1 **Title:** Prevalence and correlates of phenazine resistance in culturable bacteria from a dryland wheat field

2

3 **Running title:** Phenazine resistance in wheat rhizosphere community

4

5 **Authors:** Elena K. Perry¹, Dianne K. Newman^{1,2*}

6

7 **Affiliations:**

8 ¹Division of Biology and Biological Engineering, California Institute of Technology, Pasadena, CA

9 91125, USA

10 ²Division of Geological and Planetary Sciences, California Institute of Technology, Pasadena, CA 91125,

11 USA

12

13 **Contact information:**

14 E-mail: dkn@caltech.edu

15 Tel: 626-395-3543

16 **Abstract**

17 Phenazines are a class of bacterially-produced redox-active natural antibiotics that have
18 demonstrated potential as a sustainable alternative to traditional pesticides for the biocontrol of
19 fungal crop diseases. However, the prevalence of bacterial resistance to agriculturally-relevant
20 phenazines is poorly understood, limiting both the understanding of how these molecules might
21 shape rhizosphere bacterial communities and the ability to perform risk assessment for off-target
22 effects. Here, we describe profiles of susceptibility to the antifungal agent phenazine-1-carboxylic
23 acid (PCA) across more than 100 bacterial strains isolated from a wheat field where PCA
24 producers are indigenous and abundant. We find that Gram-positive bacteria are typically more
25 sensitive to PCA than Gram-negative bacteria, but that there is also significant variability in
26 susceptibility both within and across phyla. Phenazine-resistant strains are more likely to be

27 isolated from the wheat rhizosphere, where PCA producers are also more abundant, compared to
28 bulk soil. Furthermore, PCA toxicity is pH-dependent for most susceptible strains and broadly
29 correlates with PCA reduction rates, suggesting that uptake and redox-cycling are important
30 determinants of phenazine toxicity. Our results shed light on which classes of bacteria are most
31 likely to be susceptible to phenazine toxicity in acidic or neutral soils. In addition, the taxonomic
32 and phenotypic diversity of our strain collection represents a valuable resource for future studies on
33 the role of natural antibiotics in shaping wheat rhizosphere communities.

34 **Importance**

35 Microbial communities contribute to crop health in important ways. For example, phenazine
36 metabolites are a class of redox-active molecules made by diverse soil bacteria that underpin the
37 biocontrol of wheat and other crops. Their physiological functions are nuanced: in some contexts
38 they are toxic, in others, beneficial. While much is known about phenazine production and the
39 effect of phenazines on producing strains, our ability to predict how phenazines might shape the
40 composition of environmental microbial communities is poorly constrained; that phenazine
41 prevalence in the rhizosphere is predicted to increase in arid soils as the climate changes provides
42 an impetus for further study. As a step towards gaining a predictive understanding of phenazine-
43 linked microbial ecology, we document the effects of phenazines on diverse bacteria that were co-
44 isolated from a wheat rhizosphere and identify conditions and phenotypes that correlate with how a
45 strain will respond to phenazines.

46

47 **Introduction**

48 Diverse microorganisms produce natural antibiotics that can kill or inhibit the growth of
49 other microbes (1, 2). Several such compounds have been commercialized as antimicrobial drugs
50 for the treatment of infections, beginning with penicillin in the 1940s (3, 4). Unfortunately, the
51 selective pressures exerted by the widespread use of antibiotics in medicine and agriculture have
52 led to worrisome increases in the prevalence of antimicrobial resistance among human and animal
53 pathogens over the past several decades (5, 6). Yet while this repercussion of human antibiotic use
54 has been well documented, comparatively little is known about the ecological effects of

microbially-produced antibiotics in natural environments (7, 8). Recent studies have suggested that natural antibiotics may serve a variety of functions for their producers beyond the suppression of competing microbes (9, 10), including nutrient acquisition (11, 12), conservation of energy in the absence of oxygen (13, 14), and cell-cell signaling (15, 16). At the same time, toxicity to one or more microorganisms is by definition a feature of these molecules, but the extent to which this trait shapes their influence on microbial communities is unclear (2, 17, 18). In addition, for many if not most natural antibiotics, the determinants and prevalence of susceptibility or resistance to their toxicity remain unknown or poorly characterized (19). These gaps in knowledge hinder our ability to understand and predict the impacts of these metabolites on microbial communities of interest.

One environmental context in which natural antibiotics are thought to be particularly abundant and ecologically relevant is the rhizosphere—the narrow plane of soil immediately adjacent to plant roots (20–22). Natural antibiotics such as phenazines and 2,4-diacetylphloroglucinol have been directly detected in the rhizospheres of multiple crops, including wheat, potato, and sugar beet (22–24), and phenazines have been shown to increase the fitness of their producers when competing with other microbes in the rhizosphere (25, 26). Production of these molecules has also been demonstrated to underpin the ability of certain bacteria to control fungal crop diseases (23, 26–29), further indicating that natural antibiotics can act as agents of microbe-microbe antagonism in the rhizosphere. As a result of this activity, phenazine-producing *Pseudomonas* strains have received attention as potential biocontrol agents that could serve as a more sustainable alternative to traditional synthetic pesticides in agriculture (19, 28). However, several challenges remain with respect to the practical application of these strains, including inconsistent efficacy under field conditions (20, 28), limited understanding of the mechanisms and evolutionary dynamics of resistance to phenazines (19, 30), and the possibility of off-target effects (28).

Importantly, with regard to the latter concern, phenazines are toxic not only to fungi, but also to some bacteria (31–33). Yet while the utility of phenazine-producing pseudomonads for biocontrol of fungal crop diseases has been extensively investigated, the potential impact of these strains on non-target bacterial residents of the rhizosphere is less well understood. One study found that inoculation of *Pseudomonas* biocontrol strains shifted the rhizosphere community of maize seedlings, pushing the ratio of Gram-positive to Gram-negative bacteria in favor of the latter;

85 however, this analysis was based on profiling colony growth rates and whole-cell fatty acids from
86 pooled cultured isolates, greatly limiting the taxonomic resolution and making it difficult to rule
87 out whether the Gram-negative biocontrol strains might themselves have directly contributed to the
88 shift (34). On the other hand, at least three studies found no notable or consistent effects of
89 introduced *Pseudomonas* species on the rhizosphere bacterial communities of wheat or potato (35–
90 37), albeit the studies in wheat employed methods with limited discriminatory power (namely,
91 carbon source utilization profiling and terminal restriction fragment length polymorphism). Given
92 these mixed results and the lack of fine-grained spatial or taxonomic resolution in most studies on
93 this topic, whether phenazine-producing bacteria actively shape the surrounding rhizosphere
94 bacterial community through antibiosis, potentially at the micron or millimeter scale, remains an
95 open question.

96 In addition to the lack of clarity regarding the effects of phenazines on rhizosphere bacterial
97 communities, the taxonomic and physiological correlates of phenazine resistance in bacteria
98 remain incompletely understood. The toxicity of phenazines is generally ascribed to the generation
99 of reactive oxygen species (ROS) and interference with respiration (33, 38–40). Previous studies
100 have suggested that efflux pump expression, cell permeability, oxidative stress responses, and the
101 composition of the respiratory electron transport chain can affect bacterial susceptibility to
102 phenazines (39–44). In addition, a comparison of 14 bacterial strains indicated that Gram-negative
103 bacteria as a group may be more resistant to phenazine toxicity than Gram-positive bacteria (33).
104 However, all of these studies focused on a specific phenazine, pyocyanin, that is particularly toxic
105 (45) and best known for its role as a virulence factor during infections of humans and animals (46,
106 47). Whether the same observations hold true for more agriculturally-relevant phenazines such as
107 phenazine-1-carboxylic acid (PCA) (22, 23, 27, 48) is unknown.

108 In this study, we set out to lay a foundation for addressing unresolved questions about the
109 ecological impact of phenazine toxicity in the rhizosphere by determining the prevalence of
110 phenazine resistance among bacteria isolated from a wheat field in the Inland Pacific Northwest, a
111 region where phenazine production and the biocontrol potential of indigenous *Pseudomonas*
112 species have been studied for decades (22, 23, 25, 27). We designed a culture-based assay to
113 measure sensitivity to PCA, which is the best-studied and one of the most abundant phenazines in
114 this environment (22, 23, 48). We also performed full-length 16S rRNA gene sequencing of our

115 isolates in order to assess the relationship between taxonomy and PCA resistance. Finally, to assess
116 potential physiological correlates of PCA resistance, we measured PCA reduction rates for a subset
117 of phenotypically-diverse strains and investigated the effects of broad-spectrum efflux pump
118 inhibitors on growth in the presence of PCA.

