Dead but not forgotten: how eDNA, moisture, and space modulate the horizontal transfer of extracellular antibiotic resistance genes in soil

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Abstract

Antibiotic resistant bacteria and the spread of antibiotic resistance genes (ARGs) pose a serious risk to human and veterinary health. While many studies focus on the movement of *live* antibiotic resistant bacteria to the environment, it is unclear whether extracellular ARGs (eARGs) from *dead* cells can transfer to live bacteria to facilitate the evolution of antibiotic resistance in nature. Here, we use extracellular ARGs (eARGs) from dead, antibiotic-resistant *Pseudomonas stutzeri* cells to track the movement of eARGs to live *P. stutzeri* cells via natural transformation – a mechanism of horizontal gene transfer involving the genomic integration of eARGs. In sterile antibiotic-free agricultural soil, we manipulated the concentration of eARGs, soil moisture, and proximity to eARGs. We found that transformation occurred in soils inoculated with just 0.25 μg of eDNA g^{-1} soil, indicating that even low concentrations of soil eDNA can facilitate transformation (previous estimates suggest ~2-40 μg eDNA g^{-1} soil). When eDNA increased to 5 μg g^{-1} soil, there was a 5-fold increase in the number of antibiotic resistant *P. stutzeri* cells. We found that eARGs were transformed under soil moistures typical in terrestrial systems (5%-30% gravimetric water content), but inhibited at very high soil moistures (>30%). Overall, this work demonstrates that dead bacteria and their eARGs are an overlooked path to antibiotic resistance. More generally, the spread of eARGs in antibiotic-free soil suggests that transformation allows genetic variants to establish in the absence of antibiotic selection, and that the soil environment plays a critical role in regulating transformation.

Importance

Bacterial death can release extracellular antibiotic resistance genes (eARGs) into the environment. Agricultural soils can contain upwards of 10^9 ARGs g^{-1} soil, which may facilitate the movement of eARGs from dead to live bacteria through a mechanism of horizontal gene transfer called natural transformation. Here, we track the spread of eARGs from dead, antibiotic-resistant *Pseudomonas stutzeri* to live antibiotic-susceptible *P. stutzeri* in sterile agricultural soil. Transformation increased with the abundance of eARGs and occurred in soils ranging from 5-40% gravimetric soil moisture but was lowest in wet soils (>30%). Transformants appeared in soil after 24hr and persisted for up to 15 days even when eDNA concentrations were only a fraction of that found in field soils. Overall, our results show that natural transformation allows eARGs to spread and persist in antibiotic-free soils, and that the biological activity of eDNA past bacteria death makes environmental eARGs a public health concern.

Introduction

Antibacterial resistance is a global threat to public health (1). To reduce the impacts of antibiotic resistance on human health, we need to understand how antibiotic resistance genes (ARGs) move through the environment (2, 3). However, the evolution of antibiotic resistance has traditionally been viewed as a clinical problem, and consequently little is known about when and how novel antibiotic resistant pathogens emerge from natural systems (4, 5). ARGs in the environment are particularly concerning because they pose a significant threat to food and water resources (3) and can spread to new hosts through horizontal gene transfer (HGT) (5–8). The spread of ARGs via HGT is a major mechanism in the rise of antibiotic resistance (9). However, the environmental variables that promote the transfer of ARGs remain poorly

69 understood, despite well-documented instances of ARGs moving from the environment
70 to the clinic (2, 10–12).
71 An important, but often overlooked source of environmental ARGs is extracellular
72 DNA (eDNA) (13, 14). Extracellular ARGs (eARGs) enter the environment via active
73 secretion and through bacterial death (15, 16), where they can integrate into new
74 bacterial genomes through a mechanism of HGT called natural transformation. Soil
75 harbors one of the largest environmental reservoirs of ARGs (17, 18) and is home to
76 many antibiotic producing bacteria that could select for the maintenance of newly
77 integrated ARGs (5). Since eARGs can persist in soil for up to 90 days, the odds of
78 transfer to live cells and subsequent spread may be high in soil (19–21). In addition, 11
79 of the 12 top priority antibiotic resistant pathogens (identified by the World Health
80 Organization in 2017) are known to acquire eARGs through natural transformation (22).
81 Understanding what controls the frequency of these transfer events in soils will be
82 important for combating antibiotic resistance.
83 Outside of soil, transformation frequencies generally increase with the availability
84 of eDNA, until a saturation point when greater concentrations of eDNA no longer
85 increase the number of transformants. However, transformation may proceed differently
86 in complex and spatially heterogenous environments like soil. Spatial barriers present in
87 the soil habitat have been shown to limit but not prevent the transfer of plasmids during
88 conjugation (23, 24). However, biological processes like biofilm formation and cell
89 motility may reduce these barriers, increasing access to eARGs and the efficiency of
90 gene transfer (25, 26). The soil environment could also influence the amount of eDNA
91 present because soil properties like water content control eDNA decay rates (27).

92 Moisture could also impact the length of bacterial interactions and the induction of
93 competence – the physiological state of transforming cells (28, 29). Wetter soils tend to
94 favor active cell growth, which would stimulate transformation, but may or may not
95 overcome limitations from eDNA degradation in wet conditions (see Fig. 1 for the factors
96 likely to affect transformation in soil).

