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

Running title: Phenazine resistance in wheat rhizosphere community

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Abstract

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

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

Importance

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

Introduction

Diverse microorganisms produce natural antibiotics that can kill or inhibit the growth of other microbes (Bérdy, 2012; Granato *et al.*, 2019). Several such compounds have been commercialized as antimicrobial drugs for the treatment of infections, beginning with penicillin in the 1940s (Aminov, 2010; Hutchings *et al.*, 2019). Unfortunately, the selective pressures exerted by the widespread use of antibiotics in medicine and agriculture have led to worrisome increases in the prevalence of antimicrobial resistance among human and animal pathogens over the past several decades (Davies and Davies, 2010; Manyi-Loh *et al.*, 2018). Yet while this repercussion of human

55 antibiotic use has been well documented, comparatively little is known about the ecological effects
56 of microbially-produced antibiotics in natural environments (Aminov, 2009; Sengupta *et al.*, 2013).
57 Recent studies have suggested that natural antibiotics may serve a variety of functions for their
58 producers beyond the suppression of competing microbes (Davies, 2006; Davies *et al.*, 2006),
59 including nutrient acquisition (Wang and Newman, 2008; McRose and Newman, 2021),
60 conservation of energy in the absence of oxygen (Glasser *et al.*, 2014; Glasser *et al.*, 2017), and cell-
61 cell signaling (Dietrich *et al.*, 2006; Linares *et al.*, 2006). At the same time, toxicity to one or more
62 microorganisms is by definition a feature of these molecules, but the extent to which this trait shapes
63 their influence on microbial communities is unclear (Demain and Fang, 2000; Davies and Ryan,
64 2012; Bérdy, 2012). In addition, for many if not most natural antibiotics, the determinants and
65 prevalence of susceptibility or resistance to their toxicity remain unknown or poorly characterized
66 (Handelsman and Stabb, 1996). These gaps in knowledge hinder our ability to understand and predict
67 the impacts of these metabolites on microbial communities of interest.

68 One environmental context in which natural antibiotics are thought to be particularly
69 abundant and ecologically relevant is the rhizosphere—the narrow plane of soil immediately
70 adjacent to plant roots (Haas and Défago, 2005; Mavrodi *et al.*, 2012; Tyc *et al.*, 2017). Natural
71 antibiotics such as phenazines and 2,4-diacetylphloroglucinol have been directly detected in the
72 rhizospheres of multiple crops, including wheat, potato, and sugar beet (Thomashow *et al.*, 1990;
73 Bergsma-Vlami *et al.*, 2005; Mavrodi *et al.*, 2012), and phenazines have been shown to increase the
74 fitness of their producers when competing with other microbes in the rhizosphere (Mazzola *et al.*,
75 1992; Yu *et al.*, 2018). Production of these molecules has also been demonstrated to underpin the
76 ability of certain bacteria to control fungal crop diseases (Thomashow and Weller, 1988;
77 Thomashow *et al.*, 1990; Haas and Keel, 2003; Mazurier *et al.*, 2009; Yu *et al.*, 2018), further
78 indicating that natural antibiotics can act as agents of microbe-microbe antagonism in the
79 rhizosphere. As a result of this activity, phenazine-producing *Pseudomonas* strains have received
80 attention as potential biocontrol agents that could serve as a more sustainable alternative to traditional
81 synthetic pesticides in agriculture (Handelsman and Stabb, 1996; Haas and Keel, 2003). However,
82 several challenges remain with respect to the practical application of these strains, including
83 inconsistent efficacy under field conditions (Haas and Keel, 2003; Haas and Défago, 2005), limited
84 understanding of the mechanisms and evolutionary dynamics of resistance to phenazines (Mazzola

85 *et al.*, 1995; Handelsman and Stabb, 1996), and the possibility of off-target effects (Haas and Keel,
86 2003).

87 Importantly, with regard to the latter concern, phenazines are toxic not only to fungi, but also
88 to some bacteria (Baron and Rowe, 1981; Turner and Messenger, 1986; Costa *et al.*, 2015). Yet
89 while the utility of phenazine-producing pseudomonads for biocontrol of fungal crop diseases has
90 been extensively investigated, the potential impact of these strains on non-target bacterial residents
91 of the rhizosphere is less well understood. One study found that inoculation of *Pseudomonas*
92 biocontrol strains shifted the rhizosphere community of maize seedlings, pushing the ratio of Gram-
93 positive to Gram-negative bacteria in favor of the latter; however, this analysis was based on
94 profiling colony growth rates and whole-cell fatty acids from pooled cultured isolates, greatly
95 limiting the taxonomic resolution and making it difficult to rule out whether the Gram-negative
96 biocontrol strains might themselves have directly contributed to the shift (Kozdrój *et al.*, 2004). On
97 the other hand, at least three studies found no notable or consistent effects of introduced
98 *Pseudomonas* species on the rhizosphere bacterial communities of wheat or potato (Gagliardi *et al.*,
99 2001; Bankhead *et al.*, 2004; Roquigny *et al.*, 2018), albeit the studies in wheat employed methods
100 with limited discriminatory power (namely, carbon source utilization profiling and terminal
101 restriction fragment length polymorphism). Given these mixed results and the lack of fine-grained
102 spatial or taxonomic resolution in most studies on this topic, whether phenazine-producing bacteria
103 actively shape the surrounding rhizosphere bacterial community through antibiosis, potentially at the
104 micron or millimeter scale, remains an open question.

105 In addition to the lack of clarity regarding the effects of phenazines on rhizosphere bacterial
106 communities, the taxonomic and physiological correlates of phenazine resistance in bacteria remain
107 incompletely understood. The toxicity of phenazines is generally ascribed to the generation of
108 reactive oxygen species (ROS) and interference with respiration (Hassan and Fridovich, 1980; Baron
109 and Rowe, 1981; Voggu *et al.*, 2006; Perry and Newman, 2019). Previous studies have suggested
110 that efflux pump expression, cell permeability, oxidative stress responses, and the composition of
111 the respiratory electron transport chain can affect bacterial susceptibility to phenazines (Voggu *et*
112 *al.*, 2006; Khare and Tavazoie, 2015; Noto *et al.*, 2017; Wolloscheck *et al.*, 2018; Perry and
113 Newman, 2019; Meirelles *et al.*, 2021). In addition, a comparison of 14 bacterial strains indicated
114 that Gram-negative bacteria as a group may be more resistant to phenazine toxicity than Gram-

115 positive bacteria (Baron and Rowe, 1981). However, all of these studies focused on a specific
116 phenazine, pyocyanin, that is particularly toxic (Meirelles and Newman, 2018) and best known for
117 its role as a virulence factor during infections of humans and animals (Lau *et al.*, 2004; Liu and
118 Nizet, 2009). Whether the same observations hold true for more agriculturally-relevant phenazines
119 such as phenazine-1-carboxylic acid (PCA) (Thomashow and Weller, 1988; Thomashow *et al.*,
120 1990; Mavrodi *et al.*, 2012; Dar *et al.*, 2020) is unknown.

