrations of gluconeogenesis metabolites. Metabolomic analysis also revealed that higher anode-respiration activity resulted in a higher ATP/adenosine diphosphate (ADP) ratio and lower ratios of NADH/NAD^+ and NADPH/$NADP^+$. These findings provide direct experimental evidence that microbial anode-respiration activity controlled by the anode potential influences both the flux of central metabolic pathways and the redox balance in microbial fuel cells.

1. Introduction

Members of the deltaproteobacterial genus *Geobacter* can oxidize organic compounds to carbon dioxide during respiration using solid-state terminal electron acceptors such as iron and manganese oxides via extracellular electron transfer (EET) reactions mediated by outer membrane cytochromes [1–3]. Anodes can also serve as terminal electron acceptors during EET; consequently, *Geobacter* species have attracted considerable attention by researchers developing microbial fuel cells (MFCs) [4–8]. Improvements to MFC technology require knowledge of the parameters that influence the metabolic pathways during EET. To date, model-driven simulations of the metabolism of *Geobacter sulfurreducens* cells have been conducted to elucidate the EET-related metabolic pathways [9,10]. Meng et al. reported that respiration activity increased as the cellular growth rate decreased in a model assuming a constant rate of consumption of acetate, used as an organic substrate [10]. This negative correlation between respiration activity and cellular growth rate is attractive when considering MFC as a technology for waste water treatment (WWT). However, the control parameters for satisfying this negative correlation are currently unknown. It has been reported that the carbon flux of *G. sulfurreducens* cells can be changed by genetic manipulation [11], but such genetically-engineered cells cannot be used for practical WWT plants.

On the other hand, it has been demonstrated that the magnitude of the EET-current of *G. sulfurreducens* cells depends on the

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potential at the anode [12–14]. Since the anode potential in a practical MFC can in principle be tuned by changing the external load and/or the ratio of the anode/cathode area, it is worth investigating the metabolism patterns of *G. sulfurreducens* under active EET conditions achieved by an appropriate choice of anode potential. We here describe comprehensive intracellular metabolomic analyses of *G. sulfurreducens* cells cultured at different poised potentials aimed at evaluating how the anode potential and/or the EET activity influence metabolism.

2. Material and methods

2.1. Preparation of Geobacter cells and cultivation in an electrochemical cell

G. sulfurreducens PCA was cultured anaerobically at 30 °C for 72 h in 50 mL PS medium, as described previously [13]. Cells were collected by centrifugation for 5 min at 5000 × *g*, washed three times with PS medium, then injected into an electrochemical cell (described below) using a 1-mL syringe (Terumo, Tokyo, Japan) equipped with a 21-gauge needle. The concentration of the cell suspension in the electrochemical cell was determined by measuring the optical density at 600 nm (OD_{600}) and was adjusted to a high value of OD_{600} = 1.0 to limit the active growth of the cells. A single-chamber, three-electrode system containing PS medium supplemented with 10 mmol L^{-1} acetate and equipped with a potentiostat was used to monitor the electrochemical behavior of *G. sulfurreducens*. A glassy carbon (GC) or tin-doped indium oxide (ITO) (Niraco, Tokyo, Japan) electrode with a surface area of 3.2 cm^2 was used as the anode and was mounted on the bottom of the reactor. Further details regarding electrochemical cultivation have been described elsewhere [13].

2.2. Metabolite extraction and quenching

G. sulfurreducens cells aggregated on the bottom surface of the GC or ITO anode were collected approximately 24 h after adding the cells to the three-electrode system. The volume of each sample was adjusted to give the same cell mass based on the OD_{600}. The samples were collected by passing the culture through a polytetrafluoroethylene membrane filter (Omnipore, 0.45 μm, 47-mm diameter; Millipore, Danvers, MA, USA). The dry cell weight of each sample was estimated by multiplying the weight of an *Escherichia coli* cell by the sample OD_{600} using the following equation: dry cell weight (mg) = 0.0582 × OD_{600} × cell suspension volume (mL). Immediately after filtration, the cells were washed in place with cold phosphate-buffered saline buffer (137 mmol L^{-1} NaCl, 8.10 mmol L^{-1} Na_2HPO_4, 2.68 mmol L^{-1} KCl, and 1.47 mmol L^{-1} KH_2PO_4). The membrane filters with the washed cells were transferred to 50-mL centrifuge tubes and frozen in liquid nitrogen. Metabolites were extracted from the cells using a modified cold chloroform–methanol extraction method [15]. The water phase of the extract was dried under vacuum and stored at −80 °C until used for mass analyses [16].