97 Understanding the controls of eARG movement and transfer in soils is critical
98 because dead bacteria could be an overlooked source of antibiotic resistance. This may
99 be particularly important in agricultural systems, which can harbor 10^9 ARGs per gram
100 of dry soil (30). However, the prevalence of eARG transfer and the persistence of
101 transformants in soil has not been investigated in detail. Here, we address this
102 knowledge gap by incoulating sterile soil with eARGs under a series of environmentally
103 relevant soil conditions. We track the spread of eARGs and the evolution of antibiotic
104 resistance in single-species populations of *Pseudomonas stutzeri* – a model organism
105 for studying transformation in soil (31). We use sterile agricultural soil from perennial
106 switchgrass monocultures (*Panicum virgatum L.)* established ~6 years ago, but
107 previously in a corn–soybean rotation for more than 10 years. We manipulate the
108 concentration of soil eARGs by varying the concentration of eDNA between 0.25 and 5
109 μg g^{-1} soil. We hypothesized that increasing the availability of eARGs would increase
110 the number of transformants recovered from soil microcosms. We also hypothesized
111 that transformation would be inhibited at low (<10%) and high soil moistures (30-40%
112 gravimetric) because of biological inactivity in dry soils, and eDNA degradation in wet
113 soils (27, 32). We also manipulated the proximity of *P. stutzeri* cells to eDNA

114 introduction sites, as we expected spatial separation of transforming cells and eARGs to
115 pose a significant barrier to transformation.

116 **Results**

117 **eARG concentration affects the number of transformants in soil microcosms**

118 We predicted that the number of transformants in soil microcosms would
119 increase with the concentration of eARGs. We inoculated soil microcosms with 0.25,
120 1.25, 2.5, or 5 μg eDNA g^{-1} soil and counted the total number of transformants and cells
121 every 5 days for 15 days. *P. stutzeri* cells were counted by resuspending soil in a liquid
122 slurry, followed by serial dilution and selective plating. Every 5 days we added more
123 eDNA to soil microcosms. The antibiotic resistant *P. stutzeri* cells were genetically
124 identical to the antibiotic susceptible *P. stutzeri* cells, except for the presence of the
125 antibiotic resistance gene.

126 Antibiotic resistant transformants evolved in the presence of just 0.25 μg eDNA g^{-1}
127 soil (Fig. 2A), which, conservatively-estimated is only a fraction (~1/8) of eDNA in field
128 soil (previous work suggests that eDNA ranges from 2 to 40 μg eDNA g^{-1} soil; see
129 Agnelli et al. 2004). Increasing the amount of eDNA from 0.25 to 5 μg g^{-1} soil, increased
130 the number of antibiotic resistant bacteria 5-fold (Fig. 2B). Soil microcosms inoculated
131 with 5 μg eDNA g^{-1} soil had the most antibiotic resistant bacteria, followed by
132 microcosms inoculated with 2.5 μg eDNA g^{-1} soil. However, there was no difference
133 between transformants in soils inoculated with 1.25 versus 0.25 μg eDNA g^{-1} soil
134 ($p<0.001$, Fig. 2A,B). In soils inoculated with either 2.5 or 5 μg eDNA g^{-1} soil,
135 subsequent eDNA additions resulted in a linear increase in the number of antibiotic
136 resistant bacteria (Fig. 2B). However, inputs of small concentrations of eDNA (<1.25

137 μg), did not always increase the number of antibiotic resistant *P. stutzeri* (Fig. 2B).
138 Changes in the total number of *P. stutzeri* cells are shown in Fig. S1 to demonstrate that
139 soils with more transformants do not have larger population sizes.

140 To understand how transformation transpired over the 5 days between eDNA
141 additions, we also counted transformants and total cells every 24hr for 5 days after a
142 single addition of 5 μg eDNA g^{-1} soil (Fig. 2C,D). We found that most transformants
143 appeared in soil between 24 and 48hr after inoculation but did not increase significantly
144 after 48hr in soil ($p<0.001$, Fig. 2C). In contrast, the total number of cells followed a
145 different trajectory only increasing significantly between day 4 and 5 of the experiment
146 ($p<0.001$, Fig. 2D). In liquid LB media, antibiotic resistant *P. stutzeri* cells grow slower
147 than antibiotic susceptible cells ($p=0.037$, Fig. S2), however, we did not quantify the
148 cost of antibiotic resistance in different soil conditions.

149 **The relationship between soil moisture and number of transformants**

150 When the eDNA concentration was held constant at 2.5 μg eDNA g^{-1} soil and
151 microcosms were incubated at either 5, 10, 20, 30 or 40% soil moisture, we found that
152 transformation was highest between 5 and 20% soil moisture, and lowest at 40% soil
153 moisture (Fig. 3A). The largest number of antibiotic resistant *P. stutzeri* cells were in
154 microcosms held at 10% soil moisture though not significantly higher than at 5% and
155 20% soil moisture ($p<0.001$, Fig. 3A). However, the number of transformants at 5% soil
156 moisture did not increase until after the second eARG addition, which briefly (~1hr)
157 raised soil moisture above 5% (data not shown).

158 To elucidate if biofilm formation plays a role in facilitating transformation in soil,
159 as has been observed in laboratory populations of *P. stutzeri* (Fig. S3), we performed a

48hr assay where we disturbed the soil matrix via homogenization (using a sterile spatula) every 2hr, 8hr, or left the soil undisturbed (Fig. 3B). Because the establishment of a mature biofilm takes several hours and requires cells to surface attach and assemble into microcolonies, homogenization may prevent the progression of these steps and the formation of a mature biofilm (34). We found that homogenizing the soil every 2hr completely prevented transformation, while homogenizing soil every 8hr did not reduce transformation frequency compared to a non-homogenized control (Fig 3B, $p=0.001$). However, soils homogenized every 2hr had fewer total cells than unhomogenized controls and soils homogenized every 8hrs ($p=0.01$, 2.28×10^5 versus 2.75×10^9 cells g^{-1} soil). Additional experiments outside of soil show that changes in the total number of *P. stutzeri* cells minimally impacts transformation (Fig. S4), but low cell establishment may have reduced opportunities for transformation in soils homogenized every 2hr.

Spatial separation of *P. stutzeri* and eARG introduction sites in soil

Next, we tested how spatial separation of *P. stutzeri* cells from eARG introduction sites affected transformation (see Fig. 4A for soil microcosm design). When eDNA was introduced at different distances from live *P. stutzeri* cells (1.25, 3.8, 5 or 7cm away), we found that spatial barriers limited transformation, but interacted with moisture (Fig. 4). At 10% soil moisture there were significantly more transformants than at any other moisture level (similar to Fig. 3A), but transformants only appeared in the closest eARG introduction site, which was 1.25cm from the live *P. stutzeri* cells (transformants x soil moisture, $p<0.001$, Figure 4B,E). At 20% soil moisture, transformants were identified in 2 eARG introduction sites, and at 30% soil moisture in 3 introduction sites (Fig. 4B).

