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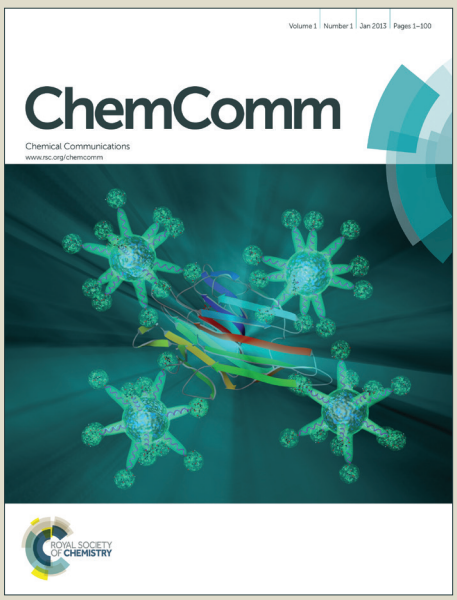

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Programming Quorum Sensing-based AND Gate in *Shewanella oneidensis* for Logic Gated-Microbial Fuel Cells

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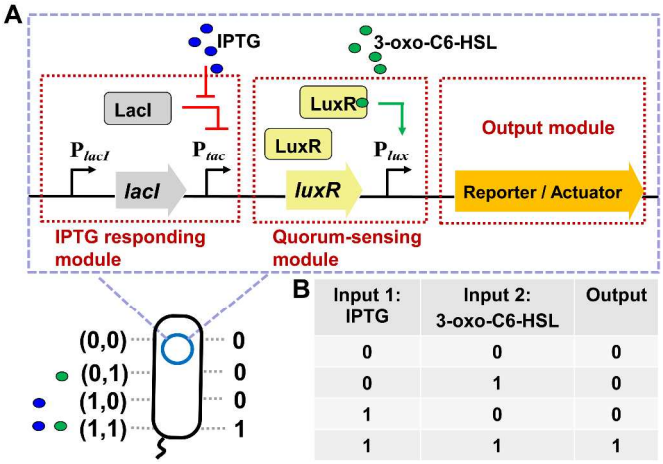
An AND logic gate based on synthetic quorum-sensing (QS) module was constructed in a *Shewanella oneidensis* MR-1 *mtrA* knockout mutant. The presence of two input signals activated the expression of a periplasmic decaheme cytochrome MtrA to regenerate the extracellular electron transfer conduit, enabling AND-gated microbial fuel cells.

Recent research in unconventional computing,[1] particularly using molecular[2] and biomolecular[3] systems, resulted in artificial (bio)chemical systems mimicking Boolean logic operations, including AND, OR, XOR, NAND, NOR and other logic gates. Biomolecular systems inspired by natural processes include a variety of species of different complexity including systems based on proteins/enzymes,[4] DNA/RNA[5] and whole cells.[6] Their operation in biological environment complementing natural biological processes was demonstrated.[7] The developed systems have already demonstrated the ability to perform simple computing operations,[8] however, real computational use of biomolecular systems is not achievable at the present level of their complexity. Instead of making computers from biomolecules, [9] which is a futuristic aim, they are considered as promising biosensors with logically processed input signals and binary YES/NO response.[10] New options for the biological information processing were received with the recent dramatic development of synthetic biology, microorganisms with sophisticated gene circuits constructed to achieve diverse logic gates-regulated responses precisely in spatial,[11] temporal,[12] and population scales.[13] However, most of the reporter genes controlled by the gene circuits were luminescence proteins, for the ease of signal detection. Interfacing biomolecular computing systems with electronics would allow for novel scientific and technological realizations, including "smart" multi-signal logically controlled bioelectronic systems.[14] Recently, owning to the development of switchable electrodes controlled by various physical and/or chemical signals,[15] information-processing systems have been employed in bioelectrochemical systems (BESs) using electrical signals as outputs.[16] Particularly, integration of biomolecular computing systems with biofuel cells[17] resulted in the development of self-powered devices logically processing biomolecular signals and producing output in the form of chemical actuation, e.g., drug release.[18] Microbial biofuel cells (MFCs)[19] are particularly attracting for interfacing electronics with the biological information processing systems.[20] Logic gates-controlled MFC according to build-in programs manipulated by external signals is of great significance, because it would allow for flexible and economic operations. Based on the control of phenazine biosynthesis, an AND logic gates was established in *Pseudomonas aerugenosa* for achieving AND gate-controlled MFC.[20] The control of the electron conduits of *Shewanella oneidensis* is of great interest owing to its greater efficiency in extracellular electron transfer (EET) and less potential pathogenesis. A gene-based AND logic gate, targeting the anaerobic respiration with DMSO reduction which was controlled by two enzyme coding genes as inputs, has been achieved in *S. oneidensis*.[21]

