Showcasing research from Zhang et al at National Institute for Materials Science, Japan and Changchun Institute of Applied Chemistry, China.

Title: Non-covalent doping of graphitic carbon nitride polymer with graphene: controlled electronic structure and enhanced optoelectronic conversion

Not merely interesting in itself, graphene was used as an interesting dopant for another semiconductor. After intercalation with a few graphene layers via π–π stacking interaction, the band-structure of graphitic carbon nitride was well modulated between more "n-type" and more "p-type".

Metabolite-based mutualism between *Pseudomonas aeruginosa* PA14 and *Enterobacter aerogenes* enhances current generation in bioelectrochemical systems†

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Understanding the ecological relationships of the microbiota in bioelectrochemical systems (BESs) is necessary to gain deeper insight into their performance. Here, we show that the fermentation product 2,3-butanediol stimulates mutually beneficial interactions between *Pseudomonas aeruginosa* PA14 and *Enterobacter aerogenes* in a BES with glucose as the initial substrate under microaerobic conditions. The experiments were conducted in potentiostatically poised 3-electrode reactors. Under these conditions: (i) the current density by a co-culture of *P. aeruginosa* and *E. aerogenes* increased at least 14-fold compared to the current density by either of these two bacteria alone; and (ii) *E. aerogenes* fermented glucose principally to 2,3-butanediol, which was subsequently consumed by *P. aeruginosa*. To determine the benefits to each microorganism in this symbiosis, we conducted experiments with pure cultures. The current production by a pure culture of *P. aeruginosa* with 2,3-butanediol was increased 2-fold compared with glucose as the carbon source. This was due to enhanced phenazine production by *P. aeruginosa*. Further, pyocyanin comprised the majority (92%) of the phenazines produced by *P. aeruginosa* with 2,3-butanediol, but only 29% with glucose. The current production by a pure culture of *E. aerogenes* increased ~19-fold when the growth medium was supplemented with 35 μg ml⁻¹ of pyocyanin as the electron mediator. We also observed that *E. aerogenes* generated maximum current densities with pyocyanin compared to the other three phenazines, indicating that *E. aerogenes* respires most effectively with pyocyanin—the phenazine which production is stimulated by this microbe’s product (2,3-butanediol). Concomitantly, a decrease in fermentation products and enhanced growth with increasing concentrations of pyocyanin implies a shift towards electrode-based respiration by

Broader context

Bioelectrochemical systems (BESs) have multiple applications, including electric current and product generation and biosensing. Microbially-catalyzed reactions are at the heart of all these devices, and therefore the microbial ecology at electrodes is of fundamental interest to increase the electric current densities. The microbiota of BES anodes broadly consists of fermenters and electrode-respiring bacteria (ERB). Despite their ubiquitous presence in mixed-culture BESs, not much attention has been paid to the ecological importance of fermenters. Here, we demonstrate that a fermenter (*Enterobacter aerogenes*) and an ERB (*Pseudomonas aeruginosa*) work in tandem via fermentation products to increase the current output in BESs. We showed a mutually-beneficial relationship: *P. aeruginosa* profits by producing more of a specific phenazine—the redox-shuttle that electronically links this organism to the anode—and *E. aerogenes* respires with this phenazine, resulting in enhanced growth. This is the first demonstration of a fermenter transforming into an ERB by virtue of ecological interactions in BESs. Further, as these two microorganisms are frequently detected in areas with natural redox gradients, it is likely that this synergism evolved in nature. Therefore, our findings have far-reaching implications, for example, in carbon cycling in soil sediments and corrosion prevention.
**E. aerogenes** rather than fermentation. Therefore, the synergism in current generation by the co-culture can be attributed to the combination of enhanced pyocyanin production by *P. aeruginosa* with 2,3-butandiol and the ability of *E. aerogenes* to efficiently respire. This study is the first to demonstrate metabolite based “inter-species communication” in BESs, resulting in enhanced electrochemical activity. It also explains how an inconsequential fermenter can become an important electrode-respiring bacterium within an ecological network at the anode.

