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Quorum sensing regulates electric current generation of *Pseudomonas aeruginosa* PA14 in bioelectrochemical systems

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ABSTRACT

Here, we show that quorum sensing (QS) modulates the current generation of the anode-respiring bacterium *Pseudomonas aeruginosa* because it controls the production of phenazines, which mediate the electron transfer to the anode. The current generation by a wildtype (WT) strain *P. aeruginosa* PA14 and the GacS/GacA protein-regulatory mutant *retS* was investigated under different environmental conditions. The *retS* mutant generated significantly higher current (45-fold) than the WT under anaerobic conditions. Anaerobic current generation by the WT was 28-fold higher with extraneously supplied lactones (a QS-signaling molecule). Compared to anaerobic conditions, the WT with some oxygen (microaerobic conditions) exhibited enhanced phenazine production (39-fold) and current levels (48-fold). Iron-rich medium and microaerobic conditions had a negative impact on current generation by *retS*. All these results were directly linked to QS activity in *P. aeruginosa*, thus, demonstrating the importance of this bacterial communication system for current generation in BESs. We also show that BESs represent a new tool for real-time investigation of phenazine-related QS activity.

1. Introduction

Bioelectrochemical systems (BESs) are gaining importance as innovative biotechnological devices for the renewable generation of electricity from wastewater in microbial fuel cells (MFCs), generation of chemical products in microbial electrolysis cells, and sequestration of CO_2. In previous studies, *Pseudomonas* sp. in the anodic microbial community of an MFC was related to current generation [1,2]. Phenazines produced by *Pseudomonas aeruginosa* (pyocyanin, 1-hydroxyphenazine, phenazine-1-carboxamide, and phenazine-1-carboxylate) act as redox-shuttles to facilitate respiration of *P. aeruginosa* with the electrode [2]. It has been recently shown that these endogeneous phenazines are responsible for survival of *P. aeruginosa* PA14 under anaerobic conditions [3].

Besides its importance in BESs, *P. aeruginosa* is also a model organism for understanding quorum sensing (QS). QS is the bacterial mode of communication via secreted signaling factors [4]. The core components of the QS system in *P. aeruginosa* are the *las* and *rhl* systems, respectively, consisting of transcriptional regulatory proteins (LasR and RhlR) and autoinducer synthases (LasI and RhlI) (Fig. 1). LasI controls the production of 3-oxo-dodecanoyl homoserine lactone (3O-C12-HSL) and RhlI controls the production of N-butyryl homoserine lactone (C4-HSL), which initiate the QS cascade (Fig. 1) [5]. In addition to controlling *rhlI*, the transcriptional regulator LasR also positively regulates the *pqsABCDE* operon, which generates the *Pseudomonas* quinolone signal (PQS), a third QS signal for *P. aeruginosa* (Fig. 1). The *pqs* operon in turn controls the *phzABCDEFG* operon, which is required for metabolizing chorismate to phenazine-1-carboxylate. The genes *phzS*, *phzM*, and *phzH* in turn metabolize phenazine-1-carboxylate to 1-hydroxyphenazine, pyocyanin, and phenazine-1-carboxamide, respectively [6]. QS in *P. aeruginosa* is also controlled by several two-component regulatory systems, such as GacS/GacA [7] (Fig. 1). GacS/GacA is negatively regulated by RetS and in the absence of RetS (*retS* mutant) a GacS/GacA signaling cascade results in the activation of the *rhl* system (we, therefore, hypothesize an upregulation of QS – see discussion).

