Bidirectional redox cycling of phenazine-1-carboxylic acid by *Citrobacter portucalensis* MBL drives increased nitrate reduction

Lev M. Tsypin[a] and Dianne K. Newman[a,b#]

[a] Division of Biology and Biological Engineering, California Institute of Technology, Pasadena, CA, USA

[b]Division of Geological and Planetary Sciences, California Institute of Technology, Pasadena, CA, USA

Running Head: *C. portucalensis* MBL links PCA and nitrate redox cycles

Address correspondence to Dianne K. Newman, dkn@caltech.edu

ABSTRACT

Phenazines are secreted metabolites that microbes use in diverse ways, from quorum sensing to antimicrobial warfare to energy conservation. Phenazines are able to contribute to these activities due to their redox activity. The physiological consequences of cellular phenazine reduction have been extensively studied, but the counterpart phenazine oxidation has been largely overlooked. Phenazine-1-carboxylic acid (PCA) is common in the environment and readily reduced by its producers. Here, we describe its anaerobic oxidation by *Citrobacter portucalensis* strain MBL, which was isolated from topsoil in Falmouth, MA, and which does not produce phenazines itself. This activity depends on the availability of a suitable terminal electron acceptor, specifically nitrate or fumarate. When *C. portucalensis* MBL is provided reduced PCA and either nitrate or fumarate, it continuously oxidizes the PCA. We compared this terminal electron acceptor-dependent PCA-oxidizing activity of *C. portucalensis* MBL to that of several other γ-proteobacteria with varying capacities to respire nitrate and/or fumarate. We found that PCA oxidation by these strains in a fumarate- or nitrate-dependent manner is decoupled from growth and correlated with their possession of the fumarate or periplasmic nitrate reductases, respectively. We infer that bacterial PCA oxidation is widespread and genetically determined. Notably, reduced PCA enhances the rate of nitrate reduction to nitrite by *C. portucalensis* MBL beyond the stoichiometric prediction, which we attribute to *C. portucalensis* MBL's ability to also reduce oxidized PCA, thereby catalyzing a complete PCA redox cycle. This bidirectionality highlights the versatility of PCA as a biological redox agent.

IMPORTANCE Phenazines are increasingly appreciated for their roles in structuring microbial communities. These tricyclic aromatic molecules have been found to regulate gene expression,

be toxic, promote antibiotic tolerance, and promote survival under oxygen starvation. In all of these contexts, however, phenazines are studied as electron acceptors. Even if their utility arises primarily from being readily reduced, they would need to be oxidized in order to be recycled. While oxygen and ferric iron can oxidize phenazines abiotically, biotic oxidation of phenazines has not been studied previously. We observed bacteria that readily oxidize phenazine-1-carboxylic acid (PCA) in a nitrate-dependent fashion, concomitantly increasing the rate of nitrate reduction to nitrite. Because nitrate is a prevalent terminal electron acceptor in diverse anoxic environments, including soils, and phenazine-producers are widespread, this observation of linked phenazine and nitrogen redox cycling suggests an underappreciated role for redox-active secreted metabolites in the environment.

OBSERVATION

Physiological studies of phenazines have focused on cellular reduction of these secreted molecules for over 120 years. Reduction of phenazines by bacteria was first proposed in the 19th century as an indicator for the presence of enteric bacteria in water supplies (1). Several decades later, pyocyanin, one of the phenazines produced by *Pseudomonas aeruginosa*, was described as an "accessory respiratory pigment" that increased the rate of oxygen consumption by *Staphylococcus*, *Pneumococcus*, and erythrocytes by shuttling electrons from the cells to oxygen (2). Once it became apparent that phenazines can have cytotoxic effects for cells that do not produce them, they were characterized as antimicrobial compounds that destructively abstract electrons from the transport chain (3). It was then discovered that phenazine reduction can greatly benefit *P. aeruginosa* by: 1) regulating gene expression in *P. aeruginosa* during quorum sensing by oxidizing a transcription factor; 2) acting as alternative terminal electron acceptors to

promote anoxic survival; and 3) facilitating iron acquisition (4–8). These reports paint a complex picture of the multifarious effects phenazines can have, but in each case the conceptual model ends with the cell reducing the phenazine. Reduced phenazines can be re-oxidized by inorganic electron acceptors like oxygen and ferric iron, and this abiotic process has been invoked to explain redox cycling of phenazines in biofilms (9, 10). However, when these electron acceptors are unavailable, biotic oxidation of reduced phenazines could close the redox cycle by regenerating oxidized phenazines. This process has not been shown to exist for secreted redox-active metabolites.

In parallel to these physiological studies, phenazines have been used as generic electron shuttles in bioelectrochemical reactor research, selected according to their chemical properties and suitability for a given application (11, 12). Electrochemically reduced neutral red (NR), a phenazine, has been successfully used as an electron donor to cells, chosen for its standard midpoint potential (very near to that of NADH/NAD^+, $E_{\frac{1}{2}}^{o\prime} = -320$ mV vs. NHE) and hydrophobicity (13–15). Anaerobic NR oxidation in BERs has been coupled to the reduction of several terminal electron acceptors, including nitrate and fumarate (16). A limitation of these studies is that NR is not found in nature. Therefore, despite NR oxidation being useful in regulating electrosynthesis, the existence of natural bacterially driven phenazine oxidation remains unexplored.

PCA is one of the mostly widely synthesized phenazines in the microbial world, from which other phenazines are derived (17, 18). PCA is known to be reduced by its producers, driving current generation in bioelectrochemical systems, in which it is re-oxidized by the anode (5, 19).

These facts make PCA a fitting candidate for microbial oxidation during anaerobic metabolism. In previous work, we enriched for PCA oxidizers from topsoil by incubating them with reduced PCA, acetate (a non-fermentable carbon source), and nitrate as the only terminal electron acceptor, and successfully isolated the PCA-oxidizing *Citrobacter portucalensis* MBL (20).

