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Endogenous Phenazine Antibiotics Promote Anaerobic Survival of *Pseudomonas aeruginosa* via Extracellular Electron Transfer[▽]

Yun Wang,[1,3] Suzanne E. Kern,[1] and Dianne K. Newman[1,2,3]*

Massachusetts Institute of Technology, Biology,[1] Earth, Atmospheric and Planetary Sciences,[2] and Howard Hughes Medical Institute,[3] Cambridge, Massachusetts

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Antibiotics are increasingly recognized as having other, important physiological functions for the cells that produce them. An example of this is the effect that phenazines have on signaling and community development for *Pseudomonas aeruginosa* (L. E. Dietrich, T. K. Teal, A. Price-Whelan, and D. K. Newman, Science 321: 1203–1206, 2008). Here we show that phenazine-facilitated electron transfer to poised-potential electrodes promotes anaerobic survival but not growth of *Pseudomonas aeruginosa* PA14 under conditions of oxidant limitation. Other electron shuttles that are reduced but not made by PA14 do not facilitate survival, suggesting that the survival effect is specific to endogenous phenazines.

Phenazines have long been recognized for their redox properties. While most attention concerning their redox activity has focused on their role in generating reactive oxygen species in the context of infection (13, 14, 19, 22), as early as 1931, Friedheim hypothesized that phenazine reduction might benefit producer cells as an alternative respiratory pigment (12). Several years ago, our group suggested that the context in which this might be most important would be in biofilms, where cell densities are high and access to oxidants is limited (16, 31); consistent with this, we recently showed that mutants unable to produce phenazines are defective in community development (8). While this phenotype is likely due to many factors, including a signaling function for phenazines in later stages of growth (9), given the 1931 hypothesis by Friedheim (12) and our related recent work demonstrating that phenazines control redox homeostasis in *Pseudomonas aeruginosa* (30), we reasoned that phenazines might contribute to the survival of cells experiencing oxidant limitation.

As a first step toward testing this, we investigated the effect of redox-active small molecules on anaerobic survival of *P. aeruginosa* PA14 in stationary-phase planktonic cultures. We justified beginning with planktonic cultures rather than biofilms based on previous studies which have suggested that cells in stationary-phase planktonic culture physiologically resemble cells in established biofilms (15, 35, 38). Moreover, by working with cells in planktonic cultures, we could build on voltammetric methods that had previously been used to determine how metabolism changes in *Escherichia coli* in the presence of the synthetic redox-shuttle ferricyanide (36). Similar voltammetric approaches have also been used to study how phenazines (32, 33) and structurally related flavins (23) mediate power generation by microbial fuel cells.

We assembled bulk electrolysis-based glass bioreactors housed within an O_2- and H_2-free glove box (MBraun) and controlled by a multichannel potentiostat (series G 300; Gamry) outside the glove box. Each bioreactor held a graphite rod working electrode (Alfa Aesar) with an operating surface area of 6 cm^2, a Ag/AgCl reference electrode (RE-5B; BASi) with a constant potential of +207 mV versus that of the normal hydrogen electrode (NHE), and about 100 ml MOPS (morpholinepropanesulfonic acid) culture medium (100 mM MOPS at pH 7.2, 93 mM NH_4Cl, 43 mM NaCl, 2.2 mM KH_2PO_4, 1 mM $MgSO_4$, 5.0 μM $FeCl_3$) (modified from reference 29). The bioreactor was joined by a fritted glass junction to a small side chamber, in which a Pt counter electrode made from Pt mesh (Alfa Aesar) soldered to a copper wire completed the circuit. In order to selectively examine different redox-active small molecules, we used the phenazine-null mutant of PA14 (Δ*phz*) which is deleted in its two phenazine biosynthetic operons (9).