121 In this study, we set out to lay a foundation for addressing unresolved questions about the
122 ecological impact of phenazine toxicity in the rhizosphere by determining the prevalence of
123 phenazine resistance among bacteria isolated from a wheat field in the Inland Pacific Northwest, a
124 region where phenazine production and the biocontrol potential of indigenous *Pseudomonas* species
125 have been studied for decades (Thomashow and Weller, 1988; Thomashow *et al.*, 1990; Mazzola *et*
126 *al.*, 1992; Mavrodi *et al.*, 2012). We designed a culture-based assay to measure sensitivity to PCA,
127 which is the best-studied and one of the most abundant phenazines in this environment (Thomashow
128 *et al.*, 1990; Mavrodi *et al.*, 2012; Dar *et al.*, 2020). We also performed full-length 16S rRNA gene
129 sequencing of our isolates in order to assess the relationship between taxonomy and PCA resistance.
130 Finally, to assess potential physiological correlates of PCA resistance, we measured PCA reduction
131 rates for a subset of phenotypically-diverse strains and investigated the effects of broad-spectrum
132 efflux pump inhibitors on growth in the presence of PCA.

133 **Results**

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

135 A total of 166 strains of bacteria were isolated from 12 soil samples collected from a wheat
136 field at Washington State University's Lind Dryland Research Station in early August 2019, shortly
137 after the wheat harvest. The samples comprised 4 replicates each of wheat rhizosphere ("Wheat"),
138 bulk soil collected between planted rows ("Between"), and bulk soil from a virgin patch of
139 uncultivated soil adjacent to the field ("Virgin"). Full-length 16S rRNA gene sequencing revealed
140 that the isolates represented 24 genera across 4 phyla: Actinobacteria, Bacteroidetes, Firmicutes, and
141 Proteobacteria. The vast majority of isolates from the bulk soil samples (both Between and Virgin)
142 were Actinobacteria or Firmicutes. In Wheat samples, on the other hand, the combined proportions

143 of these two phyla were lower, accounting for 18-50% and 10-25% of isolates respectively, while
144 Proteobacteria accounted for approximately 25-60% of isolates depending on the replicate, and 1-3
145 strains of Bacteroidetes were also detected in each replicate (5-12% of isolates) (Fig. 1).

146 *Taxonomic and spatial distribution of PCA resistance phenotypes*

147 We screened our isolates for resistance to PCA at both circumneutral and acidic pH (7.3 and
148 5.1, respectively), because the toxicity of PCA to diverse organisms is known to vary depending on
149 pH (Brisbane *et al.*, 1987; Cezairliyan *et al.*, 2013), and because the bulk soil and rhizosphere pH of
150 wheat fields in the Inland Pacific Northwest can vary by >3 units (pH 4.3-8.0) depending on
151 geographic location and fertilizer treatment status (Smiley and Cook, 1973). The pH-dependency of
152 PCA toxicity has been attributed to the fact that the deprotonated form of PCA is negatively charged
153 (Fig. S1); the negative charge on bacterial cell walls and the outer membrane of Gram-negative
154 bacteria (or the negative membrane potential of eukaryotic cells) likely hinders uptake of this species.
155 The protonated form of PCA, on the other hand, is neutral and presumably can passively diffuse
156 across cell membranes given the small size and hydrophobic nature of the molecule (Price-Whelan
157 *et al.*, 2006; Cezairliyan *et al.*, 2013). The pKa of PCA is 4.24 at 25 °C; thus, at pH 7, only 0.17%
158 of PCA in solution is protonated compared to 14.8% at pH 5, typically leading to greater toxicity at
159 the lower pH (Brisbane *et al.*, 1987). We chose 100 µM as the working concentration of PCA as this
160 is likely to be in a physiologically relevant range based on concentrations measured both in pure
161 cultures and in the field. In broth cultures of biocontrol strains of *Pseudomonas*, PCA accumulates
162 to concentrations ranging from dozens to hundreds of micromolar (Séveno *et al.*, 2001; Tagele *et al.*,
163 2019). In natural wheat rhizospheres, PCA has been detected at nanomolar concentrations (Mavrodi
164 *et al.*, 2012), but these bulk measurements almost certainly underestimate local concentrations at the
165 micron scale given that bacteria colonize the rhizosphere in a patchy manner (Thomashow *et al.*,
166 1990). Notably, PCA can accumulate in biofilms to concentrations 360-fold greater on a per volume
167 basis compared to broth cultures (Séveno *et al.*, 2001), suggesting that local concentrations of PCA
168 in the rhizosphere, where biocontrol strains form robust biofilms (LeTourneau *et al.*, 2018), may be
169 orders of magnitude higher than the reported bulk values.

170 The screen was performed using 24-well plates and 0.1x tryptic soy agar (TSA) that was
171 either left unadjusted (pH 7.3-7.5) or adjusted to pH 5.1. Each isolate was spotted onto agar in

172 separate wells to prevent crosstalk and antagonism between the strains, and image analysis was used
173 to derive quantitative information about the growth of each strain over the course of 7 days (with
174 one timepoint per day). Because several strains among our 166 isolates appeared to be duplicates of
175 each other based on 16S sequence and colony morphology, we restricted the screen to 108 strains
176 that we judged likely to be unique (Table S1A); where multiple strains appeared identical, we chose
177 a representative strain. We also included 30 strains obtained from public culture collections that
178 represented species found among our isolates (Table S1B), in order to investigate the extent to which
179 PCA resistance phenotypes are consistent within species across different strains isolated from
180 different geographical locations. Accurate quantification of growth was not possible in this screen
181 for certain strains that formed transparent colonies, produced dark pigments, or had a tendency to
182 turn mucoid and spread (Fig. S2). Nevertheless, this assay enabled us to derive detailed profiles of
183 PCA sensitivity and resistance for the vast majority of our strains.

184 To compare PCA susceptibility across strains, we first focused on a single-timepoint
185 snapshot of each strain's phenotype taken at the equivalent of early stationary phase (i.e. around the
186 time that the spots on non-PCA control plates reached their maximal density). In accordance with
187 previous reports of the pH dependency of PCA toxicity, we found that more of our strains were
188 sensitive to PCA at pH 5.1 than at pH 7.3, and strains that were mildly inhibited by PCA at pH 7.3
189 were typically inhibited more strongly at pH 5.1 (Fig. 2A-B). At pH 5.1, strains of Actinobacteria
190 and Firmicutes were strongly inhibited by PCA, while most Proteobacteria were relatively resistant.
191 Members of Bacteroidetes exhibited variable responses, ranging from mild to severe growth
192 inhibition (Fig. 2A-B). At pH 7.3, there was considerably more phenotypic variation among the
193 Actinobacteria (Fig. 2A-B), particularly within the *Streptomyces* genus. In some cases, there was
194 even noticeable variation within the same putative species of *Streptomyces* (Fig. S3). In addition,
195 two strains of *Microbacterium* (W2I7 and W4I20), as well as one strain of *Arthrobacter* (W3I6),
196 grew slightly better in the presence of PCA at pH 7.3 compared to the control condition, perhaps
197 suggesting that they can use PCA as a carbon source or otherwise benefit from its presence at a pH
198 where toxicity is limited (Fig. 2B). On the other hand, in contrast to the phenotypic variation seen
199 among Actinobacteria, Firmicutes remained almost universally sensitive to PCA at pH 7.3 (albeit
200 somewhat less so than at pH 5.1), while all members of Bacteroidetes were resistant to PCA at pH
201 7.3, in contrast to their sensitivity to PCA at pH 5.1 (Fig. 2A-B). Proteobacteria remained generally

202 resistant (Fig. 2A-B), with the most notable exception being a strain of *Sphingomonas faeni*, isolate
203 W4I17, that was inhibited by PCA at both pH 7.3 and pH 5.1 (Fig. 2A-B).