119 **Results**

120 *Taxonomic diversity of culturable bacteria from dryland wheat rhizospheres and bulk soil*

121 A total of 166 strains of bacteria were isolated from 12 soil samples collected from a wheat
122 field at Washington State University's Lind Dryland Research Station in early August 2019,
123 shortly after the wheat harvest. The samples comprised 4 replicates each of wheat rhizosphere
124 ("Wheat"), bulk soil collected between planted rows ("Between"), and bulk soil from a virgin
125 patch of uncultivated soil adjacent to the field ("Virgin"). Full-length 16S rRNA gene sequencing
126 revealed that the isolates represented 24 genera across 4 phyla: Actinobacteria, Bacteroidetes,
127 Firmicutes, and Proteobacteria. The vast majority of isolates from the bulk soil samples (both
128 Between and Virgin) were Actinobacteria or Firmicutes. In Wheat samples, on the other hand, the
129 combined proportions of these two phyla were lower, accounting for 18-50% and 10-25% of
130 isolates respectively, while Proteobacteria accounted for approximately 25-60% of isolates
131 depending on the replicate, and 1-3 strains of Bacteroidetes were also detected in each replicate (5-
132 12% of isolates) (Fig. 1).

133 *Taxonomic and spatial distribution of PCA resistance phenotypes*

134 We screened our isolates for resistance to PCA at both circumneutral and acidic pH (7.3
135 and 5.1, respectively), because the toxicity of PCA to diverse organisms is known to vary
136 depending on pH (49, 50), and because the bulk soil and rhizosphere pH of wheat fields in the
137 Inland Pacific Northwest can vary by >3 units (pH 4.3-8.0) depending on geographic location and
138 fertilizer treatment status (51). The pH-dependency of PCA toxicity has been attributed to the fact
139 that the deprotonated form of PCA is negatively charged (Fig. S1); the negative charge on bacterial
140 cell walls and the outer membrane of Gram-negative bacteria (or the negative membrane potential
141 of eukaryotic cells) likely hinders uptake of this species. The protonated form of PCA, on the other
142 hand, is neutral and presumably can passively diffuse across cell membranes given the small size

143 and hydrophobic nature of the molecule (50, 52). The pKa of PCA is 4.24 at 25 °C; thus, at pH 7,
144 only 0.2% of PCA in solution is protonated compared to 14.8% at pH 5, typically leading to
145 greater toxicity at the lower pH (49). In designing the screen, we additionally decided to focus on
146 comparing growth at a single concentration of PCA versus a PCA-free control condition, rather
147 than performing a minimum inhibitory concentration assay based on a dilution series, to enable
148 resource-efficient comparison of a large number of strains. We chose 100 μ M as the working
149 concentration of PCA as this is likely to be in a physiologically relevant range based on
150 concentrations measured both in pure cultures and in the field. In broth cultures of biocontrol
151 strains of *Pseudomonas*, PCA accumulates to concentrations ranging from dozens to hundreds of
152 micromolar (53, 54). In natural wheat rhizospheres, PCA has been detected at nanomolar
153 concentrations (22), but these bulk measurements almost certainly underestimate local
154 concentrations at the micron scale given that bacteria colonize the rhizosphere in a patchy manner
155 (23). Notably, PCA can accumulate in biofilms to concentrations 360-fold greater on a per volume
156 basis compared to broth cultures (53), suggesting that local concentrations of PCA in the
157 rhizosphere, where biocontrol strains form robust biofilms (55), may be orders of magnitude higher
158 than the reported bulk values.

159 The screen was performed using 24-well plates and 0.1x tryptic soy agar (TSA) that was
160 either left unadjusted (pH 7.3-7.5) or adjusted to pH 5.1. Each isolate was spotted onto agar in
161 separate wells to prevent crosstalk and antagonism between the strains, and image analysis was
162 used to derive quantitative information about the growth of each strain over the course of 7 days
163 (with one timepoint per day). Because several strains among our 166 isolates appeared to be
164 duplicates of each other based on 16S sequence and colony morphology, we restricted the screen to
165 108 strains that we judged likely to be unique (Table 1); where multiple strains appeared identical,
166 we chose a representative strain. We also included 30 strains obtained from public culture
167 collections that represented species found among our isolates (Table 1), in order to investigate the
168 extent to which PCA resistance phenotypes are consistent within species across different strains
169 isolated from different geographical locations. Accurate quantification of growth was not possible
170 in this screen for certain strains that formed transparent colonies, produced dark pigments, or had a
171 tendency to turn mucoid and spread (Fig. S2). Nevertheless, this assay enabled us to derive detailed
172 profiles of PCA sensitivity and resistance for the vast majority of our strains. Growth assays based

173 on planktonic cultures were precluded due to the tendency of *Streptomyces* species to form clumps,
174 as well as poor growth of *Agromyces*, *Neobacillus*, and *Paenibacillus* strains in liquid 0.1x tryptic
175 soy broth.

176 To compare PCA susceptibility across strains, we first focused on a single-timepoint
177 snapshot of each strain's phenotype taken at the equivalent of early stationary phase (i.e. around
178 the time that the spots on non-PCA control plates reached their maximal density). In accordance
179 with previous reports of the pH dependency of PCA toxicity, we found that more of our strains
180 were sensitive to PCA at pH 5.1 than at pH 7.3, and strains that were mildly inhibited by PCA at
181 pH 7.3 were typically inhibited more strongly at pH 5.1 (Fig. 2A-B). At pH 5.1, strains of
182 Actinobacteria and Firmicutes were strongly inhibited by PCA, while most Proteobacteria were
183 relatively resistant. Members of Bacteroidetes exhibited variable responses, ranging from mild to
184 severe growth inhibition (Fig. 2A-B). At pH 7.3, there was considerably more phenotypic variation
185 among the Actinobacteria (Fig. 2A-B), particularly within the *Streptomyces* genus. In some cases,
186 there was even noticeable variation within the same putative species of *Streptomyces* (Fig. S3). In
187 addition, two strains of *Microbacterium* (W2I7 and W4I20), as well as one strain of *Arthrobacter*
188 (W3I6), grew slightly better in the presence of PCA at pH 7.3 compared to the control condition,
189 perhaps suggesting that they can use PCA as a carbon source or otherwise benefit from its presence
190 at a pH where toxicity is limited (Fig. 2B). On the other hand, in contrast to the phenotypic
191 variation seen among Actinobacteria, Firmicutes remained almost universally sensitive to PCA at
192 pH 7.3 (albeit somewhat less so than at pH 5.1), while all members of Bacteroidetes were resistant
193 to PCA at pH 7.3, in contrast to their sensitivity to PCA at pH 5.1 (Fig. 2A-B). Proteobacteria
194 remained generally resistant (Fig. 2A-B), with the most notable exception being a strain of
195 *Sphingomonas faeni*, isolate W4I17, that was inhibited by PCA at both pH 7.3 and pH 5.1 (Fig.
196 2A-B).

197 Interestingly, while most strains yielded qualitatively consistent results across independent
198 biological replicates that were separated by months (Fig. S4-S7), a handful of strains displayed
199 variable growth and lag times at pH 5.1 either in the absence of PCA (mostly among
200 Actinobacteria and Firmicutes), or in the presence of PCA (a few strains among the Bacteroidetes).
201 Growth at pH 7.3 was generally more consistent, with the exception being two strains of
202 *Neobacillus niacini* (V4I10 and V4I25) that initially failed to grow in the presence of PCA, but

grew with minimal to no inhibition in subsequent replicates. The reasons for these discrepancies are unclear, but may be related to variations in how long the strains had been in stationary phase prior to inoculating the experimental cultures, which was difficult to control precisely due to differing growth rates across the large number of strains. The trace nutrient content may also have varied between different batches of media, as the first replicate was performed using a different lot of tryptic soy broth compared to subsequent replicates. Thus, the growth and PCA sensitivity of some strains may be influenced by environmental factors beyond pH that remain to be elucidated.

We next examined whether there was any evidence of correlation between PCA resistance phenotypes and the type of soil each strain was isolated from (Between, Virgin, or Wheat). A previous study based on samples taken from the same wheat field found that the relative abundance of phenazine producers was higher in the wheat rhizosphere compared to adjacent bulk soil (48), a finding that we also confirmed using shotgun metagenomic sequencing of our soil samples (Fig. 2C). We therefore hypothesized that if PCA-mediated antibiosis had shaped the bacterial community composition of this field, the prevalence of PCA resistance would be higher among isolates from the rhizosphere samples. Indeed, the Wheat samples clearly had the highest proportion of PCA-resistant isolates, regardless of whether resistance was assessed at pH 5.1 (Fig. 2D) or pH 7.3 (Fig. 2E). By contrast, all strains from Between or Virgin samples were highly sensitive to PCA at pH 5.1, though at pH 7.3, the resistance phenotypes of isolates from either type of bulk soil spanned the full range from highly sensitive to completely resistant. However, these findings are conflated with the fact that all of our Bacteroidetes strains, and all but one strain of Proteobacteria, were exclusively isolated from the Wheat samples, and that the members of these two phyla were generally relatively resistant to PCA, especially at pH 7.3. Further experiments will be necessary in order to distinguish whether the enrichment of PCA-resistant phenotypes in the rhizosphere samples is a consequence of PCA-mediated antibiosis or merely an indirect reflection of other factors that favor the growth of Proteobacteria and Bacteroidetes in the rhizosphere.

