Phenazines and Other Redox-Active Antibiotics Promote Microbial Mineral Reduction

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Natural products with important therapeutic properties are known to be produced by a variety of soil bacteria, yet the ecological function of these compounds is not well understood. Here we show that phenazines and other redox-active antibiotics can promote microbial mineral reduction. Pseudomonas chlororaphis PCL1391, a root isolate that produces phenazine-1-carboxamide (PCN), is able to reductively dissolve poorly crystalline iron and manganese oxides, whereas a strain carrying a mutation in one of the phenazine-biosynthetic genes (phzB) is not; the addition of purified PCN restores this ability to the mutant strain. The small amount of PCN produced relative to the large amount of ferric iron reduced in cultures of P. chlororaphis implies that PCN is recycled multiple times; moreover, poorly crystalline iron (hydr)oxide can be reduced abiotically by reduced PCN. This ability suggests that PCN functions as an electron shuttle rather than an iron chelator, a finding that is consistent with the observation that dissolved ferric iron is undetectable in culture fluids. Multiple phenazines and the glycopeptidic antibiotic bleomycin can also stimulate mineral reduction by the dissimilatory iron-reducing bacterium Shewanella oneidensis MR1. Because diverse bacterial strains that cannot grow on iron can reduce phenazines, and because thermodynamic calculations suggest that phenazines have lower redox potentials than those of poorly crystalline iron (hydr)oxides in a range of relevant environmental pH (5 to 9), we suggest that natural products like phenazines may promote microbial mineral reduction in the environment.

Microbial reductive dissolution of minerals can significantly affect the chemistry of soils and sedimentary environments by making inorganic compounds such as iron more available to plants and other organisms and by mobilizing adsorbed nutrients and/or pollutants into the aqueous phase (2, 11, 30). Discovering which organisms in the environment most affect iron cycling has been the subject of several previous studies (6, 44), covering which organisms in the environment most affect iron cycling has been the subject of several previous studies (6, 44), and diverse bacteria can reduce but not grow on iron, including Aerobacter aerogenes, Bacillus polymyxa, Escherichia coli, Pseudomonas spp., and Serratia marcescens (31). With the discovery that some bacteria can respire iron (hydr)oxides (32, 41), attention has focused mainly on the importance of dissimilatory iron-reducing bacteria (DIRB) and the molecular strategies by which they reduce minerals (4, 7, 13, 29, 35, 40). Evidence supporting the release of small organic molecules by DIRB for the purpose of mineral dissolution has been accumulating, and it is thought that this process may be most significant in environments in which bacteria form biofilms on mineral surfaces (19, 42, 43).

It has been appreciated for some time that naturally present complex organic molecules such as humic substances (Fig. 1a) and their functional surrogate, anthraquinone-2,6-disulfonate (AQDS) (Fig. 1b), can stimulate iron reduction by serving as electron shuttles between microbes and minerals (33). Whether natural products produced by soil and/or sedimentary bacteria can play a similar role is an unresolved question. Pseudomonas, Streptomyces, Sorangium, Arthrobacter, Nocardia, Burkholderia, Brevibacterium, and other bacterial genera are commonly found in the soil and are major producers of antibiotics (36). Among the antibiotics that they produce are those that possess redox activity, such as complex glycopeptidic antibiotics like bleomycin (Fig. 1c) and the numerous multicolored phenazine pigments (23, 54) (Fig. 1d). Structurally, these molecules resemble humic substances and AQDS in terms of their aromatic ring structure and redox-active functional groups.

To date, most ecological discussions of phenazines have focused on their crucial role in suppressing fungal pathogens of plants such as Fusarium oxysporum f. sp. radicis-lycopersici and Gaumannomyces graminis var. tritici (9). However, there has also been some discussion of a role for phenazines in aiding the ecological competence of pseudomonads in the rhizosphere (37, 45), presumably due to the ability of phenazines to generate reactive oxygen species that kill other organisms (17). Appreciable concentrations of phenazines have been detected in the rhizosphere (e.g., phenazine carboxylic acid [PCA] produced by Pseudomonas fluorescens strain 2-79 was found to be present in concentrations of 27 to 43 ng/g of root with adhering soil) (53), and the expression of phenazine-biosynthetic genes in organisms growing on tomato root seedlings and bean plants roots has been measured previously (10, 48). While little is known about the abundance of bleomycin in the soil, this compound has been the subject of intense biochemical scrutiny due to its effectiveness as an anticancer agent. Bleomycin's anticancer activity has been attributed to its ability to coordinate Fe, which then transfers electrons to DNA via an activated oxygen molecule, thereby resulting in DNA cleavage (18).