We screened seven mutants and the wildtype (WT) strain of *P. aeruginosa* PA14 for their electrochemical behavior. Six of these mutants have transposon insertions in genes involved in: GacS/GacA regulation (*retS*); in type IV pili and flagellum formation (*pilB* and *fliC*, respectively); and in phenazine production (*phzS*, *phzM*, and *phzH*). The seventh mutant (Δ*phz*) lacks both gene operons *phzA1-G1* and *phzA2-G2*, which are required for phenazine synthesis [8]. Here, we investigated the protein-regulatory mutant *retS*

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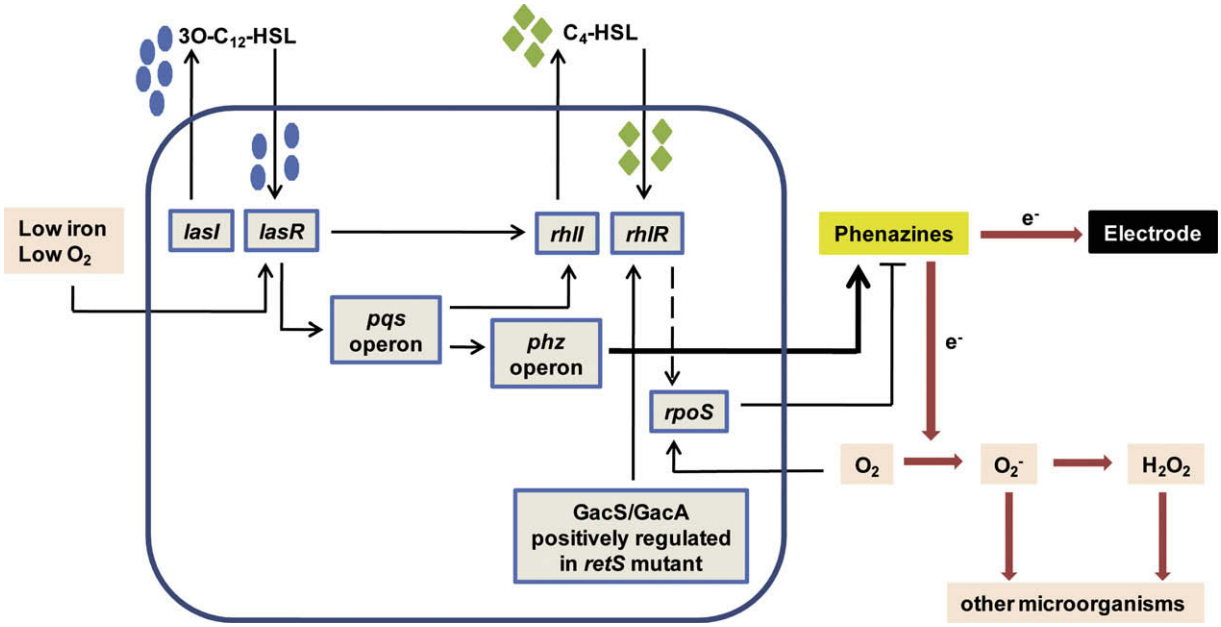

Fig. 1. Illustration of the interaction between the quorum-sensing cascade of *P. aeruginosa* PA14 with environmental factors, including the electrode.

(i.e., *retS*::TN) in further detail because of its superior performance compared to the WT, and we discovered a direct link between electricity generation of *P. aeruginosa* PA14 and QS.

2. Materials and methods

2.1. Bacterial strains and medium

P. aeruginosa PA14 wildtype and all mutants except Δ*phz* were obtained from the PA14 Transposon Insertion Mutant Library [9]. The Δ*phz* mutant was acquired from Dianne Newman (Department of Biology, MIT, Cambridge, MA). Experiments were performed in minimal AB medium [10] (30 mM glucose) at 37 °C.

2.2. Electrochemical experiments

The electrochemical cells consisted of a glass vessel with a tight rubber stopper and a three-electrode setup: working electrode (anode) – 12 cm^2 carbon paper (P50, AvCarb, www.fuelcellstore.com); counter electrode – graphite rod (Poco graphite, Decatur, TX); and reference electrode – Ag/AgCl sat. KCl (all potentials given vs. this reference). The vessel was autoclaved, filled with sterile anoxic medium (150 mL), assembled in an anaerobic hood (Coy Labs, Grass Lake, MI), and sealed. For anaerobic experiments, the headspace was maintained under sterile nitrogen gas. For microaerobic experiments, oxygen was allowed to diffuse through a 0.2-μm filter into the headspace. The electrochemical measurements (Bio-Logic VSP; Bio-Logic USA, Knoxville, TN) were structured in a repetitive loop, starting with cyclic voltammetry from −0.5 V to +0.5 V @ 2 mV s^{-1} and followed by chronoamperometry for 24 h at 0.3 V for up to 21 days. After 24 h of measurement in blank medium, the cell was inoculated with 100 μL of an overnight culture of *P. aeruginosa* in Luria-Broth (LB) medium. All experiments except the screening of mutants were performed in triplicate. 3O-C_{12}-HSL (Cayman Chemical Company, Ann Arbor, MI) was dissolved in dimethyl sulfoxide prior to addition to the medium. Iron was supplied as ferric chloride.