**Introduction**

Bioelectrochemical systems (BESs) are gaining importance to generate electricity and valuable products (*e.g.* H₂, CH₄, H₂O₂) from wastewater,⁴ to desalinate sea water,⁴ to sequester CO₂,⁵–⁷ and to use as biosensors and biocomputing devices.⁸,⁹ Typically, BESs consist of an anode (where bacteria catalyze the oxidation of wastewater/acetate), and a cathode (where O₂ is reduced or products are generated). Bacteria can transfer electrons to the anode of a BES via two mechanisms: (i) direct electron transfer—the microorganism establishes physical contact with the electrode to transfer electrons through outer membrane cytochromes, and (ii) mediated electron transfer—the microorganism produces redox-active compounds, which are oxidized at the anode and reduced by the bacteria (*i.e.* electron mediators).¹⁰ As a first step towards better understanding of the inter-species interactions at the BES anode, genome-enabled tools, such as 16S rRNA gene sequencing surveys, have been applied to characterize the diverse anodic community.¹¹–¹⁴ However, the various interactions between community members have been studied mainly with respect to syntrophy wherein metabolites produced by one species are the substrates for other species.¹⁵–¹⁷ While undefined mixed culture-based studies with BESs are suitable for environmental applications, defined mixed culture-based studies are necessary to better understand the complex ecological networks at the BES anode.

For BES research, *P. aeruginosa* has been a model organism for mediated electron transfer. *P. aeruginosa* produces four phenazines, which are electrophysiologically active compounds (pyocyanin [PYO], 1-hydroxy phenazine [1-OH-PHZ], phenazine-1-carboxylic acid [PCA], and phenazine-1-carboxamide [PCN]).¹⁸–²⁰ *P. aeruginosa* is also a model organism for studying: (i) quorum sensing—the bacterial mode of communication by secreted signaling factors; and (ii) biofilm formation.²¹ Previously, we have established that quorum sensing controls the electrochemical activity of this microorganism by regulating phenazine production.²² Lopes et al. (2011)²³ recently established that *P. aeruginosa* in biofilms could exert a selective pressure on the microbial community via its metabolites, but the authors were unable to determine the specific signals/metabolites. The authors found that *Escherichia coli* grown with the supernatant of *P. aeruginosa* exhibited lower biomass and respiratory activity.²⁴ Owing to the “social” nature of *P. aeruginosa*, multiple co-culture studies involving *P. aeruginosa* in association with fermenters in BESs have been reported in the literature. Pham et al. (2008)²⁵ demonstrated that Gram-positive *Brevibacillus* sp. could use the phenazines produced by *P. aeruginosa* for electron transfer to the electrode. Further, the rharnmolipids (a secondary metabolite) produced by *P. aeruginosa* increased the availability of phenazines for *Brevibacillus* sp., thereby asserting the ecological role of this microorganism in BESs. The synergism (two agents working together to achieve a result that cannot be attained independently) between *P. aeruginosa* and *Enterobacter faecium* in a BES was demonstrated although the authors did not investigate the mechanisms of the symbiosis.²⁶

*Enterobacter aerogenes* is being investigated for bioenergy applications due to its hydrogen production capability.²⁷,²⁸ *Enterobacter* sp. has also captured the attention of BES researchers.²⁷,²⁹ For instance, previous studies have found that hydrogen is used as a mediator by *E. aerogenes* for current generation.²⁷,²⁸ Further, Zhuang et al. (2010)²⁹ claim that *E. aerogenes* evolves over time to directly transfer electrons to the electrode via unspecified mechanisms, although the given evidence (cyclic voltammograms of the biofilm) was not substantial. *Enterobacter cloacae* was shown to degrade cellulose and generate an electric current without the addition of any exogeneous mediators.²⁸

*E. aerogenes* is a facultative aerobe capable of aerobic respiration and anaerobic fermentation while *P. aeruginosa* is an obligate respirer.³⁰ *Pseudomonas* sp. and *Enterobacter* sp. were found at the anode of a microbial fuel cell treating nitrobenzene.³¹ The co-existence of *P. aeruginosa* and *E. aerogenes* in a marine sediment has been well documented.³² The authors found these two microorganisms to exist as a synergistic culture in environmental isolates in Hawaii. A blue colored pigment (later identified as pyocyanin) was produced only when both organisms were present with *P. aeruginosa* as the producer and *Enterobacter* as the inducer. The authors found that metabolite exchange between the two organisms was necessary for the induction and synergism, but the mechanism was not elucidated. Pyocyanin also exhibits anti-microbial activity by generating superoxide radicals and/or hydrogen peroxide or nitric oxide radicals, which in turn can cause oxidative stress and damage to DNA.³³ The existence of synergism between these two species in the natural environment indicates that pyocyanin is not toxic to *E. aerogenes*. These two species were also present in a microbial community responsible for causing corrosion in water distribution systems, thus, highlighting the presence of these two microorganisms in areas with oxidation-reduction gradients.³⁴