183 Despite transformants appearing in more eDNA introduction sites, there were still fewer
184 total transformants compared to 10% soil moisture (Fig. 4E). At 40% soil moisture, there
185 were no antibiotic resistant bacteria recovered from soil, yet live *P. stutzeri* cells were
186 more abundant than in low moisture conditions and were able to disperse to every
187 eDNA introduction site (dispersal at 10% vs 40% soil moisture, p=0.0289, Fig. 4B,F).

188 **Transformation under antibiotic selective pressure**

189 To understand how antibiotic selective pressures affect the spread of ARGs, we
190 compared the success of an equal concentration of 'eARGs' vs. 'antibiotic resistant
191 cells' (i.e., dead vs alive cells, respectively) in *P. stutzeri* populations grown in liquid
192 media with 10% or 25% the lethal dose of gentamicin (Fig. 5). We found that
193 transformed eARGs only reached high abundances in populations exposed to 25% of
194 the lethal dose of gentamicin (Fig. 5**,** $p<0.001$). While live antibiotic resistant *P. stutzeri*
195 cells reached high frequencies at both 10% and 25% the lethal dose of gentamicin (Fig.
196 5, $p<0.001$). Overall, transformed eARGs took 24hr longer than live cells to establish at
197 high frequencies likely due to the time needed for transformation to transpire (Fig. 5).

198 **Discussion**

199 In order to reduce the impacts of antibiotic resistance on human health, we need
200 a better understanding of the ecological dimensions that promote the transfer of eARGs
201 through natural systems (35). In this study, we show that a 20-fold increase in the
202 concentration of soil eARGs increases the number of antibiotic resistant bacteria 5-fold.
203 We find that transformants appear under most conditions typical for terrestrial soils (5-
204 20% moisture), and that transformation efficiency decreases at high soil moistures,
205 when bacteria need to disperse to eARGs, and with soil mixing. In addition, we find that

206 eARGs can establish with the same success as live antibiotic resistant invaders in liquid
207 media supplemented with a low dose of antibiotic (25% the lethal dose). Although the
208 number of antibiotic resistant bacteria could be attributed to clonal expansion of a few
209 transformants, our results suggest that transformation and population growth occurred
210 on different timescales (Fig. 2C,D) and were optimized under different soil conditions
211 (Fig. 4E,F). Overall, our results highlight that in a single-species system, the soil
212 environment plays a critical role in modulating the spread and persistence of eARGs,
213 and that eARG removal should be incorporated into plans to combat antibiotic
214 resistance.

215 Several studies have now posited that a major cause of ARG transfer is
216 widespread HGT (8, 36). Here, we show that eARGs supplied by *dead* bacteria are also
217 readily transformed into soil bacteria, with the potential for HGT scaling with the
218 abundance of eARGs (Fig. 2A,B). This information could inform approaches to
219 combating antibiotic resistance, which disproportionately focus on killing live bacteria,
220 but may be more effective if they also reduce eARGs, which we find can be equally
221 effective at disseminating ARGs (Fig. 5). This may explain why practices that target only
222 live bacteria like composting manure prior to application on agricultural fields have been
223 found to both increase and decrease the occurrence of ARGs depending on the native
224 bacterial community and other soil conditions (37, 38). Interestingly, manures
225 composted at high temperatures, which promotes the degradation of eDNA, effectively
226 reduces ARGs (39), supporting our findings that DNA degradation is a critical factor in
227 reducing environmental concentrations of eARGs.

228 Despite the possibility of low-levels of eARGs persisting in soil for an extended
229 time, the ultimate fate of most extracellular DNA is likely degradation, not transformation
230 (40). While some eDNA can persist in soil for up to 90 days (20), previous work
231 indicates that 99% of eDNA is degraded in the first ~7 days in soil (27, 41).
232 Consequently, the most important role of soil conditions in regulating transformation
233 may be the effect of moisture on the rate of eDNA decay, and likely explains our finding
234 that transformation declined at higher soil moistures (Fig. 3A, Fig. 4B), even though
235 greater moisture also gives recipient cells access to more eARGs (Fig. 4B). Similar
236 trends have been observed for the soil bacterium *Acinetobacter calcoaceticum* whose
237 transformation frequency was highest at 18% soil moisture, but lowest at 35% soil
238 moisture (29). However, direct comparisons between *A. calcoaceticum* and *P. stutzeri*
239 are difficult because *A. calcoaceticum* transformation efficiency was not measured
240 below 18% soil moisture. Overall, our work suggests that transformation is favored at
241 low soil moistures, unlike other biological processes like extracellular enzyme activity,
242 which are positively correlated with soil moisture (42).
243 Our study quantified the relationship between soil moisture and transformation in
244 one species of bacteria and in one soil type. This relationship may vary widely across
245 bacterial species and soil type depending on how individual taxa regulate transformation
246 and the stability of eDNA in different environments (43). For instance, the frequency of
247 eARG transformation may be explained by soil physical factors, which can alter the
248 stability of eDNA (44). Agricultural soils including those from our field site typically have
249 a lower proportion of eDNA than other field soils, suggesting eDNA could have a shorter
250 half-life in these soils compared to others. Moreover, in our study, transformation was

lowest at 40% soil moisture (Fig. 3A), and soils here rarely become this wet (Fig. S5). An interesting follow-up study would be to quantify transformation in wetland or sediment soils, where water content is generally higher. We also inoculated soils with eARGs obtained from heat shocked *P. stutzeri* cells, which could have damaged eDNA, and thus affected rates of eDNA decay. However, we would not expect heat shock to differentially affect eDNA stability under various soil conditions. Nonetheless, transformation is likely much lower between different bacterial species and in multi-species communities, where competitive interactions could limit access to eARGs and limit the success of transformants (45).