Given that both phenazines and bleomycin can function as
electron carriers (in fact, phenazines are known to be part of electron transport chains in methanogenic archaea) (1), we hypothesized that redox-active antibiotics might promote reduction of minerals. To test this hypothesis, we chose to work primarily with the soil isolate *Pseudomonas chlororaphis* PCL1391, which produces phenazine-1-carboxamide (PCN) and PCA (Fig. 1d), and the DIRB *Shewanella oneidensis* MR1.

**MATERIALS AND METHODS**

**Bacterial strains and culture conditions.** The bacterial strains *P. chlororaphis* PCL1391 and *P. chlororaphis* PCL1119 used in this study were obtained from G. Bloemberg (Institute of Biology, Leiden University, Leiden, The Netherlands). PCL1391 was isolated from a tomato field in Spain, and PCL1119 is a transposon-generated mutant with a disruption in *phzB*, one of the genes in the core operon required for the biosynthesis of phenazines, making it defective in terms of phenazine production (10). *S. oneidensis* MR1 was isolated from Oneida Lake, N.Y. (41). Cultures were grown in Luria-Bertani (LB) medium; King’s A (a medium designed to elicit phenazine production) (24); basic medium (BM) with a low concentration (0.1 mM) of phosphate, 10 mM HEPES, and 20 mM glucose as the carbon source (38); or LML medium (4) as appropriate at 30°C. Other strains used in this study included additional gamma proteobacteria grown in LB (*E. coli* JM109, *P. fluorescens* WCS365, *Pseudomonas aeruginosa* PA01, and *Vibrio cholerae* El Tor) and Geobacter sp. strains Dfr1 and Dfr2 (grown as described previously by Straub et al. [52]).

**Chemicals.** AQDS, phenazine, and bleomycin were purchased from Sigma-Aldrich. Pyocyanine was prepared from phenazine methosulfate by photochemical oxidation as previously described (25).

**Purification of phenazines and high-pressure liquid chromatography analysis.** Phenazine-1-carboxamide (PCN) was purified from green precipitates in culture supernatants or from CHCl3 extracts after solvent evaporation by multiple re-crystallizations from hot water. Purity was assessed by high-pressure liquid chromatography (Beckman System Gold) with a C18 Symmetry Prep column (inside diameter, 7.8 mm; length, × 150 mm, Waters) in a gradient of water-0.01% TCA (solvent A)-acetonitrile-0.01% TCA (solvent B) (for 0 to 1 min, chromatography was in 100% solvent A, for 1 to 7 min in a linear gradient to 100% B, for 7 to 9 min in 100% solvent B, for 9 to 12 min in a linear gradient to 100% solvent A, and for 12 to 15 min in 100% solvent A) and a flow rate of 2 ml/min. Retention times for PCN and PCA were 7.45 and 8.17 min, respectively. Concentrations of PCN and PCA were estimated by using an extinction coefficient at 368 nm (ε368) of 17,600 that was calculated by measuring the absorption spectra of a PCN and of a phenazine solution of known concentration and assuming the same extinction coefficient for phenazine and PCN at 250 nm (5).

**Fe(III) reduction assays.** *P. chlororaphis* cells were grown overnight in LB medium, washed, and resuspended at about 10^7 cells/ml in 48-ml bottles with 25 ml of King’s A medium plus 10 mM poorly crystalline iron (hydr)oxide [Fe(OH)]_3 (34). Tubes were capped and incubated in a rotary shaker at 30°C. When required, the tubes were amended with purified PCN (~40 μM) from a concentrated stock in hot water. Samples were taken regularly to determine the number of CFU by plating 100 μl of serial dilutions of the sample onto LB-agar plates. Fe(II) was determined in acidified culture samples after filtration through 0.2-μm-pore-size filter tubes (nylon membrane).

