Electronic Supplementary Material (ESI) for Energy & Environmental Science

Uptake of Self-secreted Flavins as Bound Cofactors for Extracellular Electron Transfer in *Geobacter Species***

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Supplementary Information

1. Supplementary method
2. Supplementary figures S1, S2, S3, S4, S5, S6, S7, S8, S9, S10 and S11

Supplementary method

Bacterial strains and growth conditions.

G. sulfurreducens PCA and the *omcB, omcE, omcS, omcT,* quadruple mutant (gifted from Prof. D. R. Lovley, University of Massachusetts, USA) **[J. W. Voordeckers, B. –C. Kim, M. Izallalen, D. R. Lovley, *Appl. Environ. Microbiol.* 2010, *76,* 2371-2375]** were cultured anaerobically at 30 °C for 72 h in 25 mL PSN medium ($NaHCO_3$ (1.68 g), $CaCl_2 \cdot 2H_2O$ (0.02 g), NH_4Cl (0.54 g), $MgCl_2 \cdot 6H_2O$ (0.02 g), $MgSO_4 \cdot 7H_2O$ (0.02 g), KH_2PO_4 (0.14 g), HEPES (4.76 g), yeast extract (0.1 g), 1 mL trace element solution ($FeCl_2$ (10 mM), $CoCl_2$ (1 mM), $MnCl_2 \cdot 4H2O$ (1 mM), $ZnCl_2$ (1 mM), H_3BO_3 (0.1 mM), $NiCl_2$ (0.1 mM), $AlCl_3$ (0.1 mM), $Na_2MoO_4 \cdot 2H_2O$ (0.1 mM), $CuCl_2$ (0.01 mM)), and 10 μL Se/W solution (Na_2SeO_3 (1.0 mM), $Na_2WO_4 \cdot H_2O$ (1.0 mM)), per liter) supplemented with acetate (20 mM) as a carbon source and fumarate (80 mM) as an electron acceptor. Cells were then collected and washed three times with PSN medium by centrifugation for 10 min at 5000 x *g*. After the final wash, the cell suspension was centrifuged for 20 min at 5000 x *g*, and the collected cells were used for electrochemical characterization. The concentration of the cell suspension in the electrochemical cell was determined by measuring the optical density at 600 nm (OD_{600}) and adjusted to OD_{600} = 0.2, unless otherwise stated.

Fluorescence spectrophotometry. To prepare samples for the measurement, 5 ml of the supernatants from the *G. sulfurreducens* cultures that had been incubated for 3 days were passed through 0.2-μm-pore-size membrane filters (Advantec). Fluorescence spectra of the filtrates were recorded using a Hitachi F-2500 spectrometer using a 10- by 10-mm quartz cell. Emission spectra were measured using an excitation wavelength of 425 nm, and excitation spectra were monitored at 525 nm. Quantification was based on the peak intensity of fluorescence spectra, and a standard curve of fluorescence intensity was used for the calculation of FMN and RF concentration in samples. Supernatant solution from the growing media was sampled in three indivisual experiments.

Liquid chromatography mass spectrometry (LC-MS):

LC-MS was performed on the Shimadzu system with a mtakt Unison UK-C18 column [2 x 150 mm; particle size, 3 μm]. The mobile phase was water (0.1% formic acid) and acetonitrile (mobile phase B). The mobile phase started with 20% B andwas isocratic at 80% B for 12 min. The flow rate was set at 0.2 mL/min, and the injection volume was 2 μL. For quantitative analysis of FMN and RF, selected-ion monitoring (SIM) was used to record the abundance of the $[M - H]^-$ molecular ion peaks at m/z 375.35 and 455.3 for RF and FMN, respectively. Quantification was based on the LC-MS peak area of RF and FMN, and a standard curve was used for the calculation. Representative chromatograms of SIM at m/z 375.35 and 455.3 are shown in Figure 1c and S3.

