Materials and Methods

Bacteria strains and culture conditions

All *E.coli* strains were conventionally grown in Luria-Bertani (LB) broth at 37 °C with shaking at 220 rpm. When needed, ampicillin (100 μg/ml) was used to select positive clones and maintain plasmids. When the optical density of bacterial culture at 600 nm (OD600) reached 0.5, 1 mM IPTG (isopropyl-beta-D-thiogalactopyranoside) was added to induce overexpression of recombinant proteins for 24 h.

1 ml *S. oneidensis* MR-1 culture was inoculated in 100 ml LB broth and incubated at 30 °C with shaking at 220 rpm until OD600 value reached about 1.0. The bacteria culture was then harvested by centrifugation (4000 rpm×10 min). The bacteria pellets were wash three times with M9 minimal medium (6.78 g/L Na_2HPO_4; 3g/L KH_2PO_4; 1g/L NH_4Cl; 0.5g/L NaCl; 0.011 g/L $CaCl_2$; 0.12 g/L $MgSO_4$) and suspended in 100 ml anolyte (5% LB broth plus 95% M9 minimal medium containing 26mM lactate and 15 g/L glucose, PH 7.0). The bacteria suspension (10^9 cells / mL) was then transferred into the microbial fuel cell (MFC) anodic chamber and purged over nitrogen gas via a 0.2 μm filter for 35 min to remove oxygen.

DNA manipulation and recombinant construction

To initiate protein synthesis accurately from the start codon of each *rib* gene itself, the upstream start codon (ATG) next to multiple cloning sites (MCS) of pQLinkN vector was replaced with AAG, by using KOD Plus Mutagenesis Kit (Toyobo, Japan) and the primers pQlinkN-Mut-F/R (Table S1). The resulting vector was named as pQLinkNMut. Genomic DNA of *E.coli K-12* strain was purified by using GeneJET Genomic DNA Purification Kit (Thermo scientific, USA). The five genes of riboflavin (RF) biosynthetic pathway, *ribA* (Gene ID: 945763, NCBI), *ribB* (Gene ID: 947526, NCBI), *ribD* (Gene ID:945620, NCBI), *ribC* (Gene ID:945848, NCBI), and *ribE* (Gene ID:946453, NCBI), were amplified by polymerase chain reaction (PCR) using gene specific primers (Table S1), respectively. The PCR products were digested with *Bam*HI and *Not*I, and the restriction fragments were ligated into expression vector pQLinkNMut at the downstream of P_{tac} promoter (Figure S1). The recombinant vectors were electroporated into *E. coli* BL21 (DE3) electrocompetent cells. The transformants were screened by PCR and then verified by sequencing (Aitbiotech, Singapore). The plasmids of positive clones were purified and named as pQLinkNMut-RibA, pQLinkNMut-RibB, pQLinkNMut-RibD, pQLinkNMut-RibC and pQLinkNMut-RibE, respectively.

For constructing the coexpression plasmid of pQLinkNMut-RibAB, 0.4 pmol plasmid of pQLinkNMut-RibA was digested for 20 hour at 25 °C with 5 U *Swa*I in 10 μl and 0.4 pmol plasmid of pQLinkNMut-RibB was digested for 20 hour at 37 °C with 5 U *Pac*I (New England Biolabs, USA) in 10 μl. Enzymes were inactivated by incubating at 65 °C for 20 min and 0.2 pmol DNA was treated with 1 U LIC-qualified T4 DNA Polymerase (Merck, USA) in total volume of 20 μl (T4 DNA Polymerase Buffer, 10mM DTT, Nuclease-free Water, 2.5mM dGTP for *Swa*I digest and 2.5mM dCTP for *Pac*I digest). Upon incubation at 22 °C for 30 min and sequentially at 75 °C for 20 min, 2 μl of the above T4 DNA Polymerase treated solution (*Pac*I digest, containing RibB insert) was mixed thoroughly with 1 μl of that solution (*Swa*I digest, containing linearized pQLinkNMut-RibA) and heated to 65 °C for 10s and then incubated at 22 °C for 5 min. The mixture was supplemented with 1 μl 25mM EDTA, followed by incubating at 22 °C for 25 min. The resulting