204 Interestingly, while most strains yielded qualitatively consistent results across independent
205 biological replicates that were separated by months (Fig. S4-S7), a handful of strains displayed
206 variable growth and lag times at pH 5.1 either in the absence of PCA (mostly among Actinobacteria
207 and Firmicutes), or in the presence of PCA (a few strains among the Bacteroidetes). Growth at pH
208 7.3 was generally more consistent, with the exception being two strains of *Neobacillus niacini*
209 (V4I10 and V4I25) that initially failed to grow in the presence of PCA, but grew with minimal to no
210 inhibition in subsequent replicates. The reasons for these discrepancies are unclear, but may be
211 related to variations in how long the strains had been in stationary phase prior to inoculating the
212 experimental cultures, which was difficult to control precisely due to differing growth rates across
213 the large number of strains. The trace nutrient content may also have varied between different
214 batches of media, as the first replicate was performed using a different lot of tryptic soy broth
215 compared to subsequent replicates. Thus, the growth and PCA sensitivity of some strains may be
216 influenced by environmental factors beyond pH that remain to be elucidated.

217 We next examined whether there was any evidence of correlation between PCA resistance
218 phenotypes and the type of soil each strain was isolated from (Between, Virgin, or Wheat). A
219 previous study based on samples taken from the same wheat field found that the relative abundance
220 of phenazine producers was higher in the wheat rhizosphere compared to adjacent bulk soil (Dar *et*
221 *al.*, 2020), a finding that we also confirmed using shotgun metagenomic sequencing of our soil
222 samples (Fig. 2C). We therefore hypothesized that if PCA-mediated antibiosis had shaped the
223 bacterial community composition of this field, the prevalence of PCA resistance would be higher
224 among isolates from the rhizosphere samples. Indeed, the Wheat samples clearly had the highest
225 proportion of PCA-resistant isolates, regardless of whether resistance was assessed at pH 5.1 (Fig.
226 2D) or pH 7.3 (Fig. 2E). By contrast, all strains from Between or Virgin samples were highly
227 sensitive to PCA at pH 5.1, though at pH 7.3, the resistance phenotypes of isolates from either type
228 of bulk soil spanned the full range from highly sensitive to completely resistant. However, these
229 findings are conflated with the fact that all of our Bacteroidetes strains, and all but one strain of
230 Proteobacteria, were exclusively isolated from the Wheat samples, and that the members of these
231 two phyla were generally relatively resistant to PCA, especially at pH 7.3. Further experiments will

232 be necessary in order to distinguish whether the enrichment of PCA-resistant phenotypes in the
233 rhizosphere samples is a consequence of PCA-mediated antibiosis or merely an indirect reflection
234 of other factors that favor the growth of Proteobacteria and Bacteroidetes in the rhizosphere.

235 Finally, visualizing the growth of each strain over the full 7-day time course revealed more
236 nuanced variations among the PCA resistance phenotypes at both pH 5.1 and pH 7.3 (Figs. 3 and
237 S4-S7). For example, while some strains of Bacteroidetes displayed increased lag and/or slower
238 growth rates in the presence of PCA at pH 5.1, the growth on PCA often eventually caught up to the
239 PCA-free controls. At pH 7.3, the effect of PCA on *Peribacillus* species was a combination of
240 increased lag and lower final cell density, but apparently not decreased maximal growth rate, as
241 compared to no growth at all on PCA at pH 5.1. This was distinct from the slower growth rates seen
242 for certain strains of *Bacillus*, *Neobacillus*, and *Paenibacillus* at pH 7.3. A few strains of the latter
243 three genera appeared to be still unable to grow on PCA at pH 7.3 based on the values derived from
244 image analysis (Fig. S5). However, examination of the plate images by eye revealed that this
245 sometimes reflected a limitation in the sensitivity of our imaging and quantification methods to low
246 levels of growth, rather than a true lack of growth (Fig. S2).

247 *PCA reduction rates in relation to PCA susceptibility*

248 We next sought to determine whether there are physiological correlates of PCA susceptibility
249 or resistance across taxonomically-diverse bacteria. Given that phenazines are redox-active
250 molecules whose toxicity is thought to be related to ROS generation as a consequence of redox
251 cycling in the cell (Hassan and Fridovich, 1980; Mavrodi *et al.*, 2006), we hypothesized that
252 susceptibility to PCA would be correlated with higher redox-cycling rates. PCA oxidation occurs
253 rapidly and abiotically in the presence of oxygen (Wang and Newman, 2008), suggesting that the
254 rate of PCA reduction would be the primary driver of differences in redox-cycling rates across strains
255 under oxic conditions. Therefore, to test our hypothesis, we measured the rate of PCA reduction
256 under anoxia as a proxy for the true redox-cycling rate, using a subset of strains that covered a range
257 of taxonomic groups and PCA susceptibility phenotypes.

258 For each tested strain, the PCA reduction rate was typically higher at pH 5.1 than at pH 7.3
259 (Fig. 4A), matching the expectation that PCA uptake would tend to be faster at a lower pH and the

260 observation that most susceptible strains were more sensitive to PCA at pH 5.1. The exceptions were:
261 1) strains belonging to the Firmicutes, which had lower reduction rates at pH 5.1 in our assay despite
262 being more susceptible to PCA under this condition, and 2) *Sphingomonas faeni* W4I17, which was
263 in fact more susceptible to PCA at pH 7.3 than at pH 5.1 (Fig. 2A-B), matching the pH dependency
264 of its rate of PCA reduction. Notably, some of the tested Firmicutes often struggled to grow at pH
265 5.1 even in the absence of PCA (Fig. S5). This observation might account for the inhibitory effect
266 of acidic pH on PCA reduction in these strains, given that a functional metabolism would be required
267 for the generation of cellular reductants, such as NADH, that can indirectly or directly reduce PCA.
268 For the strains of Firmicutes that were able to grow at acidic pH (namely B1I1 and B1I6), the reason
269 for their lower PCA reduction rate at pH 5.1 remains unclear.

270 We also examined whether there was any correlation between PCA reduction rates and
271 resistance levels across different strains at each pH. To do so, we plotted each strain's PCA reduction
272 rate against the early stationary phase snapshot ratio of growth on PCA versus no PCA (Fig. 4B).
273 We also calculated Spearman's correlation coefficient (r_s). Interestingly, there was a statistically
274 significant negative correlation between reduction rate and resistance at pH 7.3 (r_s = -0.70, p <
275 0.001), but not at pH 5.1 (r_s = -0.16, p = 0.4485). However, there were exceptions to the trend even
276 at pH 7.3; for example, while PCA severely and consistently inhibited the growth of the four fastest
277 PCA-reducing strains, the fifth-fastest strain, W2I1 (*Pantoea agglomerans*), appeared to be
278 completely resistant to PCA in one biological replicate, though its growth was mildly inhibited by
279 PCA in another replicate (Fig. S7). In addition, strains with reduction rates in the middle range of
280 0.2-0.4 nmol/OD_{600}/min spanned the full range of resistance phenotypes, from a growth ratio of 0.12
281 (*Peribacillus simplex* B2I2) to 0.96 (*Phyllobacterium ifrigiyense* W4I11). Taken together, these data
282 suggest that a low reduction rate may be helpful in mitigating PCA toxicity but is neither necessary
283 nor sufficient for resistance.

