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OBSERVATION

Nitrate Reduction Stimulates and Is Stimulated by Phenazine-1-Carboxylic Acid Oxidation by *Citrobacter portucalensis* MBL

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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. When *C. portucalensis* MBL is provided reduced PCA and nitrate, it oxidizes the PCA at a rate that is environmentally relevant. We compared this terminal electron acceptor-dependent PCA-oxidizing activity of *C. portucalensis* MBL to that of several other gammaproteobacteria with various capacities to respire nitrate. We found that PCA oxidation by these strains in a nitrate-dependent manner is decoupled from growth and strain dependent. We infer that bacterial PCA oxidation is widespread and genetically determined. Notably, oxidizing PCA enhances the rate of nitrate reduction to nitrite by *C. portucalensis* MBL beyond the stoichiometric exchange of electrons from PCA to nitrate, 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 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.

KEYWORDS *Citrobacter*, biological oxidation, denitrification, nitrate reduction, phenazines, redox cycling

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

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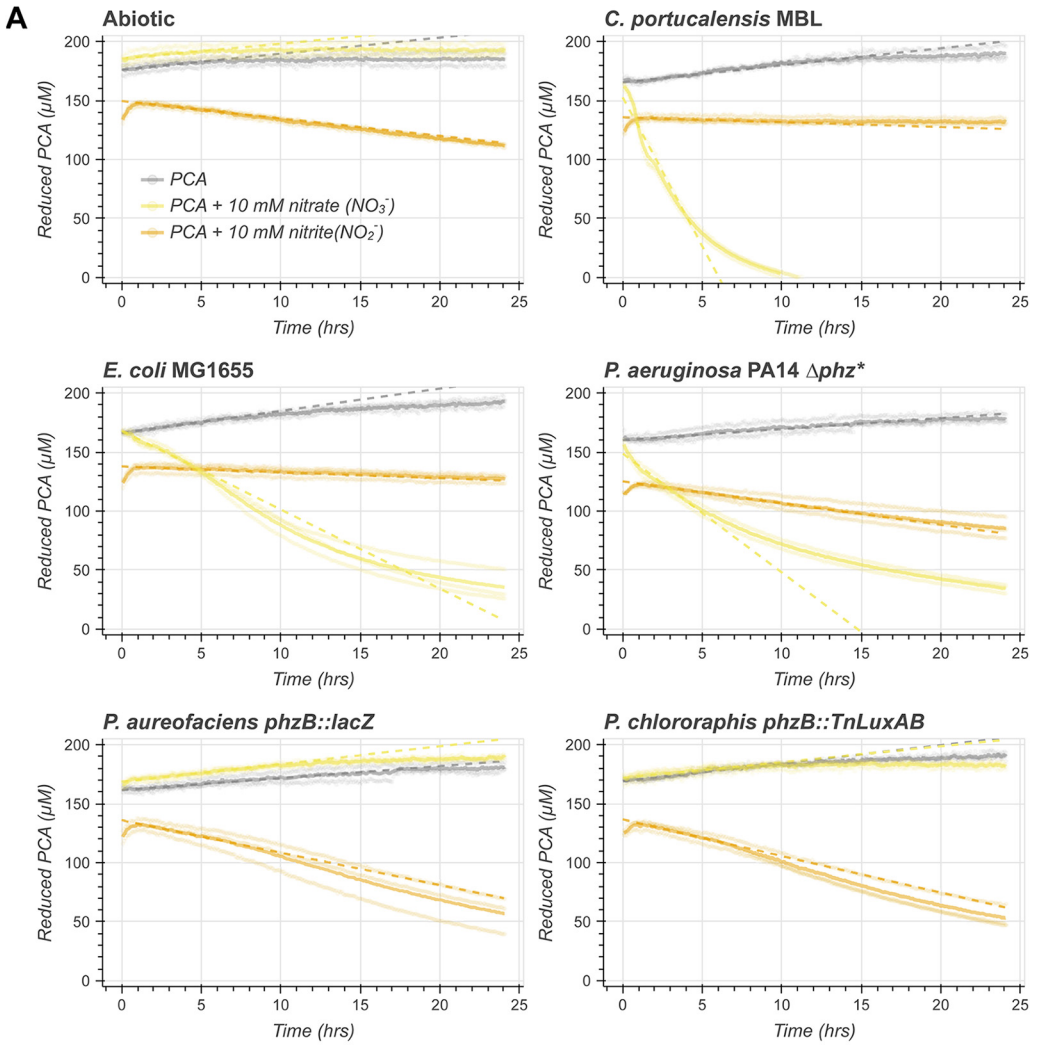
by *Pseudomonas aeruginosa*, was described as an "accessory respiratory pigment" that increased the rate of oxygen consumption by *Staphylococcus*, *Pneumococcus*, and erythrocytes by shuttling electrons from the cells to oxygen (2). Once it became apparent that phenazines can have cytotoxic effects, 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: (i) regulating gene expression in *P. aeruginosa* during quorum sensing by oxidizing a transcription factor, (ii) acting as alternative terminal electron acceptors to promote anoxic survival, and (iii) 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 reoxidized 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 (BER) 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^+, $E^{o'}_{\frac{1}{2}} = -320$ mV versus Normal Hydrogen Electrode [NHE]) and hydrophobicity (13–15). Anaerobic NR oxidation in BERs has been coupled to the reduction of several terminal electron acceptors, including nitrate (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.