**FIG. 1.** Shown are the structures of a representative humic substance molecule, drawn after those described previously (49) (a); AQDS (b); bleomycin (c); and a generic phenazine (d). If x is CONH₂, the compound is PCN; if x is COOH, the compound is PCA.
assay (50), both before [for total Fe(II)] and after [for dissolved Fe(II)] filtration through a 0.2-μm-pore-size filter. The level of dissolved Fe(III) was calculated by determining total dissolved iron as Fe(II) after a reduction step with NH₄OH and subtracting the dissolved Fe(II) concentration.

*S. oneidensis* MR1 cells were inoculated at about 10⁶ cells/ml in LML medium in anaerobic glass tubes with 30 mM Fe(OH)₃ as the electron acceptor from an overnight aerobically grown LB culture and incubated at 30°C. PCN (10 μM), filtered spent supernatants (to a final dilution of 1:10), AQDS (10 μM), and bleomycin (15 to 20 μM) were added as required. Reduction of Fe(OH)₃ was determined by the ferrozine assay. Cell counts where performed as previously described (34).

Abiotic reduction of Fe(OH)₃ was accomplished by reducing PCN with a Pd catalyst and H₂ and then mixing reduced PCN with iron citrate or poorly crystalline iron (hydr)oxide in an 80% N₂-15% CO₂-5% H₂ glove box.

Pyocyanine reduction assays. Pyocyanine reduction was measured by adapting a previously described method (12). *E. coli* JM109, *P. fluorescens* WCS365, *P. aeruginosa* PAO1, and *V. cholerae* El Tor were harvested from late exponential-early stationary phase LB culture, washed twice in saline solution (0.85% NaCl), and resuspended in the same volume of LML medium. At the beginning of the experiment, 50 μl of this cell suspension was added to 2 ml of an anoxic solution (pH 7.5) containing 10 mM HEPES; 4 mM MgCl₂; 2 mM (each) lactate, succinate, and pyruvate; and 2 μg of pyocyanine per ml. The mixture was prepared in the anaerobic chamber and placed in airight cuvettes to prevent reoxidation of pyocyanine by oxygen during the absorption measurements. Pyocyanine reduction was followed by the characteristic change of the absorption spectrum accompanying the transformation of the oxidized form to the reduced form. By using a Beckman Coulter DU7400 spectrophotometer, we measured and compared the decrease in absorption at 690 nm to that of a reference cuvette containing a cell suspension lacking pyocyanine. The optical density at 550 nm (OD₅₅₀) of each cell suspension was measured, and the rates were standardized to an OD₅₅₀ of 0.1.

Toxicity experiments. *S. oneidensis* MR1 cells were grown aerobically in LB medium overnight or anaerobically in LB-fumarate (20 mM) for a day and were inoculated in fresh medium at an OD₆₀₀ of ~0.05. Different amounts of PCN were added, and the tubes were incubated (each in duplicate) under the same conditions as those for the precursor cultures. Samples (200 μl) were taken periodically, and cell growth was determined by measuring the OD₆₀₀ in a microtiter plate reader (Odsys MR; Dynex Technologies).

RESULTS

Mineral reduction by *P. chlororaphis* strains. *P. chlororaphis* PCL1391 (wild type [WT]) and *P. chlororaphis* PCL1119 (the phzB mutant) were grown in medium that was amended with poorly crystalline manganese or iron (hydr)oxide in microtiter plates. After 1 day, mineral reduction and phenazine production in the presence of the WT but not the phzB mutant was evident by the dissolution of the manganese mineral, the change of color of the iron mineral (from a deep orange to a darker brown), and the appearance of small green crystals, which were most likely PCN precipitates (see Materials and Methods). To quantify these observations, we measured Fe(III) mineral reduction; Fe(II) production was detected only in the WT culture (Fig. 2a). However, CFU were similar in both cultures, indicating that Fe(III) mineral reduction did not have an impact on cell growth under these conditions (Fig. 2b). About half of the reduced iron in the WT cultures was in solution, as shown by iron’s presence in the filtrate fraction (data not shown); the remaining part was likely adsorbed to the mineral and/or the cells, as well as reprecipitated as Fe(II) minerals.