284 *Effects of efflux pump inhibitors on PCA susceptibility*

285 Given that PCA reduction rate alone was insufficient to account for the range of sensitivities
286 to PCA, we asked if efflux pumps might serve as an important contributor to PCA resistance in some
287 strains. To address this question, we used two efflux pump inhibitors (EPIs) in combination: 1)
288 reserpine, which is thought to target efflux pumps in the major facilitator superfamily (MFS) (Kumar

289 *et al.*, 2013), and 2) phenylalanine-arginine β-naphthylamide (PAβN), which is thought to target
290 resistance-nodulation-division (RND) efflux pumps (Jamshidi *et al.*, 2017), although it may also
291 permeabilize the outer membrane of Gram-negative bacteria (Lamers *et al.*, 2013; Schuster *et al.*,
292 2019). We tested these EPIs on a subset of the strains that were used in the PCA reduction assay; we
293 excluded the *Bacillus* strains due to their tendency to settle on the bottom of the wells during growth
294 in the plate reader, which confounded the OD_{600} readings. Preliminary experiments revealed that
295 different strains were differentially sensitive to the EPIs themselves, with growth inhibition evident
296 even in PCA-free controls. We therefore determined for each strain the maximal EPI concentration
297 that, when the two EPIs were used separately, did not markedly reduce growth of PCA-free controls
298 (up to a maximum dose of 20 μg/mL for reserpine and 50 μg/mL for PAβN) (Table S2).
299 Interestingly, for many strains, the combination of both EPIs remained highly toxic even after this
300 optimization step, particularly at pH 7.3, possibly indicating synergistic toxicity or redundant roles
301 of different efflux pumps in preventing intracellular accumulation of toxic metabolic intermediates
302 (Fig. S9); the toxicity appeared to be pH-dependent, suggesting that the protonation state of the EPIs
303 might influence their efficacy. In these cases, it was not possible to assess the impact of efflux pump
304 inhibition on PCA sensitivity. However, for the remaining strains, three types of responses to the
305 EPIs emerged. For one group of strains, treatment with both EPIs in combination did not affect their
306 resistance to PCA (Fig. 5A-B); however, we cannot distinguish whether this is because efflux does
307 not contribute to PCA resistance in these strains, or because the tested EPIs did not effectively target
308 the relevant efflux pumps. For the second group of strains, the EPIs enhanced the toxicity of PCA,
309 as would be expected if efflux is an important mechanism of resistance (Fig. 5C). Finally, for the
310 third group of strains, the EPIs counterintuitively appeared to mitigate the toxicity of PCA, or vice
311 versa (Fig. 5D). Although unexpected, the latter phenomenon indirectly suggests that PCA interacts
312 with efflux systems in these strains. For example, treatment with either PCA or the EPIs might
313 upregulate the expression of certain efflux pumps, leading to decreased toxicity relative to treatment
314 with either type of toxin alone. Alternatively, given that some pumps in the MFS family are thought
315 to be bidirectional (Pao *et al.*, 1998), it is possible that MFS pumps promote uptake rather than export
316 of PCA in certain strains.

317 **Discussion**

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

327 One of our more notable findings is that the previously reported pH dependency of PCA
328 toxicity varies across bacterial taxonomic groups. For most Actinobacteria and Firmicutes,
329 sensitivity to PCA was clearly higher at pH 5.1 than at pH 7.3, indicating greater toxicity at the lower
330 pH as expected according to the pKa of PCA. On the other hand, nearly all tested Proteobacteria
331 were completely resistant to 100 μM PCA regardless of pH, at least down to pH 5.1. Consequently,
332 at pH 5.1, PCA resistance phenotypes largely correlated with phylum—and more broadly, a Gram-
333 positive versus Gram-negative divide, which has previously been reported for the toxicity of another
334 phenazine, pyocyanin (Baron and Rowe, 1981). The Gram-positive versus Gram-negative divide at
335 pH 5.1 is perhaps not surprising, as at this pH, a significant proportion (~14%) of PCA in solution is
336 protonated and therefore would not be repelled by the negatively charged cell wall. Under this
337 condition, the outer membrane of Gram-negative species presumably presents an additional barrier
338 to the entry of PCA, helping to limit the intracellular accumulation of the toxin in the same manner
339 as for numerous other antibiotics (Nikaido, 1989). Less expected, however, was the within-phylum
340 and even within-species variation in PCA resistance phenotypes at pH 7.3 among certain taxonomic
341 groups. Interestingly, several *Streptomyces* strains exhibited at least some PCA-dependent growth
342 inhibition at pH 7.3, even though many *Streptomyces* species are capable of producing their own
343 toxic phenazines (Turner and Messenger, 1986; Dar *et al.*, 2020). Given that the major limit on PCA
344 toxicity at circumneutral pH is thought to be its ability to enter cells, the phenotypic variability at pH
345 7.3 may indicate the presence of transporters or channels capable of phenazine uptake in some PCA-
346 sensitive Actinobacteria. Alternatively, it is possible that some PCA-sensitive strains locally
347 acidified the growth medium, which was only weakly buffered (1.4 mM K_2HPO_4).

348 Importantly, despite the general Gram-positive versus Gram-negative divide in sensitivity to
349 PCA at pH 5.1, possessing an outer membrane is evidently not a leakproof shield against PCA
350 toxicity. Proteobacteria as a group were more resistant to PCA at pH 5.1 compared to strains of
351 Bacteroidetes, most of which exhibited increased lag in the presence of PCA, even though both phyla
352 comprise Gram-negative bacteria. Intriguingly, one major difference between these clades is that
353 Proteobacteria generally utilize ubiquinone as an electron carrier during aerobic growth (Collins and
354 Jones, 1981), while the Bacteroidetes genera screened in this study (*Chitinophaga*,
355 *Chryseobacterium*, and *Pedobacter*) utilize menaquinone (Lin *et al.*, 2015; Singh *et al.*, 2017; Kong
356 *et al.*, 2019). Menaquinones have a lower reduction potential compared to ubiquinones (White *et al.*,
357 2012). Although the standard reduction potential of menaquinone is still higher than that of PCA (-
358 74 mV compared to -177 mV) (Price-Whelan *et al.*, 2006; White *et al.*, 2012), indicating that PCA
359 likely is not reduced by menaquinol, this difference with ubiquinone nevertheless raises the
360 possibility that PCA may interact differently, and perhaps more readily, with the aerobic respiratory
361 electron transport chain of menaquinone-utilizing Bacteroidetes strains compared to Proteobacteria,
362 thereby generating more ROS and/or interfering with the generation of ATP. Interestingly, another
363 study has shown that the reduced form of different phenazine with a low reduction potential, neutral
364 red (3-amino-7-dimethylamino-2-methylphenazine), can directly transfer electrons to menaquinone,
365 bypassing the proton-pumping NADH dehydrogenase complex that normally transfers electrons
366 from NADH to menaquinone and thereby "short-circuiting" the electron transport chain. We
367 hypothesize that a similar phenomenon may occur with PCA in strains that rely on menaquinone. In
368 future studies, this hypothesis could be tested by 1) performing *in vitro* experiments with purified
369 menaquinone, ubiquinone, and reduced PCA to directly test whether quinones oxidize the latter and
370 if so, whether the kinetics differ between menaquinone and ubiquinone, 2) measuring ROS
371 production and steady-state ATP pools in selected strains of Bacteroidetes and Proteobacteria both
372 in the presence and absence of PCA, and 3) forcing species of Proteobacteria (such as *Escherichia*
373 *coli*) that utilize both ubiquinone and menaquinone in different branches of their electron transport
374 chains to rely only on the latter (for example, by deleting the genes for ubiquinone biosynthesis),
375 followed by reassessing their sensitivity to PCA to see if there is any effect.

