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Dead but Not Forgotten: How Extracellular DNA, 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 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 eARG concentration, 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 suggested ~2 to 40 μg eDNA g^{-1} soil). When eDNA was 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 of terrestrial systems (5 to 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 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* cells to live antibiotic-susceptible *P. stutzeri* cells in sterile agricultural soil. Transformation increased with the abundance of eARGs and occurred in soils ranging from 5 to 40% gravimetric soil moisture but was lowest in wet soils (>30%). Transformants appeared in soil after 24 h and persisted for up to 15 days even when eDNA concentrations were only a fraction of those 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 after bacterial death makes environmental eARGs a public health concern.

KEYWORDS horizontal gene transfer, natural transformation, antibiotic resistance genes, extracellular DNA, soil, agriculture

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

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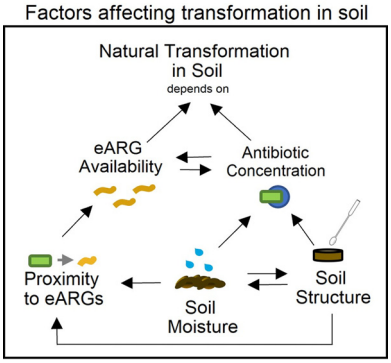

FIG 1 Soil characteristics likely to affect natural transformation. Transformation requires cellular competence and the presence of eARGs. But is also likely to depend on soil moisture, soil structure, proximity to eARGs, and antibiotic concentrations. Arrows show possible interactions between soil characteristics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

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 understood, despite well-documented instances of ARGs moving from the environment to the clinic (2, 10–12).

An important but often overlooked source of environmental ARGs is extracellular DNA (eDNA) (13, 14). Extracellular ARGs (eARGs) enter the environment through active secretion and through bacterial death (15, 16), where they can integrate into new bacterial genomes through a mechanism of HGT called natural transformation. Soil harbors one of the largest environmental reservoirs of ARGs (17, 18) and is home to many antibiotic-producing bacteria that could select for the maintenance of newly integrated ARGs (5). Since eARGs can persist in soil for up to 90 days, the odds of transfer to live cells and subsequent spread may be high in soil (19–21). In addition, 11 of the 12 top-priority antibiotic-resistant pathogens (identified by the World Health Organization in 2017) are known to acquire eARGs through natural transformation (22). Understanding what controls the frequency of these transfer events in soils will be important for combating antibiotic resistance.

Outside soil, transformation frequencies generally increase with the availability of eDNA up to a saturation point at which greater concentrations of eDNA no longer increase the number of transformants. However, transformation may proceed differently in complex and spatially heterogeneous environments like soil. Spatial barriers present in the soil habitat have been shown to limit but not prevent the transfer of plasmids during conjugation (23, 24). However, biological processes like biofilm formation and cell motility may reduce these barriers, increasing access to eARGs as well as the efficiency of gene transfer (25, 26). The soil environment could also influence the amount of eDNA present because soil properties like water content control eDNA decay rates (27). Moisture could also impact the length of bacterial interactions and the induction of competence, i.e., the physiological state of transforming cells (28, 29). Wetter soils tend to favor active cell growth, which would stimulate transformation but may or may not overcome limitations from eDNA degradation under wet conditions (see Fig. 1 for the factors likely to affect transformation in soil).

Understanding the controls of eARG movement and transfer in soils is critical because dead bacteria could be an overlooked source of antibiotic resistance. This may be particularly important in agricultural systems, which can harbor 10^9 ARGs per gram of dry soil (30). However, the prevalence of eARG transfer and the persistence of transformants in soil have not been investigated in detail. Here, we address this knowledge gap by inoculating sterile soil with eARGs under a series of environmentally relevant soil conditions. We track the spread of eARGs and the evolution of antibiotic resistance in single-species populations

