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Supporting Information

Proton Transport in the Outer-Membrane Flavocytochrome Complex Limits the Rate of Extracellular Electron Transport

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Author Contributions

A.O. designed the study. Y.T., S.K. and A.O. conducted the research. A.O., Y.T. and K.H. wrote the paper. A.O. and Y.T. contributed equally to the present work.

Supplementary Information

Supplementary Experimental

Strains and culture conditions

Shewanella oneidensis MR-1 was routinely cultured in Luria-Bertani (LB) medium at 30°C as previously described (A. Okamoto *et al, Electrochim. Acta,* **2011**, 56(16), 5526-5531). To collect cells for use in electrochemical experiments, MR-1 cells were grown aerobically in 10 mL LB medium (20 g L^{-1}) at 30°C for 20 h. The culture was then centrifuged at 6,000 × *g* for 10 min, and the resultant cell pellet was resuspended in 10 mL defined medium (DM; $NaHCO_3$ [2.5 g], $CaCl_2 \cdot 2H_2O$ [0.08 g], NH_4Cl [1.0 g], $MgCl_2 \cdot 6H_2O$ [0.2 g], NaCl [10 g], and (2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid [HEPES; 7.2 g] [per liter]; pH 7.8) supplemented with 10 mM lactate and 0.5 g yeast extract per liter as sources of carbon and trace elements, respectively. After further aerobic cultivation at 30°C for 10 h, the culture was centrifuged for 10 min, and the resultant cell pellet was washed with DM prior to being used for electrochemical experiments. Mutant strains deficient in the genes encoding F-type ATP synthase (ΔATPase; SO4746 to SO4754), acetate kinase (Δ*ackA*; SO2915), or phosphotransacetylase (Δ*pta*; SO2916) were kindly supplied by Prof. Jeffery A. Gralnick at the University of Minnesota and were constructed as previously described (Hunt. K. A. *et al, J. Bacteriol., **2010**, 192*(13), 3345-3351). The aerobic growth rate of cells in DM containing 10 mM lactate was estimated by the difference in optical density at the wavelength of 600 nm (OD_{600}) before and after 24 h of cultivation.

Electrochemical measurements

A single-chamber, three-electrode system for whole-cell electrochemistry was constructed as previously described (A. Okamoto *et al, Electrochim. Acta,* **2011**, 56(16), 5526-5531). A tin-doped In_2O_3 (ITO) substrate (surface area: 3.1 cm^2) placed at the bottom of the reactor was used as the working electrode, and Ag/AgCl (sat. KCl) and a platinum wire (approximate surface area: 10 mm^2) were used as the reference and counter electrodes, respectively. For the formation of monolayer biofilms, 5 ml DM supplemented with 10 mM lactate and 0.5 g/L yeast was added into the electrochemical cell as an electrolyte and was then deaerated by bubbling

with N_2 for more than 30 min. Oxygen concentration was monitored using a Microx TX3 trace instrument (PreSens, Munich, Germany) and was maintained below 0.1 ppm. Cell suspensions of WT, ΔATPase, Δ*ackA* or Δ*pta* strains with an optical density of 0.1 at OD_{600} were inoculated into the reactor with the ITO electrode poised at a potential of +0.4 V (vs. SHE). The reactor was then incubated at 30°C with no agitation for 24 h. Monolayer biofilm formation by WT and mutant strains was confirmed as previously described (A. Okamoto *et al, Electrochim. Acta,* **2011**, 56(16), 5526-5531).

To examine the kinetic isotope effect (KIE) on current production (I_c), the solution in the reactor was replaced with DM supplemented with 10 mM lactate and either 100 μM anthraquinone-1-sulfonate (α-AQS), 2 μM riboflavin (RF), or 2 μM flavin mononucleotide (FMN), and D_2O was then sequentially added at final concentrations ranging from 0.5 % to 4% (v/v). The ratio of I_c for each D_2O concentration was calculated using I_c values measured before and 10 min after the addition of D_2O. Cyclic voltammetry (CV) measurements were conducted using an automatic polarization system (VMP3, Bio Logic Company) with a scan rate ranging from 1 to 100 mV s^{-1}. The background current was subtracted from the measurements by fitting the baseline from regions sufficiently far from the peak and assuming the continuation of a similar and smooth charging current throughout the peak region.