376 Beyond the specific strains screened in this study, the risk assessment of phenazine-
377 producing biocontrol strains, and the understanding of phenazine biology in general, would benefit

378 greatly from the development of a platform for the prediction of phenazine resistance phenotypes
379 from genomic or phylogenetic information. The results of this study already indicate that, depending
380 on the environmental pH, phylogenetic information may be of limited utility for prediction of PCA
381 resistance, given the variation in phenotypes for Actinobacteria at circumneutral pH. However, this
382 variability may also hold the key to identifying genome-based predictive markers of phenazine
383 resistance. Given the taxonomic and phenotypic diversity of our strain collection, whole-genome
384 sequencing of our isolates, followed by comparative genomics, could potentially reveal such
385 markers.

386 In summary, this work has laid the groundwork for rectifying a major gap in studies of how
387 introduction of a phenazine-producing biocontrol strain affects rhizosphere bacterial communities.
388 Previous studies have lacked information about the baseline prevalence of resistance in the native
389 communities (Gagliardi *et al.*, 2001; Bankhead *et al.*, 2004; Kozdrój *et al.*, 2004; Roquigny *et al.*,
390 2018), and in the absence of such information, it is impossible to determine whether a negative result
391 (lack of change in the rhizosphere community) reflects a high prevalence of resistance to PCA that
392 is particular to the studied community, versus a general lack of toxicity of PCA to most bacteria or
393 fundamental abiotic constraints on the antibacterial activity of PCA in the rhizosphere (e.g. limited
394 diffusion, adsorption to soil particles, etc.). Distinguishing between these scenarios is key to
395 assessing the risk of unwanted side effects in rhizosphere communities upon the application of
396 phenazine-producing biocontrol strains. In addition, recent studies have demonstrated that
397 phenazines produced by the opportunistic pathogen *Pseudomonas aeruginosa* can promote bacterial
398 tolerance and resistance to clinical antibiotics (Schiessl *et al.*, 2019; Zhu *et al.*, 2019; Meirelles *et*
399 *al.*, 2021; VanDrisse *et al.*, 2021), and that these effects can extend to other opportunistic pathogens
400 that are resistant to phenazines (Meirelles *et al.*, 2021). Thus, understanding the prevalence and
401 genetic determinants of resistance to phenazines may have implications not only for agriculture but
402 also for human medicine and beyond, as we continue to uncover new ecological roles for these
403 multifaceted bacterial metabolites.

404 **Methods**

405 *Isolation of bacteria from wheat rhizosphere and bulk soil samples*

406 Three types of samples were collected from a non-irrigated wheat field at Washington State
407 University's Lind Dryland Research Station on August 9, 2019: wheat plants and surrounding soil,
408 bulk soil from in between the planted rows, and bulk soil from a "virgin" hillside site that has never
409 been farmed. The wheat had been harvested a few weeks prior to sample collection. All samples
410 were immediately stored on ice in clean plastic bags, and subsequently at 4 °C for four days until
411 processing. Rhizosphere soil samples were obtained by shaking the wheat roots until only 1-2 mm
412 of tightly-adhering soil remained, followed by excising the roots at the crown with a sterile razor
413 blade. The roots of 2-3 plants per replicate were placed in 50 mL conical tubes with 30 mL of sterile
414 deionized water, vortexed at top speed for 1 min, and treated in an ultrasonic water bath for 1 min to
415 dislodge bacteria from the roots. Bulk soil samples (1 g per sample) were processed in the same
416 manner. Large soil particles were allowed to settle to the bottom of the tubes on the bench top, and
417 100 µL each of a 10-fold dilution series of the supernatants was spread onto 0.1x TSA plates
418 containing 50 µg/mL nystatin to inhibit fungal growth. The plates were incubated upside down at
419 room temperature in the dark and monitored for the appearance of new colonies over the course of
420 a week. Colonies that appeared morphologically distinct in each sample were picked and restreaked
421 on 0.1x TSA until visually pure cultures were obtained. Multiple representatives were also picked
422 for the most common colony types in an attempt to account for strain variations that might not be
423 apparent to the eye. Once the streaks yielded uniform single colonies, the isolates were inoculated
424 into 1.5 mL of 0.1x tryptic soy broth (TSB) in 5 mL polycarbonate culture tubes and incubated at 30
425 °C with shaking at 250 rpm. After 1-3 days of incubation, depending on when the cultures became
426 turbid, 0.5 mL of each culture was mixed with 0.5 mL of 50% glycerol and stored at -80 °C. Some
427 cultures never became turbid under these growth conditions, but nevertheless yielded viable frozen
428 stocks.

429 *Species identification by 16S rRNA gene sequencing*

430 Single colonies or patches of morphologically pure streaks were picked and resuspended in
431 20 µL sterile nuclease-free water. Colony PCR was performed using GoTaq Green Master Mix
432 (Promega) in 50 µL reactions (1 µL of cell suspension) according to the manufacturer's instructions.
433 For putative streptomycetes (isolates that formed hard colonies rooted in agar, often with aerial
434 hyphae), the thermocycling protocol was modified to include a 10 min initial heating step at 95 °C
435 (compared to 2 minutes for other samples). The primers used were 27F

436 (AGAGTTTGATCMTGGCTCAG) and 1492R (TACGGYTACCTTGTTACGACTT) (Lane,
437 1991). The PCR products were run on a 1% agarose gel to verify the presence of a single band at the
438 expected size (~1500 bp), followed by purification with the Monarch PCR and DNA Cleanup Kit
439 (New England Biolabs). The purified products were submitted for Sanger sequencing at Laragen,
440 Inc., using the same 27F and 1492R primers. The resulting forward and reverse sequences were
441 aligned using MAFFT (https://mafft.cbrc.jp/alignment/server/) and subjected to BLAST against the
442 NCBI 16S ribosomal RNA sequences database. For a few strains, full-length sequences could only
443 be obtained following PCR on genomic DNA extracted using the DNeasy Blood & Tissue kit
444 (Qiagen), following the manufacturer's instructions for Gram-positive bacteria. In addition, for two
445 strains (B1I5 and B2I8), the forward sequence consistently appeared to contain multiple products.
446 We presumed that this was due to multiple primer binding sites or another sequencing artifact rather
447 than mixed cultures as the corresponding sequences from the other direction were clean. In these
448 cases, only the clean sequence was submitted to BLAST.

449 *PCA resistance screen*

450 The optimized version of the PCA resistance screen was performed with four conditions:
451 0.1x TSA (15 g/L agar plus 3 g/L tryptic soy broth no. 2 from MilliporeSigma) with or without 100
452 μM PCA at pH 7.3 or pH 5.1 (adjusted with HCl). PCA was purchased from Princeton BioMolecular
453 Research and dissolved in filter-sterilized 14 mM NaOH to make 10 mM stock solutions. The PCA
454 stock solution or solvent control (14 mM NaOH) was added at 1% v/v to autoclaved molten 0.1x
455 TSA; we verified that this addition did not noticeably alter the pH of the medium by pipetting
456 aliquots onto pH test strips. Subsequently, 1 mL of the medium was pipetted into each well in 24-
457 well polystyrene Cellstar Cell Culture plates (Greiner Bio-One). The plates were allowed to set and
458 dry with the lids off in a biological safety cabinet for 20-30 min, followed by storage upside with the
459 lids on at room temperature in the dark for two days prior to use.