***C. portucalensis* MBL oxidizes PCA in a nitrate- and fumarate-dependent manner.** We did not observe reduced PCA (PCA_{red}/PCA_{ox} $E_{\frac{1}{2}}^{o\prime} = -116$ mV vs. NHE (9)) oxidation in the absence of a terminal electron acceptor in both the abiotic and biotic regimes (Fig 1A, top left). When 10 mM fumarate (fumarate/succinate $E_{\frac{1}{2}}^{o\prime} = +33$ mV vs NHE (15)) or nitrate (NO_3^-/NO_2^- $E_{\frac{1}{2}}^{o\prime} = +433$ mV vs NHE (15)) were added (right panels), *C. portucalensis* MBL readily oxidized PCA_{red}. Neither fumarate nor nitrate oxidize PCA abiotically (grey curves). During both dissimilatory and assimilatory nitrate reduction, nitrate is first reduced to nitrite (21). Nitrite (NO_2^-/NO $E_{\frac{1}{2}}^{o\prime} = +350$ mV vs NHE (15)) is a more reactive nitrogen oxide than nitrate. Accordingly, we observed slow abiotic oxidation of PCA_{red} by nitrite occurs (Fig 1A, bottom left in grey).

When *C. portucalensis* MBL is incubated with PCA_{red} and nitrate or fumarate, PCA_{red} oxidation proceeds much faster than it does abiotically by nitrite. Furthermore, when *C. portucalensis* MBL is incubated with PCA_{red} and nitrite, there is no observable PCA_{red} oxidation. We interpret this to mean that *C. portucalensis* catalyzes the oxidation of PCA_{red} when an appropriate terminal electron acceptor is available but reduces oxidized PCA (PCA_{ox}) when such an electron acceptor is absent. We observed PCA_{ox} reduction when the cells started with 200 μM PCA_{ox}, but the rate

106 of reduction decreased according to the provided terminal electron acceptor (Fig 1B). Adding
107 fumarate (Fig. 1B, top right) decreased the rate of PCA_{ox} reduction by *C. portucalensis* MBL by
108 2.38 ± 0.12 μM/hr; adding nitrate caused reduction to be non-detectable; and the presence of
109 nitrite, which abiotically oxidized PCA_{red}, caused a slight dampening in the reduction rate by
110 1.25 ± 0.09 μM/hr (Fig. 1C and Supp. Fig. 4). The asymptotic steady state that *C. portucalensis*
111 MBL approaches with fumarate in Fig. 1A and B indicates that the oxidation and reduction of
112 PCA proceed simultaneously. This is further supported by the fact that PCA_{ox} reduction by *C.*
113 *portucalensis* MBL in the presence of fumarate is significantly slower than in the absence of a
114 terminal electron acceptor (Fig. 1C).
115
116 **Comparative study of nitrate- and fumarate-dependent PCA oxidation by several γ-**
117 **proteobacteria.** We assayed whether other γ-proteobacteria can also oxidize PCA in a terminal
118 electron acceptor-dependent manner (Fig. 1). Using the same assay as above, *E. coli MG1655*, *P.*
119 *aeruginosa* UCBPP-PA14 Δ*phz**, *P. chlororaphis phzB::TnluxAB*, and *P. aureofaciens*
120 *phzB::lacZ* (all of which cannot synthesize PCA either naturally or due to the specified
121 mutations) were incubated with PCA and 10 mM nitrate, nitrite, or fumarate. With the exception
122 of *P. chlororaphis* and *aureofaciens*, all strains exhibited PCA oxidation with nitrate, with *C.*
123 *portucalensis* MBL being the fastest with an initial rate of -25.23 ± 0.84 μM PCA_{red}/hr (Fig. 1C).
124 The assayed *P. aureofaciens* and *chlororaphis* oxidized PCA_{red} with nitrite faster than the abiotic
125 control: -2.75 ± 0.80 and -3.10 ± 0.58, respectively, vs. -1.48 ± 0.29 μM PCA_{red}/hr abiotically
126 (Fig. 1C). Only *C. portucalensis* MBL obviously oxidized PCA in the presence of fumarate. *E.*
127 *coli*, like *C. portucalensis* MBL, reduces PCA_{ox} more slowly with fumarate than without it,
128 indicating that it can also oxidize PCA_{red} with fumarate (Fig 1C, light blue), even if not apparent

in Fig. 1A. When the reduction rate by cells was not determined (N.D.), we interpret that the concurrent oxidation rate overwhelmed the reduction rate. None of the strains exhibited significant growth in these assays (Supp. Figs. 1 and 2).

The effect of PCA oxidation on the initial rate of nitrate reduction by *C. portucalensis* MBL. In Fig 1A it is clear that *C. portucalensis* MBL completes its oxidation of ~200 µM PCA_{red} within 10 hours when nitrate is available. We repeated this experiment in anaerobic culture tubes inoculated with *C. portucalensis* MBL, 10 mM NO_3^-, and either 200 µM PCA_{red}, 200 µM PCA_{ox}, or no PCA and measured nitrate and nitrite concentrations over time via ion chromatography. We observed that PCA_{red} oxidation significantly increased the rate of nitrate reduction to nitrite (Fig 2A). Nitrate consumption was stoichiometrically matched by nitrite production. We did not observe the production of any other nitrogen oxides or ammonium (data not shown). The no-PCA control did not show any nitrate reduction over the first eight hours (3 ± 55 µM/hr; 95% confidence interval reported for all rate measurements, calculated from a linear regression of the data from the first two timepoints for each curve in Fig. 2A). During this time, 131 ± 49 µM/hr nitrate was reduced in the PCA_{red} condition. In contrast, the PCA_{ox} control exhibited only a small increase in nitrate reduction (35 ± 35 µM/hr). To verify that nitrate was reduced to nitrite, we tracked nitrite's production. We observed that over the first eight hours the no-PCA control produced nitrite at the rate of 22 ± 3 µM/hr, the PCA_{ox} control at 58 ± 2 µM/hr, and the PCA_{red} condition at 147 ± 44 µM/hr of nitrite over the first eight hours. Thus, we estimate the effect of PCA_{red} vs PCA_{ox} to be 96 ± 60 µM/hr of increased nitrate reduction or 89 ± 44 µM/hr of increased nitrite production. The increase in nitrate reduction due to PCA_{red} was not stoichiometric: PCA redox and nitrate reduction are both two-electron processes (9, 21), and a

