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

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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 O₂- and H₂-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 cm², 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 NH₄Cl, 43 mM NaCl, 2.2 mM KH₂PO₄, 1 mM MgSO₄, 5.0 μM FeCl₃) (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 (Δphz) 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 10⁹ 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 (10⁹ 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 voltammeteries (CV) of Δphz 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 Δphz 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.

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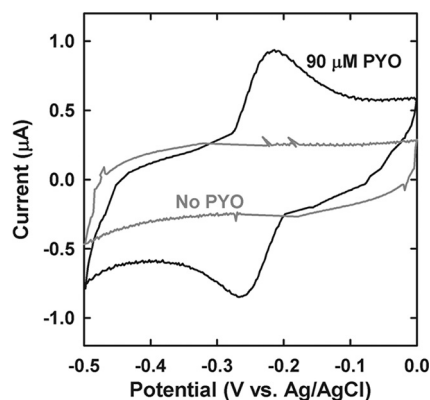


FIG. 1. Representative cyclic voltammetry (CV) of Δphz mutant cultures of *P. aeruginosa* PA14 incubated anaerobically in 100 ml MOPS medium containing 20 mM glucose, supplemented with 90 μM PYO (dark trace) or no PYO (light trace). PYO is the only electrochemically active component with single anodic (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 Δphz 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 Δphz 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 Δphz 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 Δphz survive over 7 days. In the presence of phenazine, however, the anodic current increased from the background level to $80 \pm 10 \mu\text{A}$ for PYO and PCA and $45 \pm 10 \mu\text{A}$ 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 Δphz 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

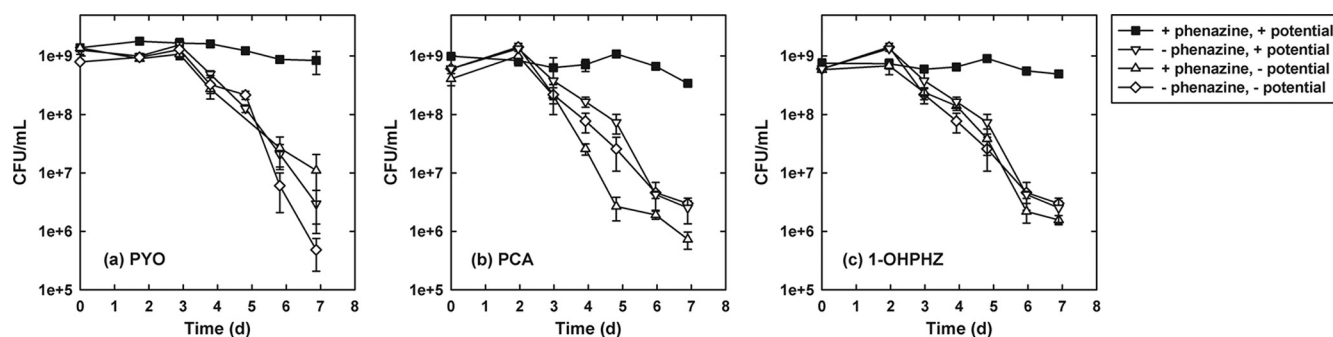


FIG. 2. PYO (a), PCA (b), and 1-OHPHZ (c) function as electron shuttles (■) to promote anaerobic survival of the Δphz mutant of *P. aeruginosa* PA14 when cells are incubated anaerobically in MOPS-buffered medium containing 20 mM D-glucose and $\sim 90 \mu\text{M}$ phenazine (PYO, PCA, or 1-OHPHZ) and 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 Δphz anaerobic incubations without phenazine (∇), poised potential (\triangle), or both (\diamond) 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.

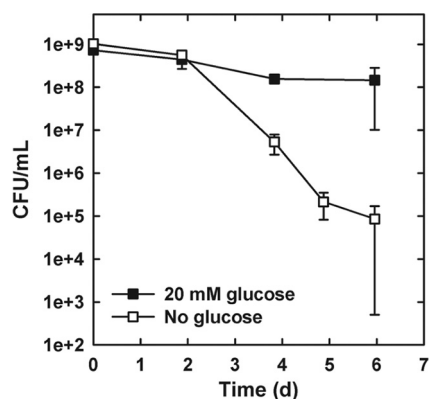


FIG. 3. Anaerobic survival of the Δphz mutant of *P. aeruginosa* PA14 without D-glucose and in the presence of 20 mM D-glucose for cells incubated in MOPS medium containing $\sim 90 \mu\text{M}$ PYO, with the graphite rod working electrode poised at +0.2 V versus that of the NHE. Survival was determined 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 ($\sim 12 \mu\text{A}$) 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 Δphz 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-OHPPHZ (Table 1). In contrast to 1-OHPPHZ, we did not observe 2,6-AQDS redox cycling between the electrode surface and Δphz 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 Δphz 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 poten-

tial, 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 Δphz cells because it cannot be reduced by Δphz . 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 Δphz , 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, auto-oxidized, 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 Δphz survival. Consequently, oxidation of HMA by the electrode was not thermodynamically feasible. As expected, HMA could not support Δphz 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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REFERENCES