2.3. Phenazine analysis

Pyocyanin (Cayman Chemical Company, Ann Arbor, MI) and 1-hydroxyphenazine (Chemos GmbH, Regenstauf, Germany) were extracted, detected, and quantified with an LC–MS (Thermo Scientific, Waltham, MA) with a protocol adapted from Dekimpe and Deziel [11] with an additional selective reaction monitoring (SRM) to enhance sensitivity using external standard calibration curves. The concentrations of pyocyanin and 1-hydroxyphenazine were added and reported as phenazine concentration.

2.4. Data analysis

Statistical analysis (pairwise ANOVA comparisons) was performed using SYSTAT 12 (Systat Software Inc., Chicago, IL).

3. Results and discussion

Seven mutants were compared to the WT with respect to their electrochemical behavior. The ratio of mean maximum current generated by the mutant to that generated by the WT was used as a performance index (PI). The Δ*phz* and *phzM* mutants had a PI of less than 1 (0.8 and 0.4, respectively), thus, performing worse than the WT. The *phzS*, *fliC*, *pilB*, *phzH*, and *retS* mutants exhibited a PI greater than 1, thus, performing better than the WT (1.1, 3.1, 4.8, 4.9, and 45, respectively).

The GacS/GacA protein-regulatory mutant *retS* (*retS*::TN) was investigated in further detail because of its superior PI value. Under anaerobic BES conditions, the *retS* mutant produced a higher current than the WT (6.08 μA cm^{-2} vs. 0.136 μA cm^{-2}; Fig. 2a). Correlating with this higher current, we found higher phenazine production by the *retS* mutant (8.94 μg mL^{-1} vs. 0.32 μg mL^{-1}; Fig. 2b), while we did not find significant differences in biofilm morphology – a single layer of bacterial cells was found on the electrodes (SEM; data not shown). The lack of regulation of the GacS/GacA system in the absence of RetS explains the higher phenazine production and current generation by the *retS* mutant. The absence of RetS results in increased autophosphorylation of GacS [12], which subsequently increases the transcription of the small RNAs RsmY and RsmZ. These small RNAs sequester the mRNA-

binding protein RsmA. This protein (when not sequestered in WT) has a negative effect on the core QS *rhl* system. Therefore, the sequestered RsmA in the *retS* mutant allows for increased phenazine synthesis [7], resulting in a higher current generation.