product was electroporated into *E. coli* BL21 (DE3) electrocompetent cells and screened by PCR and verified by sequencing. Following procedures mentioned above, the coexpression plasmid of pQLinkNMut-RibCE was produced from pQLinkNMut-RibC and pQLinkNMut-RibE. The coexpression plasmid of pQLinkNMut-RibABD was produced from pQLinkNMut-RibAB and pQLinkNMut-RibD. A ~9.6k coexpression plasmid of pQLinkNMut-RibABDCE was eventually constructed from pQLinkNMut-RibABD and pQLinkNMut-RibCE (Figure S1). The recombinant plasmid was electroporated into *E. coli* BL21 (DE3) electrocompetent cells. The positive clones were screened by PCR and sequenced using specific designed primers V-pQLinkN-F/R (Table S1) unless otherwise specified.

Anode preparation, MFC setup and operation

Dual-chamber glass MFCs (136ml each chamber) separated by Nafion 117 membrane (DuPont, USA) were constructed. The cathode was made from a carbon cloth (GasHub, Singapore) with geometric area of 6 cm^2. The anode was made from a 6 cm^2 carbon cloth. Before used, carbon cloth was treated with acetone and 1 M HCl. When required, the anode was treated with oxygen plasma atmosphere using plasma cleaner system (Harrick, USA) to enable it to become more hydrophilic for better electricigen adhesion. The cathodic chamber contains 50 mM $K_3Fe(CN)_6$ and 50 mM KCl. The catholyte level was adjusted to the anolyte level. A 2kΩ load resistor was connected into external MFC circuits, unless noted otherwise. The voltage across the load resistor was recorded by using a digital multimeter (ESCORT 3146A). MFCs were incubated at 30 ^oC.

Preparation of bio-cocatalyst beads, operation and reactivation

Recombinant *E.coli* cells cultivated in 3L LB broth at 37 ^oC (supplemented with 100 μg/ml ampicillin and 1 mM IPTG) were harvested by centrifugation at 4000 rpm for 15 min at 4 ^oC, rinsed three times with anolyte and the cell pellets (approximately 9 g/ wet weight) was suspended and mixed thoroughly with 30 ml 3% sterile alginate (completely dissolved in anolyte). The mixture was then extruded via a syringe into a sterilized 0.1 M $CaCl_2$ solution to form calcium alginate beads with the average diameter of 2 mm. The beads were cured in the same solution at 4 ^oC for 24 h. Before used, the bio-cocatalyst beads (approximately 39 g/ wet weight) were rinsed three times with anolyte to remove excess $CaCl_2$ and free cells. To test anaerobic flavinogenic activity of immobilized bio-cocatalyst, 39 g of beads (9 g recombinant *E. coli* cell pellets, wet weight) were transferred into an anaerobic culture bottle containing 100 ml anolyte, which was purged over nitrogen gas (filtered by 0.2 μm membrane) for 35 min to remove dissolved oxygen and incubated anaerobically at 30 ^oC. After completion of one batch (168 h), the beads were filtered, rinsed three times and transferred into a new bottle containing fresh anolyte for the next batch under the same experimental conditions. After completion of the eighth batch, the bio-cocatalyst beads were reactivated by replacing anolyte with 100 ml fresh LB broth (supplemented with 100 μg/ml ampicillin, 1 mM IPTG and 0.1 M $CaCl_2$) and incubated at 37 ^oC for 24 h. The beads were then filtered, rinsed three times and transferred again into a new bottle containing fresh anolyte for the ninth batch. Samples (100 μl) were taken at 12 h intervals for HPLC analysis.

After a stable output of MFC appeared (about 500 h after inoculation, electrolytes were replenished during operation when required), the bio-cocatalyst beads (39 g/wet weight) were

loaded into MFC anodic chamber containing 100 ml fresh anolyte and purged over nitrogen gas for 35 min to remove oxygen.