process without a redox cycle would predict that oxidizing PCA_{red} at a rate of 25 μM/hr (Fig. 1C) would reduce at most 25 μM/hr nitrate to nitrite. However, the lowest range of the confidence intervals suggests that at least an additional 36 μM/hr nitrate was reduced by the cells when PCA_{red} was provided, implying that the cells are redox-cycling the PCA.

Conclusions. The effect of PCA oxidation by *C. portucalensis* MBL on its rate of nitrate reduction was not stoichiometric (Fig 2A). This is consistent with two explanations: 1) a prior report argues that neutral red (a synthetic phenazine) oxidation affects electrosynthesis during anaerobic respiration primarily by changing gene regulation via menaquinone reduction (16), and so it is plausible that PCA oxidation may increase transcription of a rate-limiting factor in the electron transport chain to nitrate; 2) we observed that a PCA redox cycle by *C. portucalensis* MBL is possible (Fig 1, most evident in the case of fumarate, top right panels in A and B, where oxidation and reduction proceed simultaneously in the steady state); it is likely that PCA redox cycling may also directly stimulate nitrate reduction. We cannot determine whether reduced PCA (PCA_{red}) serves as an effective electron donor to the cells' metabolism from our above observations. Regardless, nitrate reduction driven by PCA redox-cycling has environmental ramifications far beyond the cell.

Despite all being capable of nitrate reduction, not all the γ-proteobacteria that we tested were capable of oxidizing PCA_{red} with nitrate (Fig. 1C), suggesting a genetic basis for this process. The only strains that did not readily oxidize PCA_{red} with nitrate were *P. chlororaphis* and *aureofaciens*, and, unlike the other species, they do not possess the periplasmic nitrate reductase operon *nap*. Curiously, it has been previously noted that wildtype *P. aeruginosa* UCBPP-PA14

oxidizes another phenazine, pyocyanin, in the presence of nitrate, while a *napA* transposon knockout strain does not (22). We propose that this operon may be necessary for rapid PCA_{red} oxidation and are developing genetic tools in *C. portucalensis* MBL to test this idea directly. Nitrate-dependent PCA_{red} oxidation is likely to be common in anoxic environments, such as soils, as it is not species-specific and occurs readily. If the outsized effect of PCA_{red} oxidation on nitrate reduction that we observe generalizes to other species, the production and reduction of phenazines by organisms like *Pseudomonads* likely affects the rate of nitrate consumption in their environs, adding another function to the phenazine arsenal (18). We propose that cells may catalyze a PCA redox cycle whenever they have internal stores of reducing equivalents and a usable terminal electron acceptor (Fig 2B). While both intracellular (e.g., sulfur) and extracellular (e.g., humics) bacterial redox cycles have been described (23, 24), to our knowledge this has not been appreciated for secreted metabolites, such as phenazines. Our observation implies that these molecules may act as "electron buffers", enabling cells to reduce and oxidize them according to whether they are lacking a terminal electron acceptor or an electron donor, respectively, and in so doing significantly impact other biogeochemical cycles.

MATERIALS AND METHODS

Strains and media. *Citrobacter portucalensis* MBL was isolated in our previous work (20). In the comparative PCA oxidation and reduction experiments, we used strains of γ-proteobacteria that cannot synthesize phenazines, either natively or due to mutations. We used *E. coli* MG1655, *P. aeruginosa* UCBPP-PA14 Δ*phz**, *P. chlororaphis phzB::TnLuxAB*, and *P. aureofaciens phzB::lacZ*. The wildtype *Pseudomonads* can synthesize PCA, but the used mutants cannot. All strains were grown and incubated under the same conditions. The basal medium for the

198 experiments contained 20 mM potassium phosphate buffer (final pH 7), 1 mM sodium sulfate, 10
199 mM ammonium chloride, 1x SL-10 trace elements, 1x freshwater salt solution (17.1 mM sodium
200 chloride, 1.97 mM magnesium chloride, 0.68 mM calcium chloride, and 6.71 mM potassium
201 chloride), and 1x 13-vitamin solution (10 µM MOPS pH 7.2, 0.1 µg/mL riboflavin, 0.03 µg/mL
202 biotin, 0.1 µg/mL thiamine HCl, 0.1 µg/mL L-ascorbic acid, 0.1 µg/mL d-Ca-pantothenate, 0.1
203 µg/mL folic acid, 0.1 µg/mL nicotinic acid, 0.1 µg/mL 4-aminobenzoic acid, 0.1 µg/mL
204 pyridoxine HCl, 0.1 µg/mL lipoic acid, 0.1 µg/mL NAD, 0.1 µg/mL thiamine pyrophosphate,
205 and 0.01 µg/mL cyanocobalamin). Depending on the experimental condition, as indicated in the
206 figure legends, a terminal electron acceptor would be added (10 mM of fumarate, nitrate, or
207 nitrite) or omitted. For oxidized PCA, a 10 mM stock in 20 mM NaOH was prepared. For
208 reduced PCA, an 800 µM stock in the basal medium was reduced by electrolysis. Both stocks
209 were diluted into plates to a final target concentration of 200 µM.
210
211 **Cell preparation.** All cell incubations and experiments were performed at 30 C. Cells were
212 preserved in 35% glycerol stocks at -80 C. Two days prior to the experiments, frozen cells for
213 each strain assayed were struck out on lysogeny broth (LB) agar plates and incubated overnight.
214 The evening prior to the experiment, a patch from the streaks was inoculated into liquid LB in a
215 respective culture tube and incubated slanted, shaking at 250 rpm, overnight. The morning of the
216 experiment, 1 mL of each cell culture was washed three times into the basal medium by spinning
217 for two minutes at 6000 x g, aspirating the supernatant, and gently resuspending with a pipette.
218 The OD_{600} of each washed culture was measured. The cultures were brought into a Coy glove
219 box, where they were washed three times into the same basal medium that had been made