Finally, visualizing the growth of each strain over the full 7-day time course revealed more nuanced variations among the PCA resistance phenotypes at both pH 5.1 and pH 7.3 (Figs. 3 and S4-S7). For example, while some strains of Bacteroidetes displayed increased lag and/or slower growth rates in the presence of PCA at pH 5.1, the growth on PCA often eventually caught up to the PCA-free controls. At pH 7.3, the effect of PCA on *Peribacillus* species was a combination of

233 increased lag and lower final cell density, but apparently not decreased maximal growth rate, as
234 compared to no growth at all on PCA at pH 5.1. This was distinct from the slower growth rates
235 seen for certain strains of *Bacillus*, *Neobacillus*, and *Paenibacillus* at pH 7.3. A few strains of the
236 latter three genera appeared to be still unable to grow on PCA at pH 7.3 based on the values
237 derived from image analysis (Fig. S5). However, examination of the plate images by eye revealed
238 that this sometimes reflected a limitation in the sensitivity of our imaging and quantification
239 methods to low levels of growth, rather than a true lack of growth (Fig. S2).

240 *PCA reduction rates in relation to PCA susceptibility*

241 We next sought to determine whether there are physiological correlates of PCA
242 susceptibility or resistance across taxonomically-diverse bacteria. Given that phenazines are redox-
243 active molecules whose toxicity is thought to be related to ROS generation as a consequence of
244 redox cycling in the cell (38, 56), we hypothesized that susceptibility to PCA would be correlated
245 with higher redox-cycling rates. PCA oxidation occurs rapidly and abiotically in the presence of
246 oxygen (11), suggesting that the rate of PCA reduction would be the primary driver of differences
247 in redox-cycling rates across strains under oxic conditions. Therefore, to test our hypothesis, we
248 measured the rate of PCA reduction under anoxia as a proxy for the true redox-cycling rate, using a
249 subset of strains that covered a range of taxonomic groups and PCA susceptibility phenotypes.

250 For each tested strain, the PCA reduction rate was typically higher at pH 5.1 than at pH 7.3
251 (Fig. 4A), matching the expectation that PCA uptake would tend to be faster at a lower pH and the
252 observation that most susceptible strains were more sensitive to PCA at pH 5.1. The exceptions
253 were: 1) strains belonging to the Firmicutes, which had lower reduction rates at pH 5.1 in our assay
254 despite being more susceptible to PCA under this condition, and 2) *Sphingomonas faeni* W4I17,
255 which was in fact more susceptible to PCA at pH 7.3 than at pH 5.1 (Fig. 2A-B), matching the pH
256 dependency of its rate of PCA reduction. Notably, some of the tested Firmicutes often struggled to
257 grow at pH 5.1 even in the absence of PCA (Fig. S5). This observation might account for the
258 inhibitory effect of acidic pH on PCA reduction in these strains, given that a functional metabolism
259 would be required for the generation of cellular reductants, such as NADH, that can indirectly or
260 directly reduce PCA. For the strains of Firmicutes that were able to grow at acidic pH (namely
261 B1I1 and B1I6), the reason for their lower PCA reduction rate at pH 5.1 remains unclear.

We also examined whether there was any correlation between PCA reduction rates and resistance levels across different strains at each pH. To do so, we plotted each strain's PCA reduction rate against the early stationary phase snapshot ratio of growth on PCA versus no PCA (Fig. 4B). We also calculated Spearman's correlation coefficient (r_s). Interestingly, there was a statistically significant negative correlation between reduction rate and resistance at pH 7.3 ($r_s = -0.70$, $p < 0.001$), but not at pH 5.1 ($r_s = -0.16$, $p = 0.4485$). However, there were exceptions to the trend even at pH 7.3; for example, while PCA severely and consistently inhibited the growth of the four fastest PCA-reducing strains, the fifth-fastest strain, W2I1 (*Pantoea agglomerans*), appeared to be completely resistant to PCA in one biological replicate, though its growth was mildly inhibited by PCA in another replicate (Fig. S7). In addition, strains with reduction rates in the middle range of 0.2-0.4 nmol/OD₆₀₀/min spanned the full range of resistance phenotypes, from a growth ratio of 0.12 (*Peribacillus simplex* B2I2) to 0.96 (*Phyllobacterium ifrigiyense* W4I11). Taken together, these data suggest that a low reduction rate may be helpful in mitigating PCA toxicity but is neither necessary nor sufficient for resistance.

Effects of efflux pump inhibitors on PCA susceptibility

Given that PCA reduction rate alone was insufficient to account for the range of sensitivities to PCA, we asked if efflux pumps might serve as an important contributor to PCA resistance in some strains. To address this question, we used two efflux pump inhibitors (EPIs) in combination: 1) reserpine, which is thought to target efflux pumps in the major facilitator superfamily (MFS) (57), and 2) phenylalanine-arginine β -naphthylamide (PA β N), which is thought to target resistance-nodulation-division (RND) efflux pumps (58), although it may also permeabilize the outer membrane of Gram-negative bacteria (59, 60). We tested these EPIs on a subset of the strains that were used in the PCA reduction assay; we excluded the *Bacillus* strains due to their tendency to settle on the bottom of the wells during growth in the plate reader, which confounded the OD₆₀₀ readings. Preliminary experiments revealed that different strains were differentially sensitive to the EPIs themselves, with growth inhibition evident even in PCA-free controls. We therefore determined for each strain the maximal EPI concentration that, when the two EPIs were used separately, did not markedly reduce growth of PCA-free controls (up to a maximum dose of 20 μ g/mL for reserpine and 50 μ g/mL for PA β N) (Table S2). Interestingly, for many strains, the combination of both EPIs remained highly toxic even after this optimization step,

particularly at pH 7.3, possibly indicating synergistic toxicity or redundant roles of different efflux pumps in preventing intracellular accumulation of toxic metabolic intermediates (Fig. S9); the toxicity appeared to be pH-dependent, suggesting that the protonation state of the EPIs might influence their efficacy. In these cases, it was not possible to assess the impact of efflux pump inhibition on PCA sensitivity. However, for the remaining strains, three types of responses to the EPIs emerged. For one group of strains, treatment with both EPIs in combination did not affect their resistance to PCA (Fig. 5A-B); however, we cannot distinguish whether this is because efflux does not contribute to PCA resistance in these strains, or because the tested EPIs did not effectively target the relevant efflux pumps. For the second group of strains, the EPIs enhanced the toxicity of PCA, as would be expected if efflux is an important mechanism of resistance (Fig. 5C). Finally, for the third group of strains, the EPIs counterintuitively appeared to mitigate the toxicity of PCA, or vice versa (Fig. 5D). Although unexpected, the latter phenomenon indirectly suggests that PCA interacts with efflux systems in these strains. For example, treatment with either PCA or the EPIs might upregulate the expression of certain efflux pumps, leading to decreased toxicity relative to treatment with either type of toxin alone. Alternatively, given that some pumps in the MFS family are thought to be bidirectional (61), it is possible that MFS pumps promote uptake rather than export of PCA in certain strains.

Discussion

In this study, we have characterized profiles of resistance to an agriculturally-relevant phenazine across taxonomically diverse bacteria isolated from a wheat field where phenazine producers are indigenous. We also examined potential physiological correlates of phenazine resistance in a subset of these strains. Our findings establish a basis for inferring whether intrinsic resistance is a factor that affects how phenazine production shapes bacterial communities in the rhizosphere. In particular, it will now be possible to test data-driven predictions regarding which strains, species, or even phyla are most likely to be affected by phenazine-mediated antibiosis, even though the mechanistic underpinnings of phenazine susceptibility or resistance are likely multifactorial and appear to differ across species.

One of our more notable findings is that the previously reported pH dependency of PCA toxicity varies across bacterial taxonomic groups. For most Actinobacteria and Firmicutes,

sensitivity to PCA was clearly higher at pH 5.1 than at pH 7.3, indicating greater toxicity at the lower pH as expected according to the pKa of PCA. On the other hand, nearly all tested Proteobacteria were completely resistant to 100 μ M PCA regardless of pH, at least down to pH 5.1. Consequently, at pH 5.1, PCA resistance phenotypes largely correlated with phylum—and more broadly, a Gram-positive versus Gram-negative divide, which has previously been reported for the toxicity of another phenazine, pyocyanin (33). The Gram-positive versus Gram-negative divide at pH 5.1 is perhaps not surprising, as at this pH, a significant proportion (~14%) of PCA in solution is protonated and therefore would not be repelled by the negatively charged cell wall. Under this condition, the outer membrane of Gram-negative species presumably presents an additional barrier to the entry of PCA, helping to limit the intracellular accumulation of the toxin in the same manner as for numerous other antibiotics (62). Less expected, however, was the within-phylum and even within-species variation in PCA resistance phenotypes at pH 7.3 among certain taxonomic groups. Interestingly, several *Streptomyces* strains exhibited at least some PCA-dependent growth inhibition at pH 7.3, even though many *Streptomyces* species are capable of producing their own toxic phenazines (32, 48). Given that the major limit on PCA toxicity at circumneutral pH is thought to be its ability to enter cells, the phenotypic variability at pH 7.3 may indicate the presence of transporters or channels capable of phenazine uptake in some PCA-sensitive Actinobacteria. Alternatively, it is possible that some PCA-sensitive strains locally acidified the growth medium, which was only weakly buffered (1.4 mM K_2HPO_4).