Protein Content Assay

To determine total protein content, ITO electrodes with attached cells after 24 h of I_c measurements were gently washed once with an aqueous buffer solution consisting of 4.3 mM Na_2HPO_4 and 1.4 mM KH_2PO_4 (pH 7.5). The washed electrodes were carefully broken into small pieces, placed in a 2-ml tube containing 300 μl phosphate buffered saline (PBS), and then vortexed for 5 min. The protein concentration was determined using a Micro BCA Protein Assay Kit (Pierce) according to the manufacturer's instructions.

pH measurements

The pH of the bulk solution in the electrochemical reactor was monitored during transition current production by *S. oneidensis* MR-1 from reductive intracellular energy reserves in non-buffered solution in the absence of an electron donor ($CaCl_2 \cdot 2H_2O$ [0.2 g], KCl [0.75 g], NaCl [10 g]; pH 7.8). The micro-electrode of the pH meter (D-73, Horiba, Japan) was directly introduced into the electrochemical reactor in the anaerobic chamber, in which the oxygen concentration was maintained below 0.1 ppm.

Estimation of *in vivo* and *in vitro* electron flux for a single OM Cyt *c* complex

The estimation of electron flux in single OM Cyt *c* complexes was performed as previously described (A. Okamoto *et al, Biophysics and Physicobiology,* **2016**, *13*, 71-76). Briefly, the *in vivo* electron flux for a single OM Cyt *c* complex was estimated using single-cell current production data at a poised electrode potential of +0.4 V (vs. SHE) (H. A. Liu *et al, Angew. Chem. Int. Ed.* **2010**, *49*, 6596-6599). Previous estimates of the MtrC and OmcA content in *Shewanella oneidensis* MR-1 cells ranged from 10^3–10^4 decaheme cytochromes per cell when cultured on iron oxide (Lower, B. H. *et al, J. Bacteriol.,* **2007**, 189, 4944–4952) or a poised electrode surface at +0.4 V (A. Okamoto *et al, Electrochim. Acta,* **2011**, 56(16), 5526-5531). Given that a single microbe was estimated to transport 1.2×10^6 electrons per second via OM Cyt *c*, $10^3 – 10^2$ electrons were estimated to reach the electrode surface via a single OM Cyt *c*.

Regarding the *in vitro* electron flux for purified MtrCAB complexes embedded in proteoliposomes, White *et al*. reported that 8500 ± 1000 electrons per second are transported to γ-FeOOH (White, G. F. *et al, Proc. Natl. Acad. Sci. USA*., **2013**, 110, 6346–6351), which has a redox potential that is more negative than +0.4 V (vs. SHE).

Supplementary Table

Table S1. Growth data of WT, ΔATPase, Δ*pta* and Δ*ackA* strains of *S. oneidensis* MR-1 under anaerobic electrochemical or aerobic culture conditions.

Strain	$\Delta\mu g/cm^2$ (Anaerobic)[a]	ΔOD_{600} (Aerobic)[b]
WT	12.0 ± 0.03 (n = 2)	1.50 ± 0.05 (n = 2)
ΔATPase	11.3 ± 3.09 (n = 3)	0.56 ± 0.01 (n = 2)
Δ*pta*	5.20 ± 0.67 (n = 2)	1.51 ± 0.04 (n = 2)
Δ*ackA*	1.70 ± 1.55 (n = 2)	1.62 ± 0.05 (n = 2)

a. Cell growth on the electrode was estimated by the difference in protein content before and 24 h after electrode inoculation. As the initial protein content was ~36 μg and electrode surface area was 3.1 cm^2, the protein content of WT approximately doubled in 24 h with the electrode inoculation.

b. Difference in the OD_{600} after 24 h of aerobic cultivation. The initial OD_{600} of all strains was 10^{-3}.