anoxic, following the same procedure as above. After being left to stand for 1-2 hours, the cells were inoculated into the different experimental conditions at a target starting OD_{600} of 0.1.

Measurement of PCA redox and nitrogen oxide concentrations. All PCA redox measurements were performed in a Coy chamber using a BioTek Synergy 5 plate reader. Reduced PCA concentration was measured by fluorescence (excitation 360 nm and emission 528 nm) (25). In these cultures, 1 mM acetate was provided. Plates were incubated shaking on the "medium" setting. For nitrogen oxide concentration measurements, *C. portucalensis* MBL cells were prepared as above, but incubated in culture tubes in the Coy chamber to allow for sampling. In these cultures, no acetate was provided. The tubes were kept at 30 C, but not shaking. Samples were filtered through a 0.2 µm cellulose-acetate spin filter and stored at -80 C prior to analysis. Nitrate and nitrite concentrations were measured by ion chromatography using a Dionex ICS-2000 instrument.

Data analysis. Initial redox rates in the conditions with supplied nitrite did not include the first 1.5 hours of the assay because that period showed a systematic artifact due to fluorescence quenching (Fig. 1A, lower left panel). The linear fits were calculated over the first five hours after the first detectable PCA_{red} measurement, which was determined to be appropriate based on scanning for R^2 values over increasing time frames (Supp. Figs. 4 and 5). 95% confidence intervals are calculated as the estimated value ± 1.96 * (standard error). When comparing rates, the reported error is the geometric mean of the intervals for the two measurements. All plots were generated using Bokeh and the legends and titles were adjusted using Inkscape. All the raw

data and the Jupyter notebook used for their analysis are available at https://github.com/ltsypin/Cportucalensis_observation.

ACKNOWLEDGEMENTS

We would like to thank the members of the Newman lab, and especially Scott Saunders, Darcy McRose, Avi Flamholz, John Ciemniecki, Chelsey VanDrisse, and Justin Bois for their insight and helpful discussions throughout this work. We are grateful to Nathan Dalleska at the Environmental Analysis Center at Caltech for training LMT on the Dionex instrument and providing a facility for analytical chemistry. LMT was supported by the Rosen Endowment Fellowship at Caltech and the National Science Foundation Graduate Research Fellowship (DGE-1745301). Additional support to DKN came from NIH (1R01AI127850-01A1 and 1R01HL152190-01) and ARO (W911NF-17-1-0024) grants.

REFERENCES

1. Rothberger CJ. 1898. Differential diagnostische Untersuchungen mit gefarbten Nahrboden. Centralblatt für Bakteriologie, Parasitenkunde und Infektionskrankheiten 24:513–518.
2. Friedheim EA. 1931. Pyocyanine, an accessory respiratory enzyme. J Exp Med 54:207–221.
3. Baron SS, Terranova G, Rowe JJ. 1989. Molecular mechanism of the antimicrobial action of pyocyanin. Curr Microbiol 18:223–230.
4. Dietrich LEP, Price-Whelan A, Petersen A, Whiteley M, Newman DK. 2006. The phenazine pyocyanin is a terminal signalling factor in the quorum sensing network of *Pseudomonas aeruginosa*. Mol Microbiol 61:1308–1321.
5. Glasser NR, Kern SE, Newman DK. 2014. Phenazine redox cycling enhances anaerobic survival in *Pseudomonas aeruginosa* by facilitating generation of ATP and a proton-motive force. Mol Microbiol 92:399–412.
6. Gu M, Imlay JA. 2011. The SoxRS response of *Escherichia coli* is directly activated by redox-cycling drugs rather than by superoxide. Mol Microbiol 79:1136–1150.
7. Dietrich LEP, Kiley PJ. 2011. A shared mechanism of SoxR activation by redox-cycling compounds. Mol Microbiol 79:1119–1122.
8. Wang Y, Wilks JC, Danhorn T, Ramos I, Croal L, Newman DK. 2011. Phenazine-1-carboxylic acid promotes bacterial biofilm development via ferrous iron acquisition. J Bacteriol 193:3606–3617.
9. Wang Y, Newman DK. 2008. Redox reactions of phenazine antibiotics with ferric (hydr)oxides and molecular oxygen. Environ Sci Technol 42:2380–2386.
10. Saunders SH, Tse ECM, Yates MD, Otero FJ, Trammell SA, Stemp EDA, Barton JK,

Tender LM, Newman DK. 2020. Extracellular DNA promotes efficient extracellular electron transfer by pyocyanin in *Pseudomonas aeruginosa* biofilms. Cell 182:919–932.e19.

11. Rabaey K, Boon N, Höfte M, Verstraete W. 2005. Microbial phenazine production enhances electron transfer in biofuel cells. Environ Sci Technol 39:3401–3408.

12. Pham TH, Boon N, Aelterman P, Clauwaert P, De Schamphelaire L, Vanhaecke L, De Maeyer K, Höfte M, Verstraete W, Rabaey K. 2008. Metabolites produced by *Pseudomonas sp.* enable a Gram-positive bacterium to achieve extracellular electron transfer. Appl Microbiol Biotechnol 77:1119–1129.