Importantly, despite the general Gram-positive versus Gram-negative divide in sensitivity to PCA at pH 5.1, possessing an outer membrane is evidently not a leakproof shield against PCA toxicity. Proteobacteria as a group were more resistant to PCA at pH 5.1 compared to strains of Bacteroidetes, most of which exhibited increased lag in the presence of PCA, even though both phyla comprise Gram-negative bacteria. Intriguingly, one major difference between these clades is that Proteobacteria generally utilize ubiquinone as an electron carrier during aerobic growth (63), while the Bacteroidetes genera screened in this study (*Chitinophaga*, *Chryseobacterium*, and *Pedobacter*) utilize menaquinone (64–66). Menaquinones have a lower reduction potential compared to ubiquinones (67). Although the standard reduction potential of menaquinone is still higher than that of PCA (-74 mV compared to -177 mV) (52, 67), indicating that PCA likely is not reduced by menaquinol, this difference with ubiquinone nevertheless raises the possibility that

351 PCA may interact differently, and perhaps more readily, with the aerobic respiratory electron
352 transport chain of menaquinone-utilizing Bacteroidetes strains compared to Proteobacteria, thereby
353 generating more ROS and/or interfering with the generation of ATP. Interestingly, another study
354 has shown that the reduced form of different phenazine with a low reduction potential, neutral red
355 (3-amino-7-dimethylamino-2-methylphenazine), can directly transfer electrons to menaquinone,
356 bypassing the proton-pumping NADH dehydrogenase complex that normally transfers electrons
357 from NADH to menaquinone and thereby “short-circuiting” the electron transport chain. We
358 hypothesize that a similar phenomenon may occur with PCA in strains that rely on menaquinone.
359 In future studies, this hypothesis could be tested by 1) performing *in vitro* experiments with
360 purified menaquinone, ubiquinone, and reduced PCA to directly test whether quinones oxidize the
361 latter and if so, whether the kinetics differ between menaquinone and ubiquinone, 2) measuring
362 ROS production and steady-state ATP pools in selected strains of Bacteroidetes and Proteobacteria
363 both in the presence and absence of PCA, and 3) forcing species of Proteobacteria (such as
364 *Escherichia coli*) that utilize both ubiquinone and menaquinone in different branches of their
365 electron transport chains to rely only on the latter (for example, by deleting the genes for
366 ubiquinone biosynthesis), followed by reassessing their sensitivity to PCA to see if there is any
367 effect.

368 Beyond the specific strains screened in this study, the risk assessment of phenazine-
369 producing biocontrol strains, and the understanding of phenazine biology in general, would benefit
370 greatly from the development of a platform for the prediction of phenazine resistance phenotypes
371 from genomic or phylogenetic information. The results of this study already indicate that,
372 depending on the environmental pH, phylogenetic information may be of limited utility for
373 prediction of PCA resistance, given the variation in phenotypes for Actinobacteria at circumneutral
374 pH. However, this variability may also hold the key to identifying genome-based predictive
375 markers of phenazine resistance. Given the taxonomic and phenotypic diversity of our strain
376 collection, whole-genome sequencing of our isolates, followed by comparative genomics, could
377 potentially reveal such markers.

378 In summary, this work has laid the groundwork for rectifying a major gap in studies of how
379 introduction of a phenazine-producing biocontrol strain affects rhizosphere bacterial communities.
380 Previous studies have lacked information about the baseline prevalence of resistance in the native

communities (34–37), and in the absence of such information, it is impossible to determine whether a negative result (lack of change in the rhizosphere community) reflects a high prevalence of resistance to PCA that is particular to the studied community, versus a general lack of toxicity of PCA to most bacteria or fundamental abiotic constraints on the antibacterial activity of PCA in the rhizosphere (e.g. limited diffusion, adsorption to soil particles, etc.). Distinguishing between these scenarios is key to assessing the risk of unwanted side effects in rhizosphere communities upon the application of phenazine-producing biocontrol strains. In addition, recent studies have demonstrated that phenazines produced by the opportunistic pathogen *Pseudomonas aeruginosa* can promote bacterial tolerance and resistance to clinical antibiotics (42, 68–70), and that these effects can extend to other opportunistic pathogens that are resistant to phenazines (42). Thus, understanding the prevalence and genetic determinants of resistance to phenazines may have implications not only for agriculture but also for human medicine and beyond, as we continue to uncover new ecological roles for these multifaceted bacterial metabolites.

Methods

Isolation of bacteria from wheat rhizosphere and bulk soil samples

Three types of samples were collected from a non-irrigated wheat field at Washington State University's Lind Dryland Research Station on August 9, 2019: wheat plants and surrounding soil, bulk soil from in between the planted rows, and bulk soil from a "virgin" hillside site that has never been farmed. The wheat had been harvested a few weeks prior to sample collection. All samples were immediately stored on ice in clean plastic bags, and subsequently at 4 °C for four days until processing. Rhizosphere soil samples were obtained by shaking the wheat roots until only 1-2 mm of tightly-adhering soil remained, followed by excising the roots at the crown with a sterile razor blade. The roots of 2-3 plants per replicate were placed in 50 mL conical tubes with 30 mL of sterile deionized water, vortexed at top speed for 1 min, and treated in an ultrasonic water bath for 1 min to dislodge bacteria from the roots. Bulk soil samples (1 g per sample) were processed in the same manner. Large soil particles were allowed to settle to the bottom of the tubes on the bench top, and 100 µL each of a 10-fold dilution series of the supernatants was spread onto 0.1x TSA plates containing 50 µg/mL nystatin to inhibit fungal growth. The plates were incubated upside down at room temperature in the dark and monitored for the appearance of new colonies

over the course of a week. Colonies that appeared morphologically distinct in each sample were picked and restreaked on 0.1x TSA until visually pure cultures were obtained. Multiple representatives were also picked for the most common colony types in an attempt to account for strain variations that might not be apparent to the eye. Once the streaks yielded uniform single colonies, the isolates were inoculated into 1.5 mL of 0.1x tryptic soy broth (TSB) in 5 mL polycarbonate culture tubes and incubated at 30 °C with shaking at 250 rpm. After 1-3 days of incubation, depending on when the cultures became turbid, 0.5 mL of each culture was mixed with 0.5 mL of 50% glycerol and stored at -80 °C. Some cultures never became turbid under these growth conditions, but nevertheless yielded viable frozen stocks.

Species identification by 16S rRNA gene sequencing

Single colonies or patches of morphologically pure streaks were picked and resuspended in 20 µL sterile nuclease-free water. Colony PCR was performed using GoTaq Green Master Mix (Promega) in 50 µL reactions (1 µL of cell suspension) according to the manufacturer's instructions. For putative streptomycetes (isolates that formed hard colonies rooted in agar, often with aerial hyphae), the thermocycling protocol was modified to include a 10 min initial heating step at 95 °C (compared to 2 minutes for other samples). The primers used were 27F (AGAGTTTGATCMTGGCTCAG) and 1492R (TACGGYTACCTTGTTACGACTT) (71). The PCR products were run on a 1% agarose gel to verify the presence of a single band at the expected size (~1500 bp), followed by purification with the Monarch PCR and DNA Cleanup Kit (New England Biolabs). The purified products were submitted for Sanger sequencing at Laragen, Inc., using the same 27F and 1492R primers. The resulting forward and reverse sequences were aligned using MAFFT (<https://mafft.cbrc.jp/alignment/server/>) and subjected to BLAST against the NCBI 16S ribosomal RNA sequences database. For a few strains, full-length sequences could only be obtained following PCR on genomic DNA extracted using the DNeasy Blood & Tissue kit (Qiagen), following the manufacturer's instructions for Gram-positive bacteria. In addition, for two strains (B1I5 and B2I8), the forward sequence consistently appeared to contain multiple products. We presumed that this was due to multiple primer binding sites or another sequencing artifact rather than mixed cultures as the corresponding sequences from the other direction were clean. In these cases, only the clean sequence was submitted to BLAST. The 16S rRNA gene sequences and closest BLAST hits for each strain are provided in Table S1.

440 *PCA resistance screen*

441 The optimized version of the PCA resistance screen was performed with four conditions:
442 0.1x TSA (15 g/L agar plus 3 g/L tryptic soy broth no. 2 from MilliporeSigma) with or without 100
443 μ M PCA at pH 7.3 or pH 5.1 (adjusted with HCl). PCA was purchased from Princeton
444 BioMolecular Research and dissolved in filter-sterilized 14 mM NaOH to make 10 mM stock
445 solutions. The PCA stock solution or solvent control (14 mM NaOH) was added at 1% v/v to
446 autoclaved molten 0.1x TSA; we verified that this addition did not noticeably alter the pH of the
447 medium by pipetting aliquots onto pH test strips. Subsequently, 1 mL of the medium was pipetted
448 into each well in 24-well polystyrene Cellstar Cell Culture plates (Greiner Bio-One). The plates
449 were allowed to set and dry with the lids off in a biological safety cabinet for 20-30 min, followed
450 by storage upside down with the lids on at room temperature in the dark for two days prior to use.