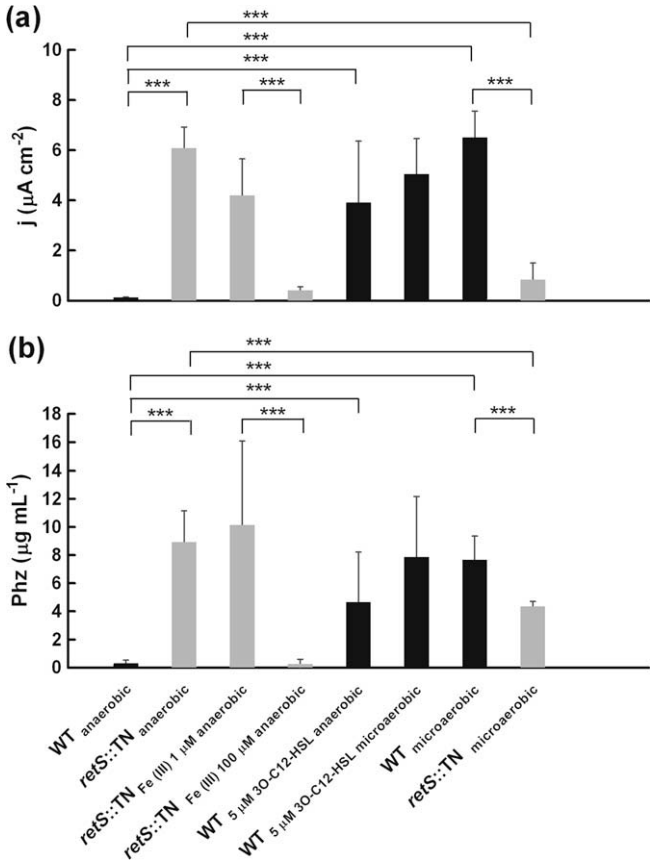

Fig. 2. (a) Mean maximum current density in μA cm^{-2} by the WT and *retS* mutant (*retS*::TN) under different experimental conditions. (b) Mean phenazine concentration (sum of pyocyanin and 1-hydroxyphenazine) in μg mL^{-1} by the WT and *retS*::TN under different experimental conditions (pairs with p-value < 0.05 are marked ***).

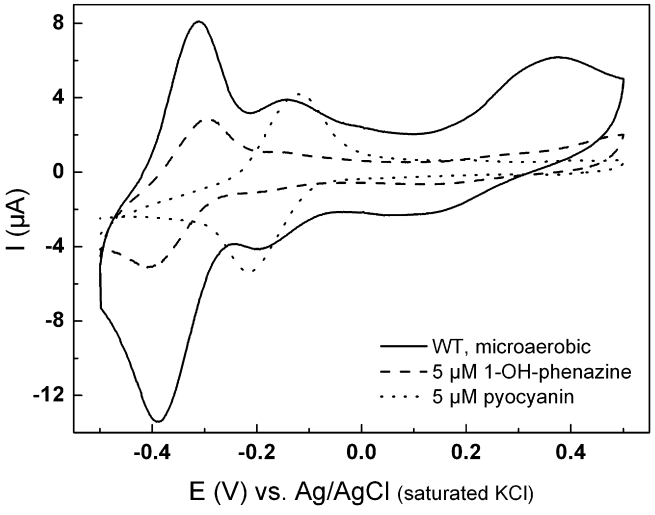

Fig. 3. Cyclic voltammograms of WT under microaerobic conditions (solid line), 5 μM 1-hydroxyphenazine (dashed line), and 5 μM pyocyanin (dotted line) at a pH of 6.5 in AB medium at a scan rate of 2 mV s^{-1}.

Cyclic voltammograms indicated redox couples for two phenazines: pyocyanin ($E_{½}$ = +29 mV vs. NHE) and 1-hydroxyphenazine ($E_{½}$ = −153 mV vs. NHE) (Fig. 3). These midpeak potentials at pH = 6.5 in AB medium agree well with those reported by Wang and Newman [13]. This prevalence of pyocyanin and 1-hydroxyphenazine was confirmed with LC–MS analysis in which these two phenazines represented the dominating mass fragments (LC peaks of the other phenazines [phenazine-1-carboxamide and phenazine-1-carboxylate] were found in some samples just above the noise signal [data not shown]). This is in agreement with our CV data (Fig. 3) because the redox peaks from phenazine-1-carboxamide ($E_{½}$ = −110 mV vs. NHE) and phenazine-1-carboxylate ($E_{½}$ = −148 mV vs. NHE) at pH = 6.5 were masked by the strong signal of 1-hydroxyphenazine ($E_{½}$ estimated from [13]). While the electrochemical analyses (CV and CA data) were recorded in real time, the phenazine production was measured after a laborious extraction procedure and LC–MS analysis. Thus, BESs represent a new tool for real-time investigation of phenazine-related QS activity because of the direct link between current generation and phenazine production (Fig. 2).