13. Park DH, Zeikus JG. 1999. Utilization of electrically reduced neutral red by *Actinobacillus succinogenes*: physiological function of neutral red in membrane-driven fumarate reduction and energy conservation. J Bacteriol 181:2403–2410.

14. Hongo M, Iwahara M. 1979. Application of electro-energizing method to L-glutamic acid fermentation. Agric Biol Chem 43:2075–2081.

15. Thauer RK, Jungermann K, Decker K. 1977. Energy conservation in chemotrophic anaerobic bacteria. Bacteriol Rev 41:100–180.

16. Harrington TD, Tran VN, Mohamed A, Renslow R, Biria S, Orfe L, Call DR, Beyenal H. 2015. The mechanism of neutral red-mediated microbial electrosynthesis in *Escherichia coli*: menaquinone reduction. Bioresour Technol 192:689–695.

17. Blankenfeldt W, Parsons JF. 2014. The structural biology of phenazine biosynthesis. Curr Opin Struct Biol 29:26–33.

18. Dar D, Thomashow LS, Weller DM, Newman DK. 2020. Global landscape of phenazine biosynthesis and biodegradation reveals species-specific colonization patterns in

agricultural soils and crop microbiomes. eLife 9.

19. Feng J, Qian Y, Wang Z, Wang X, Xu S, Chen K, Ouyang P. 2018. Enhancing the performance of *Escherichia coli*-inoculated microbial fuel cells by introduction of the phenazine-1-carboxylic acid pathway. J Biotechnol 275:1–6.

20. Tsypin LM, Saunders SH, Bar-On Y, Leadbetter JR, Newman DK. 2020. Draft genome sequence of the redox-active enteric bacterium *Citrobacter portucalensis* strain MBL. Microbiol Resour Announc 9.

21. Sparacino-Watkins C, Stolz JF, Basu P. 2014. Nitrate and periplasmic nitrate reductases. Chem Soc Rev 43:676–706.

22. Price-Whelan AM. 2009. PhD Thesis: Physiology and mechanisms of pyocyanin reduction in *Pseudomonas aeruginosa*. California Institute of Technology.

23. Schmidt TM, Arieli B, Cohen Y, Padan E, Strohl WR. 1987. Sulfur metabolism in *Beggiatoa alba*. J Bacteriol 169:5466–5472.

24. Coates JD, Cole KA, Chakraborty R, O'Connor SM, Achenbach LA. 2002. Diversity and ubiquity of bacteria capable of utilizing humic substances as electron donors for anaerobic respiration. Appl Environ Microbiol 68:2445–2452.

25. Sullivan NL, Tzeranis DS, Wang Y, So PTC, Newman D. 2011. Quantifying the dynamics of bacterial secondary metabolites by spectral multiphoton microscopy. ACS Chem Biol 6:893–899.

Main text figure legends

Figure 1. Oxidation and reduction of PCA by bacteria provided different terminal electron acceptors (TEAs). (A-B) The light circles correspond to three independent biological replicates and the dark lines to their respective means. Cells only oxidize PCA when an appropriate TEA is available. Fumarate, nitrite, or nitrate (top right, bottom left, and bottom right panels, respectively) stimulate different strains to oxidize PCA. When no TEA is provided (top left panels), no strains oxidize PCA and all reduce it at different rates (enumerated in C). With nitrate, only *P. chlororaphis* and *aureofaciens* (blue and umber curves) appear to not oxidize PCA but reduce it. Nitrite abiotically oxidizes PCA (grey curve, lower left in A), but *P. aureofaciens and chlororaphis* catalyze an even faster biological oxidation. In contrast, the *Enterics* (yellow and orange curves) reduce PCA faster than the abiotic reaction. Given fumarate, *C. portucalensis* MBL (orange) clearly oxidizes PCA, and the *Pseudomonads* reduce it. (C) This table reports the estimated initial rates of oxidation or reduction according to a linear fit over the first five hours (Supp. Figs. 3 and 4). This timeframe was determined by tracking the R^2 for the linear fit over increasing time windows (Supp. Figs. 5 and 6). N.D. signifies rates that were not detectable. We can only observe the relative magnitude of the rates of oxidation and reduction in a given condition. However, whether the oxidation of PCA overpowers its reduction depends on both the provided TEA and is associated with the presence of the periplasmic nitrate reductase (NAP) or fumarate reductase (FRD). PCA reduction is calculated as a positive rate (shades of red); PCA oxidation is calculated as a negative rate (shades of blue).

Figure 2. PCA oxidation by *C. portucalensis* MBL increases its initial rate of nitrate reduction. (A) Either 200 μM reduced PCA (PCA_{red}), 200 μM oxidized PCA (PCA_{ox}), or no

PCA was added to each condition. Ion chromatography shows that over the 10 hours that the cells are oxidizing PCA_{red} (Fig. 1A), their initial rate of nitrate reduction is substantially increased. The nitrate is stoichiometrically reduced to nitrite. Error bars are 95% confidence intervals around the mean of three independent biological replicates. When not visible, they are smaller than the circles (nitrate) or squares (nitrite) denoting the measurements. (B) Any cell that has internal stores of reducing equivalents and an appropriate terminal electron acceptor (TEA_1) may catalyze an internal PCA redox cycle (bolded arrows). We observe this for *E. coli* and *C. portucalensis* MBL with nitrate or fumarate, *P. aeruginosa* with nitrate, and *P. aureofaciens* and *chlororaphis* with nitrite. In addition to this cellularly-catalyzed reaction, the product of TEA_1's reduction (TEA_2) may abiotically oxidize PCA, which we observe when nitrate is reduced to nitrite (Fig. 1A, bottom left).