To enable higher flexible and reprogrammable design of robust gene circuit-controlled MFCs, we herein used synthetic biology method to accomplish a synthetic AND logic gate gene circuit in *Shewanella* for the first time. The AND logic gate responding machinery includes an IPTG responding module, which regulates the downstream quorum-sensing (QS) module originated from *Vibrio fischeri* (Fig. 1A). The QS module was newly introduced into *Shewanella* by synthetic biology approach. Owing to its extraordinary ability of coordinating cell behaviours within the whole population, the QS circuit could ensure consistent electro-activities among cells when response to varied input combinations. The designed gene circuit includes three modules programmed in *Shewanella*. Firstly, a constitutively expressed *lacI* and an IPTG regulated P*tac* promoter were assembled to achieve the IPTG responding module of *Shewanella* (Fig. 1A). Secondly, in the QS module,

the expression of *luxR* was controlled by the IPTG inducible P*tac* promoter. The transcriptional factor LuxR is bond and activated by N-(3-oxo-hexanoyl)-L-homoserine lactone (3-oxo-C6-HSL), a QS signalling molecule. Thirdly, the LuxR-3-oxo-C6-HSL complex (R*) is able to activate the expression of the reporter or actuator genes (the output module), accomplishing the AND logic gate gene circuit (Fig. 1B).



Input 1: IPTG	Input 2: 3-oxo-C6-HSL	Output
0	0	0
0	1	0
1	0	0
1	1	1

Figure 1 (A) Schematic diagram of the AND logic gate gene circuit programmed in *Shewanella*. (B) Truth table of the AND gate.

To characterize the performance of this AND logic gate machinery, the *gfp* gene encoding a green fluorescence protein was initially applied as the reporter gene (Fig. 2A). The gene circuit was programmed in the wild-type *S. oneidensis* MR-1. The detailed procedure of the gene circuit construction was shown in Fig. S1 (see Electronic Supplementary Information (ESI)). To accelerate the construction of gene circuit with multiple components, the three modules within this gene circuit (Fig. 2A) were individually designed as biobricks. The IPTG responding module was directly cloned from a commercial vector, and the GFP reporter module was assembled by existing biobricks. The QS module was synthesized *in vitro*. In particular, the sequence of *luxR* gene from *Vibrio fischeri* was optimized for its heterologous expression in *Shewanella* to prevent insufficient translation level due to rare codons. Additionally, double terminators were placed after *luxR* and reporter gene, in order to minimize background signal caused by the gene expression leakage.

Through orthogonal experiments of the two inducers with different concentrations, we obtained the optimal concentration of the inducers (see ESI, Fig. S3). At the optimal induction condition, 250 µl of overnight bacterial culture suspension was inoculated into 5 ml fresh LB medium (peptone, 10g/L; yeast extract, 5 g/L; NaCl 5 g/L, pH, 7.0), and incubated for 8 hours (30□, 180 rpm shaking) in four tubes with the induction of four input combinations: (1) no inducers; (2) only IPTG (0.01 mM); (3) only 3-oxo-C6-HSL (10 nM); and (4) IPTG (0.01 mM) and 3-oxo-C6-HSL (10 nM), respectively. After 8 hours, 200 µl of cells were harvested by centrifugation and washed 3 times with phosphate buffer solution (PBS: NaCl 8.0g, $Na_2HPO_4 \cdot 12H_2O$ 3.23g, $NaH_2PO_4 \cdot 2H_2O$ 0.45g, pH 7.0). Subsequently, the cell pellets were re-suspended in 200 µl PBS

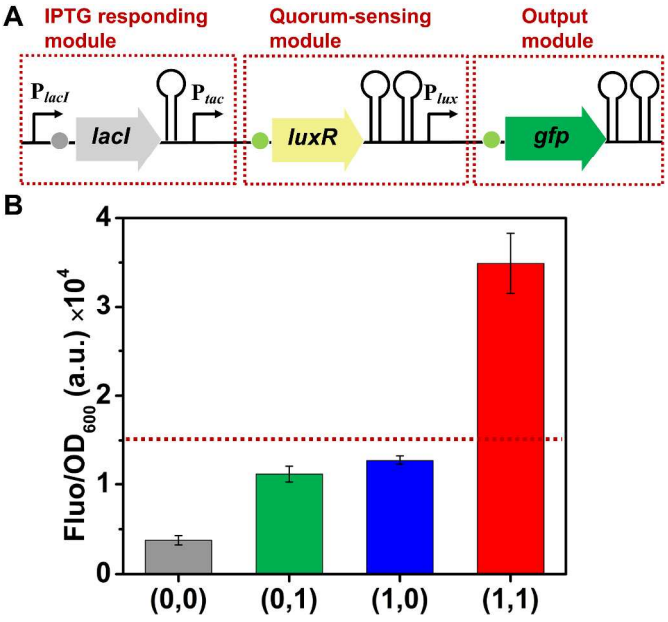


Figure 2 (A) Genetic diagram of the AND logic gate with GFP as the output reporter in *S. oneidensis* MR-1. (B) The performance of the AND logic gate gene circuit. The threshold is showed by a dash line to separate the ON and OFF states of the GFP expression.