We began by focusing on three endogenous phenazines—pyocyanin (PYO), phenazine-1-carboxylic acid (PCA), and 1-hydroxyphenazine (1-OHPHZ)—that are known to be excreted by PA14 during stationary-phase growth cycle in laboratory cultures (9, 17). We harvested cells from cultures grown aerobically on Luria-Bertani (LB; Fisher Scientific) medium at 37°C and concentrated and resuspended them in anaerobic MOPS medium at 10^9 CFU/ml. These resuspended cells were incubated in bioreactors over a period of 7 days at 30°C. To perform the survival experiments, we incubated dense suspensions (10^9 cells/ml) in the MOPS medium containing 20 mM D-glucose to ensure excess electron donor, added ~90 μM phenazine (PYO, PCA, or 1-OHPHZ), and poised the working electrode at +200 mV versus that of the NHE to make certain it was just high enough to efficiently oxidize bacterially reduced phenazine but not other medium components (e.g., D-glucose). To confirm the poised potential was appropriate to ensure that phenazine was the sole reversible redox-active component, we compared the cyclic voltammetries (CV) of Δ*phz* cultures with or without phenazine at the time point immediately prior to the start of the survival test. Using the CV method described in detail previously (39), we found that Δ*phz* cultures supplemented with phenazine exhibited single anodic (oxidation) and cathodic (reduction) peaks; these peaks were absent when phenazine was not present. This is illustrated in Fig. 1 for PYO.

* Corresponding author. Mailing address: Massachusetts Institute of Technology, Biology and Earth, Atmospheric and Planetary Sciences, Room 68-380, Cambridge, MA 02139. Phone: (617) 324-2770. Fax: (617) 324-3972. E-mail: dkn@mit.edu.

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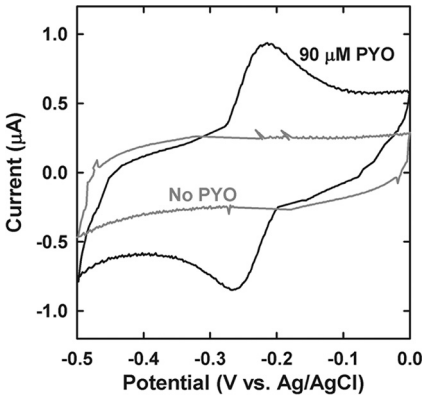

FIG. 1. Representative cyclic voltammetry (CV) of Δ*phz* mutant cultures of *P. aeruginosa* PA14 incubated anaerobically in 100 ml MOPS medium containing 20 mM glucose, supplemented with 90 μM PYO (dark trace) or no PYO (light trace). PYO is the only electrochemically active component with single anodic (oxidation) and cathodic (reduction) peaks characteristic of itself. CV experiments were performed at 100 mV/s, with electrodes consisting of a stationary gold disk working electrode (BASi), an Ag/AgCl reference electrode, and a Pt counter electrode.

For Δ*phz* cultures supplemented with phenazine, we collected supernatants at the beginning and the end of each survival test for high-performance liquid chromatography (HPLC) analyses using a previously developed method (9). In both cases, HPLC samples yielded the same single phenazine peak with characteristic peak size, implying that degradation did not occur in these experiments. Throughout the incubation period, we continuously recorded the anodic (oxidation) current as well as the charge transferred due to the oxidation of an electrochemically active component(s) at the working electrode. Periodically, we sampled to measure viability by means of counting the number of CFU on LB agar (11).

We observed that the Δ*phz* mutant maintained a constant viable cell number at the original 10^9 CFU/ml over 7 days, characteristic of survival but not growth (Fig. 2). In contrast, when we incubated the Δ*phz* mutant in the bioreactors without adding phenazine or applying a potential or both, the cells sustained their viability up to day 3 and then dropped logarithmically down to 0.1 to 1% of the original 10^9 CFU/ml by day 7 (Fig. 2). Our electrochemical observations were in agreement with the number of CFU results. Without phenazine supplementation, we observed a constant anodic current in the range of 5 to 10 μA with the poised potential, most likely reflecting a background current due to slow oxidation of medium components, which was not able to help Δ*phz* survive over 7 days. In the presence of phenazine, however, the anodic current increased from the background level to 80 ± 10 μA for PYO and PCA and 45 ± 10 μA for 1-OHPHZ within 2 h and stayed at the high current levels with slow decay (less than 20%) throughout the incubation period. The slow current decay was likely due to electrode fouling (23) and/or the accumulation of toxic metabolic by-products in the batch reactors over time. The facile reversibility of redox-active phenazines, which are reduced within the bacterial cell and oxidized outside the cell by the working electrode, led to the high current readings and was key to Δ*phz* survival.