Supplementary Figures

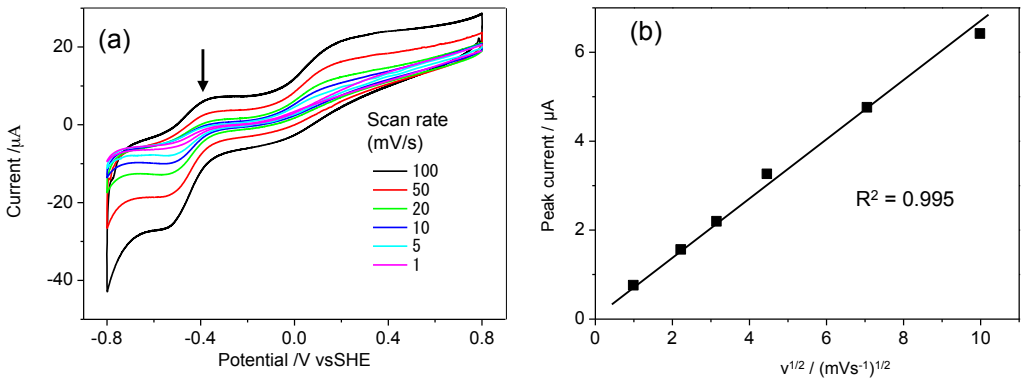

Figure S1. (a) Cyclic voltammograms (CV) of the cell-attached electrode for strain MR-1 at 30 °C in the presence of 100 μM α-AQS measured at scan rates (ν) of 1, 5, 10, 20, 50, and 100 mVs^{-1} (pH 7.8). Arrow indicate the anodic peak of α-AQS. (b) Plot of peak current in the CVs in panel (a) as a function of the square root of ν. The same trend was observed in more than two independent experiments. The anodic current was limited by the diffusion process, as the peak current showed a linear increase for scan rates in the range from 1 to 100 mVs^{-1} (E. Laviron, *J Electroanal Chem, **1979**,* 101, 19).

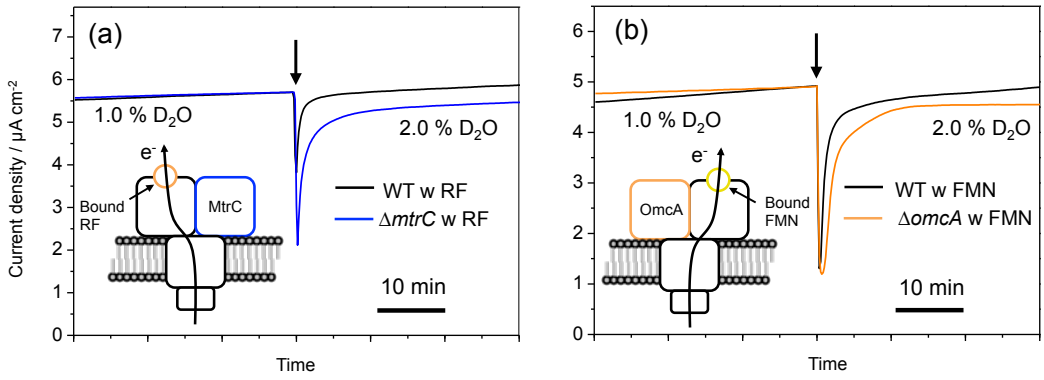

Figure S2. Effect of flavin molecules and mutation of the MtrC and OmcA proteins (strains Δ*mtrC* and Δ*omcA*, respectively) on the kinetic isotope effect (KIE) for microbial current production. (a) Time versus current production (I_c) for a monolayer biofilm of wild-type (WT; black line) and Δ*mtrC* (blue line) strains of *S. oneidensis* MR-1 in systems containing 10 mM lactate and 2 μM riboflavin (RF). (b) Time versus current production (I_c) for a monolayer biofilm of WT (black line) and Δ*omcA* (orange line) strains of *S. oneidensis* MR-1 in systems containing 10 mM lactate and 2 μM flavin mononucleotide (FMN). Data for the mutant strains Δ*mtrC* and Δ*omcA* were normalized to the tine point just prior to the addition of D_2O in the data for WT. The normalized I_c values for Δ*mtrC* and Δ*omcA* were 1.94 and 0.47 μA cm^{-2}, respectively. Inset scheme describes the location of RF, FMN, MtrC, and OmcA in the OM Cyts complex.