Electrochemical characterization

All MFC performance measurements were performed after a stable output appeared (electrolytes were replenished during operation when required). The polarization and power output curves were obtained by varying external load resistors. Both current density and power density were normalized to the geometric area of anode. All electrochemical experiments were conducted by using VersaSTAT-3F workstation (Princeton Applied Research) with an Ag/AgCl (saturated KCl) reference electrode. For the cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) measurements, the reference electrode was inserted into anodic compartment. The EIS measurement was carried out over a frequency range of 0.3 Hz to 300 kHz at open circuit potential (OCP) with ac amplitude of 10 mV. For the Tafel measurement, the reference electrode was inserted into cathodic compartment to relieve limitations to current density exerted by the cathode.[1] Tafel plots were recorded for the anode being swept at 0.2 mV/s from η =0 to 0.25 V, where η =0 is the OCP of the anode versus the reference electrode. Peak potential separation *versus* log(scan rate) for *S. oneidensis* MR-1 biofilm and RF on the carbon cloth were performed in the fresh anolyte and the fresh anolyte containing 1 μ M RF, respectively. All electrochemical tests were performed at room temperature (~30 ^{o}C).

HPLC analysis

The high-performance liquid chromatography (HPLC) analysis was performed at an Agilent 1100 HPLC system (Agilent Technologies, USA) equipped with a UV detector. The column was a Hibar 250-4 Lichrospher 100 RP-18 (Merck, USA). According to the procedures of Vasilaki *et al*[2] and Yong *et al,*[3] flavins were eluted with a mobile phase of acetonitrile and 50 mM Sodium dihydrogen phosphate (15:85, PH 3.0) at the flow rate of 0.4 ml/min and monitored at 270 nm. According to the procedure of Baere *et al,*[4] lactate was eluted with a mobile phase of acetonitrile and 25 mM Sodium dihydrogen phosphate (5:95, PH 2.2) at the flow rate of 0.4 ml/min and detected at 210 nm.

Colony forming units determination

According to the procedure of Merritt *et al,*[5] after a stable output of MFC appeared, the anodic carbon cloth was aseptically transferred into a 15 ml tube containing 10 ml sterile Phosphate buffered saline (Life technologies, USA) and vortexed thoroughly to detach all bacteria from the carbon cloth. Then the vortexed sample was 10-fold serially diluted and 100 μ l of each diluted solution was spread onto a separate LB agar plate. The plates were incubated overnight at 30 ^{o}C and the number of colony forming units (CFU) per anodic carbon cloth were determined in triplicate by using cell counter and the results were recorded only when between 50 and 400 colonies were counted each plate.

Calculation

To determine coulombic efficiency, MFC was connected to a 1kΩ load resistor. Coulombic efficiency is calculated as:[6,7]

$$\varepsilon_c = \frac{M\int_0^t Idt}{Fb_e v_{an}\Delta C} \times 100\%$$

where M is the molecular weight of the substrate lactate ($M = 90.08$), I is the current over a period of time t, F is Faraday's constant (96,485 C/mole of electrons), b_e is the theoretical number of mol of electron produced per mol of substrate during full oxidation of lactate ($b_e = 12$), v_{an} is the liquid volume of in the anodic chamber, ΔC is the substrate concentration change over the batch cycle.

Tabel S1 Primers used in this study

Primer name	Primer sequences
pQlinkN-Mut-F	5’ -GAGAAATTAACTAAGGGATCCAGTCTTCG-3'
pQlinkN-Mut-R	5’ -CTCTTTAATGAATTCTGTGTGAAATTGTTATC-3'
V-pQLinkN-F	5' -CGACCGAGTTGCTCTTGC-3'
V-pQLinkN-R	5' -ATCAGGCGGGCAAGAATG-3'
ribA-F	5’ -CG*GGATCC*ATGCAGCTTAAACG-3'
ribA-R	5’ -ATAGTTTA*GCGGCCGC*TTATTTGTTC-3'
ribB-F	5’ -CG*GGATCC*ATGAATCAGACGCTACTT-3'
ribB-R	5’- ATAGTTTA*GCGGCCGC*TCAGCTGG-3'
ribC-F	5’ -CG*GGATCC*ATGTTTACGGGGATTGTAC-3'
ribC-R	5’- ATAGTTTA*GCGGCCGC*TCAGGCTTCT-3'
ribD-F	5’ -CG*GGATCC*ATGCAGGACGAGTATTACA-3'
ribD-R	5’ -ATAGTTTA*GCGGCCGC*TCATGCACC-3'
ribE-F	5’ -CG*GGATCC*ATGAACATTATTGAAGCTAAC-3'
ribE-R	5’ -ATAGTTTA*GCGGCCGC*TCAGGCCT-3'