We then estimated the average number of redox cycles (defined as "*a*") for each phenazine molecule, which is known to undergo two-electron oxidation-reduction (39), throughout the 7-day incubation based on the equation adapted from Faraday's law for bulk electrolysis (1), $Q = 2FN = 2F(acv)$, with c as the phenazine concentration (in M), v as reaction volume (in liters), F as Faraday's constant (96,485 C/mole), N (which equals acv) as the amount of phenazine (in moles) involved in the electrolysis, and Q (in coulombs) as the net charge quantity associated with the electrochemical oxidation of reduced phenazine during electrolysis (by subtracting the background charge without phenazine from the total charge passed with phenazine). By recording Q, and knowing c and v, we calculated the number of redox cycles over 7 days for PYO, PCA, and 1-OHPHZ to be 31, 22, and 14, respectively. Moreover, each of the three phenazines showed the color characteristic of its oxidized form during redox cycling rather than that of its reduced form, which was apparent when cycling was prevented by not applying the poised potential (39); this indicated that intracellular phenazine reduction was the rate-limiting step of each redox cycle. In addition, we observed the following correlation between the reaction kinetics and phenazine thermodynamic properties: both the phenazine reduction potential

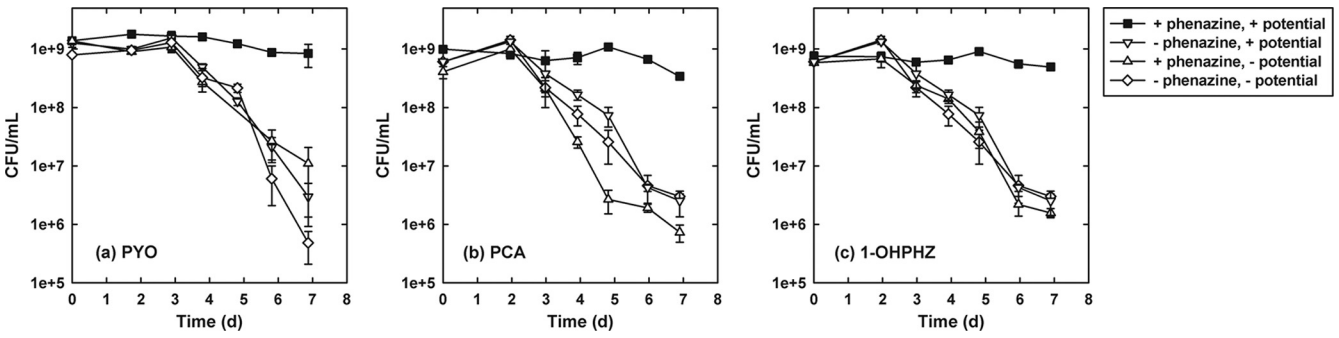

FIG. 2. PYO (a), PCA (b), and 1-OHPHZ (c) function as electron shuttles (■) to promote anaerobic survival of the Δ*phz* mutant of *P. aeruginosa* PA14 when cells are incubated anaerobically in MOPS-buffered medium containing 20 mM D-glucose and ~90 μM phenazine (PYO, PCA, or 1-OHPHZ) and with the graphite rod working electrode poised at +0.2 V versus that of the NHE. Survival was determined by measuring the number of CFU on LB agar plates. The number of CFU of Δ*phz* anaerobic incubations without phenazine (▽), poised potential (△), or both (◇) served as a control. Error bars represent standard deviations from at least triplicate samples in each experimental set. Plots represent results from at least three independent experiments.

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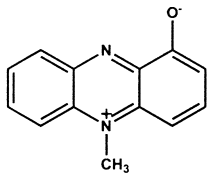
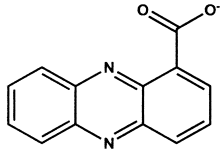
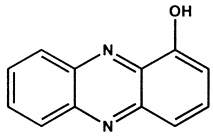
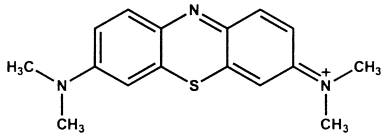
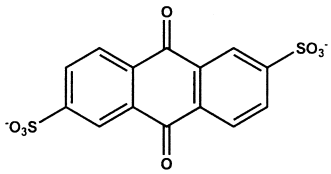
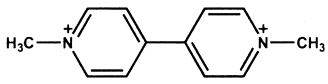
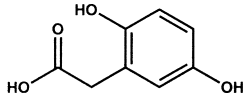
TABLE 1. Properties and results summarized from experiments for testing the roles of endogenous phenazines and other type redox-active compounds in promoting anaerobic survival of *P. aeruginosa*