- Bard, A. J., and L. R. Faulkner. 2001. Electrochemical methods: fundamentals and applications. John Wiley, New York, NY.
- Baron, S. S., and J. J. Rowe. 1981. Antibiotic action of pyocyanin. *Antimicrob. Agents Chemother.* **20**:814–820.
- Bus, J. S., and J. E. Gibson. 1984. Paraquat: model for oxidant-initiated toxicity. *Environ. Health Perspect.* **55**:37–46.
- Chatfield, C. H., and N. P. Cianciotto. 2007. The secreted pyomelanin pigment of *Legionella pneumophila* confers ferric reductase activity. *Infect. Immun.* **75**:4062–4070.
- Coates, J., E. Bluntharris, E. Phillips, J. Woodward, and D. Lovley. 1996. Humic substances as electron acceptors for microbial respiration. *Nature* **382**:445–448.
- Costerton, J. W., P. S. Stewart, and E. P. Greenberg. 1999. Bacterial biofilms: a common cause of persistent infections. *Science* **284**:1318–1322.
- Dawes, E. A., and D. W. Ribbons. 1964. Some aspects of the endogenous metabolism of bacteria. *Bacteriol. Rev.* **28**:126–149.
- Dietrich, L. E., T. K. Teal, A. Price-Whelan, and D. K. Newman. 2008. Redox-active antibiotics control gene expression and community behavior in divergent bacteria. *Science* **321**:1203–1206.
- Dietrich, L. E., A. Price-Whelan, A. Petersen, M. Whiteley, and D. K. Newman. 2006. The phenazine pyocyanin is a terminal signalling factor in the quorum sensing network of *Pseudomonas aeruginosa*. *Mol. Microbiol.* **61**:1308–1321.
- Durst, R., and M. Fultz. 1982. Mediator compounds for the electrochemical study of biological redox systems—a compilation. *Anal. Chim. Acta* **140**:1–18.
- Eschbach, M., K. Schreiber, K. Trunk, J. Buer, D. Jahn, and M. Schobert. 2004. Long-term anaerobic survival of the opportunistic pathogen *Pseudomonas aeruginosa* via pyruvate fermentation. *J. Bacteriol.* **186**:4596–4604.
- Friedheim, E. A. H. 1931. Pyocyanine, an accessory respiratory pigment. *J. Exp. Med.* **54**:207–221.
- Hassan, H. M., and I. Fridovich. 1980. Mechanism of the antibiotic action pyocyanine. *J. Bacteriol.* **141**:156–163.
- Hassett, D. J., L. Charniga, K. Bean, D. E. Ohman, and M. S. Cohen. 1992. Response of *Pseudomonas aeruginosa* to pyocyanin: mechanisms of resistance, antioxidant defenses, and demonstration of a manganese-cofactored superoxide dismutase. *Infect. Immun.* **60**:328–336.
- Hentzer, M., L. Eberl, and M. Givskov. 2005. Transcriptome analysis of *Pseudomonas aeruginosa* biofilm development: anaerobic respiration and iron limitation. *Biofilms* **2**:37–61.
- Hernandez, M. E., and D. K. Newman. 2001. Extracellular electron transfer. *Cell. Mol. Life Sci.* **58**:1562–1571.
- Ingram, J. M., and A. C. Blackwood. 1970. Microbial production of phenazines. *Adv. Appl. Microbiol.* **13**:267–282.
- Kadouri, D., E. Jurkevitch, Y. Okon, and S. Castro-Sowinski. 2005. Ecological and agricultural significance of bacterial polyhydroxyalkanoates. *Crit. Rev. Microbiol.* **31**:55–67.
- Lau, G. W., D. J. Hassett, H. Ran, and F. Kong. 2004. The role of pyocyanin in *Pseudomonas aeruginosa* infection. *Trends Mol. Med.* **10**:599–606.
- Lau, G. W., H. Ran, F. Kong, D. J. Hassett, and D. Mavrodi. 2004. *Pseudomonas aeruginosa* pyocyanin is critical for lung infection in mice. *Infect. Immun.* **72**:4275–4278.