We also observed that soils held at 10% soil moisture only supported transformation if they were not disturbed by frequent homogenization, suggesting an interaction between soil moisture and the physical structure of soil (Fig. 3B). However, homogenizing soil every 2hr also decreased the total number of cells and may have affected opportunities for transformation – although population size does not have a large effect on transformation outside of soil (Fig. S4). Alternatively, homogenization could have prevented biofilm formation, as surface-attached *P. stutzeri* cells have higher transformation efficiencies than planktonic cells (Fig. S3). Biofilms are also more common in dry soils where they increase microbial survivorship (46), further supporting the possibility that biofilm formation is key for both soil colonization and the horizontal dissemination of eARGs, particularly in drought-exposed soils (<10% soil moisture). A similar relationship between HGT and soil moisture has been observed for conjugation, which increases in dry soils due to prolonged cell-to-cell interactions (28). Future studies could use fluorescent proteins or confocal laser scanning microscopy to better

quantify the relationship between biofilm establishment and transformation efficiency in soil (47).

A major concern in the fight against antibiotic resistance is the presence of antibiotics in the environment, as antibiotics could provide positive selection for transformed eARGs to proliferate. Although antibiotics are difficult to measure in the environment and may not be prevalent enough to select for antibiotic resistant bacteria (48, 49), our experiments in liquid media demonstrated that only 25% of the lethal dose of gentamicin could select for antibiotic resistant bacteria. This shows that transformed eARGs can come to dominate a population under the right conditions, although we note dynamics are likely to differ in complex communities with more than one bacterial species. An important future research direction will be determining the antibiotic concentrations at which eARGs can establish in single and multi-species communities in soil.

Taken together, our study reveals the most important variables for understanding the transmission of eARGs in soil and sets the stage for future experiments to scale up estimates of transformation to the whole community. Here, we used sterile soil inoculated with a single bacterium to prevent competitive interactions, and to ensure the soil was antibiotic-free. Certain soil types may further alter transformation efficiencies, and future studies could probe this relationship. Regardless, this work provides novel evidence that eARGs from dead bacteria are an overlooked, but important route in the emergence of antibiotic resistance. Specifically, we find that the availability of eARGs drives the evolution of antibiotic resistance, and that transformation is prevalent under a wide range of soil conditions – only decreasing at very high soil moistures, in response

297 to spatial barriers, and with soil mixing. Together, we find that the soil environment
298 impacts the movement of eARGs from dead to live bacteria, and ultimately affects the
299 prevalence of antibiotic resistant bacteria in antibiotic-free soil. Overall, this work
300 provides novel *in situ* evidence that HGT is an evolutionary force that facilitates the
301 spread of non-selected ARGs in soil, and thus we recommend special caution in
302 releasing eARGs into the environment.

303 **Materials and Methods**

304 **Site and soil collection**

305 Soil cores (10cm depth by 5cm diameter) were collected in October 2018 and
306 April 2019 from the Great Lakes Bioenergy Research Center (GLBRC) scale-up fields
307 located at Lux Arbor Reserve Farm in southwest Michigan (42°24' N, 85°24' W). Plots
308 were established as perennial switchgrass monocultures (*Panicum virgatum L*) in 2013,
309 and before that were in a corn–soybean rotation for more than 10 years. The soils
310 developed on glacial outwash and are classified as well-drained Typic Hapludalf, fine-
311 loamy, mixed, mesic (Kalamazoo series) or coarse-loamy, mixed, mesic (Oshtemo
312 series) or loamy sand, mixed, mesic (Boyer series) (50). Soils were sieved at 2mm and
313 autoclaved in two cycles (60 minutes at 121°C; gravity cycle) separated by a 24-hr
314 window to target dormant and spore-forming cells resuscitated during the first autoclave
315 cycle.

316 **Soil microcosms**

317 Soil microcosms were established in small 60 x 15mm petri dishes using 10
318 grams of dry, sterile, switchgrass soil. Except for the experiment in Figure 4, which used
319 large 150 x 15mm petri dishes filled with 100 grams of soil. On day 0 of each

experiment, the center of the microcosm was inoculated with 2mL of wildtype *P. stutzeri* cells suspended in liquid LB at a concentration of 10^6 CFU g^{-1} soil. All LB media used throughout the manuscript followed a recipe of 10% tryptone, 5% yeast extract, and 5% NaCl (solid media contained 1.5% agarose). Immediately after adding the live *P. stutzeri* cells to soil, eDNA was slowly pipetted into the center of each soil microcosm. For the experiments shown in Fig. 4, eDNA was pipetted into each eDNA introduction site separately. eDNA and live cells were never combined before inoculation into soil. To control for contamination or evolution of gentamicin resistance via mutation, two additional treatments were included in every experiment; 1) 5µg of eDNA g^{-1} soil made from gentamicin susceptible *P. stutzeri* cells, and 2) sterile water without eDNA. Transformants never appeared in the control treatments. All microcosms were maintained at ~23°C and the soil was never mixed unless directly specified (e.g. in Fig. 3B). All microcosms were initially inoculated to ~40% soil moisture on day 0, and then dried to 20% soil moisture (except in the experiments manipulating soil moisture where the soil was dried according to the treatment-level soil moisture). Generally, we counted the number of transformants, and the population size every 5 days, and then added more eDNA to simulate periodic inputs of eARGs (only one eDNA input in Fig. 4). After eDNA additions, soils were gradually dried back to 20% soil moisture.