460 Cell suspensions for inoculation in the screen were prepared in one of two ways. First,
461 individual strains were inoculated into 5 mL TSB cultures in glass culture tubes and incubated at 25
462 °C with shaking at 250 rpm. Strains that grew overnight were then diluted to an OD_{600} of 0.05.
463 Certain strains did not grow well in this condition—in particular, strains of *Agromyces*,
464 *Streptomyces*, *Paenibacillus*, and *Neobacillus*. For these, some replicates were prepared by directly

465 scraping cells from streaks grown on 0.1x TSA plates and resuspended the cells in 200 μL TSB with
466 pipetting and brief vortexing at top speed. The OD_{600} of these cell suspensions was adjusted to 0.05,
467 except for *Streptomyces* due to their filamentous nature and tendency to clump; instead, visible cell
468 clumps were allowed to settle to the bottom and inocula were taken from the visually clear upper
469 portion. Subsequently, 10 μL of each cell suspension was pipetted onto the agar in a single well in
470 the 24-well plates. Three adjacent wells per condition were inoculated with each cell suspension,
471 representing technical replicates. After the spots dried, the plates were incubated at room temperature
472 upside in the dark for up to 7 days. Every 24 hrs, the plates were imaged in color at 600 dpi with an
473 Epson Perfection V550 Photo flatbed scanner. For strains that were prepared both from liquid
474 cultures and by resuspension from plates, we did not observe any consistent effect on the resulting
475 PCA susceptibility phenotypes.

476 *Image analysis and quantification of growth*

477 Images from the scanner were analyzed using Fiji (Schindelin *et al.*, 2012). Circular regions
478 of interest (ROIs) were drawn around each culture spot and the mean gray value was measured for
479 each ROI. We also measured the mean gray values of equivalent ROIs in the wells of blank,
480 uninoculated plates for each condition. The latter values were averaged across each 24-well blank
481 plate to give the "background" gray value, which was then subtracted from the mean gray values of
482 the culture spots. The resulting numbers were reported as the metric for growth. Importantly, while
483 this method generally worked well for comparisons across conditions within each strain, there are a
484 few caveats. First, this metric underestimated growth for strains that produced a dark pigment.
485 Second, growth was difficult to quantify for a few strains that grew as nearly transparent colonies.
486 Finally, this metric is not very sensitive to low levels of growth. Nevertheless, for the vast majority
487 of strains, this approach captured visible differences in growth across the four conditions in the
488 screen.

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

490 DNA was extracted from 250 mg of each soil sample using the DNeasy PowerSoil kit
491 (Qiagen). Illumina metagenomic DNA libraries were prepared and sequenced (150 bp single-end
492 reads) at the Millard and Muriel Jacobs Genetics and Genomics Laboratory at the California Institute

493 of Technology. The resulting sequence data was processed according to the pipeline described by
494 Dar et al. (2020).

495 *PCA reduction assay*

496 Selected strains were grown in triplicate overnight in 5 mL TSB cultures at 25 °C with
497 shaking at 250 rpm. The cells were then concentrated by serially centrifuging 1 mL aliquots at 9000
498 g for 2 min in microcentrifuge tubes, washed with 0.1x TSB (pH 7.3), and standardized to OD_{600} =
499 8 in 0.1x TSB (pH 7.3). Subsequently, 100 µL of each cell suspension was transferred into a 96-well
500 plate, and the plate was moved into a Coy anaerobic chamber (5% hydrogen/95% nitrogen
501 headspace). Using plasticware and media that had been passively degassed in the chamber for at
502 least 3 days, electrochemically-reduced PCA was added to a final concentration of 100 µM in 190
503 µL of 0.1x TSB (pH 5.1 or pH 7.3) per well in a black clear-bottom 96-well plate; a standard curve
504 was also prepared using reduced PCA at 1 µM, 10 µM, 50 µM, and 100 µM. Cells were added to
505 each well (except the standard curve) to an OD_{600} of ~0.4, and the plate was immediately transferred
506 to a Biotek Synergy 5 plate reader maintained at 30 °C. OD_{600} and fluorescence readings (360 nm
507 ex/528 nm em) were taken every 5 minutes, with continuous shaking on the "medium" setting.
508 Reduction rates were derived from the slope of a linear regression fit to the visually linear range of
509 the data using the function lm in R (see Fig. S8 for an example). The rates were converted to
510 nmol/OD/min by relating the fluorescence units to the standard curve of reduced PCA concentrations
511 and then dividing by the initial OD_{600} reading for each replicate. No appreciable change in OD_{600}
512 was observed over the course of the experiment for any of the tested strains.

513 *Efflux pump inhibition growth curves*

514 Reserpine was purchased from Sigma-Aldrich and dissolved in dimethyl sulfoxide (DMSO)
515 to make a 20 mg/mL stock solution. PAβN was purchased from MedChemExpress and dissolved
516 in distilled water to make a 50 mg/mL stock solution. Selected strains were grown in triplicate
517 overnight in 5 mL TSB cultures at 25 °C with shaking at 250 rpm, washed once with 0.1x TSB (pH
518 7.3), and then inoculated to an initial OD_{600} of 0.05 in 160 µL of 0.1x TSB (pH 5.1 or pH 7.3) in a
519 96-well plate. Each well contained either a combination of reserpine and PAβN (see Table S2 for
520 the concentrations used for each strain) or a solvent control (DMSO at 0.1% final concentration),

521 along with either 0 or 100 μM PCA. The wells were topped with 50 μL of autoclaved mineral oil to
522 prevent evaporation, and growth was monitored with OD_{600} readings every 15 minutes in a Biotek
523 Synergy 5 plate reader set to 30 °C with continuous shaking on the "medium" setting.

524 **Data availability**

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

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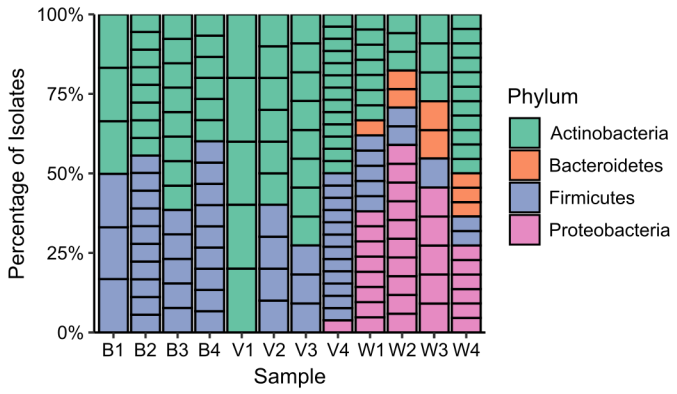

Figure 1: Taxonomic distribution of bacterial isolates from wheat rhizosphere and bulk soil samples.