451 Cell suspensions for inoculation in the screen were prepared in one of two ways. First,
452 individual strains were inoculated into 5 mL TSB cultures in glass culture tubes and incubated at
453 25 °C with shaking at 250 rpm. Strains that grew overnight were then diluted to an OD₆₀₀ of 0.05.
454 Certain strains did not grow well in this condition—in particular, strains of *Agromyces*,
455 *Streptomyces*, *Paenibacillus*, and *Neobacillus*. For these, some replicates were prepared by directly
456 scraping cells from streaks grown on 0.1x TSA plates and resuspended the cells in 200 μ L TSB
457 with pipetting and brief vortexing at top speed. The OD₆₀₀ of these cell suspensions was adjusted to
458 0.05, except for *Streptomyces* due to their filamentous nature and tendency to clump; instead,
459 visible cell clumps were allowed to settle to the bottom and inocula were taken from the visually
460 clear upper portion. Subsequently, 10 μ L of each cell suspension was pipetted onto the agar in a
461 single well in the 24-well plates. Three adjacent wells per condition were inoculated with each cell
462 suspension, representing technical replicates. After the spots dried, the plates were incubated at
463 room temperature upside in the dark for up to 7 days. Every 24 hrs, the plates were imaged in color
464 at 600 dpi with an Epson Perfection V550 Photo flatbed scanner. For strains that were prepared
465 both from liquid cultures and by resuspension from plates, we did not observe any consistent effect
466 on the resulting PCA susceptibility phenotypes.

467 *Image analysis and quantification of growth*

468 Images from the scanner were analyzed using Fiji (72). Circular regions of interest (ROIs)
469 were drawn around each culture spot and the mean gray value was measured for each ROI. We
470 also measured the mean gray values of equivalent ROIs in the wells of blank, uninoculated plates
471 for each condition. The latter values were averaged across each 24-well blank plate to give the
472 “background” gray value, which was then subtracted from the mean gray values of the culture
473 spots. The resulting numbers were reported as the metric for growth. Importantly, while this
474 method generally worked well for comparisons across conditions within each strain, there are a
475 few caveats. First, this metric underestimated growth for strains that produced a dark pigment.
476 Second, growth was difficult to quantify for a few strains that grew as nearly transparent colonies.
477 Finally, this metric is not very sensitive to low levels of growth. Nevertheless, for the vast majority
478 of strains, this approach captured visible differences in growth across the four conditions in the
479 screen.

480 *Metagenomics-based estimation of the relative abundance of PCA producers*

481 DNA was extracted from 250 mg of each soil sample using the DNeasy PowerSoil kit
482 (Qiagen). Illumina metagenomic DNA libraries were prepared and sequenced (150 bp single-end
483 reads) at the Millard and Muriel Jacobs Genetics and Genomics Laboratory at the California
484 Institute of Technology. The resulting sequence data was processed according to the pipeline
485 described by Dar et al. (2020).

486 *PCA reduction assay*

487 Selected strains were grown in triplicate overnight in 5 mL TSB cultures at 25 °C with
488 shaking at 250 rpm. The cells were then concentrated by serially centrifuging 1 mL aliquots at
489 9000 g for 2 min in microcentrifuge tubes, washed with 0.1x TSB (pH 7.3), and standardized to
490 $OD_{600} = 8$ in 0.1x TSB (pH 7.3). Subsequently, 100 μ L of each cell suspension was transferred into
491 a 96-well plate, and the plate was moved into a Coy anaerobic chamber (5% hydrogen/95%
492 nitrogen headspace). Using plasticware and media that had been passively degassed in the chamber
493 for at least 3 days, electrochemically-reduced PCA was added to a final concentration of 100 μ M
494 in 190 μ L of 0.1x TSB (pH 5.1 or pH 7.3) per well in a black clear-bottom 96-well plate; a
495 standard curve was also prepared using reduced PCA at 1 μ M, 10 μ M, 50 μ M, and 100 μ M. Cells

were added to each well (except the standard curve) to an OD₆₀₀ of ~0.4, and the plate was immediately transferred to a Biotek Synergy 5 plate reader maintained at 30 °C. OD₆₀₀ and fluorescence readings (360 nm ex/528 nm em) were taken every 5 minutes, with continuous shaking on the “medium” setting. Reduction rates were derived from the slope of a linear regression fit to the visually linear range of the data using the function lm in R (see Fig. S8 for an example). The rates were converted to nmol/OD/min by relating the fluorescence units to the standard curve of reduced PCA concentrations and then dividing by the initial OD₆₀₀ reading for each replicate. No appreciable change in OD₆₀₀ was observed over the course of the experiment for any of the tested strains.

Efflux pump inhibition growth curves

Reserpine was purchased from Sigma-Aldrich and dissolved in dimethyl sulfoxide (DMSO) to make a 20 mg/mL stock solution. PAβN was purchased from MedChemExpress and dissolved in distilled water to make a 50 mg/mL stock solution. Selected strains were grown in triplicate overnight in 5 mL TSB cultures at 25 °C with shaking at 250 rpm, washed once with 0.1x TSB (pH 7.3), and then inoculated to an initial OD₆₀₀ of 0.05 in 160 µL of 0.1x TSB (pH 5.1 or pH 7.3) in a 96-well plate. Each well contained either a combination of reserpine and PAβN (see Table S2 for the concentrations used for each strain) or a solvent control (DMSO at 0.1% final concentration), along with either 0 or 100 µM PCA. The wells were topped with 50 µL of autoclaved mineral oil to prevent evaporation, and growth was monitored with OD₆₀₀ readings every 15 minutes in a Biotek Synergy 5 plate reader set to 30 °C with continuous shaking on the “medium” setting.

Data availability

The metagenomic sequencing data generated in this study were deposited in the Sequence Read Archive (SRA) under accession PRJNA521160.

Acknowledgements

We thank Linda Thomashow and David Weller of the USDA Agricultural Research Service for providing the wheat rhizosphere and soil samples used in this study, and the members

of the Newman lab for helpful feedback throughout the process of designing and analyzing the results of the PCA resistance screen. We also thank Zevin Condiotte for assistance with the initial rounds of testing the design of the PCA resistance screen and Richard Horak for assistance with extracting genomic DNA. This material is based upon work supported by the National Science Foundation Graduate Research Fellowship under Grant No. DGE-1745301. This work was also supported by grants to DKN from the ARO (W911NF-17-1-0024), NIH (1R01AI127850-01A1) and the Resnick Sustainability Institute at Caltech.