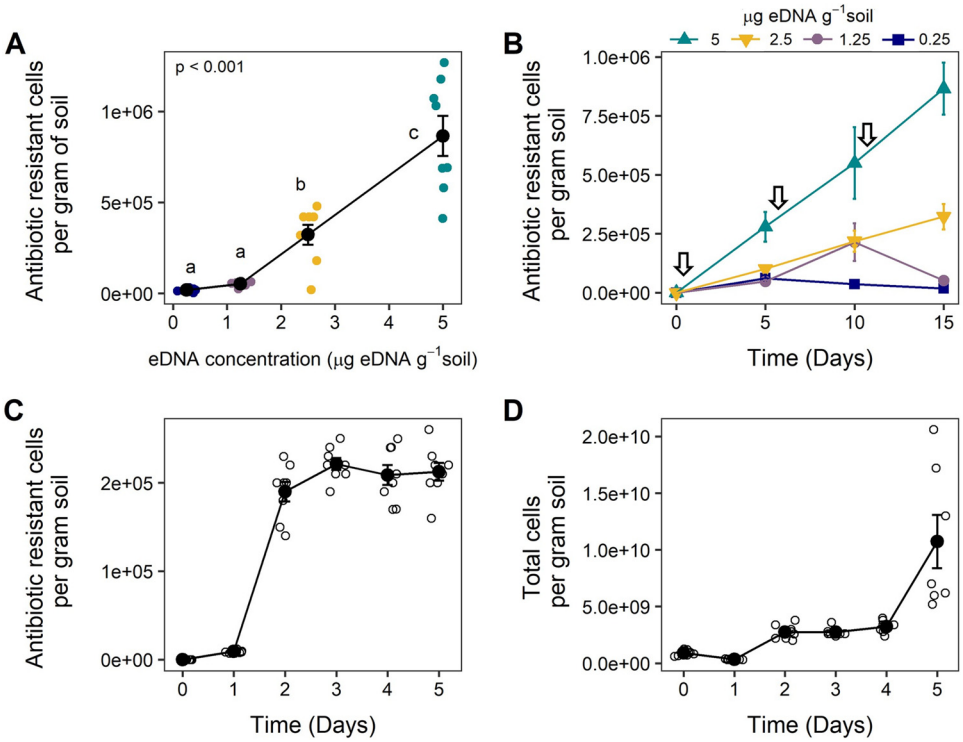

FIG 2 Relationship between the eARG concentration and the number of antibiotic-resistant *P. stutzeri* cells. (A) Number of antibiotic-resistant bacteria in soil microcosms inoculated with 0.25, 1.25, 2.5, or 5 μg eDNA per gram soil (corresponds to day 15 in panel B). Large black points represent average values and small colored points represent individual replicates. 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 24 h 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 panel C showing the total number of cells. In panels C and D, the open points represent the values for individual replicates. In panels A to D, error bars show the standard error of the mean (n = 8 replicates).

of *Pseudomonas stutzeri*, a model organism for studying transformation in soil (31). We use sterile agricultural soil from perennial switchgrass monocultures (*Panicum virgatum* L.) established ~6 years ago but previously in a corn-soybean rotation for >10 years. We manipulate the concentration of soil eARGs by varying the concentration of eDNA between 0.25 and 5 μg g^{-1} soil. We hypothesized that increasing the availability of eARGs would increase the number of transformants recovered from soil microcosms. We also hypothesized that transformation would be inhibited at low (<10%) and high (30 to 40% gravimetric) soil moistures because of biological inactivity in dry soils and eDNA degradation in wet soils (27, 32). We also manipulated the proximity of *P. stutzeri* cells to eDNA introduction sites, because we expected spatial separation of transforming cells and eARGs to pose a significant barrier to transformation.

RESULTS

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

Antibiotic-resistant transformants evolved in the presence of just 0.25 μg eDNA g^{-1} soil (Fig. 2A), which, conservatively estimated, is only a fraction (approximately one-eighth) of eDNA in field soil (previous work suggested that eDNA ranges from 2 to 40 μg eDNA g^{-1}

