

Microbial diversity determines the invasion of soil by a bacterial pathogen

Jan Dirk van Elsas^{a,1}, Mario Chiurazzi^a, Cyrus A. Mallon^a, Dana Elhottová^b, Václav Křístůfek^b, and Joana Falcão Salles^a

^aDepartment of Microbial Ecology, Center for Evolutionary and Ecological Studies, University of Groningen, 9700 CC, Groningen, The Netherlands; and ^bBiology Center, Academy of Sciences of the Czech Republic (AS CR), Public Research Institution (v.v.i.), Institute of Soil Biology, CZ-370 05 České Budějovice, Czech Republic

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Natural ecosystems show variable resistance to invasion by alien species, and this resistance can relate to the species diversity in the system. In soil, microorganisms are key components that determine life support functions, but the functional redundancy in the microbiota of most soils has long been thought to overwhelm microbial diversity–function relationships. We here show an inverse relationship between soil microbial diversity and survival of the invading species *Escherichia coli* O157:H7, assessed by using the marked derivative strain T. The invader's fate in soil was determined in the presence of (i) differentially constructed culturable bacterial communities, and (ii) microbial communities established using a dilution-to-extinction approach. Both approaches revealed a negative correlation between the diversity of the soil microbiota and survival of the invader. The relationship could be explained by a decrease in the competitive ability of the invader in species-rich vs. species-poor bacterial communities, reflected in the amount of resources used and the rate of their consumption. Soil microbial diversity is a key factor that controls the extent to which bacterial invaders can establish.

community niche | invasiveness | resource utilization

Resistance to invasion by alien species represents a major life support function of terrestrial ecosystems (1). Theoretical (2–4) and experimental (5–8) studies have indicated that biologically diverse communities are often less prone to being invaded than simpler ones, but effects of microbial diversity on invading (micro)organisms have remained underexplored. The number of microbial, in particular bacterial, species in a single gram of soil can be enormous (9–11). Because several of the functions of the soil microbiota are key to soil functioning (12), the considerable functional redundancy has been thought to overwhelm any type of diversity–function relationship (13). However, microbial diversity was found to be inversely related to invasibility of the wheat rhizosphere by *Pseudomonas aeruginosa* (14) and also affected the ability of *Ralstonia solanacearum* to induce wilting disease in tomato (15), although this was not the case in potato (16). Soil bacterial diversity exerted a positive effect on the decline of this plant pathogen. However, this effect was dependent on soil type/management, occurring only in sandy soils under conventional agricultural management (17). Confounding factors, such as soil type and origin, may have led to conflicting results in these experiments, in which microbial diversity was strongly dependent on the soil used. Only by taking a “proactive” approach, manipulating bacterial diversity in a controlled experiment, can we clearly address the effects of microbial diversity on pathogen decline in soils.

The fate of the enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 in soil is of major concern (18–20). In this context, the microbial communities that established after soil fumigation were shown to determine the fate of the invading species, whereby reduction in microbial diversity due to progressively enhanced fumigation depths resulted in higher pathogen persistence in soil (21). Similarly, in 25 different manures, the decline rate of *E. coli* O157:H7 was negatively correlated with *Enterobacteriaceae*

richness (22). Furthermore, the easily available carbon content of the manure explained this decline rate (22). A study in organic manure-amended soil showed a faster pathogen decline when rates of nutrient flow were reduced (23). Moreover, it has been shown that *E. coli* can survive at higher densities and for longer periods in sawdust than in sand livestock beddings (24). The lower survival observed in the sand was hypothesized to relate to the lower amount of organic matter and nutrients. In a follow-up experiment, evidence was provided for the contention that *E. coli* was suppressed in the sand as a result of the presence of several bacterial taxa (25). Thus, both microbial diversity and resource availability may play important roles in determining *E. coli* O157:H7 persistence in soil.

The underlying mechanisms of diversity–invasiveness relationships may lie in competition for the utilization of limiting resources [e.g., nitrate for plant communities (5)]. Theoretical tradeoff surfaces, as suggested by Tilman (4), might allow a prediction of the success of invasion. Moreover, systems harboring microbial communities with lower metabolic diversity might be more prone to invasion than those with communities capable of using a wider range of resources (26). Although competition for resources and components of diversity likely affect biological invasions, they are only pieces of the puzzle. Other mechanisms (e.g., predation and negative species interactions) might also determine the fate of invader species.