358 **Supplementary Figure Legends**

359 **Supplementary Figure 1.** OD_{600} measurements for all strains in all conditions during the
360 oxidation assays (Fig. 1A). Light circles represent all measurements from three independent
361 biological replicates corresponding to the PCA_{red} measurements in Figure 1A. The solid lines are
362 the means of the replicates. The curves are colored by condition (i.e., which, if any, terminal
363 electron acceptor was added). While there are some OD_{600} trends in the first five hours, there is
364 no evidence of growth that would indicate a doubling. In the abiotic graph, all the data points
365 overlie each other.

366

367 **Supplementary Figure 2.** OD_{600} measurements for all strains in all conditions during the
368 reduction assays (Fig. 1B). Light circles represent all measurements from three independent
369 biological replicates corresponding to the PCA_{red} measurements in Figure 1B. The solid lines are
370 the means of the replicates. The curves are colored by condition (i.e., which, if any, terminal
371 electron acceptor was added). While there are some OD_{600} trends in the first five hours, there is
372 no evidence of growth that would indicate a doubling. In the abiotic graph, all the data points
373 overlie each other.

374

375 **Supplementary Figure 3.** Oxidation assay results grouped by strain rather than by condition.
376 This is a different representation of the same data as in Fig. 1A, including the linear regressions
377 (dashed lines) that generated the initial rate estimates as reported in Fig. 1Cα.

378

379 **Supplementary Figure 4.** Reduction assay results grouped by strain rather than by condition.
380 This is a different representation of the same data as in Fig. 1B, including the linear regressions

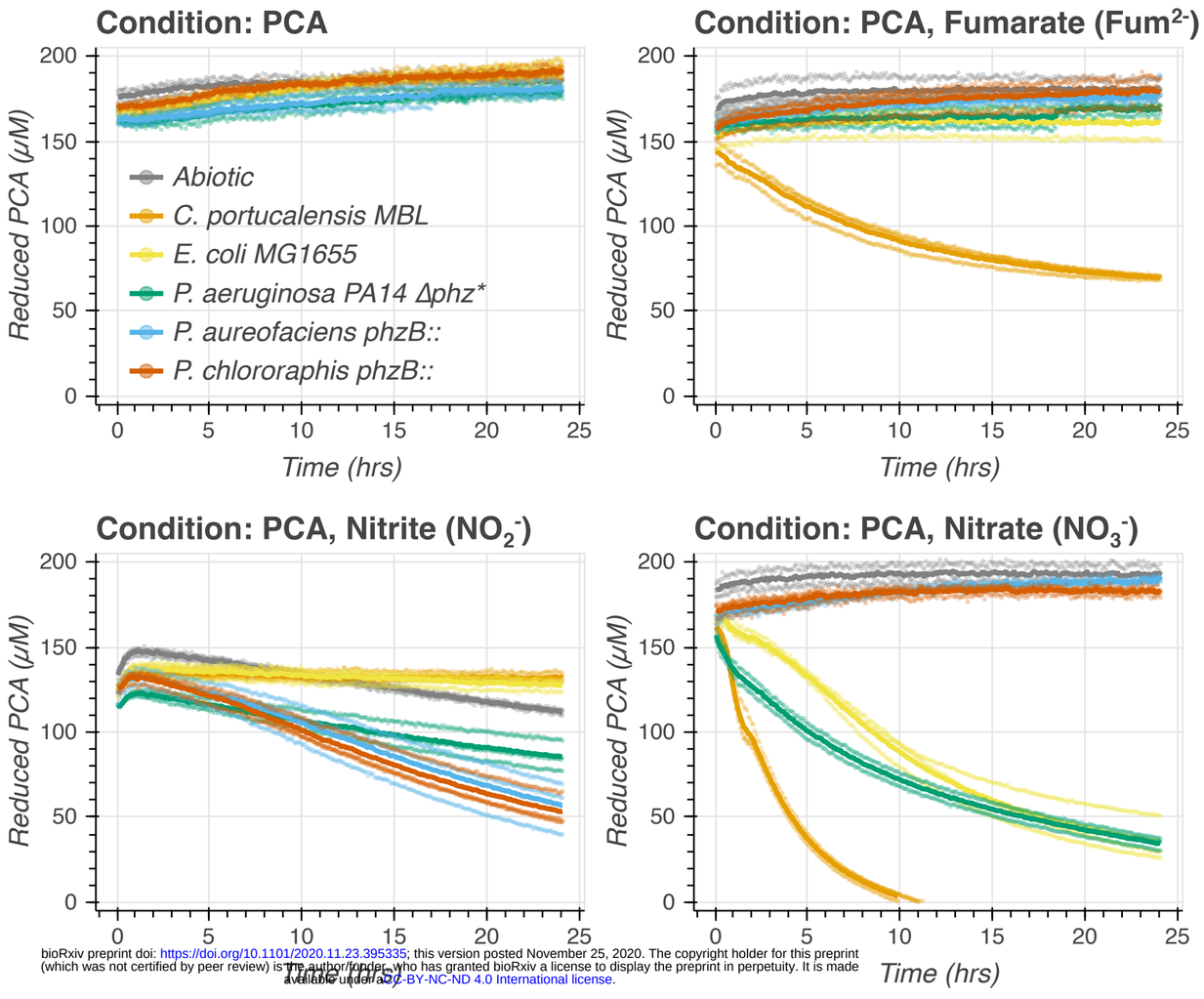
(dashed lines) that generated the initial rate estimates as reported in Fig. 1Cβ. In the abiotic conditions, no reduction was detected (N.D.).

Supplementary Figure 5. Evaluation of the appropriate window for performing the linear regressions to estimate initial redox rates in the oxidation assays (Fig. 1Cα). Starting from the first valid PCA_{red} measurement in each condition, a linear regression was performed over increasing lengths of time and the corresponding R^2 value was plotted. A time window of five hours (red line) was selected as a systematic compromise for all conditions.