Bacterial cultures and extracellular antibiotic resistance genes (eARG)

Soil microcosms were inoculated with *Pseudomonas stutzeri*, strain 28a24 (51). Prior to inoculation, the bacterial cultures were grown at 30°C on an orbital shaker at 120 rpm for 24hr in liquid LB media to a concentration of 10^6 CFU/mL. Stocks of antibiotic resistant extracellular DNA contained antibiotic resistance genes (eARGs) and

were made from a mutant *P. stutzeri* strain (see strain DAB390 Dougherty et al. 2014) encoding a gentamicin resistance gene and a LacZ gene (Tn7 transposition of pUC18-mini-Tn7T-Gm-lacZ into strain 28a24, see Choi and Schweizer 2006). eDNA was also made from the wildtype *P. stutzeri* to act as a negative control. The gentamicin resistant *P. stutzeri* cells were genetically identical to the wildtype *P. stutzeri* cells, except for the presence of the antibiotic resistance gene. The batch cultures for eDNA/eARG stocks were prepared under the same conditions specified above but were grown for 48hr and then resuspended in sterile nanopure water. The cells for eDNA stocks were then killed via heat shock (90°C for 1hr) and confirmed dead by plating. Before every assay, 100ul of stock eDNA was plated 3x to ensure the absence of live gentamicin resistant cells. We used eARGs released from dead *P. stutzeri* cells to better represent environmental eDNA, as purified genomic DNA is not found in soil. The final concentrations of eDNA ranged from 25-50 ng/µl and were appropriately diluted for each experiment (determined using Qubit fluorometric quantification and Invitrogen Quant-iT PicoGreen dsDNA Assay Kit). *P. stutzeri's* transformation efficiency plateaus at ~5ng/µl eDNA in assays conducted on agar petri dishes (Fig. S6).

Counting transformants and total cells

To determine the number of transformants in each soil microcosm, we weighed out 0.2g of soil from each microcosm or eDNA pool and placed it into a 1.5mL centrifuge tube. To each tube, we added 180µl of liquid LB and vortexed for 10 seconds (~10^{-1} dilution). After allowing the soil to settle for 10 minutes, we transferred the supernatant to a 96-well plate and diluted out to 10^{-6} or 10^{-9} depending on the experiment and the expected number of cells. In the experiments that manipulated

366 eDNA concentration and soil moisture, we plated 50µl cell suspensions. For the
367 remaining experiments we plated 10µl dots. All plating was done on petri dishes with
368 solid LB (to count the total population size) or solid LB + gentamicin (50 µg/ml) + Xgal
369 (20 µg/ml) (to count transformants in soil). Plates were incubated at 30°C and the
370 number of colonies counted after 48-72hr. The number of cells is reported g^{-1} soil,
371 except in Fig. 4 where it is reported per eARG pool (0.2g soil) and calculated according
372 to the following equation: Cells per unit = Cells μl^{-1} x [Soil slurry volume (200µl) / Soil
373 Mass in slurry (g)].

374 **Manipulation of eARG concentration in soil microcosms**

375 To understand the relationship between the availability of eDNA and
376 transformation, we varied the concentration of eDNA in soil between 5, 2.5, 1.25, 0.25
377 µg of eDNA $gram^{-1}$ soil (Fig. 2A,B). We used these concentrations as they
378 conservatively represent ~10%, 5%, 2.5% and 0.5% of the total eDNA pool in soil (43).
379 The concentration of eDNA never exceeded 10%, to account for the fact that only a
380 small percentage of soil eDNA is likely to encode antibiotic resistance genes. We ran
381 the experiment for 15 days and added eARGs to soil on day 0, 5, and 10, of the
382 experiment, with each treatment consisting of 8 replicate soil microcosms.

383 **Manipulation of soil moisture in soil microcosms**

384 To determine how soil moisture affected transformation, we maintained soil
385 microcosms at 5, 10, 20, 30 or 40% gravimetric soil moisture over a period of 10 days
386 (Fig. 3A, i.e. soil moisture = [weight after water addition – dry weight] / dry weight]*100).
387 In this experiment, all the microcosms were inoculated with an intermediate
388 concentration of eDNA (2.5µg g^{-1} soil) and eARGs were added on day 0 and 5. We

report the number of transformants present on day 10, using 8 replicate microcosms per treatment. To understand if the physical structure of the soil was important for transformation, we manipulated the physical structure of the soil by mixing the soil every 2hr, 8hr or never, throughout a 48hr period (Fig. 3B). Each treatment consisted of 4 replicates and the eDNA concentration was maintained at 5 μg g^{-1} soil, with the soil moisture remaining constant at 10%. Individual microcosms were gently mixed for approximately 30 seconds using a sterile spatula at the designated intervals.

Manipulation of eARG introduction site in soil microcosms

To determine if dispersal to eARGs and subsequent transformation events vary under different soil moistures, we introduced eDNA to different sites in large soil microcosms setup up in 150 x 15mm petri dishes with 100g of soil in each microcosm. There were 4 different soil moisture treatments: 10, 20, 30 or 40%. At each soil moisture we setup 4 microcosms (n=4, 16 soil microcosms). In each microcosm there were 8 different eDNA introduction sites which are depicted in Fig. 4A. The 8 introduction sites were located 1.25, 3.80, 5 or 7cm from the center of the petri dish and each introduction site was inoculated with 2μg eDNA g^{-1} soil. At each distance (e.g. at 1.25cm), one eDNA introduction site was inoculated with eARGs from dead gentamicin resistance *P. stutzeri* and the other with dead wildtype *P. stutzeri* – containing no eARGs and acted as a control for the movement of eARGs throughout the microcosm. eDNA was dripped onto each introduction site using a micropipette and allowed to dry (400μl total volume). Approximately, 0.5hr after eDNA introduction, *P. stutzeri* cells were dripped into the center of each microcosm (2mL total volume). Each microcosm was immediately brought to the appropriate soil moisture via drying or the introduction of sterile water,

412 depending on the soil moisture treatment. After 5 days of incubation at ~23°C, 0.2g of
413 soil was collected from each eDNA introduction site. We counted the number of
414 transformants and total cells as described in the section 'counting transformants and
415 total cells'. Transformants only appeared in one control eDNA introduction site, which
416 was 1.25cm from the center of the petri dish in microcosms incubated at 30% soil
417 moisture. However, based on our methods we cannot determine whether this was
418 caused by the movement of eDNA or the movement of transformed cells.