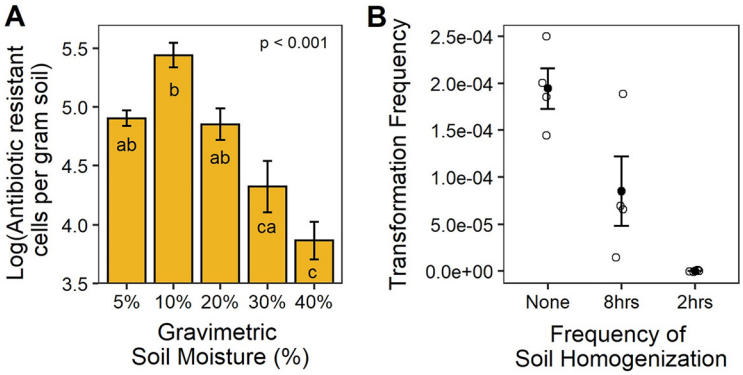

FIG 3 Relationship between soil moisture and the number of antibiotic-resistant *P. stutzeri* cells. (A) Number of antibiotic-resistant bacteria (transformants) in soil incubated at 5%, 10%, 20%, 30%, or 40% soil moisture over a 10-day experiment. Bars represent the average number of $\log_{10}$(antibiotic-resistant 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) Relationship between the frequency of soil homogenization and transformation frequency (transformants/total cells) at 10% soil moisture. Homogenization was conducted every 2 h, every 8 h, or never over a 48-h period. Closed points represent treatment averages, and open points represent individual replicates. Error bars show the standard error of the mean (n = 4 replicates).

soil [33]). Increasing the amount of eDNA from 0.25 to 5 μg g^{-1} soil increased the number of antibiotic-resistant bacteria 5-fold (Fig. 2B). Soil microcosms inoculated with 5 μg eDNA g^{-1} soil had the most antibiotic-resistant bacteria, followed by microcosms inoculated with 2.5 μg eDNA g^{-1} soil. However, there was no difference between transformants in soils inoculated with 1.25 versus 0.25 μg eDNA g^{-1} soil ($P < 0.001$) (Fig. 2A and B). In soils inoculated with either 2.5 or 5 μg eDNA g^{-1} soil, subsequent eDNA additions resulted in a linear increase in the number of antibiotic-resistant bacteria (Fig. 2B). However, inputs of small concentrations of eDNA ($<$1.25 μg) did not always increase the number of antibiotic-resistant *P. stutzeri* cells (Fig. 2B). Changes in the total number of *P. stutzeri* cells are shown in Fig. S1 in the supplemental material, to demonstrate that soils with more transformants do not have larger population sizes.

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

Relationship between soil moisture and number of transformants. When the eDNA concentration was held constant at 2.5 μg eDNA g^{-1} soil and microcosms were incubated at either 5, 10, 20, 30, or 40% soil moisture, we found that transformation was highest between 5 and 20% soil moisture and lowest at 40% soil moisture (Fig. 3A). The largest number of antibiotic-resistant *P. stutzeri* cells was in microcosms held at 10% soil moisture, although it was not significantly greater than the numbers at 5% and 20% soil moisture ($P < 0.001$) (Fig. 3A). However, the number of transformants at 5% soil moisture did not increase until after the second eARG addition, which briefly ($\sim$1 h) raised soil moisture above 5% (data not shown).

To determine whether biofilm formation plays a role in facilitating transformation in soil, as has been observed in laboratory populations of *P. stutzeri* (see Fig. S3), we performed a 48-h assay in which we disturbed the soil matrix via homogenization (using a sterile spatula) every 2 h or every 8 h or left the soil undisturbed (Fig. 3B). The establishment of a mature biofilm takes several hours and requires cells to surface attach and assemble into microcolonies, and homogenization may prevent the progression of these steps and the formation of a mature biofilm (34). We found that homogenizing the soil every 2 h completely