When we studied the bacterial community composition of a biofilm at the anode of a BES after a three-month operating period, we found that the relative presence of sequences closely related to *P. aeruginosa* PA14 (Fig. 1) was similar between the inoculum and the anode biofilm. However, sequences related to *E. aerogenes* were most abundant at the anode (Fig. 1), while they had been below detection limit in the inoculum (i.e. anaerobic granules). Under oxygen-limited conditions, such as found in our BES, *E. aerogenes* exhibits fermentation and inconsequential electrochemical activity. This poses the question: why did *E. aerogenes* emerge as a dominant member of the BES community? We hypothesized that *P. aeruginosa* and *E. aerogenes* exhibited mutualism, which is a form of symbiosis in which
Fig. 1 Maximum-likelihood phylogenetic tree (FastTree) of OTUs from this study belonging to the phylum Proteobacteria, with most similar Greengenes sequences included. Abundance of each OTU is indicated in parentheses (N sequences); taxonomy following reference sequences is the deepest taxonomic assignment available in the Greengenes taxonomy ("s = species, g = genus, f = family"). Greek letters indicate the class of the proteobacteria. Percent support values are listed for higher-order nodes. Highlighted regions include OTUs similar to *Pseudomonas* sp. and *Enterobacter* sp..
both agents benefit from the relationship. Our experiments with pure and defined cultures, substantiated this hypothesis and, in addition, also ascertained that the synergism between *P. aeruginosa* and *E. aerogenes* was regulated by the fermentation product—2,3-butanediol.

**Materials and methods**

**Bacterial strains and medium**

We obtained *P. aeruginosa* PA14 wildtype from the PA14 Transposon Insertion Mutant Library and *E. aerogenes* from NRRL (strain B-115). Experiments were performed in minimal AB medium supplemented with 30 mM of one carbon source (2,3-butanediol/glucose/ethanol) at 37 °C.

**16S rRNA gene sequencing**

A 16S rRNA gene survey characterized the anodic mixed culture of an upflow microbial fuel cell (UMFC) operated for three months (see He et al. (2006)²³). Bulk DNA from the inoculum and anode biofilm was extracted using bead beating and phenol: chloroform extraction; 16S rRNA genes were amplified with a 30-cycle PCR protocol using bacterial primers 8F and 1391R. The 20 µl reaction utilized AmpliTaq-Gold (Promega Corp, Madison, WI), 0.4 pmol µl⁻¹ forward and reverse primers, 0.5 mM MgCl₂, 0.2 mM dNTPs, 0.8 mg ml⁻¹ BSA, 5 mg ml⁻¹ aceticamide, and 1 µl of template. Amplicons were gel purified (Qiagen, Valencia, CA), cloned into TOPO TA pCR4.0, and transformed into *E. coli* TOP10 cells (Invitrogen, Carlsbad, CA). Clones were sequenced using vector-specific primers and an additional selective reaction monitoring (SRM) to enhance sensitivity using external standard calibration curves.

**Metabolite and phenazine measurement**

Metabolites were detected and measured with a HPLC (Shimadzu, Columbia, MD), using an Aminex HPX-87H column (BioRad, Hercules, CA). 5 mM sulfuric acid was used as the eluent at a flow rate of 0.6 ml min⁻¹ at 60 °C. Pyocyanin (Cayman Chemical Company, Ann Arbor, MI), 1-hydroxy phenazine (Chemos GmbH, Regenstauf, Germany), phenazine-1-carboxylic acid, and phenazine-1-carboxamid (Princeton Building Blocks, Monmouth Junction, NJ) were extracted and detected and quantified via a LC/MS (Thermo Scientific, Waltham, MA) with a protocol adapted from Dekimpe and Deziel (2009) with an additional selective reaction monitoring (SRM) to enhance sensitivity using external standard calibration curves.