Chemical name (abbreviation)	Structure[g]	E_0' vs. that of NHE (mV)	No. of redox cycles over 7 days	Support survival	Reduction by PA14
Pyocyanin (PYO)		−40[a]	31	Yes	Yes
Phenazine-1-carboxylate (PCA)		−114[a]	22	Yes	Yes
1-Hydroxyphenazine (1-OHPHZ)		−174[a]	14	Yes	Yes
Methylene blue (MB)		0[b] +11[c]	3	No	Yes
2,6-AQDS		−184[d]	0	No	Yes, but very slowly
Paraquat		−446[e]	0	No	No
Homogentisic acid (HMA)		+306[b]	0	No	—[f]

[a] From reference 39.
[b] E_0' values were measured in aqueous solution at pH 7 in this study.
[c] From reference 10.
[d] From reference 16.
[e] From references 25 and 26.
[f] —, not applicable; present in its reduced form.
[g] The oxidized form is shown for all entries except homogentisic acid, for which the reduced form is shown.

(Table 1) and the intracellular reduction rate decreased in the order PYO > PCA > 1-OHPHZ. In summary, despite different electron-shuttling efficiencies, all three phenazines supported Δ*phz* survival equally well within the testing period by acting as electron acceptors (Fig. 2).

By comparing the survival of Δ*phz* in medium with and without D-glucose in pairwise experiments, we confirmed that D-glucose was the electron donor promoting survival in the presence of phenazines. As shown in Fig. 3, without D-glucose but with added PYO and a poised potential, Δ*phz* maintained a constant viable cell number of 10^9 CFU/ml for just 2 days and then dropped by 4 orders of magnitude by day 6. These results also indicate that survival over the first 2 to 3 days is independent of phenazine electron shuttling and glucose utilization. As has been observed, bacterial cells are able to store internal energy reserves to support their short-term survival, which might explain this effect (7, 18). Phenazine electron shuttling supported survival but not growth, even when cells were suspended at much lower initial concentrations (10^7 CFU/ml), indicating that the survival effect was independent of the concentration of cells.

To determine whether the observed electron shuttling-promoted survival was specific to the endogenous phenazines of *P. aeruginosa* or more general, we performed analogous bioreactor experiments with four other redox-active compounds (listed in Table 1). Methylene blue (MB) is a synthetic compound that shares the core redox-active structure of natural phenazines. Its cyclic voltammogram at pH 7 exhibited reversible voltammetry peaks centered at 0 mV (versus that of the NHE) (Table 1), about 40 mV higher than the phenazine

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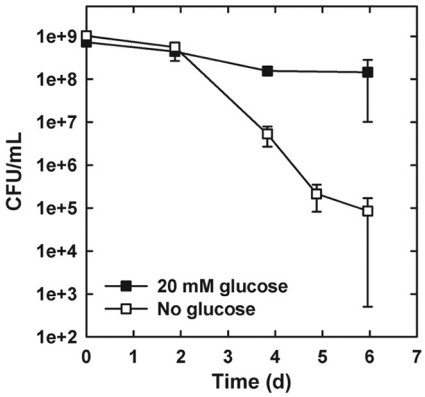

FIG. 3. Anaerobic survival of the Δ*phz* mutant of *P. aeruginosa* PA14 without D-glucose and in the presence of 20 mM D-glucose for cells incubated in MOPS medium containing ~90 μM PYO, with the graphite rod working electrode poised at +0.2 V versus that of the NHE. Survival was determined by the number of CFU on LB agar plates. Error bars represent standard deviations from triplicate samples in each experimental set. Plots represent results from two independent experiments.

PYO, indicating that MB was electrochemically redox active. In the survival control experiments without a poised potential, the color of MB changed from blue (its oxidized form) to colorless (its reduced form), confirming that MB was reduced intracellularly. In contrast, during the survival experiments with the poised potential, MB remained blue, suggesting that reduced MB can be readily oxidized at the electrode surface. Unlike PYO, however, the redox cycling of MB was so inefficient that the current (~12 μA) with MB was only marginally higher than the background current (5 to 10 μA), and we estimated that MB oxidation-reduction cycled only three times over 7 days. The viable cells dropped 3 orders of magnitude regardless of bioreactor experimental conditions, i.e., with or without MB being added and/or a potential being applied, revealing that MB redox cycling cannot support Δ*phz* survival.