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

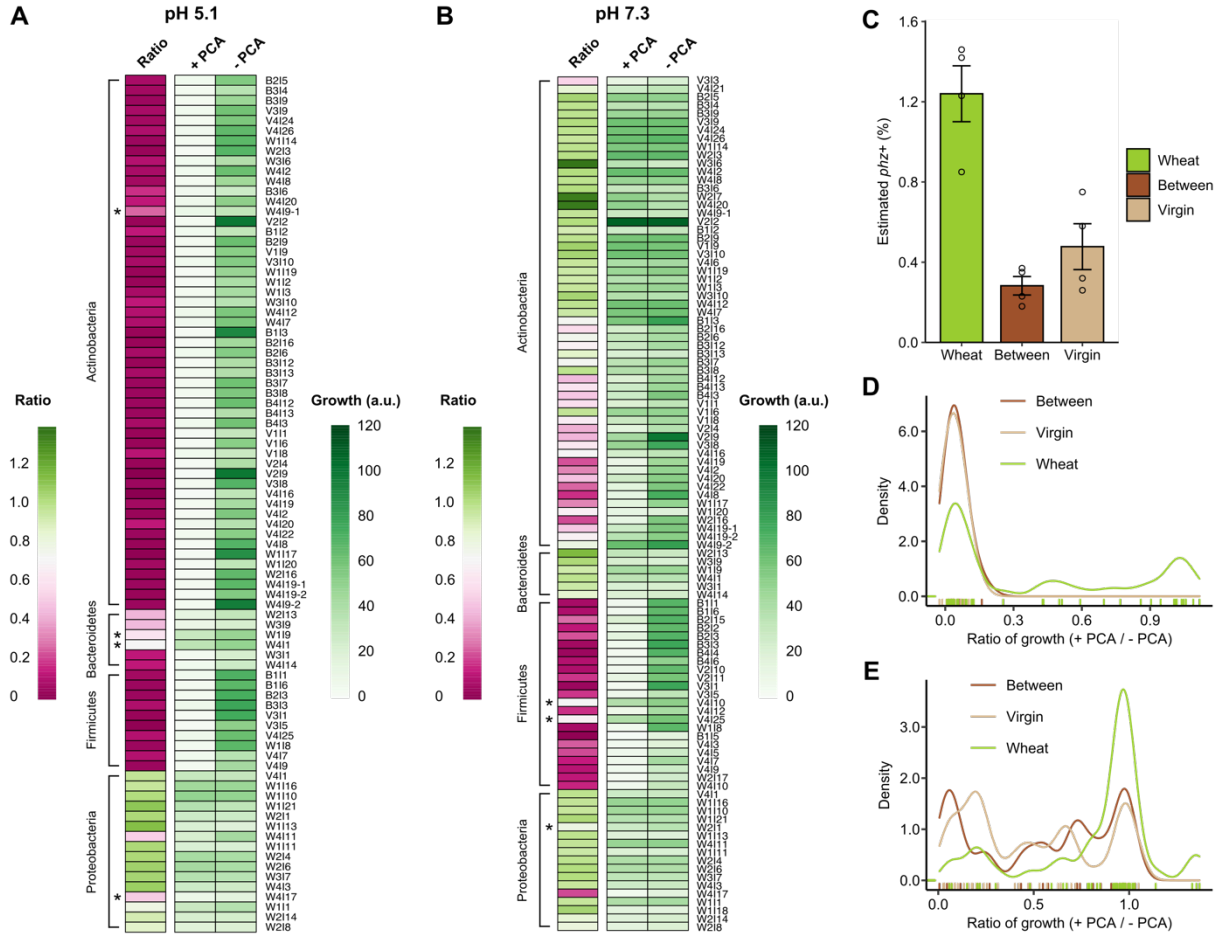

Figure 2: Distribution of PCA resistance phenotypes across phyla and soil sample type. **A-B.** Heat maps depicting the growth of the strains at pH 5.1 (A) and pH 7.3 (B). Each row represents a strain. The leftmost columns are colored according to the ratio of growth on PCA-containing agar versus PCA-free agar; magenta indicates sensitivity to PCA while green indicates resistance to PCA. The right two columns in each heat map are colored according to the separate values for growth on PCA-containing agar (+ PCA) or solvent control agar (- PCA), with darker green indicating more growth. Values are the mean of two to four biological replicates that each comprised three technical replicates. Asterisks indicate strains that displayed markedly variable susceptibility to PCA across biological replicates. Strains for which fewer than two biological replicates grew to stationary phase in the PCA-free control at the given pH, or for which color interfered with growth quantification, were omitted from this analysis. See Methods for a description of how growth was quantified. **C.** Relative abundance of putative phenazine producers (*phz*+ bacteria) across the three types of soil samples. Error bars represent the standard deviation. **D-E.** Density plots (i.e. smoothed histograms) representing the distribution of PCA resistance phenotypes at pH 5.1 (D) and pH 7.3 (E) among strains isolated from Between (bulk), Virgin (bulk), or Wheat (rhizosphere) soil**.** Higher values along the x-axis indicate greater resistance to PCA. If multiple identical strains were isolated from the same soil type, only one representative was counted; thus, 26 strains were isolated from Between, 39 from Virgin, and 45 from Wheat. Colored tick marks above the x-axis represent where individual isolates fall along the range of PCA resistance phenotypes.

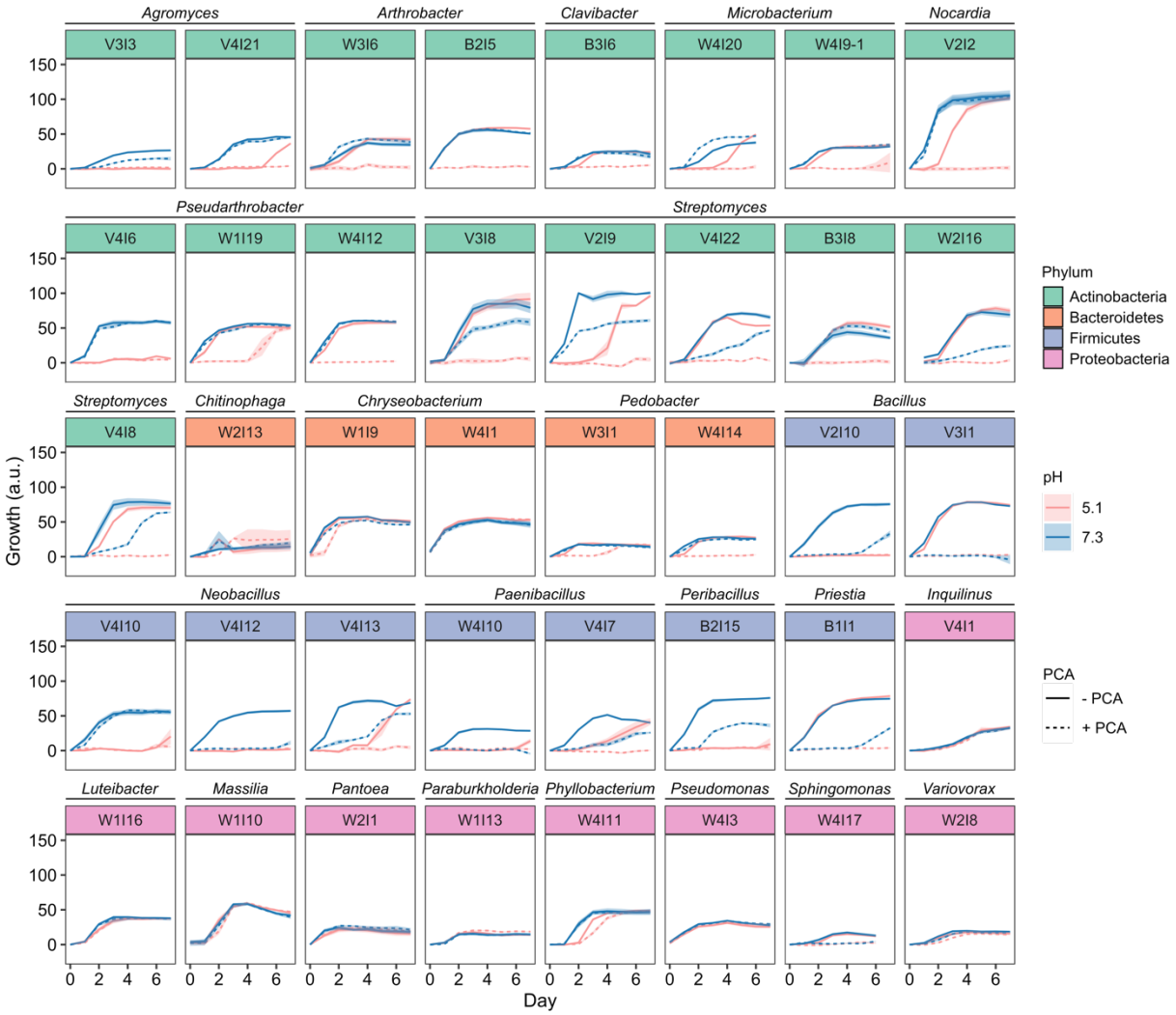

Figure 3: Growth of representative strains over time with and without PCA.
Growth was quantified as described in the Methods. Solid lines represent the growth of spotted cultures on PCA-free agar, and dashed lines represent the growth of spotted cultures on agar containing 100 µM PCA. Blue represents growth at pH 7.3 and pink represents growth at pH 5.1. Data points are the mean of three technical replicates from a representative biological replicate for each strain, and the shaded ribbon represents the standard deviation. The selected strains depicted here cover the range of PCA susceptibility phenotypes observed in each genus. The full set of biological replicates for all tested isolates is displayed in supplementary Figs. S4-S7.