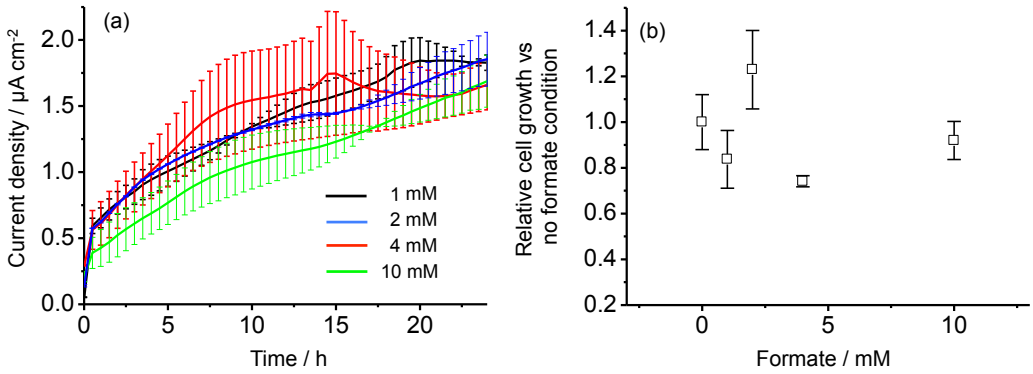

Figure S3. Effect of formate on current production (I_c) and growth of *S. oneidensis* MR-1 on a potential-poised electrode. Time course of current production (I_c) during lactate oxidation (a) and the growth rate (b) measured at +0.4 V (vs. SHE) in the presence of 10 mM lactate and 1, 2, 4, or 10 mM formate. The cell growth on the electrode was estimated by determining the difference in protein content before and after 24 h of cultivation. The I_c and growth data are shown as the mean ± standard error of mean (n = 2).

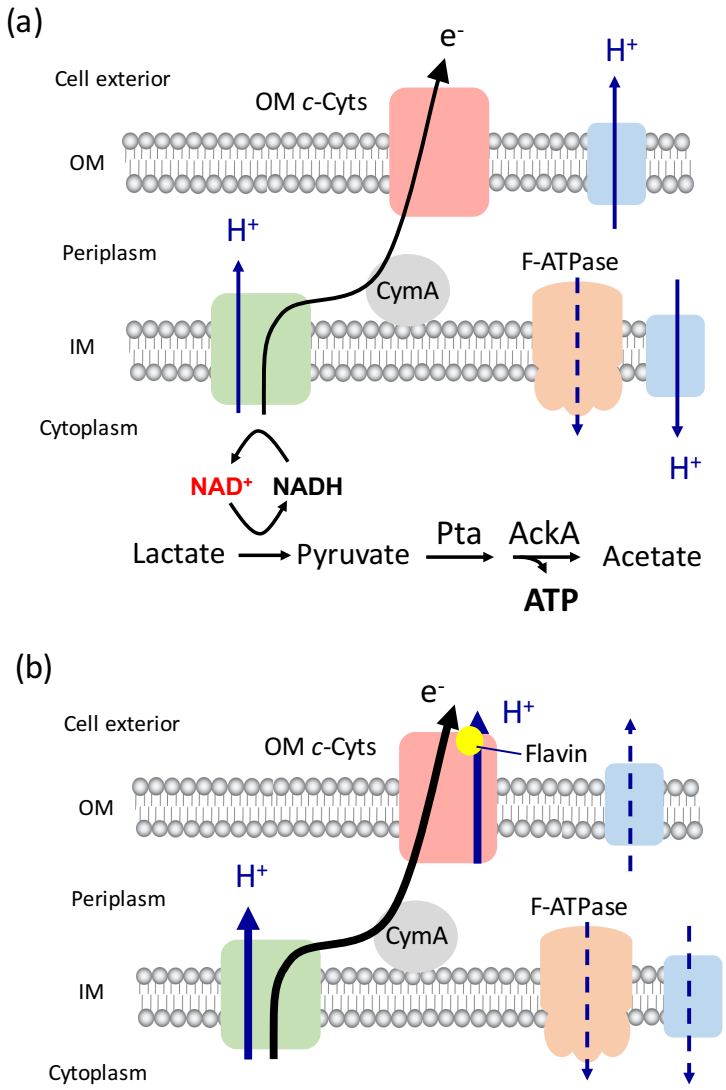

Figure S4. Hypothetical model of ATP production and proton transport pathway across inner- (IM) and outer-membrane (OM) associated with extracellular electron transport via OM *c*-type cytochromes (OM *c*-Cyts) in *S. oneidensis* MR-1 in (a) the absence and (b) the presence of the bound flavin cofactor. Black and blue arrows represent electron (e^-) and proton (H^+), respectively. For both cases, the proton export from the periplasm reduces proton motive force, and not F-type ATP synthase (F-ATPase) but substrate-level phosphorylation is the main mechanism for ATP production. In the absence of the bound flavin cofactor (panel a), proton transport is mediated by hypothetical membrane proton transporters (blue box) with large kinetic isotope effect. In the presence of the bound flavin cofactor (panel b), the flavin bound OM *c*-Cyts function as rapid electron and proton pathway.