- Maddula, V. S., Z. Zhang, E. A. Pierson, and L. S. Pierson III. 2006. Quorum sensing and phenazines are involved in biofilm formation by *Pseudomonas chlororaphis* (*aureofaciens*) strain 30–84. *Microb. Ecol.* **52**:289–301.
- Mahajan-Miklos, S., M. W. Tan, L. G. Rahme, and F. M. Ausubel. 1999. Molecular mechanisms of bacterial virulence elucidated using a *Pseudomonas aeruginosa*-*Caenorhabditis elegans* pathogenesis model. *Cell* **96**:47–56.
- Marsili, E., D. B. Baron, I. D. Shikhare, D. Coursolle, J. A. Gralnick, and D. R. Bond. 2008. *Shewanella* secretes flavins that mediate extracellular electron transfer. *Proc. Natl. Acad. Sci. U. S. A.* **105**:3968–3973.
- Mazzola, M., R. J. Cook, L. S. Thomashow, D. M. Weller, and L. S. Pierson III. 1992. Contribution of phenazine antibiotic biosynthesis to the ecological competence of fluorescent pseudomonads in soil habitats. *Appl. Environ. Microbiol.* **58**:2616–2624.
- Michaelis, L., and E. S. Hill. 1933. Potentiometric studies on semiquinones. *J. Am. Chem. Soc.* **55**:1481–1494.
- Michaelis, L., and E. S. Hill. 1933. The viologen indicators. *J. Gen. Physiol.* **16**:859–873.
- Newman, D. K., and R. Kolter. 2000. A role for excreted quinones in extracellular electron transfer. *Nature* **405**:94–97.
- Nosanchuk, J. D., and A. Casadevall. 2003. The contribution of melanin to microbial pathogenesis. *Cell Microbiol.* **5**:203–223.
- Palmer, K. L., L. M. Mashburn, P. K. Singh, and M. Whiteley. 2005. Cystic fibrosis sputum supports growth and cues key aspects of *Pseudomonas aeruginosa* physiology. *J. Bacteriol.* **187**:5267–5277.
- Price-Whelan, A., L. E. Dietrich, and D. K. Newman. 2007. Pyocyanin alters redox homeostasis and carbon flux through central metabolic pathways in *Pseudomonas aeruginosa* PA14. *J. Bacteriol.* **189**:6372–6381.
- Price-Whelan, A., L. E. Dietrich, and D. K. Newman. 2006. Rethinking 'secondary' metabolism: physiological roles for phenazine antibiotics. *Nat. Chem. Biol.* **2**:71–78.
- Rabaey, K., N. Boon, M. Hofte, and W. Verstraete. 2005. Microbial phenazine production enhances electron transfer in biofuel cells. *Environ. Sci. Technol.* **39**:3401–3408.
- Rabaey, K., N. Boon, S. D. Siciliano, M. Verhaege, and W. Verstraete. 2004. Biofuel cells select for microbial consortia that self-mediate electron transfer. *Appl. Environ. Microbiol.* **70**:5373–5382.
- Reference deleted.
- Spoering, A. L., and K. Lewis. 2001. Biofilms and planktonic cells of *Pseudomonas aeruginosa* have similar resistance to killing by antimicrobials. *J. Bacteriol.* **183**:6746–6751.
- Swain, A., B. Schink, and R. Emde. 1989. Anaerobic oxidation of glycerol by *Escherichia coli* in an amperometric poised-potential culture system. *Appl. Microbiol. Biotech.* **32**:170–175.
- Turick, C. E., L. S. Tisa, and F. Caccavo, Jr. 2002. Melanin production and use as a soluble electron shuttle for Fe(III) oxide reduction and as a terminal electron acceptor by *Shewanella* algae BrY. *Appl. Environ. Microbiol.* **68**:2436–2444.
- Waite, R. D., A. Papakonstantinou, E. Littler, and M. A. Curtis. 2005. Transcriptome analysis of *Pseudomonas aeruginosa* growth: comparison of gene expression in planktonic cultures and developing and mature biofilms. *J. Bacteriol.* **187**:6571–6576.
- Wang, Y., and D. K. Newman. 2008. Redox reactions of phenazine antibiotics with ferric (hydr)oxides and molecular oxygen. *Environ. Sci. Technol.* **42**:2380–2386.