To better understand whether and how microbial diversity might hinder pathogen establishment in soil, we performed three experiments using a derivative of *E. coli* O157:H7 (strain T). Strain T is a genetically marked nontoxigenic *E. coli* O157:H7 (20), allowing survival and competition studies in soil. The aim of the first two experiments was to assess the effect of microbial diversity on invader establishment and survival. Along with assessing the effects of soil microbial diversity on invasibility of the system, we investigated whether protozoa exerted effects on the invader. The third experiment aimed to elucidate the mechanism behind the diversity–invasibility relationship that was found.

Results and Discussion

Assembly Experiment. In the first experiment, random bacterial isolates were obtained from a grassland soil in The Netherlands. Then, using batches of the same presterilized soil, bacterial communities consisting of 5, 20, or 100 random isolates were assembled in the soil, by adding isolate mixes in equal total cell numbers ($\approx 10^6$ g⁻¹ dry soil) to the soil. In each treatment, 20% of the total added diversity encompassed actinobacterial morphs, which are known to produce antimicrobial compounds (27, 28). “Zero” control (no cells added; sterile soil) as well as natural soil

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¹To whom correspondence should be addressed. E-mail: j.d.van.elsas@rug.nl.

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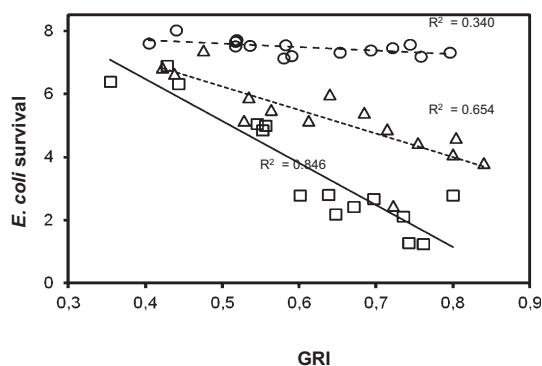


Fig. 4. Relationship between *E. coli* strain T population dynamics (log cfu g⁻¹ soil) and the global richness indicator (GRI) at different times after introduction of the invader. The GRI (34) was derived from the sum of five richness values (number of bands on DGGE gels) obtained from the following microbial groups: total bacteria, total fungi, pseudomonads, actinobacteria, and bacilli, each normalized per group, and divided by 5. Linear correlations between *E. coli* survival and GRI increased over time ($R^2 = 0.34$, $R^2 = 0.654$, and $R^2 = 0.846$ for 3, 30, and 60 d after pathogen inoculation, respectively). Open circles, day 3; open triangles, day 30; open squares, day 60.

confirm the previous findings obtained with simpler communities (assembly approach), clearly indicating that the reduction in pathogen density is not observed only in fast-growing bacterial communities. In fact, by creating more realistic soil microbial communities, containing oligotrophic and nonculturable organisms, a steeper decline over time in pathogen population size was observed.

Inverse Relationships Between Invader Survival and Richness of Different Groups. Significant inverse linear relationships between survival of the invader and richness at day 60 were found for most microbial groups under study (i.e., bacteria, fungi, pseudomonads, and Actinobacteria; Fig. S2), survival being particularly affected by bacterial and actinobacterial richness. To a lower extent, it was also negatively correlated with the *Pseudomonas* and fungal richness values, whereas it did not show a clear relationship with *Bacillus* richness. The negative correlations were corroborated by similar regression performed with the Shannon diversity indices. Multiple regressions, in which richness for all five microbial groups at day 60 was related to invader survival, were also run. The correlation between invader survival and actinobacterial richness was highly significant ($R^2 = 0.893$; $P = 0.0001$), indicating that Actinobacteria are major contributors to this model. Actinobacteria have the capacity to produce ample secondary metabolites involved in soil microbiostasis (27, 28), indicating that negative interspecies interactions play an important role in determining the fate of invading species. The notion that specific groups of bacteria play a specific role in *E. coli* suppression is not new (25). It was not clear, however, whether the microbial suppression of *E. coli* was due to competition for vital resources or to direct antagonism. To confirm the presence of suppressors of strain T, future experiments should harness the assemblage approach, selecting either antagonists or competitors for the same resources.