Electrochemical Measurements:

A single-chamber three-electrode system with a working electrode (W.E.) on the bottom surface of the reactor was used for the electrochemical studies of intact *G. sulfurreducens* cells. An indium-doped SnO_2 (ITO) substrate was used as the W.E., and Ag/AgCl (KCl sat.) and platinum wire were used as the reference (R.E.) and counter (C.E.) electrodes, respectively. The area of the W.E. was 3.14 cm^2. A current versus time curve for microbial current generation was measured under a potentiostatic condition at 0.2 V (Ag/AgCl) using an automatic polarization system (HZ-5000, Hokuto Denko, Tokyo, Japan). Differential pulse voltammetry measurements were conducted using 5.0 mV pulse increments, a 50 mV pulse amplitude, a 300 ms pulse width and a 5.0 s pulse period. Measurements were performed for sampling for 10 ms after each pulse. The sets of electrochemical measurements were at least triplicated. The background current was subtracted using SOAS software in Fig. 4a, which fits the baseline from regions sufficiently far from the peak assuming continuation of a similar and smooth charging current throughout the peak region **[Fourmond, V. *et al. Bioelectrochemistry* 2009 76, 141-147]**.

Scanning Electron Microscopy:
The ITO electrode with attached cells was carefully removed from the electrochemical reactor and was then washed with HEPES buffer. Samples were fixed with 2% glutaraldehyde and subjected to a serial dehydration protocol using increasing concentrations of ethanol. After three final changes in 100% ethanol, samples were subjected to critical point drying (VFD-21S, Vacuum Device). Desiccated samples were coated with evaporated platinum, and viewed using a YE-9800 electron microscope (Keyence) at an operating voltage of 10 kV. Representive images of the electrode surface were shown in Fig. 3.

Fluorescence Spectrometory of Outer Membrane Fraction
Outer membrane fractions of WT and Δ*omcBEST* strains were prepared by previously described methods **[Inoue, K. et al. Appl. Environ. Microbiol. 2010, *76*, 3999-4007]**. Followed by 100 mM Tris-HCl buffer dilution (pH 7.5), emission spectra were measured using an excitation wavelength of 440 nm, and excitation spectra were monitored at 520 nm. Emission and excitation peak assignment was based on the fluorescence spectra of 10 nM riboflavin in 100 mM Tris-HCl buffer solution.

Supplementary Figures

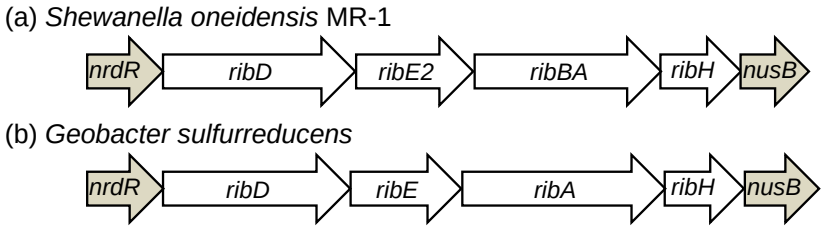

Fig. S1. Organization of the nrdR-ribDE2BAH-nusB flavin synthesis region in strain *S. oneidensis* MR-1 (a) and a homologous region in *G. sulfurreducens* strain PCA. This region corresponds to the genome coordinates 3611310 to 3615848 in MR-1 (accession no. NC_004347.2) and 1846788 to 1850727 in *G. sulfurreducens* (NC_002939.5). In addition, *G. sulfurreducens* has homologs of strain MR-1 SO_0702, which encodes flavin exporter protein **[N. J. Kotloski, J. A. Gralnick, Mbio 2013,*4*, 1-4.]**. These regions correspond to the genome coordinates 719084 to 720442 in MR-1 (NC_004347.2) and. 2923198 to 2924751 in *G. sulfurreducens* (NC_002939.5).

