Chemical warfare and survival strategies in bacterial range expansions

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Dispersal of species is a fundamental ecological process in the evolution and maintenance of biodiversity. Limited control over ecological parameters has hindered progress in understanding of what enables species to colonize new areas, as well as the importance of interspecies interactions. Such control is necessary to construct reliable mathematical models of ecosystems. In our work, we studied dispersal in the context of bacterial range expansions and identified the major determinants of species coexistence for a bacterial model system of three \textit{Escherichia coli} strains (toxin-producing, sensitive and resistant). Genetic engineering allowed us to tune strain growth rates and to design different ecological scenarios (cyclic and hierarchical). We found that coexistence of all strains depended on three strongly interdependent factors: composition of inoculum, relative strain growth rates and effective toxin range. Robust agreement between our experiments and a thoroughly calibrated computational model enabled us to extrapolate these intricate interdependencies in terms of phenomenological biodiversity laws. Our mathematical analysis also suggested that cyclic dominance between strains is not a prerequisite for coexistence in competitive range expansions. Instead, robust three-strain coexistence required a balance between growth rates and either a reduced initial ratio of the toxin-producing strain, or a sufficiently short toxin range.

1. Introduction

The fate of a species depends on the abilities of its members to colonize new areas and to outperform competitors [1,2]. A central theme of ecological research is to understand these abilities and to explain how many competing species still manage to live in lasting coexistence, especially during arms races over common resources [3–10]. Structured environments were theoretically proposed to be facilitators of biodiversity [3,11–19]. However, experimental verification of the proposed mechanisms promoting biodiversity is hard to come by. Ecological studies traditionally focused on systems of mammals and plants, but the long reproduction times and large spatial scales involved impede experimental progress [20]. To circumvent these problems, recent studies have turned to microbial model systems in which both spatial and temporal scales are experimentally better accessible [3,21–23]. New methods of genetic engineering even admit the possibility to modify the behaviour of test species. These methods stimulated further research on microbial systems and increased our knowledge about their transient and long-term dynamics [24]. For microbial life in well-mixed culture, for example, experimental and theoretical models have recently shown how transient processes can be amplified by recurring life cycles to change a system’s long-term fate [25,26]. In spatial environments, long-term limits are more difficult to attain. We followed a previous study on competitions of three bacterial strains of \textit{Escherichia coli} (toxin-producing, sensitive and resistant) in fixed spatial environments [3] and
identified traits that ensure the transient coexistence of strains during the course of range expansions.

What determines whether a bacterial species thrives or fal ters as it explores new areas can be studied systematically after droplet inoculation on an agar plate [5,27–30]. Recent experimental studies have highlighted the importance of random genetic drift in driving population differentiation along the expanding fronts of bacterial colonies—an effect that gives rise to monoclonal sectoring patterns [5,31]. Natural microbial colonies and biofilms are characterized by a complex community structure [21,22,32], which is shaped by competition between strains for resources such as nutrients and space [2,5,27–30], interference competition through the production of toxins [3,8,22,29,33,34], and different forms of mutualism, cooperation and cheating [4,6,22,35]. Only a few recent studies, most of them theoretical, have explored the role of such interactions for expanding populations [36–38]. Experimental studies are appearing just recently [10,39,40] and are much needed to identify and characterize the key principles that drive population dynamics in expanding systems. In our work, we investigated range expansions for a bacterial model system comprising three Escherichia coli strains: a toxic strain, a sensitive strain (facing death upon the encounter of toxins) and a resistant strain. By genetically altering strain growth rates, we created three different ecological scenarios, including a hierarchical scenario and a scenario that mimicked a cyclic rock–paper–scissors game (figure 1) [3,18]. Control over strain growth rates also enabled us to acquire sufficient experimental data to construct and validate a computational model of the expansion process. The model was used to predict parameter regimes for which coexistence of all three strains was observed in experiments. Furthermore, we identified the factors that determined a strain’s chance of survival (composition of the inoculum, relative growth rates and effective toxin range), and quantified the relationship between these factors in terms of phenomenological ‘biodiversity laws’. Our work highlights the central importance of bacterial interactions in the evolution and maintenance of biological diversity, and pursues the theoretical aim to understand how interactions affect coexistence [41].