Supplementary Figure 6. Evaluation of the appropriate window for performing the linear regressions to estimate initial redox rates in the reduction assays (Fig. 1Cβ). Starting from the first valid PCA_{red} measurement in each condition, a linear regression was performed over increasing lengths of time and the corresponding R^2 value was plotted. A time window of five hours (red line) was selected as a systematic compromise for all conditions.

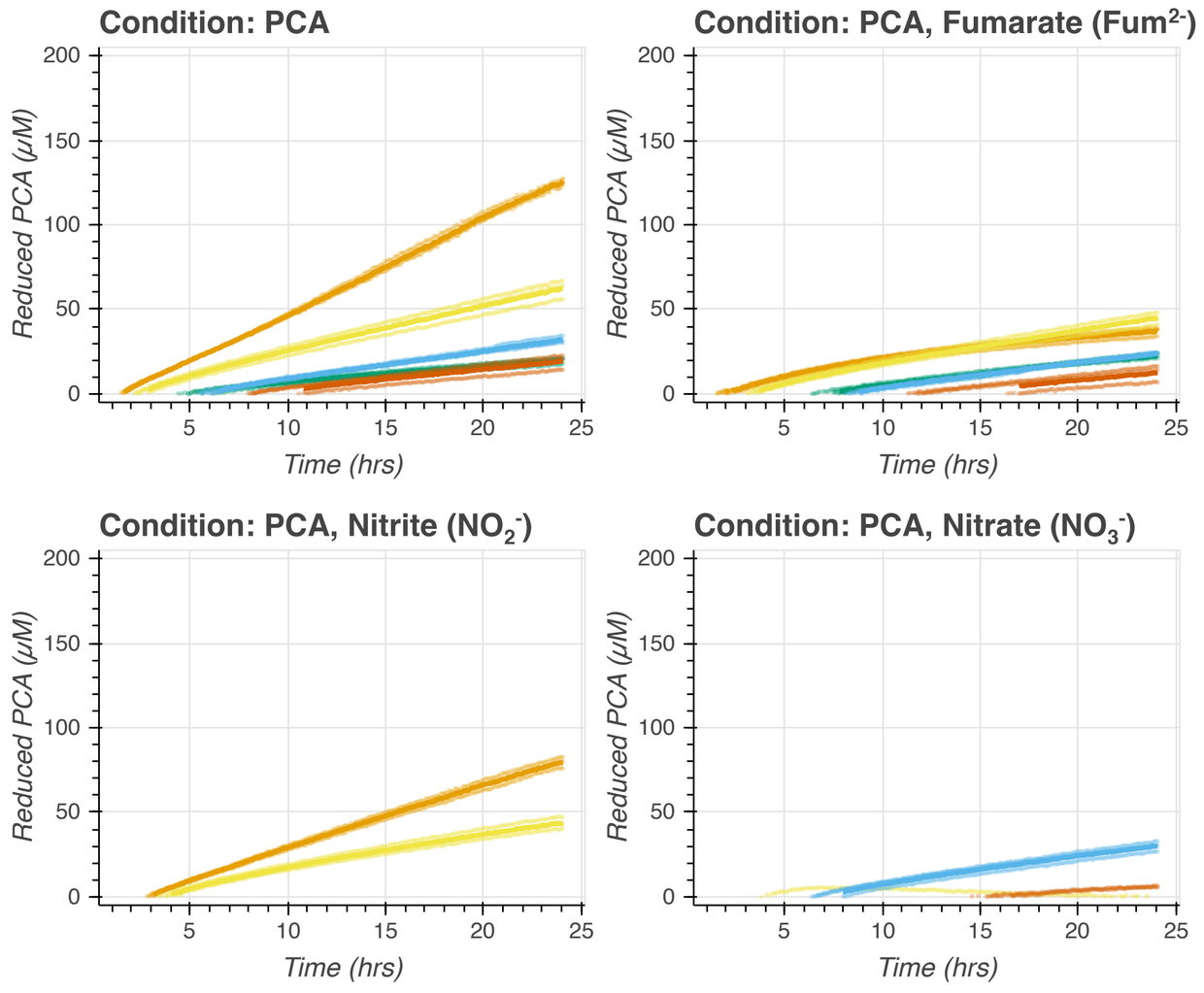
A

Initial Condition: All reduced PCA (oxidation assay)



B

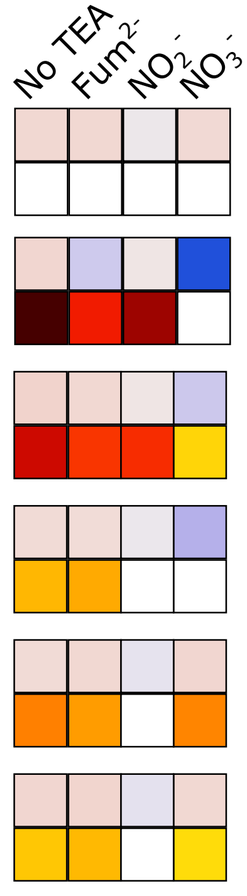
Initial Condition: All oxidized PCA (reduction assay)