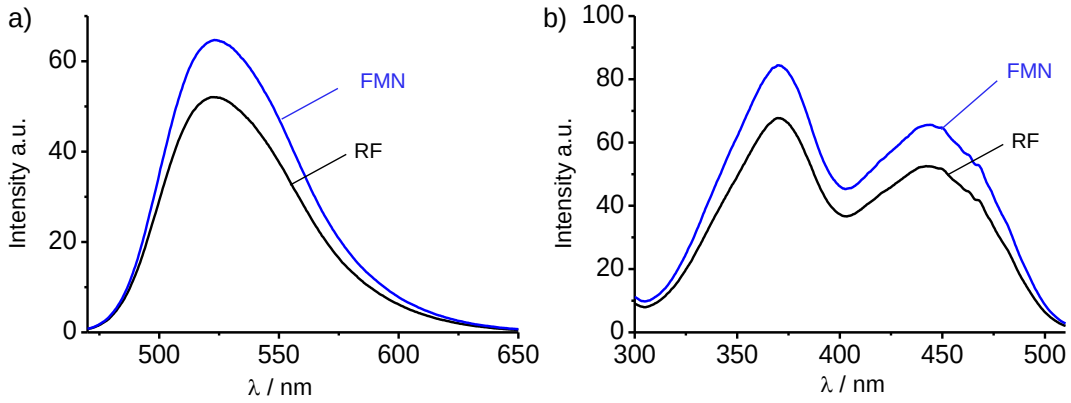

Fig. S2. Emission (a) and excitation (b) spectra of PSN media containing 10 μM FMN (blue line) or RF (black line). The excitation wavelength was 445 nm and the emission wavelength is 525 nm.

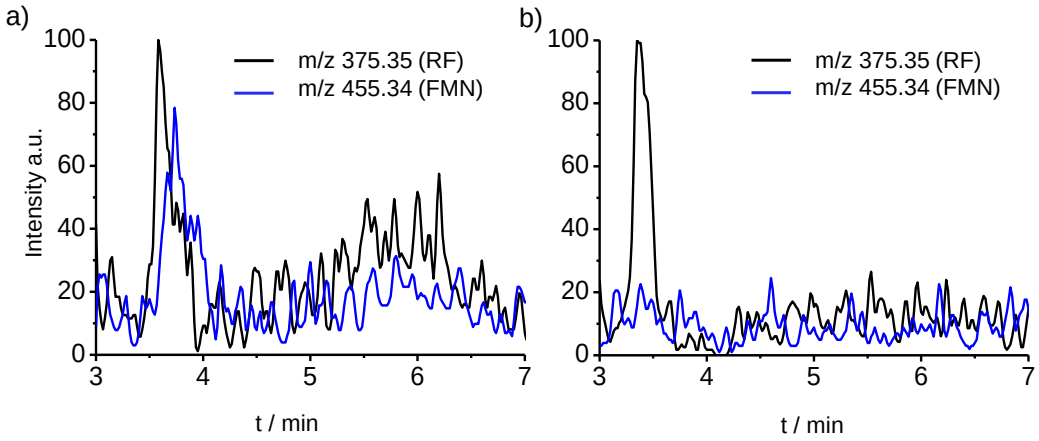

Fig. S3. Mass chromatography patterns of cell culture supernatants containing 100 nM FMN (a) and 100 nM riboflavin (b) in SIM mode. Note that the supernatant containing 100 nM flavin was prepared by adding flavin under the assumption that the cell culture supernatant did not contain flavin.