References

1. Granato ET, Meiller-Legrand TA, Foster KR. 2019. The evolution and ecology of bacterial warfare. *Curr Biol* 29:R521–R537.
2. Bérdy J. 2012. Thoughts and facts about antibiotics: where we are now and where we are heading. *J Antibiot* 65:385–395.
3. Aminov RI. 2010. A brief history of the antibiotic era: lessons learned and challenges for the future. *Front Microbiol* 1:134.
4. Hutchings MI, Truman AW, Wilkinson B. 2019. Antibiotics: past, present and future. *Curr Opin Microbiol* 51:72–80.
5. Manyi-Loh C, Mamphweli S, Meyer E, Okoh A. 2018. Antibiotic use in agriculture and its consequential resistance in environmental sources: potential public health implications. *Molecules* 23.
6. Davies J, Davies D. 2010. Origins and evolution of antibiotic resistance. *Microbiol Mol Biol Rev* 74:417–433.
7. Sengupta S, Chattopadhyay MK, Grossart H-P. 2013. The multifaceted roles of antibiotics and antibiotic resistance in nature. *Front Microbiol* 4:47.
8. Aminov RI. 2009. The role of antibiotics and antibiotic resistance in nature. *Environ Microbiol* 11:2970–2988.
9. Davies J, Spiegelman GB, Yim G. 2006. The world of subinhibitory antibiotic concentrations. *Curr Opin Microbiol* 9:445–453.
10. Davies J. 2006. Are antibiotics naturally antibiotics? *J Ind Microbiol Biotechnol* 33:496–499.
11. Wang Y, Newman DK. 2008. Redox reactions of phenazine antibiotics with ferric (hydr)oxides and molecular oxygen. *Environ Sci Technol* 42:2380–2386.
12. McRose DL, Newman DK. 2021. Redox-active antibiotics enhance phosphorus bioavailability. *Science* 371:1033–1037.
13. Glasser NR, Saunders SH, Newman DK. 2017. The colorful world of extracellular electron shuttles. *Annu Rev Microbiol* 71:731–751.
14. 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.
15. Linares JF, Gustafsson I, Baquero F, Martinez JL. 2006. Antibiotics as intermicrobial signaling agents instead of weapons. *Proc Natl Acad Sci USA* 103:19484–19489.
16. Dietrich LE, 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.
17. Demain AL, Fang A. 2000. The natural functions of secondary metabolites. Adv Biochem Eng Biotechnol 69:1–39.
18. Davies J, Ryan KS. 2012. Introducing the parvome: bioactive compounds in the microbial world. ACS Chem Biol 7:252–259.
19. Handelsman J, Stabb EV. 1996. Biocontrol of soilborne plant pathogens. Plant Cell 8:1855–1869.
20. Haas D, Défago G. 2005. Biological control of soil-borne pathogens by fluorescent pseudomonads. Nat Rev Microbiol 3:307–319.
21. Tyc O, Song C, Dickschat JS, Vos M, Garbeva P. 2017. The ecological role of volatile and soluble secondary metabolites produced by soil bacteria. Trends Microbiol 25:280–292.
22. Mavrodi DV, Mavrodi OV, Parejko JA, Bonsall RF, Kwak Y-S, Paulitz TC, Thomashow LS, Weller DM. 2012. Accumulation of the antibiotic phenazine-1-carboxylic acid in the rhizosphere of dryland cereals. Appl Environ Microbiol 78:804–812.
23. Thomashow LS, Weller DM, Bonsall RF, Pierson LS. 1990. Production of the antibiotic phenazine-1-carboxylic acid by fluorescent *Pseudomonas* species in the rhizosphere of wheat. Appl Environ Microbiol 56:908–912.
24. Bergsma-Vlami M, Prins ME, Raaijmakers JM. 2005. Influence of plant species on population dynamics, genotypic diversity and antibiotic production in the rhizosphere by indigenous *Pseudomonas* spp. FEMS Microbiol Ecol 52:59–69.
25. Mazzola M, Cook RJ, Thomashow LS, Weller DM, Pierson LS. 1992. Contribution of phenazine antibiotic biosynthesis to the ecological competence of fluorescent pseudomonads in soil habitats. Appl Environ Microbiol 58:2616–2624.
26. Yu JM, Wang D, Pierson LS, Pierson EA. 2018. Effect of producing different phenazines on bacterial fitness and biological control in *Pseudomonas chlororaphis* 30-84. Plant Pathol J 34:44–58.
27. Thomashow LS, Weller DM. 1988. Role of a phenazine antibiotic from *Pseudomonas fluorescens* in biological control of *Gaeumannomyces graminis* var. *tritici*. J Bacteriol 170:3499–3508.
28. Haas D, Keel C. 2003. Regulation of antibiotic production in root-colonizing *Pseudomonas* spp. and relevance for biological control of plant disease. Annu Rev Phytopathol 41:117–153.
29. Mazurier S, Corberand T, Lemanceau P, Raaijmakers JM. 2009. Phenazine antibiotics produced by fluorescent pseudomonads contribute to natural soil suppressiveness to *Fusarium* wilt. ISME J 3:977–991.
30. Mazzola M, Fujimoto DK, Thomashow LS, Cook RJ. 1995. Variation in sensitivity of *Gaeumannomyces graminis* to antibiotics produced by fluorescent *Pseudomonas* spp. and effect on biological control of take-all of wheat. Appl Environ Microbiol 61:2554–2559.
31. Costa KC, Bergkessel M, Saunders S, Kurlach J, Newman DK. 2015. Enzymatic degradation of phenazines can generate energy and protect sensitive organisms from toxicity. mBio 6:e01520-15.
32. Turner JM, Messenger AJ. 1986. Occurrence, biochemistry and physiology of phenazine pigment production. Adv Microb Physiol 27:211–275.
33. Baron SS, Rowe JJ. 1981. Antibiotic action of pyocyanin. Antimicrob Agents Chemother 20:814–820.

- 611 34. Kozdrój J, Trevors JT, van Elsas JD. 2004. Influence of introduced potential biocontrol
612 agents on maize seedling growth and bacterial community structure in the rhizosphere. *Soil*
613 *Biol Biochem* 36:1775–1784.
- 614 35. Bankhead SB, Landa BB, Lutton E, Weller DM, Gardener BBM. 2004. Minimal changes
615 in rhizobacterial population structure following root colonization by wild type and
616 transgenic biocontrol strains. *FEMS Microbiol Ecol* 49:307–318.
- 617 36. Roquigny R, Novinscak A, Léger G, Marcoux N, Joly DL, Filion M. 2018. Deciphering
618 the rhizosphere and geocaulosphere microbiomes of potato following inoculation with the
619 biocontrol agent *Pseudomonas fluorescens* strain LBUM223. *Phytobiomes* 2:92–99.
- 620 37. Gagliardi JV, Buyer JS, Angle JS, Russek-Cohen E. 2001. Structural and functional
621 analysis of whole-soil microbial communities for risk and efficacy testing following
622 microbial inoculation of wheat roots in diverse soils. *Soil Biol Biochem* 33:25–40.
- 623 38. Hassan HM, Fridovich I. 1980. Mechanism of the antibiotic action pyocyanine. *J Bacteriol*
624 141:156–163.
- 625 39. Voggu L, Schlag S, Biswas R, Rosenstein R, Rausch C, Götz F. 2006. Microevolution of
626 cytochrome *bd* oxidase in *Staphylococci* and its implication in resistance to respiratory
627 toxins released by *Pseudomonas*. *J Bacteriol* 188:8079–8086.
- 628 40. Perry EK, Newman DK. 2019. The transcription factors ActR and SoxR differentially
629 affect the phenazine tolerance of *Agrobacterium tumefaciens*. *Mol Microbiol* 112:199–218.
- 630 41. Wolloscheck D, Krishnamoorthy G, Nguyen J, Zgurskaya HI. 2018. Kinetic control of
631 quorum sensing in *Pseudomonas aeruginosa* by multidrug efflux pumps. *ACS Infect Dis*
632 4:185–195.
- 633 42. Meirelles LA, Perry EK, Bergkessel M, Newman DK. 2021. Bacterial defenses against a
634 natural antibiotic promote collateral resilience to clinical antibiotics. *PLoS Biol*
635 19:e3001093.
- 636 43. Noto MJ, Burns WJ, Beavers WN, Skaar EP. 2017. Mechanisms of pyocyanin toxicity and
637 genetic determinants of resistance in *Staphylococcus aureus*. *J Bacteriol* 199.
- 638 44. Khare A, Tavazoie S. 2015. Multifactorial competition and resistance in a two-species
639 bacterial system. *PLoS Genet* 11:e1005715.
- 640 45. Meirelles LA, Newman DK. 2018. Both toxic and beneficial effects of pyocyanin
641 contribute to the lifecycle of *Pseudomonas aeruginosa*. *Mol Microbiol* 110:995–1010.
- 642 46. Liu GY, Nizet V. 2009. Color me bad: microbial pigments as virulence factors. *Trends*
643 *Microbiol* 17:406–413.
- 644 47. Lau GW, Ran H, Kong F, Hassett DJ, Mavrodi D. 2004. *Pseudomonas aeruginosa*
645 pyocyanin is critical for lung infection in mice. *Infect Immun* 72:4275–4278.
- 646 48. Dar D, Thomashow LS, Weller DM, Newman DK. 2020. Global landscape of phenazine
647 biosynthesis and biodegradation reveals species-specific colonization patterns in
648 agricultural soils and crop microbiomes. *eLife* 9.
- 649 49. Brisbane PG, Janik LJ, Tate ME, Warren RF. 1987. Revised structure for the phenazine
650 antibiotic from *Pseudomonas fluorescens* 2-79 (NRRL B-15132). *Antimicrob Agents*
651 *Chemother* 31:1967–1971.
- 652 50. Cezairliyan B, Vinayavekhin N, Grenfell-Lee D, Yuen GJ, Saghatelian A, Ausubel FM.
653 2013. Identification of *Pseudomonas aeruginosa* phenazines that kill *Caenorhabditis*
654 *elegans*. *PLoS Pathog* 9:e1003101.
- 655 51. Smiley RW, Cook RJ. 1973. Relationship between take-all of wheat and rhizosphere pH in
656 soils fertilized with ammonium vs. nitrate-nitrogen. *Phytopathology* 63:882–890.