and transferred into 96-wells plate. Fluorescence intensity (excitation: 485 nm, emission: 520 nm) and cell density (OD_{600}) were measured by a microplate reader (SpectraMax M2, Molecular Devices, USA), and three biological replicates were performed for each condition. To verify that GFP fluorescence responses to different inductions were caused by the inherent programmed gene circuit machinery, the total GFP fluorescence intensity was normalized to cell density (OD_{600}). The normalized fluorescence intensity was used as the performance index of the gene circuit (shown in Fig. 2B).[22] The LuxR-3-oxo-C6-HSL complex was unable to be formed in the absence of either of the two inputs (IPTG and 3-oxo-C6-HSL). The logic gate was considered to be "OFF" when fluorescence was below the threshold (as shown in the dash line, Fig. 2B). Apparently, the AND logic gate was "ON" when IPTG and 3-oxo-C6-HSL were applied simultaneously. As expected, the GFP output with input (1,1) was dramatically higher compared to other input combinations: (0,0), (0,1) and (1,0). These results demonstrated the functional performance of our AND logic gate gene circuit with an IPTG responding module and a heterologous QS system in *S. oneidensis* MR-1.

Upon verification of the successful construction of the AND logic gate gene circuit in *Shewanella* (Fig. 2), we furthermore programed the expression of MtrA (a key component of the MtrCAB-OmcA membrane conduit) as an actuator to replace GFP in a *S. oneidensis mtrA* knockout mutant strain (Fig. 3A), which was applied in microbial fuel cells (MFCs) to achieve the finely regulated electricity output with the AND logic gate (see Fig. 3D). *S. oneidensis* exchanges electrons with electrodes through direct contact-based extracellular electron transfer (EET) *via* outer-membrane (OM) *c*-type cytochromes (*c*-Cyts) or nanowires in the biofilm, and electron shuttle (flavins)-mediated EET (Fig. S4). Deletion of *mtrA* could disrupt the EET pathways of *Shewanella* and block

electron exchange between cells and electrodes, which severely impair the outward current.[23] The *S. oneidensis mtrA* knockout mutant harbouring the gene circuit (shown in Fig. 3A) was inoculated in LB medium, and incubated overnight in four tubes with the induction of the four inputs (0,0), (0,1), (1,0) and (1,1), respectively. Then, 5 ml of each of these overnight bacterial culture suspensions were transferred into 100 ml fresh LB broth and incubated for 8 hours at the same condition. Then, the cells were washed twice in PBS and injected into the MFC anode chamber, with a seeding optical density at 600 nm (OD_{600}) of 0.6. The dual-chamber MFCs (140 ml for both anode and cathode chambers) were separated by Nafion 117, and carbon cloth was used as the working electrode (2.5 cm × 2.5 cm) and counter electrode (2.5 cm × 3.0 cm). The catholyte was 50 mM $K_3[Fe(CN)_6]$ in 50 mM KCl solution. The anode medium was M9 medium (Na_2HPO_4, 6g/L; KH_2PO_4, 3g/L; NaCl, 0.5g/L; NH_4Cl, 1g/L; $MgSO_4$, 1mM; $CaCl_2$, 0.1 mM) supplemented with 5% LB broth and 20 mM lactate. Each induction condition in MFCs was triplicated. The MFCs were incubated at 30°C, and the output voltage (V) was recorded across a 2 kΩ resistor. All electrochemical characterizations, including MFC polarization discharge curves (Fig. 3B), power density output curves (Fig. 3C) and cyclic voltammetry (Fig. S4), were conducted at room temperature using CHI600E electrochemical workstation with an Ag/AgCl (KCl saturated) reference electrode (CH Instrument, Shanghai, China).