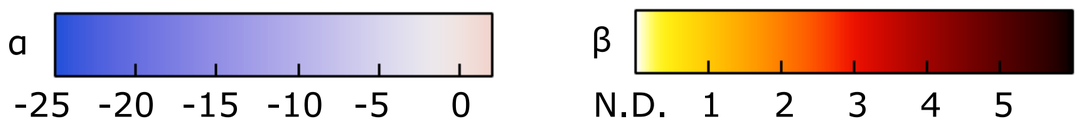
C

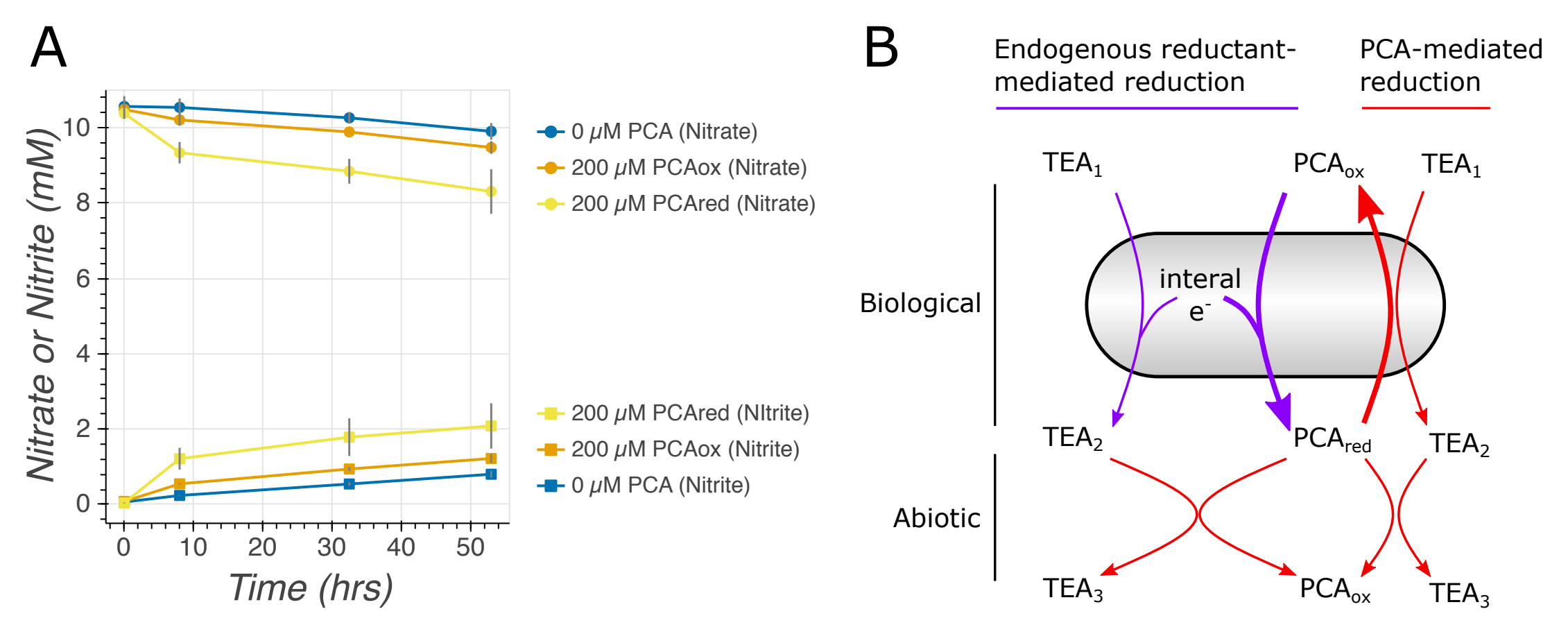
Initial redox rates (μM PCA_{red}/hr) ± 95% confidence interval:
(α) oxidation assay; (β) reduction assay

Strain	FRD?	NAP?	PCA alone	PCA with Fum^{2-}	PCA with NO_2^-	PCA with NO_3^-
Abiotic	-	-	(α) 1.36 ± 0.32 (β) N.D.	(α) 1.41 ± 0.63 (β) N.D.	(α) -1.48 ± 0.29 (β) N.D.	(α) 1.26 ± 0.45 (β) N.D.
C. portucalensis MBL	+	+	(α) 1.45 ± 0.15 (β) 5.30 ± 0.05	(α) -6.49 ± 0.50 (β) 2.92 ± 0.11	(α) -0.41 ± 0.39 (β) 4.05 ± 0.07	(α) -25.23 ± 0.84 (β) N.D.
E. coli MG1655	+	+	(α) 1.87 ± 0.21 (β) 3.42 ± 0.16	(α) 1.43 ± 0.57 (β) 2.70 ± 0.12	(α) -0.50 ± 0.60 (β) 2.78 ± 0.13	(α) -6.70 ± 0.27 (β) 0.80 ± 0.15
P. aeruginosa PA14 *Δphz**	-	+	(α) 0.93 ± 0.32 (β) 1.24 ± 0.13	(α) 0.80 ± 0.34 (β) 1.44 ± 0.13	(α) -1.84 ± 0.42 (β) N.D.	(α) -10.09 ± 0.42 (β) N.D.
P. aureofaciens phzB::	-	-	(α) 1.02 ± 0.23 (β) 1.97 ± 0.08	(α) 1.41 ± 0.26 (β) 1.63 ± 0.09	(α) -2.75 ± 0.80 (β) N.D.	(α) 1.49 ± 0.21 (β) 1.91 ± 0.18
P. chlororaphis phzB::	-	-	(α) 1.52 ± 0.16 (β) 1.02 ± 0.19	(α) 1.79 ± 0.50 (β) 1.22 ± 0.07	(α) -3.10 ± 0.58 (β) N.D.	(α) 1.33 ± 0.35 (β) 0.71 ± 0.09



Color maps for initial redox rates (μM PCA_{red}/hr)




A
Nitrate or Nitrite (mM)
Time (hrs)
0 μM PCA (Nitrate)
200 μM PCAox (Nitrate)
200 μM PCAred (Nitrate)
200 μM PCAred (NItrite)
200 μM PCAox (Nitrite)
0 μM PCA (Nitrite)
B
Endogenous reductant-mediated reduction
PCA-mediated reduction
Biological
Abiotic
TEA1
PCAox
TEA1
interal e-
TEA2
PCAred
TEA2
TEA3
PCAox
TEA3