Competition Between Invader and Resident Community. Competition for nutrients is an important mechanism that may limit invasions in highly diverse communities (5). In resource-based niche theories, the establishment of invading species is dependent on the amount of (limiting) resources that are left unconsumed by native species, as well as by the rate at which native and invader species consume the existing resources (4, 37). We hypothesized that the uptake of diverse resources plays important

roles in the invasibility of microbial communities by strain T. Thus, to explain the effect of microbial diversity on strain T, we evaluated the competitive ability of the latter in the presence of bacterial communities of increasing richness, focusing on single niche factors (i.e., the carbon source). Thus, we compared the resource utilization patterns of communities varying in richness (1, 5, 10, and 20 species), with or without strain T, on 31 individual carbon sources typical for soil (38). The dissimilarity in resource utilization patterns of bacterial communities in the presence and absence of strain T was used as a proxy for the competitive ability of strain T, with higher dissimilarities indicating higher dominance of strain T in the community. The competitive ability of strain T indeed decreased with increasing species richness (Fig. 5). The significant negative correlation observed between strain T's competitive ability and species richness, for both the amount of each resource ($R^2 = 0.456$, $P = 0.0001$) and the rates of utilization ($R^2 = 0.334$, $P = 0.0001$), indicated that the more diverse communities were, the better they were able to acquire resources, and at a higher rate, than strain T. As expected, in the absence of the invader, the communities with higher species richness were able to consume more of the individual resources (Fig. S3; $R^2 = 0.39$, $P < 0.0001$).

Rate of Resource Utilization. Considering that species with higher consumption rates will be more efficient in extracting resources, and thus more competitive, we could make a parallel to the resource requirement parameter R^* proposed by Tilman (4, 37). In his work, it was proposed that species with lowest R^* would outcompete those that require higher resource levels. Briefly, considering a fixed amount of resource, species with higher growth rates will have a lower R^* (assuming that loss rate due to

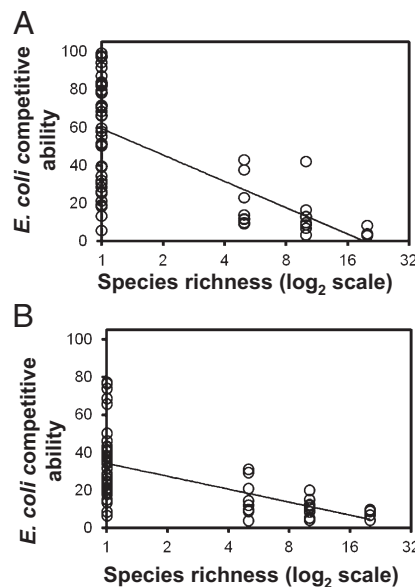


Fig. 5. Relationship between manipulated species richness and the competitive ability of *E. coli* strain T. The competitive abilities are expressed both in relation to the amount of resources consumed (A) and their rate of consumption (B) and represent the dissimilarity in resource utilization patterns, obtained by pairwise comparisons of bacterial communities of increasing richness (1, 5, 10, and 20 species), in the presence and absence of strain T. Each circle represents a pairwise comparison. Resource utilization patterns correspond to the average in the amount (A, $R^2 = 0.46$, $P = 0.0001$) or in the rate of consumption (B, $R^2 = 0.33$, $P = 0.0001$) of 31 carbon sources present in the Biolog Ecoplate. Similar data were obtained when only the 10 carbon sources used by *E. coli* strain T were analyzed ($R^2 = 0.47$, $P < 0.0001$ and $R^2 = 0.43$, $P < 0.0001$, for amount and rate of consumption, respectively).