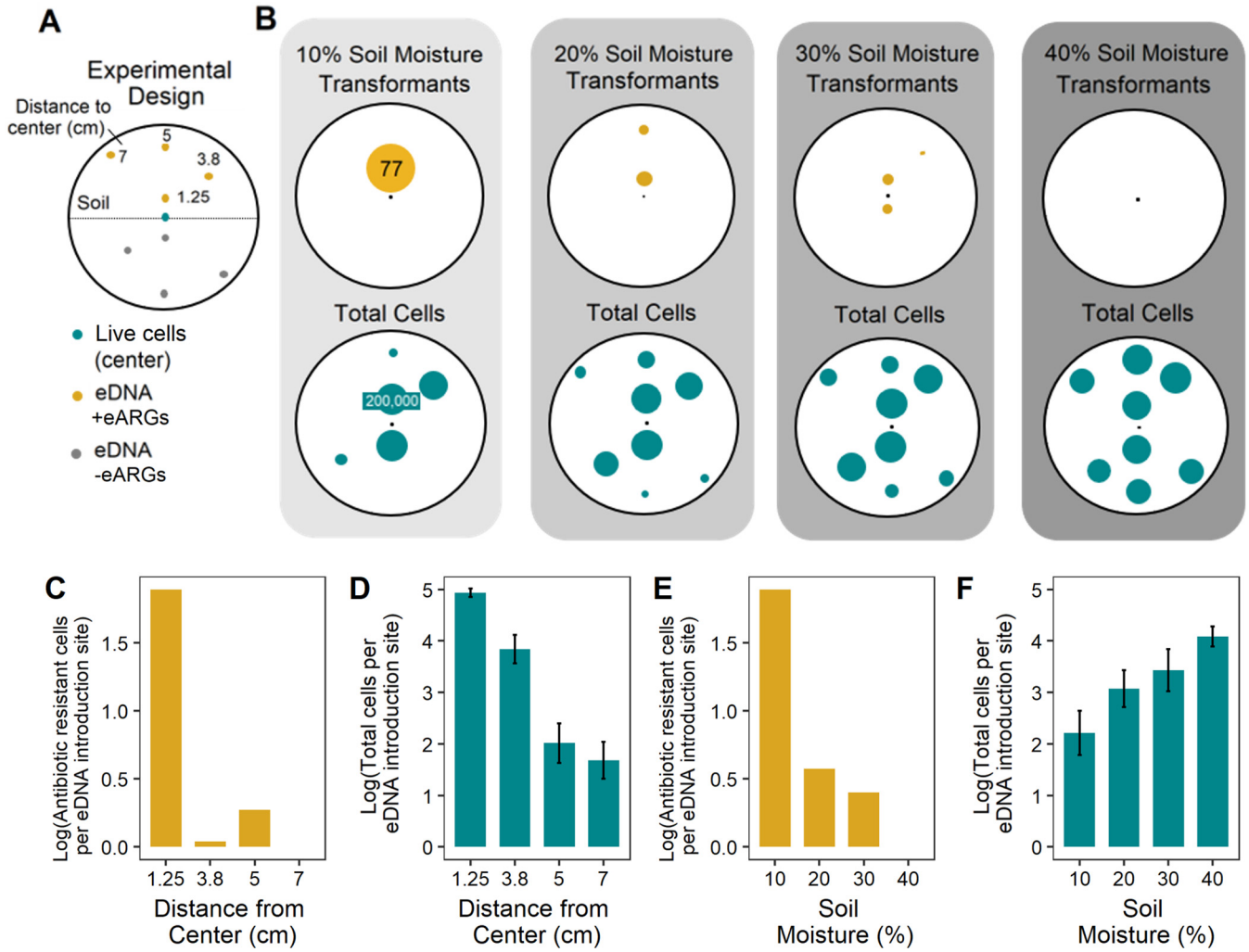

FIG 4 Relationship between dispersal and transformation at different soil moistures. (A) Bird's eye view of the soil microcosm setup. The entire microcosm was filled with soil. The eDNA introduction sites are 1.25, 3.8, 5, or 7 cm from the location of live *P. stutzeri* cells. Yellow points indicate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

prevented transformation, while homogenizing the soil every 8 h did not reduce transformation frequency, compared to a nonhomogenized control ($P = 0.001$) (Fig. 3B). However, soils homogenized every 2 h had fewer total cells than nonhomogenized controls and soils homogenized every 8 h (2.28×10^5 versus 2.75×10^9 cells g^{-1} soil; $P = 0.01$). Additional experiments outside soil showed that changes in the total number of *P. stutzeri* cells minimally affected transformation (see Fig. S4), but low cell establishment might have reduced opportunities for transformation in soils homogenized every 2 h.

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 7 cm away), we found that spatial barriers limited transformation but interacted with moisture (Fig. 4). There were significantly more transformants at 10% soil moisture than at any other moisture level (similar to Fig. 3A), but transformants appeared only in the closest eARG introduction site, which was 1.25 cm from the live *P. stutzeri* cells (transformants $\times$ soil moisture, $P < 0.001$) (Fig. 4B and E). Transformants were identified in two eARG introduction sites