 Extraction and quantification of phenazines in the WT culture showed that PCN production was correlated with cell numbers, and its concentration was constant once the cells reached stationary phase. Up to 20 μM PCN and 5 μM PCA were produced by the WT (Fig. 3a). No phenazines were detected in the phzB mutant culture. The amount of PCN produced by the WT was positively correlated with the amounts of oxygen initially present in the system (defined by the size of the bottle’s headspace) and varied with the medium used in the experiment (e.g., King’s A, LB, or BM), but a similar trend was observed under all conditions. The final concentration of Fe(II) (3 mM) (Fig. 3b) was about 120 times higher than was the total phenazine concentration (25 μM). Because two electrons are available per reduced phenazine molecule and only one electron is required to reduce each iron ion, the phenazines must have been cycled at least 60 times to account for the observed iron reduction. Adding PCN to the phzB mutant restored the ability of the strain to reduce the iron mineral, thereby indicating that PCN is essential for the reduction process (Fig. 3b). Again, the amount of iron reduced per amount of phenazine added was large, demonstrating redox shuttling of phenazines between the bacteria and the minerals. That phenazines were serving as electron shuttles was confirmed by control experiments in which chemically reduced PCN was found to reduce and solubilize Fe(III) minerals in the absence of bacteria. Moreover, no soluble Fe(III) could be detected in the filtrate fractions of WT cultures, suggesting that chelation of Fe(III) is not a significant mechanism for phenazine stimulation of Fe(III) mineral reduction.

FIG. 2. Total Fe(II) produced in culture (a) and CFU (b) of *P. chlororaphis* PCL1391 (WT) (●) and PCL1119 (phzB mutant) (○) grown in the presence of poorly crystalline iron(III) hydr(oxides). Data are representative of at least three independent experiments.
phenazines tested (e.g., pyocyanine, phenazine methosulfate, and phenazine) stimulated Fe(III) mineral reduction by MR1 in the same way. When PCN was added to cultures of different DIRB (e.g., Geobacter strains Dfr1 and Dfr2 [51]), acceleration of iron reduction was also observed (data not shown). Because reduced phenazines react abiotically with insoluble Fe(III) minerals, any strain that can reduce phenazines has the potential to promote mineral reduction. Diverse gamma proteobacteria that we tested, including E. coli JM109, P. fluorescens WCS365, P. aeruginosa PAO1, and V. cholerae strain El Tor, were able to reduce pyocyanine.

To determine whether phenazines play a significant role in extracellular electron transfer that is differentiable from the contribution of other small molecules released by P. chlororaphis PCL1391, we tested whether Fe(III) mineral reduction by S. oneidensis MR1 could be stimulated by spent supernatants from the WT and from the phzB mutant. Figure 4b shows that rapid and significant production of Fe(II) by S. oneidensis MR1 occurs in the presence of spent supernatants from the WT, whereas spent supernatants from the phzB mutant had a reduced effect that was evident only at later stages in the experiment. Because P. chlororaphis PCL 1119 does not produce phenazines, these results suggest that phenazines are the primary natural products released by P. chlororaphis PCL1391 that promote iron reduction by S. oneidensis MR1; however, it is likely that other bacterially produced compounds in the culture can stimulate iron reduction as well.

In addition to phenazines, bleomycin, a glycopeptidic redox-active antibiotic produced by Streptomyces verticillus (Fig. 1c), also greatly increased the rate of Fe(III) mineral reduction by MR1 cultures (Fig. 4c). Controls with bleomycin in the absence of cells showed no iron reduction (data not shown).