predation is similar in the experiment). Thus, species with higher consumption rates would use the resources faster and, therefore, reduce the probability that less-competitive strains would thrive. In this context, we calculated, for each individual strain and on each carbon source, the rate at which the resources were consumed. The growth rates of strain T were, indeed, significantly lower compared with those of the set of 40 bacterial species used in the experiments. Considering the 10 resources used by *E. coli*, its average growth rate was 4.17 ± 0.77 omnilog units h^{-1} (average \pm SE), whereas the average rate of resource utilization of the other 40 strains on the same set of resources was 14.51 ± 0.63 omnilog units h^{-1} (average \pm SE). These results support the view that efficiency of resource utilization by the invader vs. that by the established community was at the heart of the effect of microbial diversity seen in the soil microcosms, at least when considering species interactions by one niche factor at a time. It is important to note that the relative rates of resource utilization observed do not necessarily reflect the actual in situ rates in soil, where complex interactions between substrates and resident microbial communities are likely to be found. Nevertheless, they provide an indication of the potential activities of representatives of the soil microflora. See *SI Discussion*.

Materials and Methods

Soil and Microcosms. Fresh samples of a species-rich loamy sand soil denoted Wildekamp (W; pH-KCl 5.5; approximately 2% organic matter, 16–18% moisture upon sampling), obtained from the upper 10 cm of the Wildekamp field (Wageningen, The Netherlands) in autumn (September 2006), were used throughout. The field was covered by permanent grass, and regular mowing in summertime was the only treatment applied. Part of the soil was sterilized by γ irradiation (50 kGy), and another part was kept for <7 d at room temperature in closed plastic bags that were regularly aerated. The first was used as the matrix of soil microcosms, whereas the latter was used, in the dilution-to-extinction experiment, as an inoculum.

Experimental Setup. Varying treatments were established in soil microcosms. One type of manipulation consisted of the addition, to the γ -sterilized W soil, of consortia consisting of mixtures of random bacterial isolates, of increasing complexity, whereas a second one consisted of the addition of microbial communities obtained via a dilution-to-extinction approach. Microcosms were constructed in 200-mL bottles that contained 50 g of W soil at final moisture content of 65% of water-holding capacity (WHC). Control microcosms contained natural or sterilized soil without added cells. Three replicates were used per treatment per time. Flasks were distributed in plastic trays following a randomized design. The soil microcosms were incubated at 20 °C in the dark and at constant moisture. Microcosms were destructively sampled at each time point during the experimental period (3, 7, 14, 30, and 60 d) and monitored with respect to invader population density and microbial diversity (details below).

In a first experiment, the sterilized W soil was inoculated with 0, 5, 20, or 100 bacterial strains isolated from the same soil, before sterilization. For each diversity level, 20% of the bacterial strains encompassed typical actinobacterial morphs. Bacterial isolation was performed by shaking 10 g of soil in 90 mL of sterile 0.1% sodium pyrophosphate plus 10 g of gravel (2–4 mm diameter) for 30 min. Serial 10-fold dilutions of this mother suspension were spread onto R2A medium (Oxoid), and colonies appearing at up to 12 d were randomly picked, purified by streaking, and stored until their utilization in the bacterial mixtures. For introduction, cells obtained from individual pure fresh colonies were suspended in sterile water to obtain an absorbance at 600 nm (A_{600}) of 1. Assuming that an A_{600} of 1 corresponds to 10^9 cells mL^{-1} , we set up inocula for each treatment by adding different amounts of each bacterial suspension to obtain mixtures with the same amount of cells but different strain richness (5, 20, or 100 strains). As expected, approximately 5×10^8 to 10^9 bacterial cfu g^{-1} soil were established before the introduction of strain T in each microcosm (checked by plating on R2A).

In the second experiment, a dilution-to-extinction technique was adopted (30, 31). Specifically, microcosms containing 50 g of γ -sterilized W soil were inoculated with 5-mL aliquots of different suspensions obtained by serially diluting (1:10) natural W soil suspensions in sterile water. The systems were carefully mixed avoiding contamination, and covered to prevent water evaporation. As inocula, the 10^1 , 10^3 , and 10^6 dilutions were used. The 10^1 dilution was also used after vacuum-filtration through membranes with progressively smaller pore sizes (5, 3, 2, and 1 μm), thus yielding treatment

" 10^1 -filtered" (10-F). The aim of this successive filtration was to exclude protozoa and other soil mesofauna, but it is likely that larger (>1 μm) bacterial and fungal cells have been left out as well. Natural soil did not receive inoculant cells, but soil humidity was maintained at 65% of WHC.