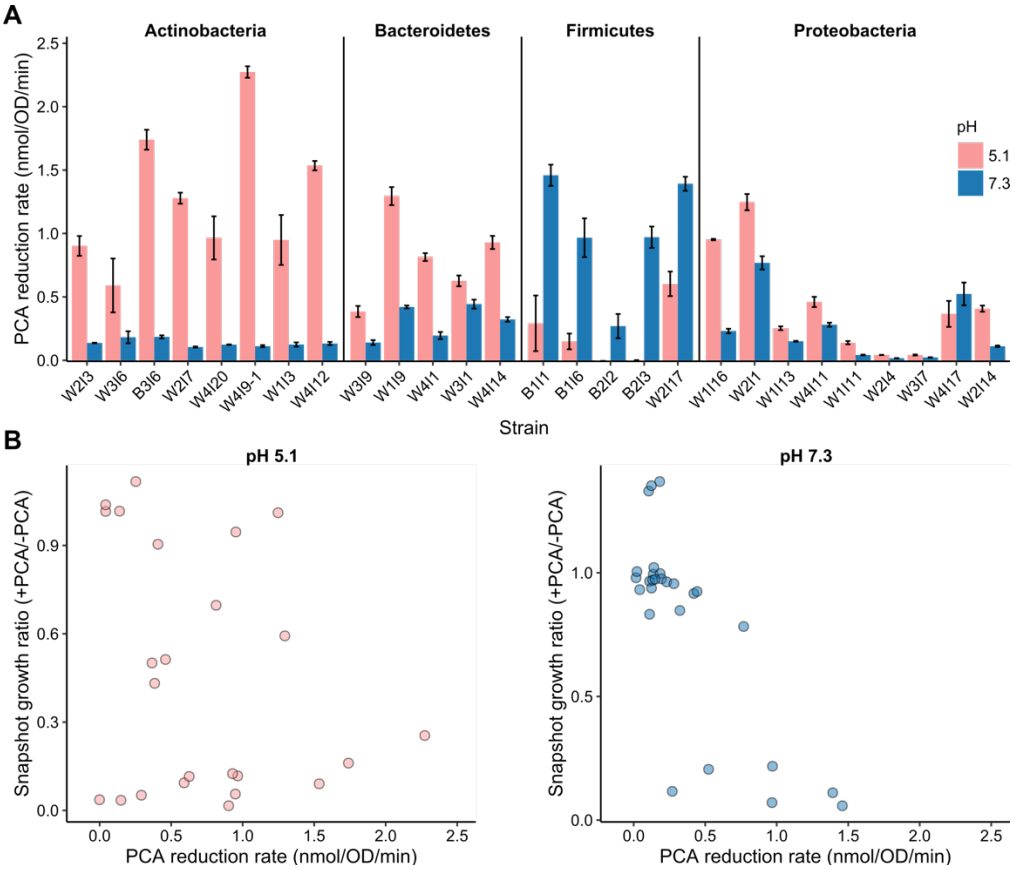

Figure 4: PCA reduction rates of selected strains at pH 5.1 and pH 7.3.
A. PCA reduction rates of representative strains from each phylum at pH 5.1 and pH 7.3. The values represent the mean of three biological replicates and error bars represent the standard deviation. Reduction rates were normalized to OD_{600} values. **B.** PCA reduction rates versus each strain's growth ratio (+PCA/-PCA) at early stationary phase. The growth ratios are the same values calculated and used in Fig. 2.

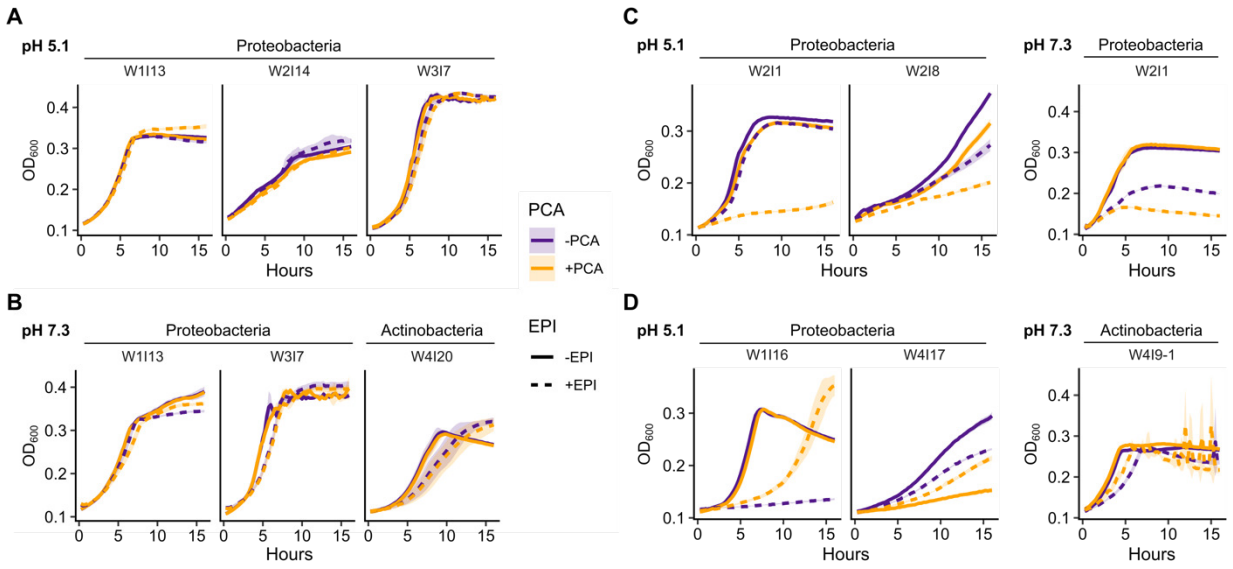

Figure 5: Effects of efflux pump inhibitors on PCA susceptibility.
A-B. Growth curves of strains for which treatment with a combination of the efflux pump inhibitors (EPI) reserpine and PAβN did not affect susceptibility to PCA at pH 5.1 (A) or pH 7.3 (B). **C.** Growth curves of strains for which treatment with reserpine and PAβN potentiated the toxicity of PCA. **D.** Growth curves of strains for which treatment with reserpine and PAβN improved growth in the presence of PCA (strain W4I17) or vice versa (strains W1I16 and W4I9-1). In all panels, data points are the mean of three biological replicates and the shaded ribbon represents the standard deviation. The concentrations of reserpine and PAβN used for each strain can be found in Table S2.