2. Material and methods

2.1. Bacterial strains and fluorescent proteins

The strains used in our study represent the Escherichia coli Colicin E2 system (BZB1011 (sensitive ‘S’ strain), E2−BZB1011 (toxic ‘C’ strain) and E2−BZB1011 (resistant ‘R’ strain)) [3]. For visualization of distinct strains, plasmids expressing either the green fluorescent protein (GFP), the red fluorescent protein mCherry (mCh) or no fluorescent protein (nfp) were introduced into S, R and C, respectively. The resulting strains were named: S:GFP, S:mCh, S:nfp, R:GFP, etc. All fluorescent proteins were expressed from the arabinose-inducible promoter pBAD as present in the plasmid pBAD24. Introduction of the fluorescent proteins resulted in the plasmids pBAD24-GFP [42] and pBAD24-mCherry [43]. To prevent plasmid loss, all plasmids, including the plasmid not expressing a fluorescent protein, carried an ampicillin antibiotic resistance.

2.2. Preparation of the system and growth conditions

Bacteria were grown in overnight cultures of liquid M63 medium at 37°C, supplemented with glycerol (0.2%), casein hydrolysate (0.2%) and arabinose (0.2%) for fluorescence induction, and with ampicillin (100 µg ml⁻¹). Analysis of colony development was performed on M63 agar plates (1.5% agar) that were prepared as above for the liquid culture.

Strain mixtures were diluted from the overnight culture to OD 0.1 at different initial ratios as indicated in the next sections. Ratios S : R : C (shorthand for r₅ : r₉ : r₁₀) of 1 : 1 : 1 represent an equal amount of all three strains. Ratios 5 : 1 : 1 indicate that the S strain was initially added five times more than the R and C strains, whereas ratios of 1 : 1 : 0.1 indicate that the C strain was added at one-tenth of the other two strains. Droplets of the resulting mixture (1 µl) were applied to M63 agar plates in triplicate. The time between mixing of strains and inoculation had to be kept short, because droplets of inoculum temporarily form well-mixed environments. Tuning the pH level of our agar plates resulted in slow colony growth at pH 6 (slow growth condition ‘S’) compared with fast colony growth at optimal pH 7 (fast growth condition ‘F’). Each experiment (for slow and fast growth conditions) was performed two times and revealed qualitatively the same result.

2.3. Analysis of colony development

Colony development was recorded using an upright microscope (90i, Nikon, Düsseldorf, Germany). Fluorescence was analysed using filter sets with 472/30 nm excitation for GFP (DM: 495, BA: 520/35 BP), whereas excitation for mCherry was 562/25 nm (DM: 593, BA: 641/45 BP). Images were taken with a DS-Qi1MC digital camera (Nikon). Background correction and image analysis were performed using the free software IMAGEJ.

In order to quantify the growth dynamics of the three strains, we recorded the expansion of single-strain colonies for each combination of strain, fluorescent marker and growth condition in parallel by taking bright field images. For slow growth conditions, these pictures were recorded every 2 h from 4 to 34 h after inoculation. A final picture was recorded 48 h after inoculation. For fast growth conditions, the pictures were recorded...
3. Results and discussion

3.1. Design of distinct ecological scenarios

As detailed in the Material and methods section, we studied competitive microbial range expansion for a prominent model system that comprises three genetically distinct strains of *Escherichia coli* [3]: a toxin-producing strain (C), a sensitive strain (S) and a resistant strain (R). During growth, around 3% [45] of the C cells undergo lysis and release colicin E2 (diffusion constant of the order of $10^{-7}$ cm$^2$ s$^{-1}$ [46]). The colicins subsequently diffuse through the extracellular fluid around bacterial cells until possibly being absorbed by sensitive *E. coli* cells. The sensitive cells are prone to the endonuclease activity of colicin and suffer a degeneration of their DNA, which inhibits further cell divisions [48]. Eventually, the cells lyse. Inhibition zones around toxic C cells may be as large as 100–400 μm in radius [33,47]. Because colicin production is costly, the growth rate of these cells is significantly lower than those of the other two strains. We genetically engineered two of our three strains to express either GFP or the red fluorescent protein mCherry (the strain not expressing a fluorescent protein is marked as nfp). We observed that while a strain expressing GFP could expand at roughly the same speed as its non-fluorescent counterpart, this did not hold for strains expressing mCherry. We discovered that their growth rate was significantly reduced by the expression of mCherry (see the electronic supplementary material, figure S1a). This effect was also observed for growth in liquid culture [43]. The fluorescent proteins thereby allowed us to design different ecological scenarios by changing the order in which the proteins were assigned to our strains (figure 1). Every scenario differed from one another by changes in relative strain growth rates as described in the following. Furthermore, the fluorescent proteins allowed us to visualize each strain independently during its expansion in range (see Material and methods).