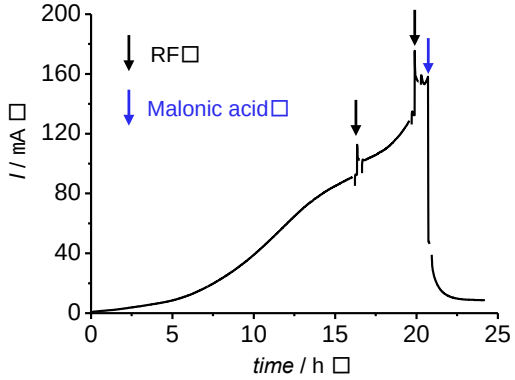

Fig. S4. Current (I_c) versus time (t) measurements of microbial current generation for *G. sulfurreducens* on an ITO electrode. Arrows indicate the time when 1 μM riboflavin (RF) or 100 mM malonic acid were injected into the electrochemical cell. Note that the pH of the solution containing malonic acid was adjusted to 7 by adding NaOH to avoid changing the pH of the medium.

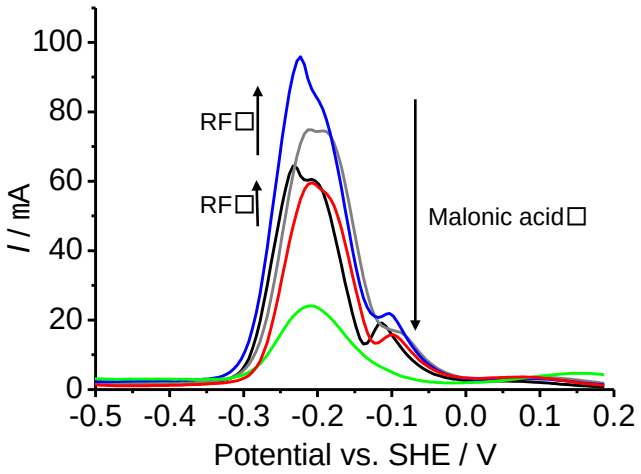

Fig. S5. Differential pulse (DP) voltammograms measured before and after the addition of 1 μM RF (red and black lines, respectively), before and after the another addition of 1 μM RF (gray and blue lines, respectively), and after 100 mM malonic acid (green line).

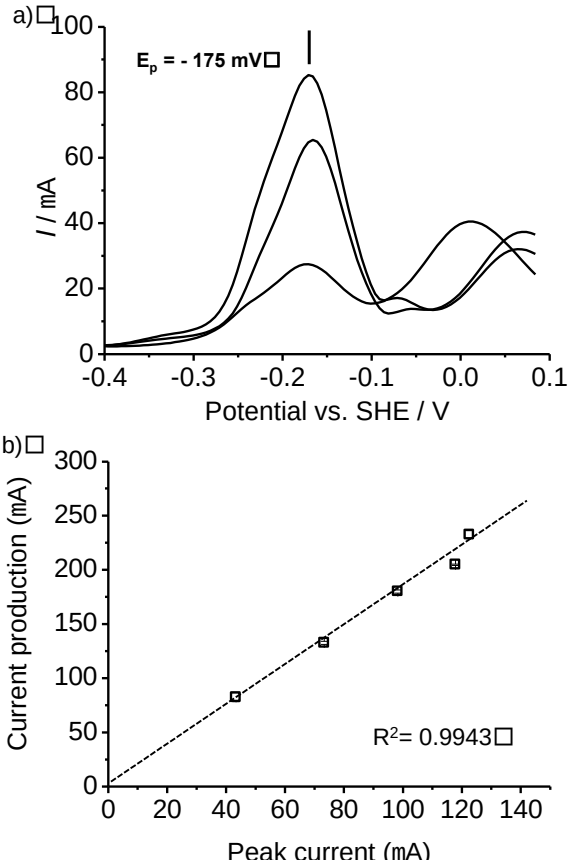

Fig. S6. (a) DP voltammograms of *G. sulfurreducens* biofilm on ITO electrodes in the presence of 10 mM acetate and 2 uM FMN. The peak current (I_p) at a peak potential (E_p) of −0.175 V increased with time. (b) Plot of I_c in panel a against I_p at E_p of −0.175 V. The squares of the correlation coefficients were estimated by the addition of the point of origin to the obtained data.