Phenazine-1-carboxylic acid (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 reoxidized 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 nonfermentable carbon source), and nitrate as the only terminal electron acceptor, and successfully isolated the PCA-oxidizing *Citrobacter portucalensis* MBL, which is unable to synthesize its own phenazines (20).

In this Observation, we performed three sets of experiments to study biological redox transformations of PCA: (i) an oxidation assay in which reduced PCA (PCA_{red}) was incubated with or without cells and with or without a terminal electron acceptor (Fig. 1), (ii) a reduction assay in which oxidized PCA (PCA_{ox}) was incubated with or without cells and with or without a terminal electron acceptor (see Fig. S1 in the supplemental material), and (iii) an ion chromatography experiment in which we measured the reduction of nitrate to nitrate by *C. portucalensis* MBL depending on $PCA_{red/ox}$ availability (Fig. 2A).

***C. portucalensis* MBL oxidizes PCA in a nitrate-dependent manner.** We did not observe PCA (PCA_{red}/PCA_{ox} $E^{o'}_{\frac{1}{2}} = -116$ mV versus NHE [9]) oxidation in the absence of a terminal electron acceptor in either the abiotic and biotic regimes (Fig. 1A, gray curves; Fig. 1B, PCA-only column). When 10 mM nitrate (NO_3^-/NO_2^- $E^{o'}_{\frac{1}{2}} = +433$ mV versus NHE [15]) was added (right panels), *C. portucalensis* MBL readily oxidized PCA_{red} at an initial rate of $-25.23 \pm 0.84\,\mu$M/h (Fig. 1A, top right panel, yellow curve, and Fig. 1B). Nitrate did not oxidize PCA abiotically (Fig. 1A, top left panel, yellow curve). During both dissimilatory and assimilatory nitrate reduction, nitrate is first reduced to nitrite (21). Nitrite (NO_2^-/NO $E^{o'}_{\frac{1}{2}} = +350$ mV versus NHE [15]) drove slow abiotic oxidation of PCA_{red} at a steady rate of $-1.48 \pm 0.29\,\mu$M/h after an initial short period of reduction (Fig. 1A, top left panel, orange curve, and Fig. 1B). This abiotic



B

Strain	Initial PCA redox rate (μM/hr): positive = reduction; negative = oxidation		
	PCA	PCA + NO_2^-	PCA + NO_3^-
Abiotic	1.36 +/- 0.32	-1.48 +/- 0.29	1.26 +/- 0.45
C. portucalensis MBL	1.45 +/- 0.15	-0.41 +/- 0.39	-25.23 +/- 0.84
E. coli MG1655	1.87 +/- 0.21	-0.50 +/- 0.60	-6.70 +/- 0.27
P. aeruginosa PA14 Δ*phz**	0.93 +/- 0.32	-1.84 +/- 0.42	-10.09 +/- 0.42
P. aureofaciens phzB::lacZ	1.02 +/- 0.23	-2.75 +/- 0.80	1.49 +/- 0.21
P. chlororaphis phzB::TnLuxAB	1.52 +/- 0.16	-3.10 +/- 0.58	1.33 +/- 0.35

FIG 1 Oxidation of PCA_{red} by bacteria provided different terminal electron acceptors (TEAs). (A) The light circles correspond to three independent biological replicates, and the dark lines to their respective means. Cells oxidize PCA only when an appropriate TEA is available. Nitrate and nitrite (yellow and orange curves, respectively) stimulate different strains to oxidize PCA_{red}. When no TEA is provided (gray curves), no strains oxidize PCA_{red}. With nitrate, only *P. chlororaphis* and *P. aureofaciens* appear to not oxidize PCA_{red}. Nitrite abiotically oxidizes PCA_{red} (orange curve, top left panel), but *P. aureofaciens and chlororaphis* catalyze an even faster biological oxidation. In contrast, the enterics (*C. portucalensis* MBL and *E. coli* MG1655)

(Continued on next page)

rate of oxidation is insufficient to be responsible for the rate of PCA_{red} oxidation by cells in the presence of nitrate.

