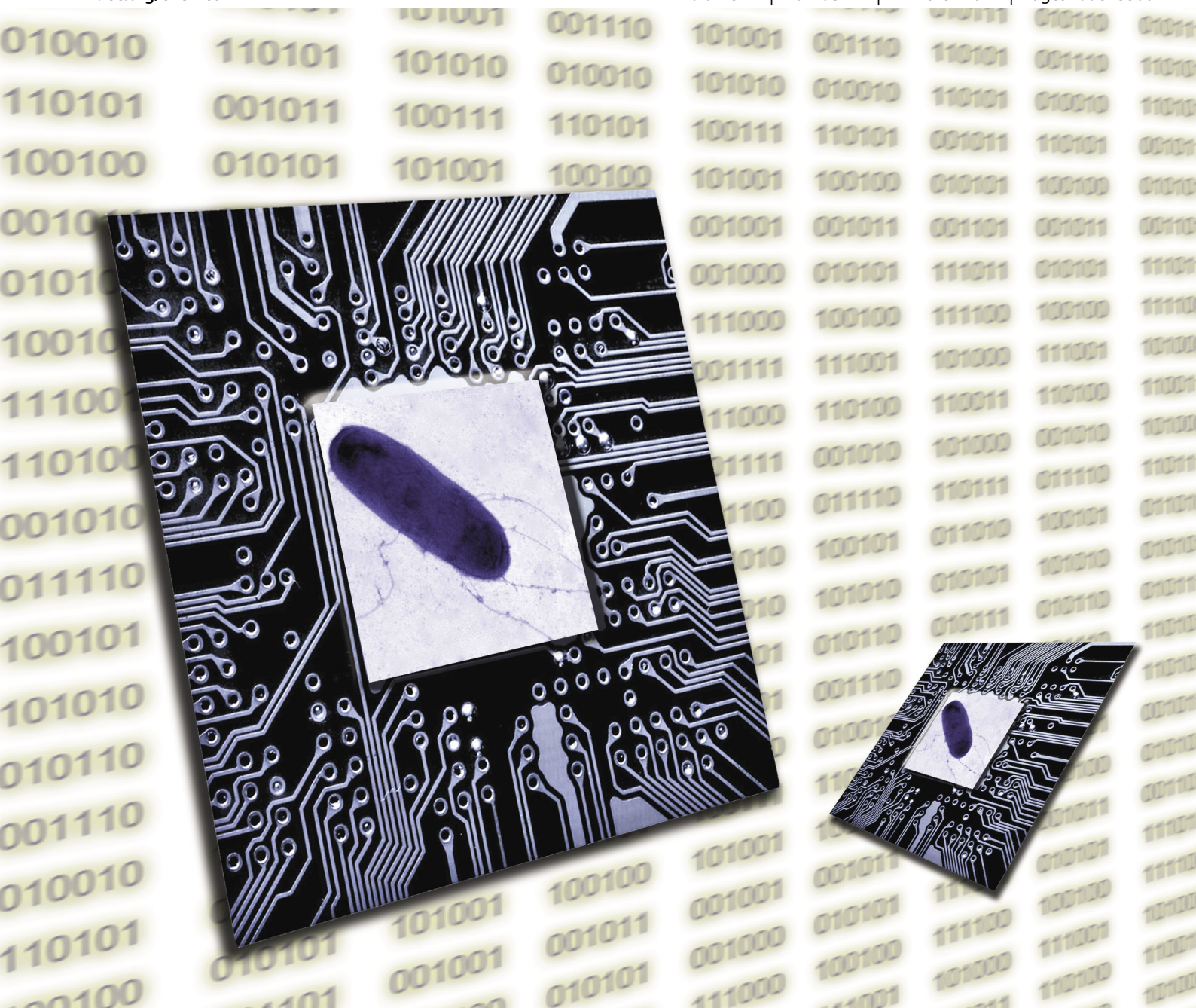


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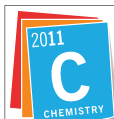


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COMMUNICATION

Bacteria-based AND logic gate: a decision-making and self-powered biosensor

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We developed a bacteria-based AND logic gate using a *Pseudomonas aeruginosa lasI/rhlI* double mutant with two quorum-sensing signaling molecules as the input signals. We showed a distinct electrical output signal, despite the complexity and continuous regulation of metabolic reactions of living cells.

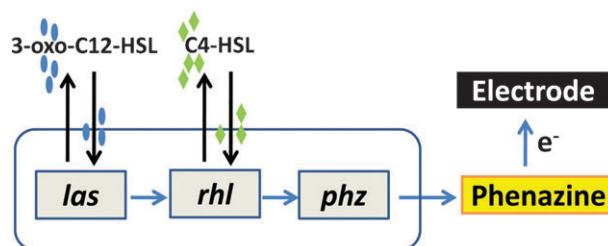
Here, we present bioelectrochemical systems (BESs) based on the electrochemical activity of living bacterial cells to control the electric current generation *via* built-in biomolecular Boolean logic operations. Biomolecular computing is a next-generation, complex computing approach (*i.e.*, unconventional computing), which is still in its infancy.¹ However, enzymatic BESs (*e.g.*, biofuel cells) in which enzymatic reactions were the core parts of various Boolean logic gates (*i.e.*, AND, OR, XOR) to process biochemical input signals by defined chemical reactions have been intensively studied.^{2–4} In this communication, we report, for the first time, a bioelectrochemical AND logic gate completely based on bacterial signal transduction. Different from enzymatic logic gates, the input signals are processed by living bacteria with all their regulatory and metabolic complexity.