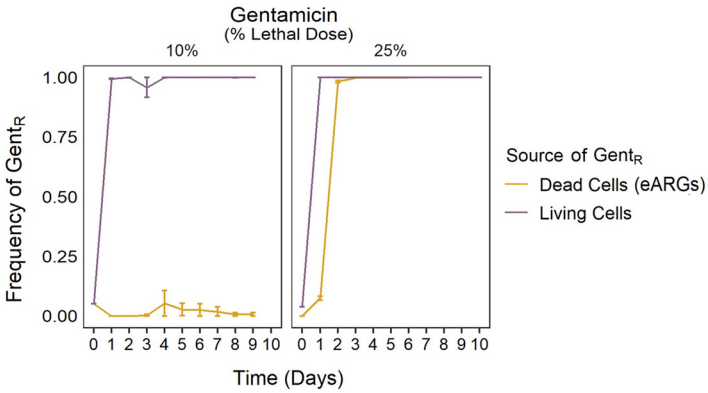

FIG 5 Frequency of gentamicin-resistant bacteria (Gent$_R$) over 10 days in *P. stutzeri* populations provided with live or dead gentamicin-resistant cells. Living cell treatments started at 4% gentamicin-resistant cells; dead cell treatments started at 0% gentamicin-resistant cells, but eARGs for gentamicin resistance were provided. *P. stutzeri* populations were grown at either 10% (left) or 25% (right) of the lethal dose of gentamicin. Points show daily averages, and error bars show the standard error of the mean (n = 4 replicates).

at 20% soil moisture and in three introduction sites at 30% soil moisture (Fig. 4B); despite transformants appearing in more eDNA introduction sites, there were still fewer total transformants, compared to 10% soil moisture (Fig. 4E). At 40% soil moisture, no antibiotic-resistant bacteria were recovered from soil, but live *P. stutzeri* cells were more abundant than under low-moisture conditions and were able to disperse to every eDNA introduction site (dispersal at 10% versus 40% soil moisture, $P = 0.0289$) (Fig. 4B and F).

Transformation under antibiotic selective pressure. To understand how antibiotic selective pressures affected the spread of ARGs, we compared the success of an equal concentration of eARGs versus antibiotic-resistant cells (i.e., dead versus alive cells, respectively) in *P. stutzeri* populations grown in liquid medium with 10% or 25% of the lethal dose of gentamicin (Fig. 5). We found that transformed eARGs reached high abundances only in populations exposed to 25% of the lethal dose of gentamicin ($P < 0.001$) (Fig. 5), while live antibiotic-resistant *P. stutzeri* cells reached high frequencies at both 10% and 25% of the lethal dose of gentamicin ($P < 0.001$) (Fig. 5). Overall, transformed eARGs took 24 h longer than live cells to establish high frequencies, likely due to the time needed for transformation to transpire (Fig. 5).

DISCUSSION

In order to reduce the impacts of antibiotic resistance on human health, we need a better understanding of the ecological dimensions that promote the transfer of eARGs through natural systems (35). In this study, we show that a 20-fold increase in the concentration of soil eARGs increases the number of antibiotic-resistant bacteria 5-fold. We find that transformants appear under most conditions typical for terrestrial soils (5 to 20% moisture) and that transformation efficiency decreases at high soil moistures, with soil mixing, and when bacteria need to disperse to eARGs. In addition, we find that eARGs can establish with the same success as live antibiotic-resistant invaders in liquid medium supplemented with a low dose of antibiotic (25% of the lethal dose). Although the number of antibiotic-resistant bacteria could be attributed to clonal expansion of a few transformants, our results suggest that transformation and population growth occurred on different time scales (Fig. 2C and D) and were optimized under different soil conditions (Fig. 4E and F). Overall, our results highlight that, in a single-species system, the soil environment plays a critical role in modulating the spread and persistence of eARGs, and that eARG removal should be incorporated into plans to combat antibiotic resistance.

Several studies have posited that a major cause of ARG transfer is widespread HGT (8, 36). Here, we show that eARGs supplied by dead bacteria are also readily transformed into soil bacteria, with the potential for HGT scaling with the abundance of eARGs (Fig. 2A

and B). This information could inform approaches to combating antibiotic resistance, which disproportionately focus on killing live bacteria but may be more effective if they also reduce eARGs, which we find can be equally effective at disseminating ARGs (Fig. 5). This may explain why practices that target only live bacteria, such as composting manure prior to application on agricultural fields, have been found to both increase and decrease the occurrence of ARGs, depending on the native bacterial community and other soil conditions (37, 38). Interestingly, manure composting at high temperatures, which promotes the degradation of eDNA, effectively reduces ARGs (39), supporting our findings that DNA degradation is a critical factor in reducing environmental concentrations of eARGs.