When *C. portucalensis* MBL is incubated with PCA_{red} and nitrite, there is less PCA_{red} oxidation than in the abiotic case (Fig. 1A, top right panel, orange curve). We interpret this to mean that *C. portucalensis* catalyzes the oxidation of PCA_{red} when an appropriate terminal electron acceptor is available but reduces 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 of reduction decreased according to the provided terminal electron acceptor (Fig. S1). Adding nitrate to the PCA_{ox} condition caused reduction to be nondetectable, and the presence of nitrite, which abiotically oxidizes PCA_{red}, caused a slight dampening in the reduction rate by 1.25 $\pm$ 0.09 μM/h (Fig. S1).

Comparative study of nitrate-dependent PCA oxidation by several gammaproteobacterial. We assayed whether other gammaproteobacteria can also oxidize PCA in a terminal electron acceptor-dependent manner (Fig. 1). Using the same assay as described above, *Escherichia coli* MG1655, *P. aeruginosa* UCBPP-PA14 Δ*phz**, *Pseudomonas chlororaphis phzB*::*TnluxAB*, and *Pseudomonas aureofaciens phzB*::*lacZ* (all of which cannot synthesize PCA either naturally or due to the specified mutations) were incubated with PCA and 10 mM nitrate or nitrite, or no terminal electron acceptor. With the exception of *P. chlororaphis* and *P. aureofaciens*, all strains exhibited PCA oxidation with nitrate, with *C. portucalensis* MBL being the fastest (Fig. 1B). The assayed *P. aureofaciens* and *P. chlororaphis* oxidized PCA_{red} with nitrite faster than the abiotic control: -2.75 ± 0.80 and -3.10 ± 0.58 μM/h, respectively, versus -1.48 ± 0.29 μM/h abiotically (Fig. 1B). The reduction assay for the other strains reflected our observations with *C. portucalensis* MBL: when a terminal electron acceptor that stimulated PCA_{red} oxidation was present, no reduction was observed (Fig. S1). None of the strains exhibited significant growth in these assays (Fig. S2 and S3).

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 h 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 8 h (3 $\pm$ 55 μM/h; 95% confidence interval reported for all rate measurements, calculated from a linear regression of the data from the first two time points for each curve in Fig. 2A). During this time, 131 $\pm$ 49 μM/h nitrate was reduced in the PCA_{red} condition. In contrast, the PCA_{ox} control exhibited only a putative increase in nitrate reduction (35 $\pm$ 35 μM/h). To verify that nitrate was reduced to nitrite, we tracked nitrite production. We observed that over the first 8 h the no-PCA control produced nitrite at the rate of 22 $\pm$ 3 μM/h, the PCA_{ox} control at 58 $\pm$ 2 μM/h, and the PCA_{red} condition at 147 $\pm$ 44 μM/h of nitrite over the first 8 h. Thus, we estimate the effect of PCA_{red} versus PCA_{ox} to be 96 $\pm$ 60 μM/h of increased nitrate reduction or 89 $\pm$ 44 μM/h of increased nitrite production. The increase in nitrate reduction due to PCA_{red} was greater than the absolute number of electrons the PCA_{red} could provide: 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/h (Fig. 1B) would reduce at most 25 μM/h nitrate to nitrite. However, the lowest range of the confidence intervals suggests that at least an extra

FIG 1 Legend (Continued)

reduce PCA_{ox} faster than the abiotic reaction with nitrite can compensate. The dashed lines correspond to the linear fits reported in panel B. (B) This table reports the estimated initial rates of oxidation according to a linear fit over the first 5 h. This time frame was determined by tracking the R^2 for the linear fit over increasing time windows (see Fig. S4 in the supplemental material). PCA_{ox} reduction is calculated as a positive rate; PCA_{red} oxidation is calculated as a negative rate.