52. Price-Whelan A, Dietrich LEP, Newman DK. 2006. Rethinking “secondary” metabolism: physiological roles for phenazine antibiotics. *Nat Chem Biol* 2:71–78.
53. Séveno NA, Morgan JAW, Wellington EMH. 2001. Growth of *Pseudomonas aureofaciens* PGS12 and the dynamics of HHL and phenazine production in liquid culture, on nutrient agar, and on plant roots. *Microb Ecol* 41:314–324.
54. Tagele SB, Lee HG, Kim SW, Lee YS. 2019. Phenazine and 1-undecene producing *Pseudomonas chlororaphis* subsp. *aurantiaca* strain KNU17Pc1 for growth promotion and disease suppression in Korean maize cultivars. *J Microbiol Biotechnol* 29:66–78.
55. LeTourneau MK, Marshall MJ, Cliff JB, Bonsall RF, Dohnalkova AC, Mavrodi DV, Devi SI, Mavrodi OV, Harsh JB, Weller DM, Thomashow LS. 2018. Phenazine-1-carboxylic acid and soil moisture influence biofilm development and turnover of rhizobacterial biomass on wheat root surfaces. *Environ Microbiol* 20:2178–2194.
56. Mavrodi DV, Blankenfeldt W, Thomashow LS. 2006. Phenazine compounds in fluorescent *Pseudomonas* spp. biosynthesis and regulation. *Annu Rev Phytopathol* 44:417–445.
57. Kumar S, Mukherjee MM, Varela MF. 2013. Modulation of bacterial multidrug resistance efflux pumps of the major facilitator superfamily. *International Journal of Bacteriology* 2013.
58. Jamshidi S, Sutton JM, Rahman KM. 2017. Computational study reveals the molecular mechanism of the interaction between the efflux inhibitor PAβN and the AdeB transporter from *Acinetobacter baumannii*. *ACS Omega* 2:3002–3016.
59. Lamers RP, Cavallari JF, Burrows LL. 2013. The efflux inhibitor phenylalanine-arginine beta-naphthylamide (PAβN) permeabilizes the outer membrane of gram-negative bacteria. *PLoS One* 8:e60666.
60. Schuster S, Bohnert JA, Vavra M, Rossen JW, Kern WV. 2019. Proof of an Outer Membrane target of the efflux inhibitor Phe-Arg-β-Naphthylamide from random mutagenesis. *Molecules* 24.
61. Pao SS, Paulsen IT, Saier MH. 1998. Major facilitator superfamily. *Microbiol Mol Biol Rev* 62:1–34.
62. Nikaido H. 1989. Role of the outer membrane of Gram-negative bacteria in antimicrobial resistance, p. 1–34. *In* Bryan, LE (ed.), *Microbial resistance to drugs*. Springer Berlin Heidelberg, Berlin, Heidelberg.
63. Collins MD, Jones D. 1981. Distribution of isoprenoid quinone structural types in bacteria and their taxonomic implication. *Microbiol Rev* 45:316–354.
64. Singh P, Singh H, Kim Y-J, Yang D-C. 2017. *Pedobacter panacis* sp. nov., isolated from *Panax ginseng* soil. *Antonie Van Leeuwenhoek* 110:235–244.
65. Kong X-K, Chen D, Huang J-W, Cheng X-K, Jiang J-D. 2019. *Chitinophaga deserti* sp. nov., isolated from desert soil. *Int J Syst Evol Microbiol* 69:1783–1788.
66. Lin S-Y, Hameed A, Wen C-Z, Liu Y-C, Shen F-T, Hsu Y-H, Lai W-A, Young C-C. 2015. *Chryseobacterium echinoideorum* sp. nov., isolated from sea urchins (*Tripneustes gratilla*). *Int J Syst Evol Microbiol* 65:3985–3990.
67. White D, Drummond J, Fuqua C. 2012. Electron transport, p. 146–174. *In* *The physiology and biochemistry of prokaryotes*, 4th ed. Oxford University Press, New York, New York.
68. Schiessl KT, Hu F, Jo J, Nazia SZ, Wang B, Price-Whelan A, Min W, Dietrich LEP. 2019. Phenazine production promotes antibiotic tolerance and metabolic heterogeneity in *Pseudomonas aeruginosa* biofilms. *Nat Commun* 10:762.
69. Zhu K, Chen S, Sysoeva TA, You L. 2019. Universal antibiotic tolerance arising from

- antibiotic-triggered accumulation of pyocyanin in *Pseudomonas aeruginosa*. PLoS Biol 17:e3000573.
70. VanDrise CM, Lipsh-Sokolik R, Khersonsky O, Fleishman SJ, Newman DK. 2021. Computationally designed pyocyanin demethylase acts synergistically with tobramycin to kill recalcitrant *Pseudomonas aeruginosa* biofilms. Proc Natl Acad Sci USA 118.
71. Lane DJ. 1991. 16S/23S rRNA sequencing, p. 115–176. In Stackebrandt, E, Goodfellow, M (eds.), Nucleic acid techniques in bacterial systematics. John Wiley & Sons, Inc., New York, New York.
72. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, Tinevez J-Y, White DJ, Hartenstein V, Eliceiri K, Tomancak P, Cardona A. 2012. Fiji: an open-source platform for biological-image analysis. Nat Methods 9:676–682.

Table 1: Organisms used in this study

Species	Strain ID	Source
<i>Agromyces albus</i>	V4I21	This study
<i>Agromyces ramosus</i>	V3I3	This study
<i>Arthrobacter agilis</i>	W3I6	This study
<i>Arthrobacter globiformus</i>	V3I9	This study
<i>Arthrobacter globiformus</i>	V4I26	This study
<i>Arthrobacter globiformus</i>	W2I3	This study
<i>Arthrobacter humicola</i>	B3I4	This study
<i>Arthrobacter pascens</i>	V4I24	This study
<i>Arthrobacter pascens</i>	W1I14	This study
<i>Arthrobacter pascens</i>	W4I8	This study
<i>Arthrobacter pokkali</i>	B2I5	This study
<i>Arthrobacter woluwensis</i>	W4I2	This study
<i>Clavibacter michiganensis</i>	B3I6	This study
<i>Microbacterium murale</i>	W2I7	This study
<i>Microbacterium natoriense</i>	W4I9-1	This study
<i>Microbacterium phyllosphaerae</i>	W4I20	This study
<i>Microbacterium sp.</i>	W4I4	This study
<i>Nocardia salmonicida</i>	V2I2	This study
<i>Pseudarthrobacter chlorophenolicus</i>	B2I9	This study
<i>Pseudarthrobacter defluvii</i>	B1I2	This study
<i>Pseudarthrobacter defluvii</i>	W1I2	This study
<i>Pseudarthrobacter equi</i>	W3I10	This study
<i>Pseudarthrobacter phenanthrenivorans</i>	W4I12	This study

<i>Pseudarthrobacter siccitolerans</i>	B3I9	This study
<i>Pseudarthrobacter siccitolerans</i>	V1I9	This study
<i>Pseudarthrobacter siccitolerans</i>	V3I10	This study
<i>Pseudarthrobacter siccitolerans</i>	W1I19	This study
<i>Pseudarthrobacter siccitolerans</i>	W1I3	This study
<i>Pseudarthrobacter siccitolerans</i>	W4I7	This study
<i>Pseudarthrobacter sp.</i>	V4I6	This study
<i>Streptomyces afghaniensis</i>	W1I20	This study
<i>Streptomyces africanus</i>	B3I12	This study
<i>Streptomyces bobili</i>	V4I16	This study
<i>Streptomyces bungoensis</i>	B4I12	This study
<i>Streptomyces canus</i>	B4I3	This study
<i>Streptomyces canus</i>	V4I20	This study
<i>Streptomyces canus</i>	V4I22	This study
<i>Streptomyces cyaneofuscatus</i>	W4I9-2	This study
<i>Streptomyces ederensis</i>	V2I4	This study
<i>Streptomyces fildesensis</i>	B3I13	This study
<i>Streptomyces galbus</i>	W1I17	This study
<i>Streptomyces luteogriseus</i>	W4I19-1	This study
<i>Streptomyces novaecaesareae</i>	B3I7	This study
<i>Streptomyces novaecaesareae</i>	B3I8	This study
<i>Streptomyces phaeochromogenes</i>	V1I8	This study
<i>Streptomyces plumbiresistens</i>	V4I2	This study
<i>Streptomyces rishiriensis</i>	B2I10	This study
<i>Streptomyces rishiriensis</i>	B2I6	This study
<i>Streptomyces rishiriensis</i>	B4I13	This study
<i>Streptomyces rishiriensis</i>	W4I19-2	This study
<i>Streptomyces sp.</i>	B1I3	This study
<i>Streptomyces sp.</i>	B2I16	This study
<i>Streptomyces sp.</i>	V1I6	This study
<i>Streptomyces sp.</i>	V3I7	This study
<i>Streptomyces sp.</i>	V4I23	This study
<i>Streptomyces sp.</i>	V4I8	This study
<i>Streptomyces sp.</i>	V1I1	This study
<i>Streptomyces sp.</i>	V2I9	This study
<i>Streptomyces tauricus</i>	V3I8	This study
<i>Streptomyces turgidiscabies</i>	V4I19	This study
<i>Streptomyces turgidiscabies</i>	W2I16	This study
<i>Chitinophaga ginsengisegetis</i>	W2I13	This study