**Results and discussion**

**Community analysis of a UMFC anode biofilm**

The electrochemical cells consisted of a glass reactor with a three-electrode setup: working electrode (anode)—150 cm² carbon cloth (PAN®—PW06, Zoltek Corp., St Louis, MO) mounted to a graphite rod (Poco graphite, Decatur, TX) with carbon cement (CCC Carbon Adhesive, EMS, Hatfield, PA); counter electrode—graphite rod (Poco graphite); and reference electrode—Ag/AgCl sat. KCl (all potentials given vs. this reference). The vessel was autoclaved and filled with sterile medium (350 mL). For anaerobic experiments, the reactors were continuously sparged with N₂ via a 0.22 µm filter. For microaerobic experiments, oxygen was allowed to diffuse through a 0.22 µm filter into the headspace. The electrochemical measurements with a potentiostat (BioLogic VSP; BioLogic 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 chronocoulometry for 24 h at 0.3 V vs. Ag/AgCl sat. KCl for up to 7 days. After a 24 h measurement in blank medium, the reactor was inoculated with 100 µl of an overnight culture of *P. aeruginosa* and/or *E. aerogenes* in Luria–Bertani broth (LB) medium. For abiotic experiments, H₂ was sparged through a diffuser continuously. Triplicates were conducted for each experiment and the average values along with standard deviations were reported throughout the text. The current and pyocyanin concentration vs. time profile for one experiment is shown in the ESI (Fig. S1†).

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the lower complexity of the artificial wastewater solution (sucrose) that was fed to the UMFC biomass compared to the real brewery wastewater that was fed to the granular sludge (inoculum). The ability of bacteria to transfer electrons to the anode in a BES has been shown to be phylogenetically widespread; known electrode-respiring bacteria include species in the phyla Proteobacteria, which increased in our study from 27.2% to 69.5% for the inoculum and the UMFC culture, respectively (Fig. 2).

The enrichment of Proteobacteria in the UMFC was supported by an increased representation of the subgroups Alphaproteobacteria and Gammaproteobacteria, while the subgroup Delta-proteobacteria decreased compared to the inoculum. However, the known electrode-respiring bacterium Geobacter sp. within the Deltaproteobacteria increased in abundance from 1.6% to 3.3% (3 OTUs; 11 UMFC sequences). We identified 53% of the UMFC sequences as Gammaproteobacteria (160 sequences out of 302). P. aeruginosa remained at similar levels between the inoculum and the UMFC (3 OTUs; 18 UMFC sequences). Among the Gammaproteobacterial sequences in the UMFC anode biomass, however, the largest representation (95 sequences) was found for the 3 OTUs that are closely related to E. aerogenes (Fig. 1). This genus had been below the level of detection in our sequencing survey of the inoculum.

**BES experiments with P. aeruginosa and E. aerogenes**

*E. aerogenes* is known to primarily ferment under microaerobic or anaerobic conditions with H2 as a product. H2 can subsequently be electrochemically oxidized with metal catalysts at the anode. However, electrochemical oxidation of H2 is not directly energetically beneficial to *E. aerogenes*. In addition, there is no conclusive proof of direct electron transfer by *E. aerogenes*.22,29 Therefore, the success of *Enterobacter* sp. under the environmental conditions of an UMFC anode was unexpected, and we raised the question whether respiration with the anode in association with *P. aeruginosa* can be an important physiological process of *E. aerogenes*. To investigate this we performed bioelectrochemical experiments in triplicate under microaerobic and anaerobic conditions with: (i) a co-culture of *P. aeruginosa* and *E. aerogenes*; (ii) a pure culture of *P. aeruginosa*; and (iii) a pure culture of *E. aerogenes*. The co-culture of both bacteria generated at least 14-fold higher current densities than the pure cultures under microaerobic conditions (46.53 ± 6.4 μA cm−2 with the co-culture vs. 3.25 ± 0.14 μA cm−2 with *P. aeruginosa* and 2.53 ± 1.3 μA cm−2 with *E. aerogenes*; Fig. 3); thereby conclusively exhibiting synergism between the two microorganisms. Under anaerobic conditions, the co-culture also generated higher current densities than the pure cultures (0.45 ± 0.07 μA cm−2 with the co-culture vs. 0.14 ± 0.02 μA cm−2 with *P. aeruginosa* and 0.2 ± 0.13 μA cm−2 with *E. aerogenes*; Fig. 3). The current densities under microaerobic conditions were two orders of magnitude higher than under anaerobic conditions (Fig. 3). This confirms our previous findings with *P. aeruginosa* that microaerobic conditions are more conducive to electric current generation by this electrode-respiring bacterium.22 Importantly, the long-standing notion that BESs have to be operated under strict anaerobic conditions for optimum performance has been recently questioned.16,50,51