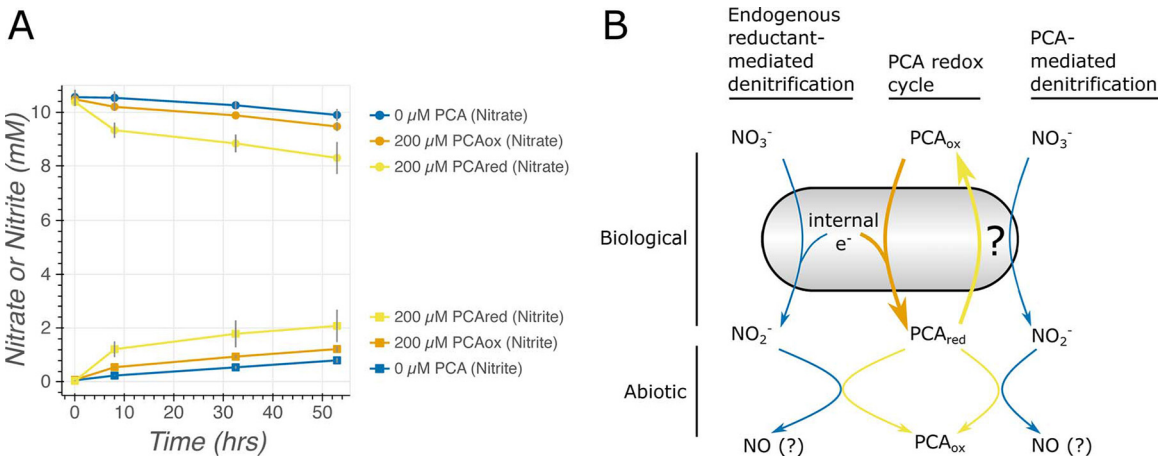


FIG 2 PCA oxidation by *C. portucalensis* MBL increases its initial rate of nitrate reduction. (A) Either 200 μM reduced PCA (PCA_{red}), 200 μM oxidized PCA (PCA_{ox}), or no PCA was added to each condition. Ion chromatography shows that over the 10 h that the *C. portucalensis* MBL cells are oxidizing 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 values of three independent biological replicates. When not visible, the intervals are smaller than the circles (nitrate) or squares (nitrite) denoting the measurements. (B) Blue arrows denote denitrification, yellow arrows denote observed paths of PCA_{red} oxidation, and the orange arrow denotes the observed path of PCA_{ox} reduction. Any cell that has internal stores of reducing equivalents and an appropriate terminal electron acceptor (for *C. portucalensis* MBL, *E. coli* MG1655, and *P. aeruginosa* Δ*phz** in our experiments—nitrate) may catalyze an internal PCA redox cycle (thick arrows). *P. aureofaciens* and *P. chlororaphis* may also do this with nitrite. In addition to this cellularly catalyzed reaction, the product of nitrate's reduction (nitrite) may abiotically oxidize PCA (Fig. 1A, top left panel, orange curve). The nature of the biological oxidation of PCA coupled to nitrate reduction remains unknown but will be amenable to genetic experiments.

36 μM/h nitrate was reduced by the cells when PCA_{red} was provided, implying that PCA is stimulating nitrate reduction by some other means than a one-to-one electron donation; our data showing PCA reduction capability (Fig. S1) suggests that the cells may be cycling PCA under these conditions, though this conclusion awaits definitive demonstration.

Conclusions. The effect of PCA oxidation by *C. portucalensis* MBL on its rate of nitrate reduction was outsized (Fig. 2A). This is consistent with two explanations. (i) 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. (ii) We observed that a PCA redox cycle by *C. portucalensis* MBL is possible: the cells naturally reduce PCA_{ox}, but this is not detectable while nitrate is present, potentially meaning that the PCA is being reoxidized as soon as it is reduced. If this is the case, it is possible that PCA redox cycling may also shunt more electrons to nitrate reduction. An alternative explanation for why PCA_{ox} reduction is not observed in the presence of nitrate is that the cells are preferentially reducing nitrate instead of PCA. While we cannot directly test this hypothesis given our current experimental set up, this will be possible once we develop methods to genetically modify *C. portucalensis* MBL. We have not been able to determine whether reduced PCA (PCA_{red}) serves as an effective electron donor to the cell's metabolism from our above observations. Even if there is no direct physiological benefit from PCA oxidation, that cells can catalyze this reaction and stimulate nitrate reduction has environmental ramifications far beyond the cell.

Despite all being capable of nitrate reduction, not all the gammaproteobacteria that we tested were capable of oxidizing PCA_{red} with nitrate (Fig. 1), suggesting certain enzymes are responsible for this process. We are developing genetic tools in *C. portucalensis* MBL to test this 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. Our observed rate of PCA_{red} oxidation (25 μM/h) is significant relative to the measured concentration and half-life of PCA in the rhizosphere (~2.5 μM and

~3.5 days, respectively) (22). Allowing that the conditions of our experiments may have resulted in an overestimate of the natural PCA_{red} oxidation rate in the environment, even if the laboratory PCA_{red} oxidation rate is orders of magnitude faster than in nature, this process would still be environmentally relevant. Furthermore, if the outsized effect of PCA_{red} oxidation on nitrate reduction that we observed generalizes to other species, the production and reduction of phenazines by organisms like pseudomonads likely affect 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., humic) bacterial redox cycles have been described (23, 24), to our knowledge this has not been appreciated for secreted redox active metabolites, such as phenazines. Our observation underscores the possibility 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, such as the nitrogen cycle.