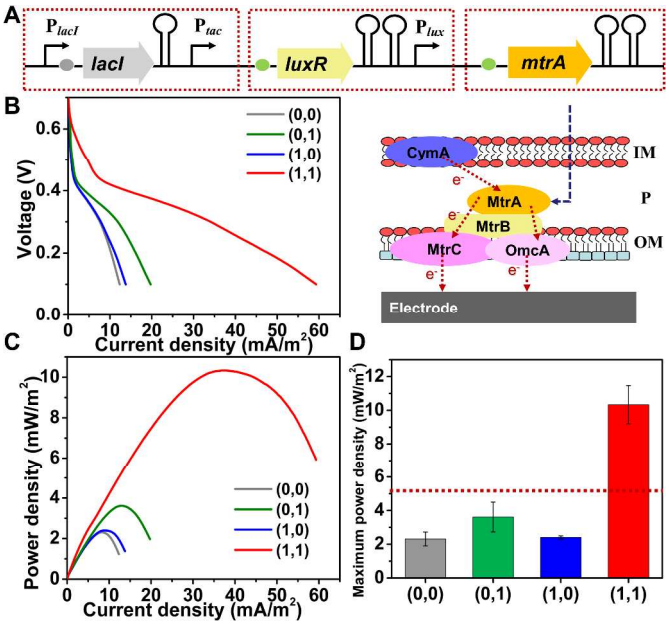


Figure 3 (A) Genetic diagram of the AND gate and schematic of the controlled membrane conduit in *S. oneidensis*. A cellular conduit system is composed of conductive membrane proteins (CymA and MtrCAB-OmcA), which allow electrons to flow from intracellular space to external electron acceptor (electrode). IM: inner membrane, P: periplasm, and OM: outer membrane. (B) Polarization curves of MFCs at the four input combinations. (C) Power density output curves at the four input combinations. (D) Maximum output power densities of the AND logic gate-controlled MFCs obtained by polarization discharge curves. The dashed line shows the threshold that differentiates the ON and OFF states of MFCs.

To obtain the polarization curves of MFCs (voltage-current density (V-I) curves) at the four combinations of inputs (Fig. 3B), the linear sweep voltammetry (LSV) with the scan rate of 0.1 mV/s was implemented. The MFCs' output power density curves were calculated by multiplying the voltage with its corresponding current on the V-I curves, and normalized by the geometric area of the carbon cloth electrode (Fig 3C). Then, the maximum power densities of the MFCs that correspond to the four input combinations were obtained (Fig. 3D). The MFCs showed low power densities at the conditions of (0,0), (0,1) and (1,0), *i.e.*, 2.31 ± 0.42 mW m^{-2} (no induction), 3.61 ± 0.88 mW m^{-2} (3-oxo-C6-HSL only), and 2.40 ± 0.09 mW m^{-2} (IPTG only), respectively. While, a 4-folds' increase in the maximum power density (10.33 ± 1.33 mW m^{-2}) was detected when both inputs were present, evidencing the presence of both inputs switched on the *lux* promoter (P_{lux}) to elicit the expression of MtrA. In the *mtrA* knockout mutant, the EET pathway is interrupted by the absence of MtrA, which severely impaired the power output in MFC. While, the presence of both inputs activates the expression of MtrA, rescues the MtrA deficiency in the mutant strain and restores the electron conduit (Fig. 3A). We further noticed that the system performances of the AND logic gate using GFP as a reporter (Fig. 2) and the MFC electrical output using *mtrA* as the downstream gene controlled by our gene circuit (Fig. 3) were consistent, indicating the reprogrammable character of our AND logic gate in the gene circuit design.

In conclusion, we developed a reprogrammable gene circuit in recombinant *S. oneidensis* to accomplish AND logic gate-controlled extracellular electron transfer for the first time. The modular structure of our gene circuit enabled flexibility and potential to create more QS-based AND logical gates by changing the downstream genes (reporter or actuator), which would greatly expand the possibilities of system output. For example, the genes involved in the production of flavins (as electron shuttles in *Shewanella*), the formation of biofilm or redox enzymes could be incorporated in the downstream genes, which would enable other design of gene circuit-controlled MFCs. In future efforts, development of promoters with high specificity in response to certain inducers should be crucial to ensure applications of gene circuit-controlled MFCs in nature ecological environments.

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Notes and references

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†Electronic supplementary information (ESI) available: Experimental method of *in vitro* gene synthesis; Plasmid maps ; The orthogonal experiment of inducers concentration with fluorescence output; The BES performance of electrical output controller with various combinations of the input signals.

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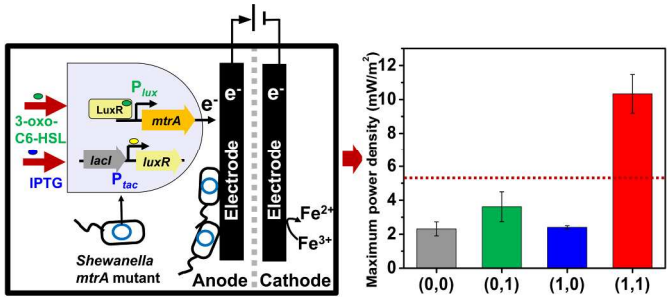

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A modularly structured, flexible, and reprogrammable AND logic gate gene circuit-controlled microbial fuel cell.