*E. aerogenes* has a higher growth rate than *P. aeruginosa* (doubling time in AB media with glucose for *E. aerogenes* is 16 min vs. 50 min for *P. aeruginosa*). Therefore, it is likely that in BESs, *E. aerogenes* grows faster than *P. aeruginosa* and initially ferments glucose to metabolites that subsequently act as substrates for *P. aeruginosa*. Indeed, the metabolic analysis of the co-culture experiment for both microaerobic and anaerobic conditions over the operating period (Fig. 4) revealed that: (i) under microaerobic conditions, *E. aerogenes* had already depleted glucose by day 1, and *P. aeruginosa* consumed 2,3-butanediol (the major metabolite) during days 3–7; and (ii) under anaerobic conditions glucose consumption by *E. aerogenes* was much slower and lactate and acetate were the main fermentation products. This is in accordance with previous studies asserting the importance of microaerobic conditions for 2,3-butanediol...
fermentation by E. aerogenes. 2,3-Butanediol is a pH-neutral metabolite, thereby, preventing lethal media acidification and also acts as an energy storage mechanism for E. aerogenes under conditions of glucose deprivation.

Because microaerobic conditions stimulated high current densities, all the subsequent experiments were performed under microaerobic conditions.

Effect of 2,3-butanediol on P. aeruginosa

After establishing the synergism between the two species via increased current generation, our objective became to determine the mutually beneficial compound. Fermentation products link the two species in a food chain; thus, it is possible that such a product confers benefits to P. aeruginosa. Therefore, we assessed the electrochemical activity of P. aeruginosa in triplicate under microaerobic conditions with glucose (control), 2,3-butanediol, or ethanol (the latter two are fermentation products of E. aerogenes under microaerobic conditions) as the carbon source. The current densities were increased two-fold with 2,3-butanediol compared to glucose (control) and they were seven times higher with 2,3-butanediol compared to ethanol (5.23 ± 0.19 μA cm⁻² with 2,3-butanediol vs. 3.25 ± 0.14 μA cm⁻² with glucose vs. 0.73 ± 0.07 μA cm⁻² with ethanol; Fig. 5), indicating that 2,3-butanediol was the most beneficial fermentation product for P. aeruginosa. In addition, the coulombic efficiencies were maximum with 2,3-butanediol (12.95 ± 0.39% with 2,3-butanediol vs. 6.39 ± 0.79% with glucose vs. 0.68 ± 0.48% with ethanol; Fig. 5). The phenazines produced by P. aeruginosa possess different colors, for example, pyocyanin is blue, and 1-hydroxy phenazine is yellow under the environmental conditions of our BESs. Interestingly, the reactors with P. aeruginosa grown on 2,3-butanediol turned green in color whereas those grown on glucose turned yellow in color (Fig. 6a). We have previously shown that phenazine concentrations correlate well with current densities in P. aeruginosa. Because of the higher current generation and varying reactor colors, we hypothesized that 2,3-butanediol stimulated both higher phenazine production and a change in the phenazine spectrum of P. aeruginosa, respectively. Indeed, the phenazine analysis indicated an increase in phenazine concentrations with 2,3-butanediol compared to glucose (sum of all phenazines: 20.8 ± 4.38 μg ml⁻¹ with 2,3-butanediol vs. 6.4 ± 2.1 μg ml⁻¹ with glucose; Fig. 6b). In addition, 92% of the phenazines produced with 2,3-butanediol were pyocyanin, resulting in the green color (mixture of the blue color of pyocyanin and the yellow colors of 1-hydroxy phenazine and the other two phenazines).

With glucose as the carbon source, 65% of the phenazines were 1-hydroxy phenazine with much lower concentrations of pyocyanin, thus, resulting in the yellow color (Fig. 6b).

Under anaerobic conditions, the relatively higher phenazine concentrations with the co-culture were also related to 2,3-butanediol, but were 3 orders of magnitude lower than...
microaerobic conditions (0.03 ± 0.014 μg ml⁻¹ under anaerobic conditions vs. 37.25 ± 4.67 μg ml⁻¹ under microaerobic conditions). The concentration of 2,3-butanediol in the co-culture under anaerobic conditions was 1/13th compared to microaerobic conditions (1.06 ± 1.29 mM under anaerobic conditions vs. 13.45 ± 1.32 mM under microaerobic conditions).