* Restriction enzyme sites for *Bam*HI or *Not*I are shown in italics

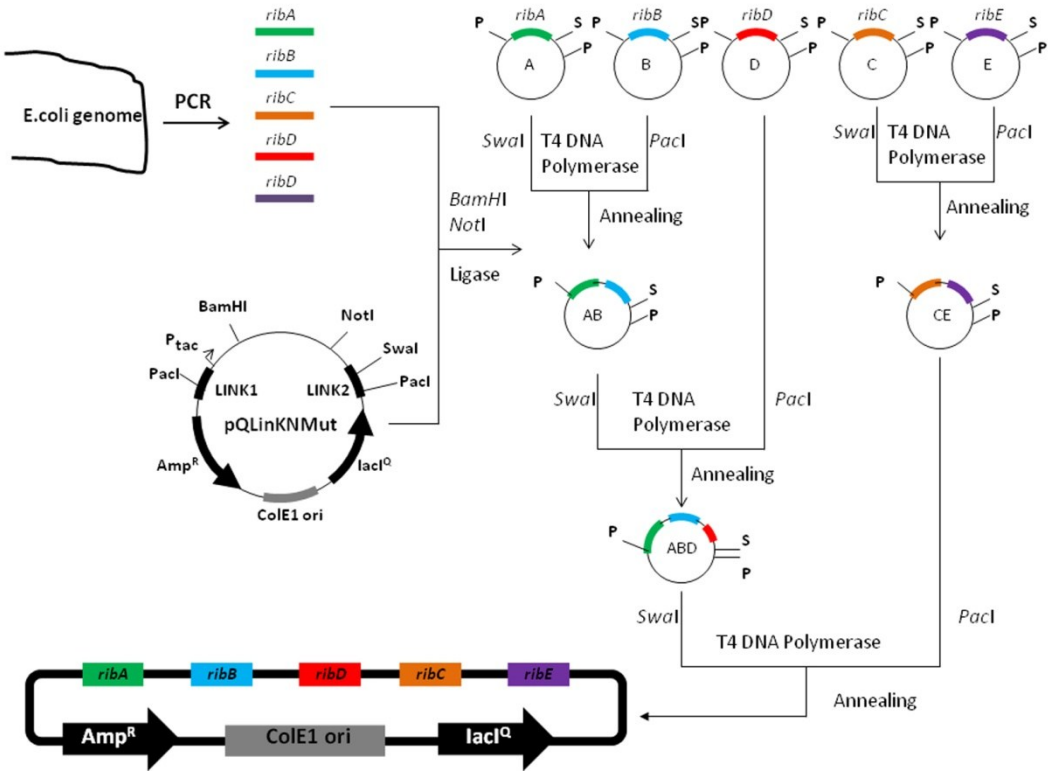

Fig. S1. Flowchart of DNA manipulation and construction of co-expression vectors. A: pQLinkNMut-RibA, B: pQLinkNMut-RibB, C: pQLinkNMut-RibC, D: pQLinkNMut-RibD, E: pQLinkNMut-RibE, AB: pQLinkNMut-RibAB, CE: pQLinkNMut-RibCE, ABD: pQLinkNMut-RibABD