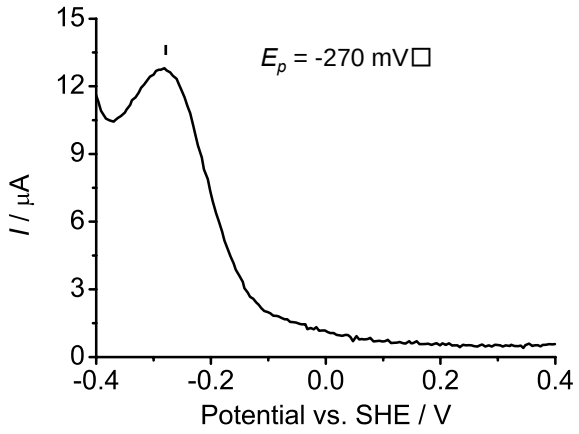

Fig. S7. Differential pulse (DP) voltammograms for cell-free 10 µM riboflavin solution.

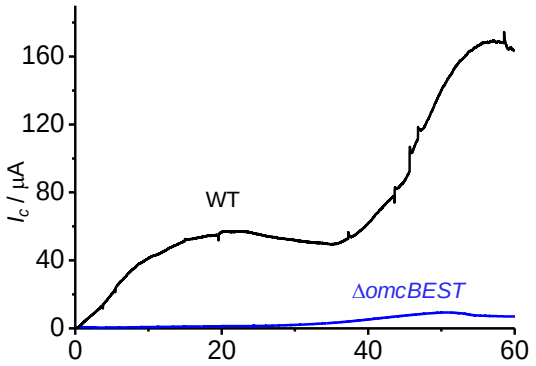

Fig. S8. Current (I_c) versus time (t) measurements of microbial current generation for Δ*omcBEST* of *G. sulfurreducens* on ITO electrode.

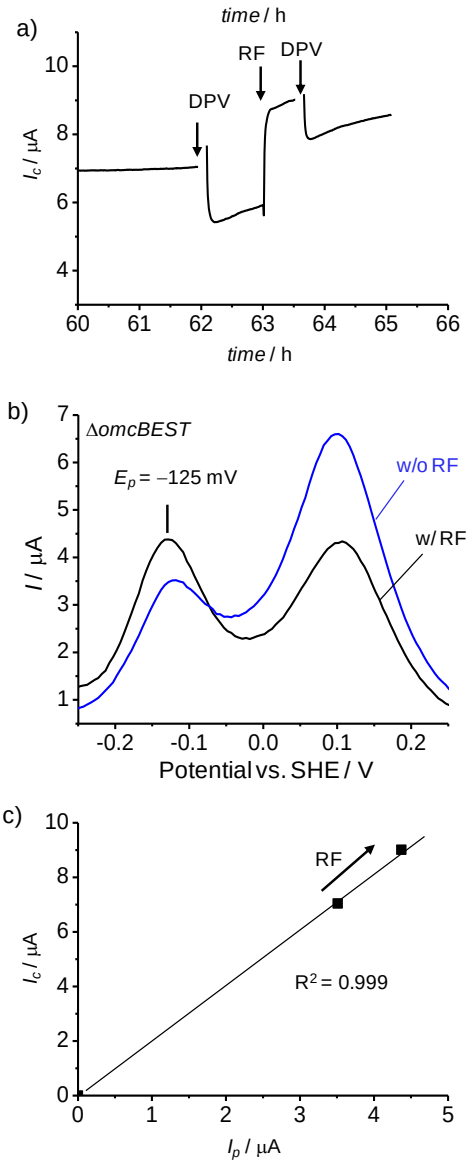

Fig. S9. Current (I_c) versus time (t) measurements of microbial current generation for Δ*omcBEST* of *G. sulfurreducens* on ITO electrode before (a) and after the addition of RF (b). (c) Plot of I_c in panel b against the peak current (I_p) at a peak potential (E_p) of −0.125 V. The squares of the correlation coefficients were estimated by the addition of the point of origin to the obtained data.