To further study the relationship between QS and current generation, experiments were performed to downregulate and upregulate phenazine production in the *retS* mutant and in WT, respectively, by introducing suitable environmental factors (i.e., iron-rich medium and 3O-C12-HSL, respectively). Previous researchers found that iron deficiency results in enhanced production of extracellular virulence factors (i.e., phenazines) [14] because the expression of *lasR* is enhanced by iron limitation (Fig. 1) [5]. Therefore, to downregulate phenazine production under anaerobic conditions we increased the iron concentration in *retS*-mutant experiments (from 1 μM to 100 μM $FeCl_3$). As predicted, the current and phenazine levels with 100-μM iron were significantly lower than with 1-μM iron (Fig. 2). To upregulate phenazine production in WT experiments under anaerobic conditions, we added 3O-C12-HSL, which is an initial inducer of QS in *P. aeruginosa* [8]. As predicted, the WT showed increasing current generation and phenazine production with exogeneous 3O-C12-HSL (Fig. 2).

We performed our experiments under anaerobic conditions that are common in BESs, while most QS studies are performed with oxygen. To understand the effect of oxygen on phenazine production, we performed experiments under microaerobic conditions. Current and phenazine levels for the WT under microaerobic conditions were significantly higher than under anaerobic conditions (Fig. 2), which can be attributed to a faster growth rate of *P. aeruginosa* with O_2 [15]. We also performed WT experiments under microaerobic conditions with and without exogenous addition of 3O-C12-HSL, but no significant difference was observed (Fig. 2). Most likely, the enhanced growth under microaerobic conditions overshadowed the effect of 3O-C12-HSL.

The enhanced electrochemical activity of WT under microaerobic vs. anaerobic conditions is an important finding for BES research. It has already been reported that oxygen enhances the electrochemical activity of *Shewanella oneidensis* MR-I [16,17]. The presence of oxygen is also responsible for the antibiotic activity of the phenazine pyocyanin, which is attributed to its ability to reduce oxygen to superoxide (O_2^-), leading to the formation of hydrogen peroxide. Both these oxidants are toxic to most microorganisms (Fig. 1) [18]. *P. aeruginosa* overcomes this toxicity by expressing elevated levels of the enzyme superoxide dismutase [18]. In the anodic microbial ecology of a mixed-culture BES with oxygen intrusion, this may have broad implications because phenazines introduce a pressure on microbial survival and selection. Therefore, phenazines will have a dual role as a redox-shuttle and as an antibiotic in BESs under microaerobic conditions.

Conversely, the *retS* mutant exhibited significantly lower current and phenazine levels under microaerobic conditions com-

pared to anaerobic conditions (Fig. 2). Oxidative stress controls phenazine production in *P. aeruginosa* via the stress factor RpoS (Fig. 1), which also protects the bacterium against various other stresses [18]. Suh et al. further verified this with a *P. aeruginosa* mutant with low *rpoS* expression; which, accordingly, produced elevated concentrations of phenazines compared to the WT in the presence of oxygen [19]. The negative feedback effect of oxygen on phenazine production via RpoS could explain why we observed a significantly lower current and phenazine level for the *retS* mutant than WT under microaerobic conditions (Fig. 2). We hypothesize that RpoS is more dominant in the *retS* mutant due to the positive reinforcement of the GacS/GacA system, which can be indirectly linked to the production of RpoS (transcription regulation via the *rhl* system) [20]. However, further studies are necessary to conclude that the *retS* mutant is more susceptible to oxidative stress than the WT.

4. Conclusions

Here, we showed that QS and current generation in BESs for *P. aeruginosa* are closely linked via phenazine production. The presence of environmental factors positively or negatively affected certain parts of QS. Subsequently, this resulted in the modulation of phenazine production, thereby, controlling the current generation by this microorganism. *P. aeruginosa* is an important organism in the anodic community of BESs, [2] and it can influence community selection via the antibiotic action of pyocyanin, especially in the presence of oxygen. Through the real-time electrochemical detection of changes in the phenazine levels, BESs represent a new laboratory tool to study QS-related responses in *P. aeruginosa*.

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