Pathogen Introduction. In both microcosm experiments, after allowing 30 d for the establishment and stabilization of the microbial community at a comparable level across all of the treatments, an invading bacterial pathogen [i.e., the genetically marked, nonpathogenic *E. coli* O157:H7 derivative strain Tn5 *luxCDAEB* (20)] was introduced into the soil microcosms at approximately 10^8 cfu g^{-1} dry soil. This established a soil moisture content of 75% of WHC.

Monitoring of Survival of the Invader as Well as Total Bacterial Communities.

After *E. coli* O157:H7 derivative strain T addition, we monitored the microcosms at different time points over a 60-d period, during which all cultivation-dependent and -independent analyses were performed. The survival of strain T, expressed as the number of cfu g^{-1} dry soil, was then determined by selective dilution plating on tryptic soy agar (TSA) plus respective antibiotics, in accordance with van Elsas et al. (21). *E. coli* counts were determined after 24 h incubation at 37 °C. Survival of strain T was thus based on the cfu numbers observed on selective medium at each sampling time, during a 60-d period. The number of total culturable bacteria, expressed as cfu g^{-1} dry soil, was determined by dilution plating onto unselective R2A medium followed by colony counting after prolonged incubation (up to 12 d) at 28 °C. Total bacterial biomass was determined using dichlorotriazinylamino fluorescein-assisted microscopy, and total protozoa were estimated using the most-probable-number method of Darbyshire et al. (33). We further determined the prevalence of different indicator PLFAs by using MIDI-FAME analysis (21).

The modified microbial diversities established in the two types of microcosms were assessed using soil-DNA-based PCR-DGGE. The richness (number of different amplicon types) and diversity (Shannon index) of total fungi and bacteria, as well as of specific bacterial groups (only for the dilution-to-extinction experiment), were evaluated on the basis of PCR-DGGE analyses performed at day 3, 30, and 60 after invasion by *E. coli* strain T. In the group-specific PCR-DGGE approach, we assayed those microbial groups of which members were likely responsible for antagonism toward invading microorganisms (i.e., *Pseudomonas* spp., *Bacillus* spp., and *Actinobacteria*). In all cases, DNA was extracted from soil using standard soil DNA extraction (MoBio Ultraclean Extraction Kit). PCR amplifications and DGGE separation procedures were applied as indicated for total bacteria, fungi, and *Actinobacteria*, *Pseudomonas*, and *Bacillus* (27). The patterns obtained were compared across treatments by using GelCompar version 4.0 (Applied Biosystems) and diversity measures, such as band (taken as indicative for species) richness and the Shannon index of diversity (including a richness and evenness component), were calculated. On the basis of the observed richness values, a global richness indicator (GRI) was calculated (36), by adding the normalized richness values obtained for five microbial groups (total bacteria, total fungi, actinobacteria, pseudomonads, and bacilli) and dividing it by 5. A range of statistical analyses was performed to assess the relationship between the survival of strain T and the diversity parameters established.

Competition Experiment. Bacterial strains were isolated from soil (as described previously), and a set of 40 different strains, distinguished according to colony morphology and BOX-PCR (39), was selected. Briefly, the strains were used to create communities of increasing species richness, following a broken stick model (39, 40) to create communities varying in species richness and composition. Bacterial species were randomly ordered, creating one stick, which was then subsequently "broken", giving two assemblages of 20 species, four of 10 species, and eight of 5 species. Extra assemblages were constructed by creating a third and fourth stick of 20 species, containing the species located at the center or ends of the first 20-species sticks. Further division of these sticks provided an additional four assemblages containing 10 species. Thus, four, eight, and eight assemblages containing respectively 20, 10, and 5 species were created, in addition to the 40 monocultures. Microcosms consisted of Ecoplates (Biolog) containing 31 carbon sources commonly found in soil, replicated three times (38). Microcosms were inoculated with the assemblages at similar final cell densities ($\text{OD}_{600} = 0.1$). Thus, each microcosm received the species at a cell density of $\text{OD}_{600} = 0.1/s$, where s is the number of species in the community inoculum (1–20). For microcosms containing strain T, the latter was introduced at $\text{OD}_{600} = 0.02$, regardless of the established species richness. The data based on monocultures in the absence of the pathogen were used to calculate the growth rates of individual strains (using slopes, see below) on the respective C sources. *E. coli* strain T was also used as a single strain at initial densities corresponding to $\text{OD}_{600} = 0.02$ and $\text{OD}_{600} = 0.1$, for comparisons in the com-