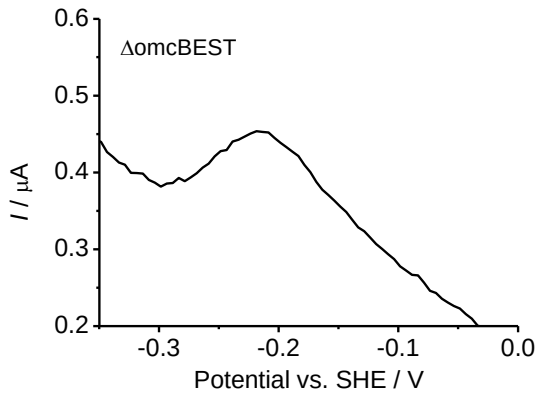

Fig S10. Differential pulse voltammograms of strain ΔomcBEST in the presence of 2 μM FMN.

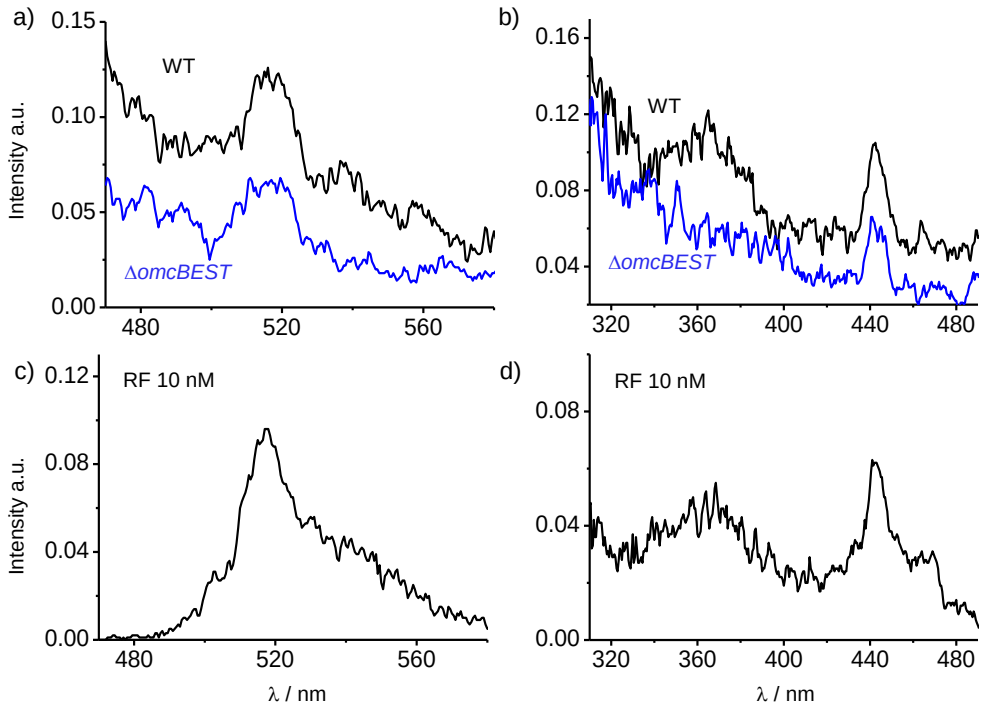

Fig S11. Emission (a) and excitation (b) spectra of outer membrane fraction of *G. sulfurreducens* wild type (WT) (black line) and Δ*omcBEST* (blue line) strains in 100 mM Tris-HCl buffer solution at pH 7.5. Emission (c) and excitation (d) spectra of 100 mM Tris-HCl buffer solution containing 10 nM RF. The excitation wavelength was 440 nm and the emission wavelength is 520 nm.