Microbial BESs harbor electrochemically-active bacteria at electrodes to catalyze oxidation and reduction reactions. When a naturally-occurring potential difference between, for example, an anaerobic microbial anode and an aerobic cathode is maintained, electric power can be generated with such BESs (*i.e.*, microbial fuel cells [MFCs]).⁵ For research purposes, or to foster anaerobic reduction reactions at the cathode, an artificial potential is applied to increase the potential difference between anodes and cathodes.⁶ By maintaining an appropriate potential at the anode to support a community of microbes that oxidize organic material in wastewater and by artificially elevating the potential at the cathode to reduce CO₂, BES has been hailed as a sustainable technology to treat wastewater with low greenhouse gas emissions.⁷ While three separate MFCs have been electronically connected

to provide a Boolean logic output,⁸ direct bacterial signal transduction in microbial BESs has never been used as a logic system. Boolean AND logic gates based on whole cells of bacteria that were responding to external conditions in an analog mode by, for example, fluorescence signals have been reported.^{9–11} Here, we report a bacteria-based AND logic gate with a digital response through an electrochemical signal output.

We built a bacteria-based AND logic gate using potentiostatically-controlled and self-powered BESs with a *Pseudomonas aeruginosa* (PA 14) *lasI/rhlI* double mutant as the electrochemically-active bacterium. The *las* and *rhl* cascade systems are important regulatory systems of cell–cell communication (*i.e.*, quorum sensing [QS]) for *P. aeruginosa*.

QS is usually defined as a cell-density-dependent regulation system *via* secreted signaling factors.¹² For the *P. aeruginosa* wildtype strain, the two QS signaling molecules, 3-oxo-dodecanoyl homoserine lactone (3-oxo-C12-HSL) and *N*-butanoyl-L-homoserine lactone (C4-HSL), are self-secreted by the LasI and RhlI inducers, respectively. Upon high enough cell density, these secreted compounds are sensed by the LasR and RhlR receptors, respectively, to initiate a concerted cell-response strategy for pathogenesis *via* the production of various extracellular virulence factors (*e.g.*, for cystic fibrosis).¹³ One of the important gene systems under the control of the *las/rhl* regulatory system is the *phz* operon, which codes for the enzymes required for phenazine biosynthesis.¹⁴ Phenazines are redox-compounds that act as a reversible redox mediator for electric current generation in our engineered BESs.¹⁵ The presence of 3-oxo-C12-HSL and C4-HSL initiates the QS cascade, resulting in current generation in BES (Scheme 1).¹⁶



Scheme 1 Simplified illustration of homoserine lactone-controlled phenazine synthesis by *P. aeruginosa*.

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The *lasI/rhlI* double mutant cannot synthesize 3-oxo-C12-HSL and C4-HSL. Therefore, using this double mutant provided us with a bacterial system that is stimulated by biochemical signals.

The bacteria-based **AND** logic gate was implemented in two different BESs: (1) a potentiostatically-controlled BES (0.3 V vs. Ag/AgCl; VSP, Bio-Logic USA, Knoxville, TN); and (2) a self-powered MFC. For the former BES, we used a temperature-controlled and sealed glass vessel (300 mL, 37 °C), containing a three-electrode setup: a working electrode (carbon fiber cloth with a geometric surface area of 162 cm², PANEX[®] 30; PW06, Zoltek, St. Louis), a counter electrode (graphite rod), and a reference electrode (Ag/AgCl sat. KCl). For the latter BES, we used an H-type MFC containing a Nafion 117 membrane (23 cm²), separating the anode and cathode chambers (220 mL). Both the MFC anode and cathode were made out of the carbon fiber cloth that was similar to the working electrode in the potentiostatically-controlled BES. The three-electrode system and the anode chamber of the MFC were operated with AB bacterial minimum medium (pH 6.8, 8 mM (NH₄)₂SO₄, 21 mM Na₂HPO₄, 11 mM KH₂PO₄, 26 mM NaCl, 0.39 mM Na₂SO₄, 0.1 mM CaCl₂, 2 μM FeCl₃, 30 mM glucose) under oxygen-limited conditions (no active aeration, but headspace was open to atmosphere via a sterile vent filter). The cathode chamber of the MFC was filled with 0.1 M phosphate buffer solution, containing 0.1 M potassium ferricyanide as a terminal electron acceptor. The *P. aeruginosa lasI/rhlI* mutant was pregrown in Luria-Bertani (LB) medium. 3-oxo-C12-HSL and C4-HSL were applied as input signals. Statistical analysis (a 2-factorial, 2-leveled ANOVA analysis) was performed using Minitab 16 (Minitab Inc., State College, PA).