Concentrations of phenazines inhibitory for strain MR1. Phenazines are known to have antibacterial properties; therefore, the effect of adding PCN to growing MR1 cultures was tested. PCN reduced the growth rate and yield of MR1 growing aerobically in LB and anaerobically in LB-fumarate as follows. Aerobically grown cells eventually reached stationary-phase cell densities comparable to those of controls without PCN up to a concentration of 75 μM. When PCN was added at a concentration of 150 μM, the cell yield was only 25% of that of the no-PCN control, and growth of S. oneidensis MR1 was completely inhibited at concentrations equal to or greater than 300 μM PCN. S. oneidensis MR1 cells growing anaerobically on fumarate generally reached lower stationary-phase cell density levels than did the aerobically grown cultures. The inhibitory effect of PCN in cultures growing on fumarate increased with increasing concentrations of added PCN. Small amounts of PCN (e.g., 2.4 μM) reduced the final cell density of the culture to 77% of that of the no-PCN control, and the final cell density achieved in the presence of added 300 μM PCN was 6% relative to that of the no-PCN control. No inhibitory effects were observed for P. chlororaphis PCL1391 growing aerobically in LB with concentrations of up to 300 μM PCN.

Thermodynamic constraints on phenazine-mediated iron mineral reduction. To assess the conditions under which iron mineral reduction by phenazines would be thermodynamically feasible, we constructed an E°/pH diagram. The total iron concentration was assumed to be 10⁻⁵ M, carbonate species were neglected for simplicity, and a value of E° of −114 mV

FIG. 3. (a) Total phenazine production (▲) in a PCL1391 culture representing the sum of PCN (■) and PCA (▲). Data represent the average of two independent extractions of each time point in the same experiment; bars show the data range. (b) Total Fe(II) production by PCL1391 (WT) (▲), PCL1119 (phzB mutant) (■) and PCL1119 with PCN added (40 μM, added at time point indicated by arrow) (△). Data are representative of at least two independent experiments.
for PCN was taken from the literature (14). Given these parameters, we determined that the reaction between PCN and Fe(OH)₃ is favorable at pH values of 7 and lower but becomes unfavorable at higher pH values (Fig. 5). As shown in Fig. 5, the more Fe²⁺ that accumulates in the system, the smaller the pH range becomes in which phenazines can act as an electron shuttle. On the other hand, in the presence of more reduced phenazines relative to oxidized phenazines in the system, electron transfer via phenazines is also possible at higher Fe²⁺ concentrations and/or higher pH values.

DISCUSSION

That common soil bacteria such as *Pseudomonas* species can reduce (but not grow on) iron minerals has long been appreciated (31); however, the mechanism(s) responsible for this phenomenon has been unknown. Here we show that phenazines and other redox-active antibiotics produced by soil bacteria can serve as electron shuttles, being reduced microbiologically and subsequently oxidized by poorly crystalline iron and manganese (hydr)oxides.

Phenazine production has been well studied for a variety of organisms because of their function in the biocontrol of plant pathogens and because they serve as virulence factors in the human host (9, 23). It is noteworthy that the environmental factors that are known to stimulate phenazine production make sense in the context of Fe(III) mineral reduction. These factors include low phosphate levels (15), oxygen limitation (26), and the presence of Fe(III) (26, 27) and organic components of root and/or seedling exudates such as fructose, ribose, and citric acid (T. F. Chin-A-Woeng, personal communication). Microaerophilic or anaerobic zones have been reported to occur in rhizosphere environments and soil micropockets (22). Oxygen regulation of phenazine production may thus ensure that phenazines are produced under the most favorable conditions for mineral reduction. Moreover, oxygen tensions drop when bacteria are at a high cell density (e.g., as in biofilms), and quorum sensing appears to be an additional mechanism for the regulation of phenazine production (8, 28, 46, 56). Regulating phenazine production by quorum sensing may limit competition by nonproducer strains that might directly or indirectly benefit from the production of phenazines.

There are multiple ways in which phenazines may affect ecological fitness. Phenazines can function as antibiotics (3), as accessory respiratory pigments (16), and, as shown in this study, as agents of mineral reduction. With respect to the last role, it seems likely that phenazines may act in concert with siderophores to make iron bioavailable. Although siderophores are thermodynamically able to dissolve iron minerals due to their high binding constants for Fe(III), this process can, from a kinetic standpoint, be very slow, and whether
siderophores alone can account for iron acquisition in the environment is questionable (21, 55). Evidence in support of this hypothesis comes from previous studies of Pseudomonas mendocina, in which unidentified low-molecular-weight reductants and siderophores were thought to account for iron acquisition from minerals (20).