The concentration of phenazines produced by *P. aeruginosa* in the co-culture with *E. aerogenes* (37.28 ± 4.67 μg ml⁻¹) was higher than produced by a pure culture of *P. aeruginosa* with 2,3-butanediol (20.8 ± 4.38 μg ml⁻¹). Although we cannot explain this difference definitively, it is possible that: (i) a higher cell density of *P. aeruginosa* was achieved in the co-culture compared to the pure culture due to the availability of multiple substrates, resulting in a higher phenazine concentration; and (ii) *P. aeruginosa* had to compete with *E. aerogenes* for phenazines as electron acceptors in the co-culture, resulting in enhanced phenazine production.

**Benefit for *E. aerogenes* in a co-culture with *P. aeruginosa***

*E. aerogenes* exhibits respiration with O₂ as the electron acceptor under completely aerobic conditions and fermentation under microaerobic/anaerobic conditions. *E. aerogenes* is not known to use nitrate/sulfate or other electron acceptors for anaerobic respiration. Since respiration has often a higher energy gain than fermentation (38 ATPs with O₂ as the electron acceptor for respiration vs. a maximum of 4 ATPs for acetogenesis), respiration is more energetically beneficial to *E. aerogenes*. Therefore, we investigated if *E. aerogenes* could use the phenazines in their oxidized state as an electron acceptor for respiration and we determined the most effective phenazine.

Electrochemical experiments were conducted in triplicate with *E. aerogenes* in AB media with glucose in the presence of each of the four phenazines under microaerobic conditions. *E. aerogenes* generated the maximum current densities with pyocyanin (the major phenazine produced by *P. aeruginosa* with 2,3-butanediol as the carbon source) as the electron acceptor under the applied experimental conditions (18.52 ± 6.62 μA cm⁻² with 5 μg ml⁻¹ PYO, 2.67 ± 0.46 μA cm⁻² with 5 μg ml⁻¹ 1-OH-PHZ, 8.42 ± 1.52 μA cm⁻² with 5 μg ml⁻¹ PCA, and 1.78 ± 0.38 μA cm⁻² with 5 μg ml⁻¹ PCN; Fig. 7). When the growth medium was supplemented with 35 μg ml⁻¹ pyocyanin (the concentration encountered by *E. aerogenes* in the co-culture; Fig. 6b), the current density (49.11 ± 14 μA cm⁻²) increased 19-fold compared to the absence of phenazines (2.53 ± 1.3 μA cm⁻²), indicating that *E. aerogenes* was using the pyocyanin produced by *P. aeruginosa* for respiration with the electrode. This switch to respiration was also supported by a growth benefit for *E. aerogenes* with increasing pyocyanin concentrations as seen from the optical density at 600 nm (OD₆₀₀ with 0 μg ml⁻¹ pyocyanin = 0.62 ± 0.03 vs. OD₆₀₀ with 35 μg ml⁻¹ pyocyanin = 1.83 ± 0.09), verifying that *E. aerogenes* is not susceptible to the antibiotic effects of pyocyanin. Further, this effect of enhanced growth of *E. aerogenes* due to respiration via pyocyanin might also be responsible for the abundance of this organism in our UMFC (95 sequences; Fig. 1). If fermentation products, such as H₂, were responsible for reducing the phenazines then this would have: (i) theoretically contributed to current generation; and (ii) eliminated the energetic gain for *E. aerogenes*. To eliminate this possibility, we conducted cyclic voltammetry and chronoamperometry in AB medium sparged with H₂ in the presence and absence of pyocyanin. Similar experiments were also performed with the
P. aeruginosa produced by in energy gain would result in a high growth rate that may implications on BES microbiota sequences; Fig. 1).

soil horizons, sediment-water interfaces, and marine sediments. In nature, they exist in BESs, these gradients are generated from the anode surface into oxidation gradients are analogous to anode environments. In natural ecosystems characterized by the presence of reduction– oxidation, and a 1323% increase in the number of coulombs transferred as electric current concomitant with a 3-fold increase in growth (between 0 μg ml⁻¹ and 35 μg ml⁻¹ pyocyanin). This indicates an increase in respiration and decrease in fermentation of E. aerogenes in a BES with pyocyanin, resulting in harnessing more energy by respiration with the electrode via the pyocyanin produced by P. aeruginosa than by fermentation. Such advantage in energy gain would result in a high growth rate that may explain the abundance of E. aerogenes in the UMFC (95 sequences; Fig. 1).