The control over the growth rates of our three strains enabled us to design three different ecological scenarios and to study how the composition of expanding colonies depended on the interplay between resource and interference competition. In a first scenario (I), we arranged the bacterial growth rates such that $\mu_S > \mu_R > \mu_C$ (mCherry expressed by the R strain). As detailed in the electronic supplementary material, appendix S1, we determined these growth rates by measuring the maximal radial expansion velocity ($\mu$ h$^{-1}$) of single-strain S, R, and C colonies. These rates were thus independent of the toxin action of C on S, which was quantified independently as described further below. It followed from the above hierarchy $\mu_S > \mu_R > \mu_C$ and from the toxin action of C on S that our first ecological system exhibited a cyclic (non-transitive) dominance: C dominated S by killing
that were present along these fronts after 48 h were con- 
terial colonies over 48 h and identified the strains that 
agar plates (supplemented with minimal M63 medium; see 
(figure 1 and C dominated S by interference competition, but the 
strictly hierarchical: R dominated C by resource competition, 
conditions, the competition network was neither cyclic nor 
equal (mCherry expressed by the C strain). Under these con- 
intermediate scenario (III), the toxic strain had by far the 
action network was 
transitive 
$\mathcal{R}$ dominated 
$\mathcal{C}$ by 
interaction between strains and can only be applied to cases in 
which either the $\mathcal{S}$ or the $\mathcal{C}$ strain has already disappeared 
from a colony’s front. In such situations, the strain that 
eventually dominates may be inferred from the radial expansion 
velocities of single-strain colonies that are listed in the elec- 
tronic supplementary material, table S1.

### 3.2. Cyclic dominance is not sufficient to ensure 
coexistence of all strains

We first sought to determine the surviving strains when a 
droplet of inoculum that contained an equal number of all 
three strains (initial ratios $\mathcal{S} : \mathcal{R} : \mathcal{C} = 1 : 1 : 1$) expanded in 
range. Surprisingly, in the cyclic rock–paper–scissors scen- 
ario I, we found no evidence for coexistence of all three 
strains. In a previous report, such three-strain coexistence 
was observed for spatially extended systems with a regular 
arrangement of neighbouring single-strain colonies [3]. Com- 
petitive exclusion with dominance of the fastest-growing 
strain ($\mathcal{S}$) was not observed either. Instead, we found that $\mathcal{S}$ 
was driven to extinction, whereas strains $\mathcal{R}$ and $\mathcal{C}$ dominated 
the colony front, where they formed monoclonal sectors 
(figure 2a). Notably, in the non-cyclic scenarios II and III, 
coexistence was completely lost. Here, the $\mathcal{R}$ strain outcom- 
peted both $\mathcal{S}$ and $\mathcal{C}$, and was the only survivor with access 
to uncolonized area (figure 2b,c). Hence, ‘survival of the fast- 
est’ [10] could only be observed in hierarchical scenario II, 
whereas who survived in the other two scenarios was more 
subtle and was heavily affected by the long-range toxin 
action. The outcomes of our bacterial competitions were 
shown to be robust against small changes in relative growth 
rates of the strains (induced by reassigning the fluorescent 
protein GFP while keeping the assignment of mCherry; see 
the electronic supplementary material, appendix S1), and 
robust against changes in overall growth conditions (slow 
growth at pH 6, fast growth at pH 7; see Material and methods 
and the electronic supplementary material, figure S1). The 
results of supporting experiments are listed in the electronic 
supplementary material, figures S3 and S4.

### 3.3. Identification of biodiversity zones

To elucidate the above findings and to identify the factors that 
promote or jeopardize survival of the competing strains, we 
developed a stochastic agent-based model to capture the 
system dynamics on a coarse-grained scale (see Material and 
methods and the electronic supplementary material, appendix 
S1). Our theoretical approach rested on a lattice-based
description of range expansions and extended previous models [31] by considering the long-range nature of the toxin interaction. We performed additional experiments on the expansion of single-strain colonies to adjust the model’s parameters. Comparisons between experimental and simulated growth curves enabled us to determine all model parameters except for the toxin interaction. We modelled this interaction based on a source and degradation process, and estimated its range and strength by measuring the distance between the front of a growing C colony and the front of a neighbouring S colony (see the electronic supplementary material, appendix S1). The estimate complies with literature values [33, 47]. Even though our theoretical model simplified the bacterial dynamics (e.g. by considering only a single bacterial layer, whereas the real colony piled up in hundreds of them in its interior), the model captured the essential parameters. We successfully applied the model to reproduce experimentally observed segregation patterns and to predict the strains that survived a range expansion (figure 2). Let us emphasize that the model’s parametrization rested on independent experiments as described above.