<i>Chitinophaga ginsengisegetis</i>	W3I9	This study
<i>Chryseobacterium ginsenosidimutans</i>	W1I9	This study
<i>Chryseobacterium shigense</i>	W4I1	This study
<i>Pedobacter kyongii</i>	W3I1	This study
<i>Pedobacter kyongii</i>	W4I14	This study
<i>[Brevibacterium] frigoritolerans</i>	B2I3	This study
<i>Bacillus atrophaeus</i>	V3I1	This study
<i>Bacillus idriensis</i>	V2I10	This study
<i>Bacillus pumilus</i>	B3I3	This study
<i>Neobacillus drentensis</i>	B4I4	This study
<i>Neobacillus drentensis</i>	B4I6	This study
<i>Neobacillus drentensis</i>	V3I5	This study
<i>Neobacillus niacini</i>	V4I10	This study
<i>Neobacillus niacini</i>	V4I12	This study
<i>Neobacillus niacini</i>	V4I25	This study
<i>Paenibacillus alginolyticus</i>	V4I5	This study
<i>Paenibacillus alginolyticus</i>	V4I7	This study
<i>Paenibacillus marchantiophytorum</i>	V4I3	This study
<i>Paenibacillus mobilis</i>	W2I17	This study
<i>Paenibacillus mobilis</i>	W4I10	This study
<i>Paenibacillus nebraskensis</i>	V4I9	This study
<i>Paenibacillus rhizoryzae</i>	B1I5	This study
<i>Paenibacillus rhizoryzae</i>	B2I8	This study
<i>Peribacillus simplex</i>	B2I15	This study
<i>Peribacillus simplex</i>	B2I2	This study
<i>Peribacillus simplex</i>	V2I11	This study
<i>Priestia megaterium</i>	B1I1	This study
<i>Priestia megaterium</i>	B1I6	This study
<i>Priestia megaterium</i>	W1I8	This study
<i>Inquilinus ginsengisoli</i>	V4I1	This study
<i>Luteibacter jiangsuensis</i>	W1I16	This study
<i>Massilia niastensis</i>	W1I21	This study
<i>Massilia plicata</i>	W1I10	This study
<i>Pantoea agglomerans</i>	W2I1	This study
<i>Paraburkholderia graminis</i>	W1I13	This study
<i>Phyllobacterium ifrigiyense</i>	W4I11	This study
<i>Pseudomonas brenneri</i>	W2I6	This study
<i>Pseudomonas cedrina</i>	W1I11	This study
<i>Pseudomonas fluorescens</i>	W3I7	This study

<i>Pseudomonas orientalis</i>	W4I3	This study
<i>Pseudomonas synxantha</i>	W2I4	This study
<i>Sphingomonas faeni</i>	W4I17	This study
<i>Variovorax boronicumulans</i>	W1I1	This study
<i>Variovorax boronicumulans</i>	W2I14	This study
<i>Variovorax paradoxus</i>	W1I18	This study
<i>Variovorax paradoxus</i>	W2I8	This study
<i>Agromyces agilis</i>	DSM 20550	DSMZ
<i>Massilia niastensis</i>	DSM 21313	DSMZ
<i>Massilia plicata</i>	DSM 17505	DSMZ
<i>Pseudarthrobacter equi</i>	DSM 23395	DSMZ
<i>Pseudarthrobacter phenanthrenivorans</i>	DSM 18606	DSMZ
<i>Pseudomonas brenneri</i>	DSM 15294	DSMZ
<i>Sphingomonas faeni</i>	DSM 14747	DSMZ
<i>Streptomyces luteogriseus</i>	DSM 40483	DSMZ
<i>Agromyces albus</i>	NBRC 103057	NBRC
<i>Agromyces ramosus</i>	NBRC 13899	NBRC
<i>Arthrobacter globiformus</i>	NBRC 12137	NBRC
<i>Chitinophaga ginsengisegetis</i>	NBRC 109750	NBRC
<i>Neobacillus drenensis</i>	NBRC 102427	NBRC
<i>Peribacillus simplex</i>	NBRC 15720	NBRC
<i>Priestia megaterium</i>	NBRC 15308	NBRC
<i>Streptomyces rishiriensis</i>	NBRC 13407	NBRC
<i>Variovorax boronicumulans</i>	NBRC 103145	NBRC
<i>Variovorax paradoxus</i>	NBRC 15149	NBRC
<i>Bacillus atrophaeus</i>	NRS-213	NRRL
<i>Bacillus pumilus</i>	NRS-272	NRRL
<i>Streptomyces africanus</i>	B-24243	NRRL
<i>Streptomyces aurantiacus</i>	ISP-5412	NRRL
<i>Streptomyces bobili</i>	B-1338	NRRL
<i>Streptomyces bungoensis</i>	B-24305	NRRL
<i>Streptomyces canus</i>	B-3980	NRRL
<i>Streptomyces ciscaucasicus</i>	ISP-5275	NRRL
<i>Streptomyces cyaneofuscatus</i>	B-2570	NRRL
<i>Streptomyces novaecaesareae</i>	B-1267	NRRL
<i>Streptomyces peucetius</i>	B-3826	NRRL
<i>Streptomyces phaeochromogenes</i>	B-1248	NRRL

720

721

722 **Figure legends**

723

724 **Figure 1: Taxonomic distribution of bacterial isolates from wheat rhizosphere and bulk soil**
725 **samples.**

726 This plot depicts the proportion of isolates from each sample that belonged to the 4 represented
727 phyla. Each column represents one soil sample, and each box within the columns represents an
728 individual isolate colored by the phylum to which it belongs (e.g. 6 boxes comprising one column
729 indicates that 6 strains were isolated from that sample). B = Between (bulk soil), V = Virgin
730 (bulk soil), W = Wheat (rhizosphere).

731

732 **Figure 2: Distribution of PCA resistance phenotypes across phyla and soil sample type.**

733 **A-B.** Heat maps depicting the growth of the strains at pH 5.1 (A) and pH 7.3 (B). Each row
734 represents a strain. The leftmost columns are colored according to the ratio of growth on PCA-
735 containing agar versus PCA-free agar; magenta indicates sensitivity to PCA while green indicates
736 resistance to PCA. The right two columns in each heat map are colored according to the separate
737 values for growth on PCA-containing agar (+ PCA) or solvent control agar (- PCA), with darker
738 green indicating more growth. Values are the mean of two to four biological replicates that each
739 comprised three technical replicates. Asterisks indicate strains that displayed markedly variable
740 susceptibility to PCA across biological replicates. Strains for which fewer than two biological
741 replicates grew to stationary phase in the PCA-free control at the given pH, or for which color
742 interfered with growth quantification, were omitted from this analysis. See Methods for a
743 description of how growth was quantified. **C.** Relative abundance of putative phenazine
744 producers (*phz+* bacteria) across the three types of soil samples. Error bars represent the standard
745 deviation. **D-E.** Density plots (i.e. smoothed histograms) representing the distribution of PCA
746 resistance phenotypes at pH 5.1 (D) and pH 7.3 (E) among strains isolated from Between (bulk),
747 Virgin (bulk), or Wheat (rhizosphere) soil. Higher values along the x-axis indicate greater
748 resistance to PCA. If multiple identical strains were isolated from the same soil type, only one
749 representative was counted; thus, 26 strains were isolated from Between, 39 from Virgin, and 45
750 from Wheat. Colored tick marks above the x-axis represent where individual isolates fall along
751 the range of PCA resistance phenotypes.

752

753 **Figure 3: Growth of representative strains over time with and without PCA.**

754 Growth was quantified as described in the Methods. Solid lines represent the growth of spotted
755 cultures on PCA-free agar, and dashed lines represent the growth of spotted cultures on agar
756 containing 100 μ M PCA. Blue represents growth at pH 7.3 and pink represents growth at pH 5.1.
757 Data points are the mean of three technical replicates from a representative biological replicate
758 for each strain, and the shaded ribbon represents the standard deviation. The selected strains
759 depicted here cover the range of PCA susceptibility phenotypes observed in each genus. The full
760 set of biological replicates for all tested isolates is displayed in supplementary Figs. S4-S7.

761

762 **Figure 4: PCA reduction rates of selected strains at pH 5.1 and pH 7.3.**

763 **A.** PCA reduction rates of representative strains from each phylum at pH 5.1 and pH 7.3. The
764 values represent the mean of three biological replicates and error bars represent the standard
765 deviation. Reduction rates were normalized to OD₆₀₀ values. **B.** PCA reduction rates versus each
766 strain's growth ratio (+PCA/-PCA) at early stationary phase. The growth ratios are the same
767 values calculated and used in Fig. 2.

768

769 **Figure 5: Effects of efflux pump inhibitors on PCA susceptibility.**

770 **A-B.** Growth curves of strains for which treatment with a combination of the efflux pump
771 inhibitors (EPI) reserpine and PA β N did not affect susceptibility to PCA at pH 5.1 (A) or pH 7.3
772 (B). **C.** Growth curves of strains for which treatment with reserpine and PA β N potentiated the
773 toxicity of PCA. **D.** Growth curves of strains for which treatment with reserpine and PA β N
774 improved growth in the presence of PCA (strain W4I17) or vice versa (strains W1I16 and W4I9-
775 1). In all panels, data points are the mean of three biological replicates and the shaded ribbon
776 represents the standard deviation. The concentrations of reserpine and PA β N used for each strain
777 can be found in Table S2.

778