Our thermodynamic calculations show that phenazine-mediated reduction of poorly crystalline iron (hydr)oxides would be favored at pH values of 7 to 8, whereas higher pH values would make the process endergonic. If Fe(II) accumulates, as occurred in our experimental system, the reaction will reach equilibrium and further reduction will not occur. On the other hand, if the Fe(II) produced is scavenged by active uptake, binding to chelators, reoxidation by oxygen, or precipitation as a mineral phase, such as siderite (FeCO$_3$), phenazine-mediated Fe(III) reduction will be favored. Although our model does not explicitly include carbonate species, their presence would enhance Fe(III) reduction because siderite precipitation would maintain dissolved Fe(II) at low levels, and this would affect the potential of the Fe(II)-Fe(III) redox couple so as to favor Fe(III) reduction by phenazine. The redox potential of the specific redox-active antibiotic produced, the ratio of reduced to oxidized antibiotic, and the redox potential of the mineral are key parameters that constrain the occurrence of reduction. For example, PCA is reported to have a lower redox potential than that of PCN (47) and would thus be expected to reduce minerals at higher pH and higher Fe(II) concentrations compared to those of PCN. Due to their lower redox potentials compared to those of poorly crystalline ferric hydr(oxide) (39), other common Fe(III) mineral phases in soils, like hematite and goethite, would be predicted to be reduced by phenazines only at lower pH values. Because adsorption of reductants to minerals is required for electron transfer to occur, the specific chemical structure of the reductant is also important since it will determine its adsorption properties and thus the rate of reductive dissolution.

In addition to promoting iron reduction by the producer strains, redox-active antibiotics may positively affect iron reduction and/or acquisition by other microorganisms. The stimulation of Fe(III) reduction by S. oneidensis MR1 and Geobacter species by phenazines and bleomycin suggests that DIRB may reduce in situ-produced antibiotics when soils and sediments become anaerobic. Because these compounds are more bioavailable than are ferric minerals, organisms that can reduce them may gain more energy per unit time, thus providing them with a growth advantage. From the literature we can estimate that phenazine concentrations in the rhizosphere may be on the order of 0.1 mM to several micromolar, assuming that 1 g of soil fills a volume of 1 ml (52). Although it is difficult to know exactly how much will be bioavailable (i.e., not adsorbed onto minerals or natural organic matter), it seems likely that at these concentrations phenazines may function more effectively as electron shuttles than as antibiotics. Moreover, any bacterium that can reduce phenazines or bleomycin—regardless of whether it is a DIRB—can be expected to indirectly promote Fe(III) reduction.

In summary, our results show that redox-active natural products produced by soil bacteria can function as electron shuttles to minerals and can also be utilized by non-antibiotic producing species. These findings suggest that the production and cycling of redox-active antibiotics may affect bacterial and plant access to iron and a variety of other nutrients, such as phosphate, trace metals, and organics that are associated with mineral phases. Such nutrient mobilization may provide an ecological advantage for the producer strains—a possibly important and underappreciated role for redox-active antibiotics that can now be tested. Moreover, given their facility for electron transfer, it is important to consider whether redox-active

![FIG. 5. $E_h$/pH diagram showing the thermodynamic stability region of iron species and PCN as a function of pH and $E_h$. The dashed lines represent the redox potential for the PCN redox couple at PCN$_{red}$/PCN$_{oxid}$ ratios of 1 to 1 and 100 to 1, respectively. To draw the Fe(OH)$_3$/Fe$^{3+}$ boundary line, we assumed Fe$^{3+}$ concentrations of 10 $\mu$M, 1 mM, and 10 mM. $H_2$ and $O_2$ lines frame the stability field for water.](image-url)
antibiotic-producing strains can derive energy from their re-
duction; we are currently exploring this possibility.

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