Despite the possibility of low levels of eARGs persisting in soil for an extended time, the ultimate fate of most eDNA is likely degradation and not transformation (40). While some eDNA can persist in soil for up to 90 days (20), previous work indicates that 99% of eDNA is degraded in the first ~7 days in soil (27, 41). Consequently, the most important role of soil conditions in regulating transformation may be the effect of moisture on the rate of eDNA decay, which likely explains our finding that transformation declined at higher soil moistures (Fig. 3A and Fig. 4B), even though greater moisture also gives recipient cells access to more eARGs (Fig. 4B). Similar trends have been observed for the soil bacterium *Acinetobacter calcoaceticum*, whose transformation frequency was highest at 18% soil moisture but lowest at 35% soil moisture (29). However, direct comparisons between *A. calcoaceticum* and *P. stutzeri* are difficult because *A. calcoaceticum* transformation efficiency was not measured below 18% soil moisture. Overall, our work suggests that transformation is favored at low soil moistures, unlike other biological processes such as extracellular enzyme activity, which are positively correlated with soil moisture (42).

Our study quantified the relationship between soil moisture and transformation in one species of bacteria and in one soil type. This relationship may vary widely across bacterial species and soil types, depending on how individual taxa regulate transformation and eDNA dynamics in different environments (43). For instance, the frequency of eARG transformation may be explained by soil physical factors, which can alter the stability of eDNA (44). Agricultural soils, including those from our field site, typically have smaller proportions of eDNA than other field soils, suggesting that eDNA could have a short half-life in these soils. Moreover, in our study, transformation was lowest at 40% soil moisture (Fig. 3A), and soils here rarely become this wet (see Fig. S5 in the supplemental material). An interesting follow-up study would be to quantify transformation in wetland or sediment soils, where water content is generally higher. In addition, we inoculated soils with eARGs obtained from *P. stutzeri* cells exposed to heat shock, which could have damaged eDNA and thus affected the 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 for different bacterial species and in multispecies communities, where competitive interactions could limit access to eARGs and limit the success of transformants (45).

We observed that soils held at 10% soil moisture supported transformation only 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 2 h also decreased the total number of cells and might have affected opportunities for transformation, although population size does not have a large effect on transformation outside soil (see Fig. S4). Alternatively, homogenization could have prevented biofilm formation, because surface-attached *P. stutzeri* cells have higher transformation efficiencies than planktonic cells (see 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-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, because 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 medium 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 that 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-species and multispecies communities in soil.

Taken together, our findings reveal the most important variables for understanding the transmission of eARGs in soil and set 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 that the soil was free of antibiotics. 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, decreasing only at very high soil moistures, in response to spatial barriers, and with soil mixing. Together, we find that the soil environment impacts the movement of eARGs from dead to live bacteria and ultimately affects the prevalence of antibiotic-resistant bacteria in antibiotic-free soil. Overall, this work provides novel *in situ* evidence that HGT is an evolutionary force that facilitates the spread of nonselected ARGs in soil; therefore, we recommend special caution in releasing eARGs into the environment.

MATERIALS AND METHODS

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

Soil microcosms. Soil microcosms were established in small (60 by 15 mm) petri dishes using 10 g of dry, sterile, switchgrass soil except for the experiment in Fig. 4, which used large (150 by 15 mm) petri dishes filled with 100 g of soil. On day 0 of each experiment, the center of the microcosm was inoculated with 2 mL of wild-type *P. stutzeri* cells suspended in liquid LB medium at a concentration of 10^6 CFU g^{-1} soil. All LB media used throughout the study followed a recipe of 10% tryptone, 5% yeast extract, and 5% NaCl (solid media contained 1.5% agarose). Immediately after addition of 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, i.e., (i) 5 μg of eDNA g^{-1} soil made from gentamicin-susceptible *P. stutzeri* cells and (ii) 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, in which 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 (there was only one eDNA input in Fig. 4). After eDNA additions, soils were gradually dried back to 20% soil moisture.