Implications on BES microbiota

Multiple studies have characterized the anode microbiota and discovered unknown electrode-respiring bacteria. This may suggest a unique microbial community in BESs, however, natural ecosystems characterized by the presence of reduction– oxidation gradients are analogous to anode environments. In BESs, these gradients are generated from the anode surface into the bulk reactor liquid, sludge, or biofilm. In nature, they exist in soil horizons, sediment-water interfaces, and marine sediments. For example, the inverse correlation between oxygen concentration and depth in marine sediments supports a wide range of energy yielding reactions, including both anaerobic and aerobic microbial processes. Benthic microbial fuel cells harvest energy from this natural gradient, thereby already challenging the notion of the “artificial” character of BES ecology. In regards to P. aeruginosa, the use of phenazines for long-range electron transfer to reduce Fe(III) in soil is already well established, and thus the electrochemical function of these compounds is not unique to BESs. P. aeruginosa and E. aerogenes have also been found to co-exist in: (i) ocean floor sediments; (ii) soil microbiota; and (iii) microbiota causing corrosion in water distribution systems. It is, therefore, likely that the metabolite-based mutualism, which we describe here, evolved in nature. This also implies that our findings have implications beyond BES research to, for example, better understand carbon cycles in natural environments or corrosion prevention in engineered systems.

The functional anode in our UMFC, then, would have selected for this mutualistic partnership. This resulted in the enrichment of E. aerogenes at the anode compared to the inoculum, especially since redox gradients and E. aerogenes populations were not important in the environmental conditions of the inoculum. In part, this was possible due to microaerobic conditions at the UMFC anode because of dissolved oxygen in the substrate solution and oxygen diffusion from the catholyte to the anolyte through the membrane. The dual nature of phenazines as redox shuttles and antibiotics likely applied an additional selection pressure for the enrichment of E. aerogenes, because they are capable of tolerating this stress.

The respiration of E. aerogenes with pyocyanin under microaerobic conditions resulted in a higher growth rate (Fig. 8), which would explain the dominant presence of E. aerogenes at the anode of our UMFC. We observed in the three-electrode BESs of this study that the bulk of phenazine-based respiration was performed by E. aerogenes rather than P. aeruginosa; under analogous conditions compared to the co-culture (46.53 μA cm⁻²), E. aerogenes obtained a maximum current density of 49.11 μA cm⁻² with 35 μg ml⁻¹ pyocyanin while this was only 5.23 μA cm⁻² for P. aeruginosa with 30 mM 2,3-butanediol. Therefore, we predict that P. aeruginosa is more important within our described ecological network to support the production of pyocyanin rather than to respire. The resulting abundance of E. aerogenes (especially compared to P. aeruginosa) in the UMFC biofilm would be explained by this phenomenon, but of course we cannot be sure as E aerogenes is also able to ferment sugars without a symbiotic partner. In fact, a complete shift from fermentation to respiration for E. aerogenes would prevent the formation of 2,3-butanediol and nullify the symbiosis. Indeed, even at a pyocyanin concentration of 35 μg ml⁻¹, 10% of the glucose was still fermented to 2,3-butanediol and ethanol. Besides the likely important E. aerogenes respiration that we described here, Geo-bacter sp., which was also enriched within the UMFC anode biofilm compared to the inoculum, respired with the anode with acetate as the electron donor (possibly from P. aeruginosa [Fig. 4]).

Conclusions

Here, we showed that P. aeruginosa and E. aerogenes are interlinked via a fermentation metabolite (i.e. inter-species communication) and that this relationship was not just restricted to syntrophy, but involved metabolite-based mutualism. This translated into synergism with respect to electric current generation in BESs. Both microorganisms benefited from the pH-neutral nature of 2,3-butanediol, thus, preventing lethal
media acidification commonly encountered in fermentation. In addition, *P. aeruginosa* benefited by producing more phenazines (and shifting towards the production of pyocyanin) with 2,3-butanediol as the carbon source compared with glucose, resulting in higher respiration activity. *E. aerogenes* benefited by using pyocyanin as an electron acceptor to respire with the electrode rather than to ferment, resulting in higher cell growth that may explain its abundance in the undefined mixed culture of an anode biofilm.

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**Notes and references**