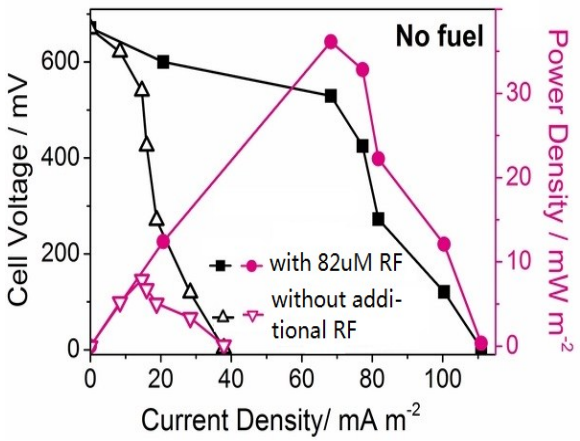

Fig. S2. Power output and polarization curves of MFCs without fuel using oxygen plasma treated carbon cloth anodes. 82μM RF was added into the MFC anodic chamber containing anolyte without lactate.

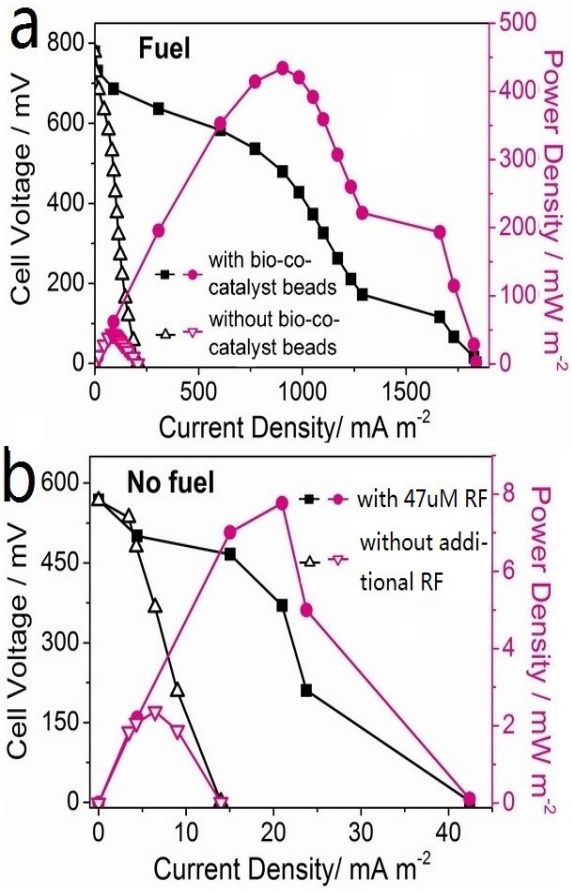

Fig. S3. Power output and polarization curves of MFCs (a) with fuel using carbon cloth anodes without oxygen plasma treatment. RF concentration in MFC anodic chamber containing bio-cocatalyst beads accumulated to 46.84±0.24 μM (n=3) after loading these beads for about 66h. (b) without fuel. 47 μM RF was added into the MFC anodic chamber containing anolyte without lactate.

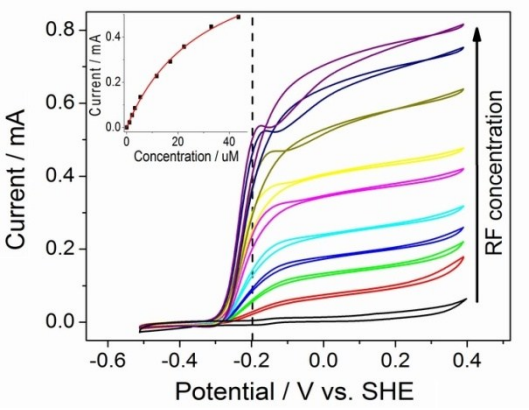

Fig. S4. Typical cyclic voltammograms (1 mV/s) from a *S. oneidensis* MR-1 biofilm residing on anodic carbon cloth without O_2 plasma treatment in 0, 1.1, 2.16, 3.22, 5.34, 11.8, 17, 22.3, 32.9 and 43.5 μM RF. The electrochemical potential (-0.2 V) where catalytic current was analyzed is illustrated by a dash line. Inset: Variation of the magnitude of the catalytic current, measured at -

0.2 V, with RF concentrations (background value at 0 μ M RF was subtracted). The line shows the catalytic current arising from a Michaelis-Menten type of kinetics with a K_m value of 30.5 μ M.

References

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