Bacterial cultures and eARGs. 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 24 h in liquid LB medium to a concentration of 10^6 CFU/mL. Stocks of antibiotic-resistant eDNA contained ARGs (eARGs) and were made from a mutant *P. stutzeri* strain (strain DAB390 [52]) containing a gentamicin resistance gene and a *lacZ* gene (Tn*7* transposition of pUC18-mini-Tn*7*T-Gm-*lacZ* into strain 28a24 [53]). eDNA was also made from the wild-type *P. stutzeri* to act as a negative control. The gentamicin-resistant *P. stutzeri* cells were genetically identical to the wild-type *P. stutzeri* cells except for the presence of the ARG. The batch cultures for eDNA/eARG stocks were prepared under the same conditions as specified above but were grown for 48 h and then resuspended in sterile nanopure water. The cells for eDNA stocks were then killed via heat shock (90°C for 1 h) and confirmed dead by plating.

Before every assay, 100 μL of stock eDNA was plated three times to ensure the absence of live gentamicin-resistant cells. We used eARGs released from dead *P. stutzeri* cells to better represent environmental eDNA, because purified genomic DNA is not found in soil. The final concentrations of eDNA ranged from 25 to 50 ng/μL and were appropriately diluted for each experiment (determined using Qubit fluorometric quantification and the Invitrogen Quant-iT PicoGreen double-stranded DNA [dsDNA] assay kit). The transformation efficiency of *P. stutzeri* plateaus at ~5 ng/μL eDNA in assays conducted on agar petri dishes (see Fig. S6 in the supplemental material).

Counting of transformants and total cells. To determine the number of transformants in each soil microcosm, we weighed out 0.2 g of soil from each microcosm or eDNA pool and placed it into a 1.5-mL centrifuge tube. We added 180 μL of liquid LB medium to each tube and vortex-mixed the tubes for 10 s (~10^{-1} dilution). After allowing the soil to settle for 10 min, we transferred the supernatant to a 96-well plate and diluted it to 10^{-6} or 10^{-9} depending on the experiment and the expected number of cells. In the experiments that manipulated eDNA concentration and soil moisture, we plated 50-μL cell suspensions. For the remaining experiments, we plated 10-μL dots. All plating was done on petri dishes with solid LB medium (to count the total population size) or solid LB medium with gentamicin (50 μg/mL) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (20 μg/mL) (to count transformants in soil). Plates were incubated at 30°C, and the number of colonies was counted after 48 to 72 h. The number of cells is reported per gram of soil except in Fig. 4, where it is reported per eARG pool (0.2 g soil), and was calculated according to the following equation: cells per unit = cells μL^{-1} × (soil slurry volume [200 μL]/soil mass in slurry [g]).

Manipulation of eARG concentrations in soil microcosms. To understand the relationship between the availability of eDNA and transformation, we varied the concentration of eDNA in soil between 5, 2.5, 1.25, and 0.25 μg of eDNA g^{-1} soil (Fig. 2A and B). We used these concentrations because they conservatively represent ~10%, 5%, 2.5%, and 0.5% of the total eDNA pool in soil (43). The concentration of eDNA never exceeded 10%, to account for the fact that only a small percentage of soil eDNA is likely to contain ARGs. We ran the experiment for 15 days and added eARGs to soil on days 0, 5, and 10 of the experiment, with each treatment consisting of 8 replicate soil microcosms.

Manipulation of soil moisture in soil microcosms. To determine how soil moisture affected transformation, we maintained soil microcosms at 5, 10, 20, 30, or 40% gravimetric soil moisture over a period of 10 days (Fig. 3A) (i.e., soil moisture = [weight after water addition − dry weight/dry weight] × 100). In this experiment, all of the microcosms were inoculated with an intermediate concentration of eDNA (2.5 μg g^{-1} soil) and eARGs were added on days 0 and 5. We report the number of transformants present on day 10, using 8 replicate microcosms per treatment. To determine whether the physical structure of the soil was important for transformation, we manipulated the physical structure of the soil by mixing the soil every 2 h, every 8 h, or never throughout a 48-h 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 s, using a sterile spatula, at the designated intervals.