Materials and methods. (i) 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 gammaproteobacteria that cannot synthesize phenazines, either natively or due to mutations. We used *E. coli* MG1655, *P. aeruginosa* UCBPP-PA14 Δ*phz** (25), *P. chlororaphis phzB*::*TnLuxAB* (strain PCL1119 obtained from G. Bloemberg, Leiden University [26]), and *P. aureofaciens phzB*::*lacZ* (strain 30-84Z obtained from L. Pierson, University of Arizona [27]). The wild-type pseudomonads can synthesize PCA, but the mutants used cannot. All strains were grown and incubated under the same conditions. The basal medium for the experiments contained 20 mM potassium phosphate buffer (final pH 7), 1 mM sodium sulfate, 10 mM ammonium chloride, 1× SL-10 trace elements, 1× freshwater salt solution (17.1 mM sodium chloride, 1.97 mM magnesium chloride, 0.68 mM calcium chloride, and 6.71 mM potassium chloride), and 1× 13-vitamin solution (10 μM morpholinepropanesulfonic acid [MOPS] [pH 7.2], 0.1 μg/ml riboflavin, 0.03 μg/ml biotin, 0.1 μg/ml thiamine HCl, 0.1 μg/ml L-ascorbic acid, 0.1 μg/ml D-Ca-pantothenate, 0.1 μg/ml folic acid, 0.1 μg/ml nicotinic acid, 0.1 μg/ml 4-aminobenzoic acid, 0.1 μg/ml pyridoxine HCl, 0.1 μg/ml lipoic acid, 0.1 μg/ml NAD, 0.1 μg/ml thiamine pyrophosphate, and 0.01 μg/ml cyanocobalamin). Depending on the experimental condition, as indicated in the figure legends, a terminal electron acceptor would be added (10 mM nitrate or nitrite) or omitted. For oxidized PCA, a 10 mM stock in 20 mM NaOH was prepared. For reduced PCA, an 800 μM stock in the basal medium was reduced by electrolysis. Both stocks were diluted into wells in 96-well plates (BRAND Cat./No 781671) to a final target concentration of 200 μM.

(ii) Cell preparation. All cell incubations and experiments were performed at 30°C. Cells were preserved in 35% glycerol stocks at −80°C. Two days prior to the experiments, frozen cells for each strain assayed were struck out on lysogeny broth (LB) agar plates and incubated overnight. The evening prior to the experiment, a patch from the streaks was inoculated into liquid LB in a respective culture tube and incubated slanted, shaking at 250 rpm, overnight. The morning of the experiment, 1 ml of each cell culture was washed three times into the basal medium by spinning for 2 min at 6,000 × *g*, aspirating the supernatant, and gently resuspending with a pipette. The optical density at 600 nm (OD_{600}) of each washed culture was measured. The cultures were brought into a Coy glove box, where they were washed three times into the same basal medium that had been made anoxic, following the same procedure as described above. After being left to stand for 1 to 2 h, the cells were inoculated into the different experimental conditions at a target starting OD_{600} of 0.1.

(iii) Measurement of PCA redox and nitrogen oxide concentrations. All PCA redox measurements were performed in a Coy chamber (5% hydrogen/95% nitrogen headspace) using a BioTek Synergy 5 plate reader. Reduced PCA concentration was measured by fluorescence (excitation 360 nm and emission 528 nm) (28). Plates were incubated shaking on the "medium" setting. For nitrogen oxide concentration

measurements, *C. portucalensis* MBL cells were prepared as described 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.

(iv) Data analysis. Initial redox rates under the conditions with supplied nitrite did not include the first 1.5 h 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 5 h after the first detectable PCA_{red} measurement, which was determined to be appropriate based on scanning for R^2 values over increasing time frames (Fig. S4 and S5). Ninety-five percent confidence intervals are calculated as the estimated value $\pm$ 1.96 $\times$ 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.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

FIG S1, EPS file, 0.7 MB.

FIG S2, EPS file, 1.5 MB.

FIG S3, EPS file, 1.5 MB.

FIG S4, EPS file, 0.4 MB.

FIG S5, EPS file, 0.4 MB.

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