Anthraquinone-2,6-disulfonate (2,6-AQDS), the well-studied anthraquinone-type exogenous electron shuttle used by *Shewanella* and *Geobacter* species, among others (5, 27), has a reduction potential similar to that of the phenazine 1-OHPHZ (Table 1). In contrast to 1-OHPHZ, we did not observe 2,6-AQDS redox cycling between the electrode surface and Δ*phz* cells, due to apparently slow intracellular 2,6-AQDS reduction. After 7 days of incubation, the cell cultures of the control conditions (no potential applied) showed a faint orange color. Considering that oxidized 2,6-AQDS is colorless and that the reduced form is bright red orange in the 100 μM concentration range (27), this indicated that only a small portion of 2,6-AQDS was reduced. Consistent with this observation, 2,6-AQDS was not able to support Δ*phz* survival, as measured by viable cell counts.

Paraquat is a redox-active compound that undergoes reversible single-electron transfer between the colorless oxidized form and the blue-colored reduced radical, with a reduction potential (−446 mV versus that of the NHE, pH 7) lower than those of most cellular reducing equivalents (e.g., NAD[P]H, reduced glutathione) (25, 26). Despite its low reduction potential, paraquat is known for its ability to undergo in vivo redox cycling in some eukaryotic and bacterial cells (3). The reduced paraquat radical produced during this process can react with intracellular oxygen and catalyze the formation of toxic superoxide radical (3) via a mechanism similar to that of PYO-induced toxicity under aerobic conditions (13, 14, 19, 22). In contrast to PYO, we did not observe paraquat electron shuttling between the electrode surface and Δ*phz* cells because it cannot be reduced by Δ*phz*. We did not observe any reduction-associated color change or current readings higher than the background level. Not surprisingly, paraquat supplementation did not support anaerobic survival of Δ*phz*, according to the CFU measurements.

The last putative electron-shuttling compound we tested was homogentisic acid (HMA), a phenolic small molecule known as the primary precursor for synthesizing (pyo)melanin (4, 37). For (pyo)melanin-producing organisms, including some *P. aeruginosa* strains, HMA is secreted in its reduced form, autoxidized, and polymerized into a red-brown humic-like compound, (pyo)melanin (4, 28, 37), which has been reported to function as an electron shuttle for enhanced Fe(III) reduction in *Shewanella* species (37). By performing CV as described previously (39), we determined that HMA is subject to reversible oxidation-reduction via single-electron transfer, resulting in a reduction potential of +306 mV versus that of the NHE (pH 7), higher than the potential applied to test for Δ*phz* survival. Consequently, oxidation of HMA by the electrode was not thermodynamically feasible. As expected, HMA could not support Δ*phz* survival. Together, these results imply that electron shuttling-promoted *P. aeruginosa* survival is likely to be specific to endogenous phenazines but not to other type redox-active molecules. This is likely because sophisticated systems are necessary for controlling the reactivity of these molecules within the cell, and this machinery has evolved in pseudomonads to be specific for the electron shuttles it produces.

In conclusion, this work indicates that "enabling survival" can now be added to the list of roles performed by phenazines for their producers, which includes altering the intracellular redox state (30), making iron more bioavailable by reducing ferric (hydr)oxides (39), serving as a signaling compound (9), facilitating biofilm development (8, 21; A. I. Ramos-Solis, L. E. Dietrich, A. Price-Whelan, and D. K. Newman, submitted for publication), contributing to virulence (20), and killing microbial competitors (2, 13). In mixed species communities where pseudomonads live, be they on the surfaces of plant roots (24) or in the mucus-filled lungs of patients with cystic fibrosis (6), it seems possible that phenazines might benefit other organisms in the community as well. Indeed, support for this idea comes from work with *Pseudomonas* species in the context of microbial fuel cells, where it was suggested that other organisms in these consortia engage in redox shuttling using the phenazines produced by *P. aeruginosa* (32, 33). Whether these types of beneficial effects contribute to shaping the ecological structure of the communities that phenazine-producing pseudomonads inhabit remains to be determined.

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