419 **Antibiotic selective pressure in assays outside of soil**

420 Outside of soil, we tested how an equal concentration of 'eARGs' and 'live
421 antibiotic resistant cells' establish in populations of *P. stutzeri* challenged with a low
422 dose of antibiotic (Fig. 5). Initially, we established two equal populations of *P.stutzeri*
423 cells carrying kanamycin resistance (Kan_R) (see strain DAB386 Dougherty et al. 2014).
424 To one of the two populations, we added 60,000 'live' gentamicin resistant *P. stutzeri*
425 cells ($Gent_R$). To the other population, we added 60,000 'dead $Gent_R$' cells which
426 provided a source of eARGs. Therefore, on day 0 of the experiment, the two treatments
427 contained either 4% $Gent_R$ and 96% Kan_R cells, or 0% $Gent_R$ and 100% Kan_R cells,
428 respectively. We used kanamycin resistant cells for the founding population instead of
429 the antibiotic susceptible strain *P. stutzeri* 28a24 (used in previous experiments), so that
430 we could track the evolution of multi-drug resistance, which we found was quite rare and
431 only occurred at 25% the lethal dose of gentamicin (Fig. S7). Populations were founded
432 in 1mL of LB media, and the experiment ran for 10 days, with each population receiving
433 1 mL of fresh LB media every 24hr. Each day we counted the number of $Gent_R$
434 genotypes and the total number of cells using serial dilution and selective plating. Data

435 is missing for day 10 in populations grown at 10% the lethal dose of gentamicin due to
436 an accidental loss of sample. We counted the total number of cells on solid LB media
437 (no antibiotic) and the number of $Gent_R$ cells on solid LB media with gentamicin (50
438 µg/ml) + Xgal (20 µg/ml). We report the frequency of $Gent_R$ genotypes ($Gent_R$ cells/total
439 cells) in Fig. 5.

440 **Statistical analyses**

441 Prior to analyses all data were verified to meet assumptions of normality and
442 homogeneity of variance. Data shown in Fig. 3A and Fig. 4 did not conform to
443 assumptions of homogeneity of variance and were log transformed. The results from the
444 soil microcosm studies were analyzed by either one-way or two-way ANOVA followed
445 by Tukey's post hoc with test variable (i.e. soil manipulation and sampling day) as a
446 fixed effect using the R *stats* package (R core team 2018). Experiments with multiple
447 sampling days were analyzed by two-way ANOVA, except in certian instances, when
448 the test variables were analyzed individually on the final sampling day (e.g.Fig. 3A).
449 Results from the soil microcosm experiment in (Fig 4) were analyzed by two-way
450 ANOVA with the 'distance to eARGs' and 'soil moisture' as fixed effects. When
451 signficant, interactions between test variables were included in the model. The
452 frequency of antibiotic resistant bacteria present in each laboratory population at the
453 end of the experiment were compared using two-way ANOVA with the treatment (Live
454 vs Dead cells) and selection regime (10 or 25% lethal dose gentamicin) as fixed effects
455 (Fig. 5). Differences between all test variable groups were considered significant at $\alpha \leq$
456 0.05. Raw data is on the open science framework data repository (https://osf.io/7jp3b/).

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Author Contributions HK, KD and SE conceptualized the experimental design for the soil microcosms. HK performed all soil microcosm incubations and laboratory work. HK and SE performed the analyses and wrote the manuscript. HK, KD, SE provided comments on the manuscript.

Conflict of Interest The authors declare no competing interests.

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Figure Legends

FIG 1 The soil characteristics likely to affect natural transformation. Transformation requires cellular competence and the presence of eARGs. But is also likely to depend on soil moisture, soil structure, proximity to eARGs, and antibiotic concentrations. Arrows show possible interactions between soil characteristics and point towards the effected variable.

FIG 2 The relationship between eARG concentration and number of antibiotic resistant *P. stutzeri* cells. (A) The number of antibiotic resistant bacteria in soil microcosms inoculated with 0.25, 1.25, 2.5 or 5 µg eDNA per gram soil (corresponds to day 15 in B). The different letters indicate significant differences based on Tukey's post hoc test. (B) Time series showing the effect of sequential eARG additions (indicated by arrows) in microcosms inoculated with different concentrations of eARGs. (C) Time series showing the number of antibiotic resistant bacteria every 24hr over 5 days in soil microcosms inoculated with a single addition of 5 µg eDNA per gram soil. (D) Data from the same time series as C showing the total number of cells. (A-D) Solid points represent the average number of cells per gram soil and error bars show the standard error of the mean (n=8 replicates). (A,C-D) The open points represent the values for individual replicates.

FIG 3 The relationship between soil moisture and the number of antibiotic resistant *P. stutzeri* cells. (A) The number of antibiotic resistant bacteria (transformants) in soil incubated at 5%, 10%, 20%, 30% or 40% soil moisture over a 10-day experiment. Bars represent the average number of $\log_{10}$(antibiotic resistant bacteria per gram soil) and error bars show the standard error of the mean (n=8 replicates). The different letters indicate significant differences based on Tukey's post hoc test. (B) The relationship between the frequency of soil homogenization and transformation frequency (transformants/total cells) at 10% soil moisture. Homogenization was conducted every 2hr, 8hr or never over a 48hr window. Closed points represent treatment averages and open points represent individual replicates. Error bars show the standard error of the mean (n=4 replicates).