To build the **AND** logic gate using the potentiostatically-controlled BES, the absence of 3-oxo-C12-HSL or C4-HSL was considered as logic **0**, while their presence at operational concentration (15 μM) was considered as logic **1**. The output was defined as **1** (TRUE) when the current produced by the BES was above the threshold value of 0.29 mA and **0** (FALSE) when it was below 0.29 mA (Fig. 1). Application of the input **0,0** did not activate the global gene regulation system due to the absence of both QS signals. Thus, the expression of the *phz* operon was not upregulated, resulting in the absence of phenazines, and therefore in a low current of 0.04 ± 0.01 mA. In the presence of both 3-oxo-C12-HSL and C4-HSL

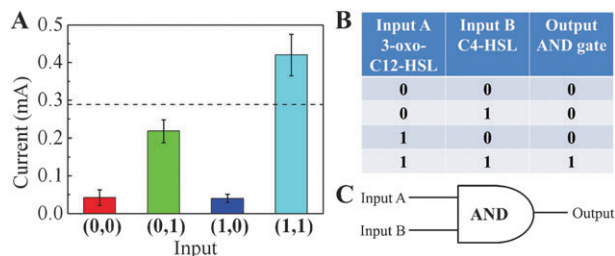


Fig. 1 (A) Phenazine-based current production in potentiostatically-controlled BESs for four different input combinations; standard deviation from at least triplicate experiments. The dashed line shows the threshold that separates output **0** and **1**. (B) Truth table for the bacteria-based **AND** logic gate. (C) Equivalent circuit of an **AND** logic gate.

(input **1,1**), the *las* and the *rhl* systems were activated. Consequently, the expression of the *phz* operon was fully upregulated by the QS system, which resulted in the highest phenazine production. The resulting current generation was higher than 0.29 mA (0.42 ± 0.06 mA), and therefore the BES generated the output **1**. In the presence of only C4-HSL (input **0,1**), the *rhl* system was activated, and the phenazine production was somewhat upregulated, leading to a current generation of 0.22 ± 0.03 mA. With only 3-oxo-C12-HSL (input **1,0**), the *las* system was activated, but the level of upregulation of phenazine production by the *las* system was lower than by the *rhl* system (*las* and *rhl* work in a cascade mechanism; Scheme 1). For that reason, the current was lower for the input **1,0** compared to **0,1**. The current of either of these signal combinations was not high enough to meet the threshold of 0.29 mA, thus, resulting in the output **0** (99.0% confidence). Therefore, the feature of the double mutant-based sensing system corresponds to the equivalent circuit of the **AND** logic gate (Fig. 1). We confirmed the concerted reaction of the two input signals in the double mutant (leading to **AND** gate activation) with a 2-factorial, 2-leveled ANOVA analysis ($p = 0.001$).

The same logic operations for the **AND** logic gate were used in the self-powered MFC for which the power density was logically controlled by phenazine regulation. We used maximum power densities (MPDs), which are obtained from polarization curves, to determine the output. The highest MPDs for all MFC tests were obtained for input **1,1** (1.69 ± 0.41 mW m⁻², Fig. 2). The threshold of the Boolean logic **AND** gate for our MFC system was 1.19 mW m⁻² (88.9% confidence). The confidence interval for our self-powered biosensor is lower than for our potentiostatically-controlled BES because the batch-mode MFCs showed experimental limitations (ferricyanide diffusion and accumulation in the anode chamber negatively affected bacterial performance and, therefore, the resulting MPDs). Even under these limitations, however, the ANOVA analysis confirmed the concerted interaction of our input signals ($p = 0.015$).

Bacterial metabolic reactions are very complex. They are comprised of constant changes in multiple gene expression and protein levels. Despite this complex metabolic network, we showed that two simple input signals were converted into one clear digital output signal. However, the time scale of this bacteria-based biocomputing system is long (~115 h). The MFC integration with a logic gate has the potential to fit the requirement of a self-powered and decision-making biosensor. *P. aeruginosa* is an opportunistic pathogen, which infects

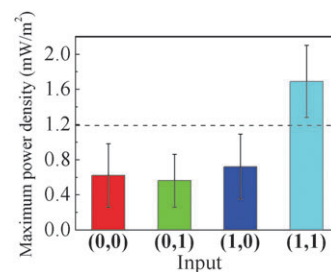


Fig. 2 Phenazine-based MPDs in MFCs for four different input combinations; standard deviation from quadruplicate experiments. The dashed line shows the threshold that separates output **0** and **1**.

humans, other animals, and plants. Based on the detection and biomolecular computing of two chemical input signals, our work demonstrated the feasibility of developing a biosensor for the detection or long-term monitoring of *P. aeruginosa* infections (e.g., in *in vitro* human tissue of the respiratory tract) via homoserine lactone sensing. Other highly promising applications for bacteria-based biocomputing systems may be the monitoring and controlling of fermentation, wastewater treatment, or remediation processes, possibly with other electrochemically-active bacteria.

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