2.3. Metabolomic analyses

The dried (water phase) extracts containing intracellular metabolites were thawed on ice and then derivatized at 30 °C for 90 min with 100 μL of 20 mg mL^{-1} methoxyamine hydrochloride in pyridine. After the addition of 50 μL *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (GL Sciences, Tokyo, Japan) to each sample, the derivatized extracts were incubated at 37 °C for 30 min [17]. Samples (1 μL) of the derivatives were subjected to gas chromatography-quadrupole-mass spectrometry (GC-Q-MS) analysis (GCMSQP-2010 system; Shimadzu, Kyoto, Japan) to detect metabolites relating to the TCA cycle, glutamate, glutamine, and glucose. Separate aliquots of the dried extract samples were dissolved in 50 μL Milli-Q water and analyzed using a liquid chromatography triple-stage quadrupole-mass spectrometry (LC-QqQ-MS) system (high-performance liquid chromatography: Agilent 1200 series; MS: Agilent 6460 with Jet Stream Technology; Agilent Technologies, Waldbronn, Germany) controlled by MassHunter Workstation Data Acquisition software (v. B. 04.01; Agilent Technologies) to detect intracellular metabolites relating to the gluconeogenesis, pentose phosphate (PP) pathway, acetyl-CoA, ATP, adenosine diphosphate (ADP), NAD(P)H, and $NAD(P)^+$ [18,19]. Details of the GC-Q-MS and LC-QqQ-MS operating conditions and methods have been described previously [20–22]. Extracellular acetate concentration in the supernatant was determined using a high-performance liquid chromatograph system (Shimadzu, Kyoto, Japan), as described previously [22].

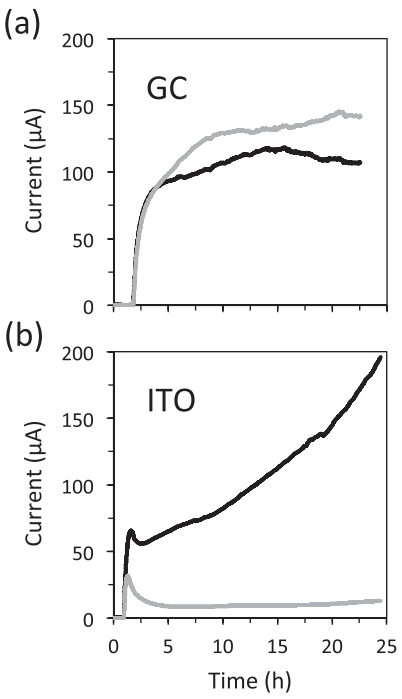

Fig. 1. Time courses of extracellular electron transfer (EET) current for *G. sulfurreducens* cells at −0.2 V (black lines) and +0.2 V (gray lines) on an (a) glassy carbon (GC) or (b) tin-doped indium oxide (ITO) anode.

3. Results and discussion

Fig. 1(a) shows representative time courses of the microbial current generated by *G. sulfurreducens* cells cultured at −0.2 V (black lines) and +0.2 V (gray lines) on a GC anode. The microbial catalytic current was 16.5 ± 11.0% lower at −0.2 V than at +0.2 V. As a reference for later discussion, the current vs. time curves obtained on an ITO anode are shown in Fig. 1(b) and show that the potential of the microbial current tended to be opposite to that obtained using the GC anode. Consumptions of supernatant acetate at the 24 h cultivations were not so changed and 88.1–92.5% using GC and ITO anodes at −0.2 V and +0.2 V. We hypothesized that the difference in the microbial current affects the metabolic state of the *G. sulfurreducens* cells. To test this, we conducted intracellular metabolomic analyses of cells cultured at −0.2 V and +0.2 V for about 24 h on the GC anode (Supplementary Table S1). The relative concentration ratios of the intracellular central metabolites of cells cultured at −0.2 V to those cultured at +0.2 V are shown as a heat-map visualization (Fig. 2(a)). The results in Fig. 2(a) indicate that almost all of the metabolites in