Manipulation of eARG introduction sites in soil microcosms. To determine whether dispersal of eARGs and subsequent transformation events varied under different soil moistures, we introduced eDNA at different sites in large soil microcosms set up in petri dishes (150 by 15 mm) with 100 g of soil in each microcosm. There were four different soil moisture treatments, i.e., 10, 20, 30, and 40%. At each soil moisture, we set up 4 microcosms (n = 4 [16 soil microcosms]). In each microcosm, there were eight different eDNA introduction sites, which are depicted in Fig. 4A. The introduction sites were located 1.25, 3.80, 5, or 7 cm from the center of the petri dish, and each introduction site was inoculated with 2 μg eDNA g^{-1} soil. At each distance (e.g., at 1.25 cm), one eDNA introduction site was inoculated with eARGs from dead gentamicin-resistant *P. stutzeri* cells and the other with dead wild-type *P. stutzeri* cells, which contained 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 (400-μL total volume). Approximately, 0.5 h after eDNA introduction, *P. stutzeri* cells were dripped into the center of each microcosm (2-mL total volume). Each microcosm was immediately brought to the appropriate soil moisture via drying or the introduction of sterile water, depending on the soil moisture treatment. After 5 days of incubation at ~23°C, 0.2 g of soil was collected from each eDNA introduction site. We counted the number of transformants and total cells as described in "Counting of transformants and total cells." Transformants appeared in only one control eDNA introduction site, which was 1.25 cm from the center of the petri dish in microcosms incubated at 30% soil moisture. However, based on our methods, we cannot determine whether this was caused by the movement of eDNA or the movement of transformed cells.

Antibiotic selective pressure in assays outside soil. Outside soil, we tested how equal concentrations of eARGs and live antibiotic-resistant cells establish in populations of *P. stutzeri* cells challenged with a low dose of antibiotic (Fig. 5). Initially, we established two equal populations of *P. stutzeri* cells carrying kanamycin resistance (strain DAB386 [52]). To one of the two populations, we added 60,000 live gentamicin-resistant *P. stutzeri* cells. To the other population, we added 60,000 dead gentamicin-resistant cells, which provided a source of eARGs. Therefore, on day 0 of the experiment, the two treatments contained either 4% gentamicin-resistant cells and 96% kanamycin-resistant cells or 0% gentamicin-resistant cells and 100% kanamycin-resistant cells. We used kanamycin-resistant cells for the founding population instead of the antibiotic-susceptible strain *P. stutzeri* 28a24 (used in previous experiments) so that we could track the evolution of multidrug resistance, which we found was quite rare and occurred only at 25% of the lethal dose of gentamicin (see Fig. S7). Populations were founded in 1 mL of LB medium, and the experiment ran for 10 days, with each population receiving 1 mL of fresh LB medium every 24 h. Each day we counted the number of gentamicin-resistant genotypes and the total number of cells using serial dilution and selective plating. Data are missing for day 10 for populations grown at 10% of the lethal dose of gentamicin due to an accidental loss of sample. We counted the total number

of cells on solid LB medium (no antibiotic) and the number of gentamicin-resistant cells on solid LB medium with gentamicin (50 μg/mL) and X-Gal (20 μg/mL). We report the frequency of gentamicin-resistant genotypes (gentamicin-resistant cells/total cells) in Fig. 5.

Statistical analyses. Prior to analyses, all data were confirmed to meet assumptions of normality and homogeneity of variance. Data shown in Fig. 3A and Fig. 4 did not conform to assumptions of homogeneity of variance and were log transformed. The results from the soil microcosm studies were analyzed by either one-way or two-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test with test variable (i.e., soil manipulation and sampling day) as a fixed effect, using the R *stats* package. Experiments with multiple sampling days were analyzed by two-way ANOVA except in certain instances when the test variables were analyzed individually on the final sampling day (e.g., Fig. 3A). Results from the soil microcosm experiment in Fig. 4 were analyzed by two-way ANOVA with the distance to eARGs and soil moisture as fixed effects. When significant, interactions between test variables were included in the model. The frequency of antibiotic-resistant bacteria present in each laboratory population at the end of the experiment were compared using two-way ANOVA with the treatment (live versus dead cells) and selection regimen (10 or 25% of the lethal dose of gentamicin) as fixed effects (Fig. 5). Differences among all test variable groups were considered significant at α values of $\leq$0.05.

Data availability. Raw data are available at the Open Science Framework (OSF) data repository (https://osf.io/7jp3b).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

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H.A.K., K.M.D., and S.E.E. conceptualized the experimental design for the soil microcosms. H.A.K. performed all soil microcosm incubations and laboratory work. H.A.K. and S.E.E. performed the analyses and wrote the manuscript. H.A.K., K.M.D., and S.E.E. provided comments on the manuscript.

We declare no conflict of interest.

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