petition and growth rate experiments, respectively. Incubation was in the omnilog apparatus (Biolog) for 48 h at 25 °C, and reading was every 15 min. The quantity of substrate used and the rates of consumption were calculated according to the area under the curve and the slopes of increase, respectively, for each of the 31 individual C sources, using the software provided. Pairwise comparisons of the bacterial assemblages, in the presence and absence of strain T, were performed by using Bray Curtis dissimilarity implemented in the

software PRIMER 5 (PRIMER-E). Similar relationships were obtained by using the distance measures Gower and Euclidean Distance.

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Supporting Information

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SI Discussion

Previous experiments, in which the fate of *Escherichia coli* in soils has been addressed, have indicated that the indigenous microbiota is an important factor in *E. coli* survival (1–5). In none of these cases, however, was the microbial diversity of the system manipulated, resulting in the premise that the effects observed could also be attributed to factors such as soil type, origin, and management. In contrast with other studies, the goal of our experiment was to use manipulated diversity (species richness) in one system to understand the effect on invasibility, instead of comparing different environments that *E. coli* could inhabit. In summary, our collective data indicate that the survival of the bacterial pathogen exemplified by strain T is strongly limited in microbiologically diverse soil but higher in biologically compromised soil. This inverse relationship between survival of the invading *E. coli* strain and the extant microbial diversity extends a central hypothesis concerning the ecosystem biodiversity–invasibility issue to microorganisms (i.e., that at progressively lowered soil microbial diversity values increasing establishment

of an invading species is possible). Resource-based niche theory provided a plausible explanation for microbial community invasibility.

By addressing species interactions in a multitude of niches, for instance, by determining the niche breadth and niche overlap (6), it might be possible to determine the exact extent to which resource competition determines the invasibility of soil communities by bacterial pathogens. Moreover, as suggested in the main text, mechanisms additional to competitiveness in resource capture and utilization may be invoked to explain the fate of the invader, as well as its progressive disappearance in later stages in low-diversity communities. Antagonism against the invader, such as exhibited by antibiotic-producing organisms or predators, may have played one role. Assembling bacterial communities that vary both in terms of their niche (6) and in the numbers and types of antagonists and predators will determine the contribution of each of these mechanisms to the microbial diversity–invasibility relationship as well as community invasibility in response to these factors and tradeoffs therein.

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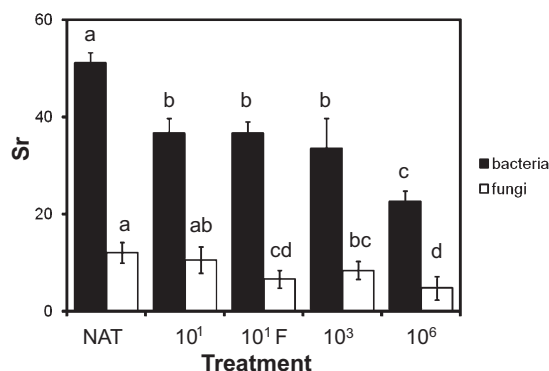


Fig. S1. PCR-denaturing gradient gel electrophoresis-based estimated richness of dominant types (S_r ; numbers of bands) across treatments for 16S rRNA gene-based bacterial (filled bars) and 18S rRNA gene-based fungal (open bars) patterns in the dilution-to-extinction experiment. Bars represent mean values (\pm SDs) of three replicates from three sampled experimental points (3, 30, and 60 d). NAT, natural soil; 10¹, 10¹-F, 10³, and 10⁶ treatments, see legend to Fig. 3 (main text). a, b, c, d, and combinations: statistical class at $P < 0.05$.