FIG 4 The relationship between dispersal and transformation at different soil moistures. (A) Bird's eye view of the soil microcosm setup. The entire microcosm was filled with soil. The location of eDNA introduction sites are 1.25, 3.8, 5 or 7cm from the location of live *P. stutzeri* cells. Yellow points indicate an eDNA introduction site with eARGs and gray points depict eDNA introduction sites without eARGs (sourced from antibiotic-susceptible *P. stutzeri*). (B) Top panel: the average number of antibiotic resistant transformants per eDNA introduction site. Bottom panel: the average number of total cells. The size of the dot increases as the number of cells increase. For reference, 2 dots in the 10% soil moisture panel are labeled with the number of *P. stutzeri* cells per eDNA introduction site. (C) The total transformants and (D) the average cells at each distance, pooled across the four soil moistures. (E) The total transformants and (F) the average cells at each soil moisture, pooled across the four distances. Error bars show the standard error of the mean (n=4 replicates).

686

687

FIG 5 The frequency of gentamicin resistant bacteria ($Gent_R$) over 10 days in *P. stutzeri* populations provided live or dead gentamicin resistant cells. 'Living Cells' treatments started at 4% $Gent_R$ and 'Dead Cells' treatments started at 0% $Gent_R$ but were provided eARGs encoding $Gent_R$. *P. stutzeri* populations were grown at either 10% (left) or 25% (right) the lethal dose of gentamicin. Points show daily averages and error bars show the standard error of the mean (n=4 replicates).

Figure 1

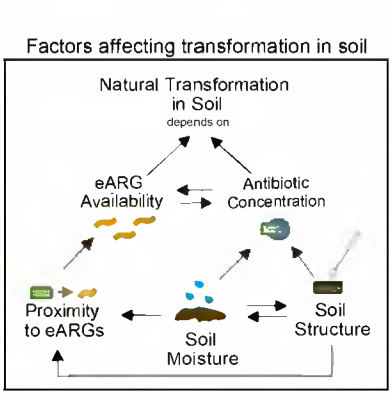

Factors affecting transformation in soil
Natural Transformation in Soil
depends on
eARG Availability
Antibiotic Concentration
Proximity to eARGs
Soil Moisture
Soil Structure

Figure 2

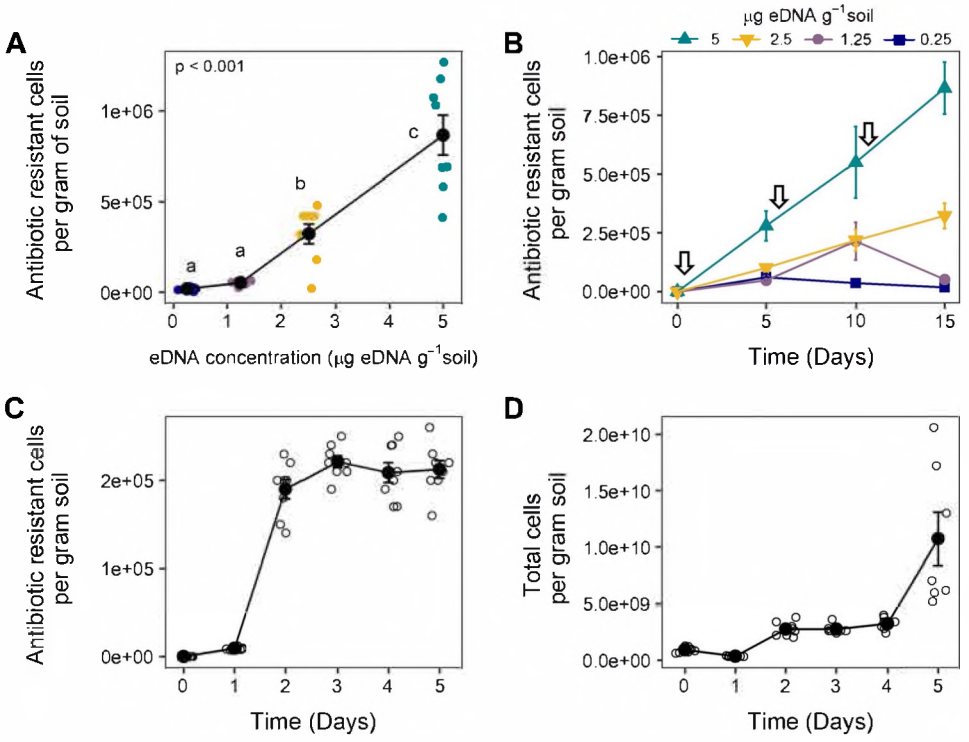

A
p < 0.001
Antibiotic resistant cells per gram of soil
eDNA concentration (µg eDNA g^{-1}soil)
a
a
b
c
B
µg eDNA g^{-1}soil
5
2.5
1.25
0.25
Antibiotic resistant cells per gram soil
Time (Days)
C
Antibiotic resistant cells per gram soil
Time (Days)
D
Total cells per gram soil
Time (Days)

Figure 3

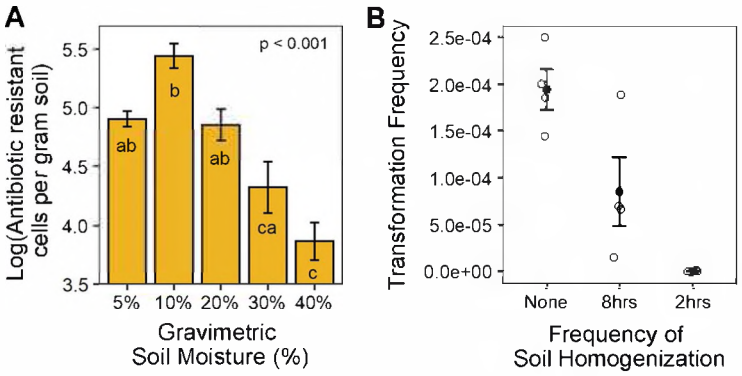

A
p < 0.001
Log(Antibiotic resistant cells per gram soil)
5.5
5.0
4.5
4.0
3.5
ab
b
ab
ca
c
5%
10%
20%
30%
40%
Gravimetric Soil Moisture (%)
B
Transformation Frequency
2.5e-04
2.0e-04
1.5e-04
1.0e-04
5.0e-05
0.0e+00
None
8hrs
2hrs
Frequency of Soil Homogenization

Figure 4

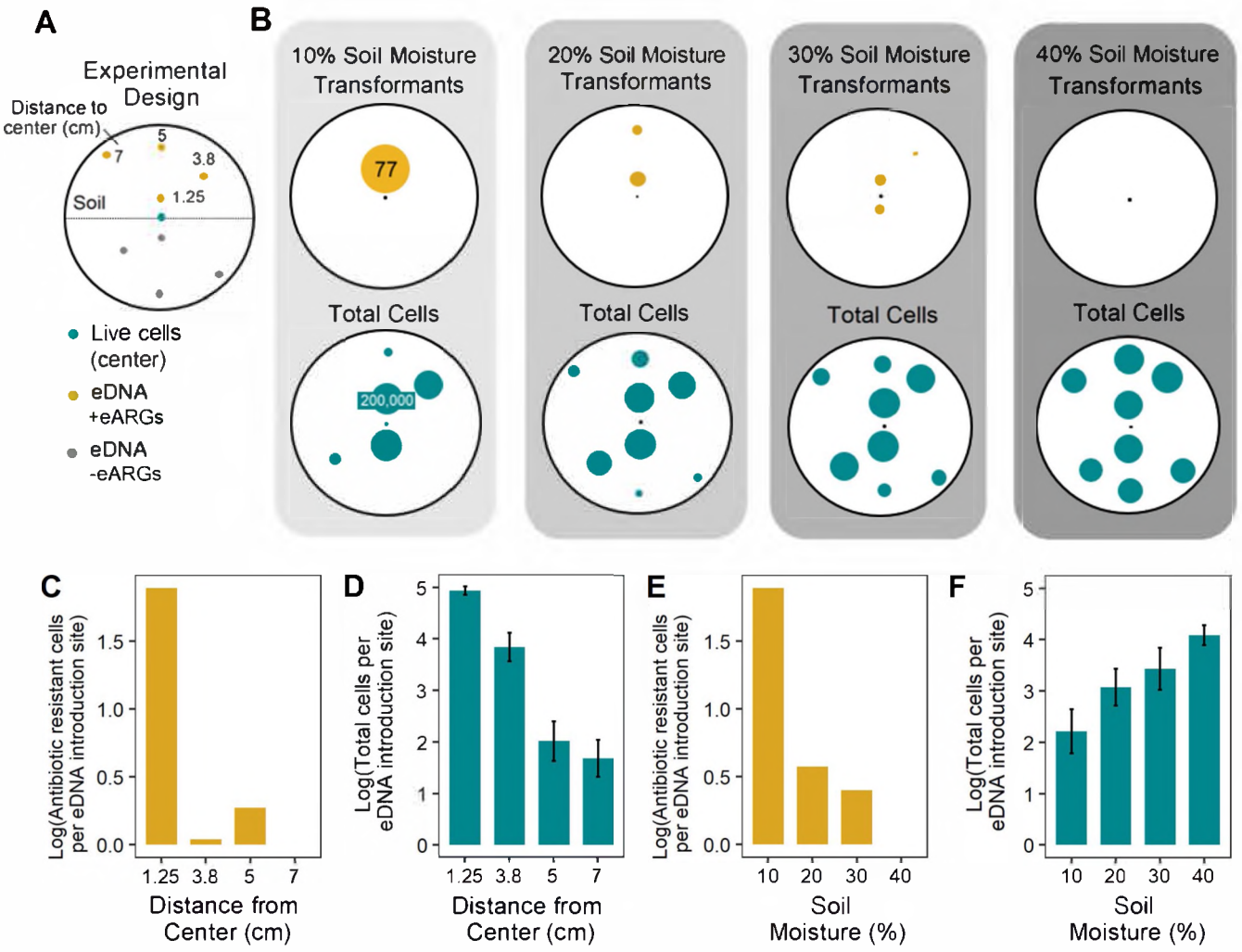
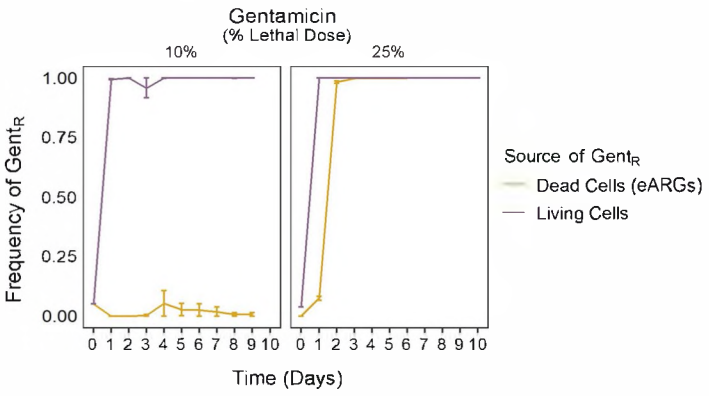

A
Experimental Design
Distance to center (cm)
5
7
3.8
Soil
1.25
Live cells (center)
eDNA +eARGs
eDNA -eARGs
B
10% Soil Moisture Transformants
20% Soil Moisture Transformants
30% Soil Moisture Transformants
40% Soil Moisture Transformants
77
Total Cells
200,000
C
Log(Antibiotic resistant cells per eDNA introduction site)
Distance from Center (cm)
D
Log(Total cells per eDNA introduction site)
Distance from Center (cm)
E
Log(Antibiotic resistant cells per eDNA introduction site)
Soil Moisture (%)
F
Log(Total cells per eDNA introduction site)
Soil Moisture (%)

Figure 5


Gentamicin
(% Lethal Dose)
10%
25%
Frequency of Gent$_R$
1.00
0.75
0.50
0.25
0.00
0 1 2 3 4 5 6 7 8 9 10
Time (Days)
Source of Gent$_R$
Dead Cells (eARGs)
Living Cells