After having established and validated a reliable theoretical model that reproduced our experimental observations, we investigated whether it was possible to rescue the S strain. As the survival of the S strain was directly coupled to the C strain’s presence, we analysed how reductions in the initial ratio of the C strain affected the other strains’ survival (in particular of the S strain). Simulation data for the cyclic ecological scenario I predicted that reduction of its initial ratio should lead to the formation of broader R sectors at the expense of C (figure 3). The same effect was seen in experiments with initial ratios of \( S : R : C = 1 : 1 : 0.5 \) (see the electronic supplementary material, figure S5). Further reduction of the initial ratio of C in our simulations revealed a regime of three-strain coexistence centred around \( S : R : C = 1 : 1 : 0.1 \) (figure 3). This permissive zone of biodiversity in parameter space coincided remarkably well with our experimental observations of transient three-strain coexistence at ratios \( 1 : 1 : 0.1 \) (figure 4a). For ecological scenarios with a more hierarchical interaction relationship between strains (scenarios II and III), the R strain was clearly dominant (figure 4b,c). Hence, toxin resistance is apparently a more effective survival strategy than either rapid growth or toxin production if the hierarchical order in the competition network is enhanced.

Whether a bacterial strain manages to survive a range expansion and to populate a colony’s expanding front depends on two aspects: first, on its ability to form initial clusters in the inoculum from which outward sectors may emerge; second, on the stability of the arising sectors to the annihilation of neighbouring sector boundaries [5]. Both of these aspects are subjected to random genetic drift and may prevent the establishment of stable sectors in a simulation (figure 3). However, whether a specific outcome of the
bacterial competition is possible in principle depended solely on the interplay between three factors in our experiments: (i) on the initial strain ratios in the inoculum (demographic noise owing to low absolute cell numbers was of minor importance), (ii) on the relative growth rates of the three strains, and (iii) on the effective range of colicin toxicity. On the other hand, differences in lag-times between strains played only a minor role in deciding whether a particular strain survived along the expanding front of a colony. To gain insights into the mechanisms responsible for the dependence of biodiversity on the three factors (i)–(iii) and into how they are correlated with each other, we extended our simulations to explore broad parameter ranges.

If the initial ratios of R and C were varied with respect to the initial ratio of S in cyclic ecological scenario I, then our simulations showed that biodiversity was most pronounced when the initial ratio $r_C$ of the C strain was reduced to 5–20% of that of the S strain (figure 5a). Higher initial ratios of C suppressed growth of the S strain completely, but the R strain ended up dominating the expanding front. In this case, toxin resistance may be seen as a ‘cheating’ strategy: the R strain could profit from colicin production by the ‘cooperating’ C strain without having to pay the associated metabolic costs. By cheating, the R strain managed to beat S, even though it would have been the loser in a direct pairwise competition. Conversely, at lower initial ratios of the C strain, the S strain could still bear the incurred costs. Both R and S outgrew the C strain and eventually shared the expanding front. Our results indicated that a narrow range of initial ratios delineated a regime of maximal biodiversity. Biodiversity required that increases in the initial ratio of C were compensated for by even larger increases in the initial ratio of R. The correlation was quantified by the saturation law—a ‘biodiversity law’—shown in figure 5b. We attributed the saturation to the finite range of colicin toxicity: dense swathes of R cells were needed to shield sensitive cells from the toxin emitted by the C strain. Behind these barriers, surviving S cells could give rise to sectors, leading to the eventual coexistence of all three strains.

Subsequently, we set the initial ratios of the three strains to the rescue window $S : R : C = 1 : 1 : 0.1$ and investigated how changes in the relative growth rates of the strains (i.e. changes in the interaction hierarchy) affected the degree of coexistence. Our simulations showed that three-strain coexistence was most pronounced when the growth rates were of comparable size and when the growth rates of strains C and R were varied in a correlated fashion: $\mu_R \sim \mu_C$ (figure 5b). As our model predicted two- and three-strain coexistence (as well as its absence) in full accordance with experimental results (R and S in the intermediate scenario III, all three strains in the cyclic scenario I, but only the R strain in the hierarchical scenario II), we expect our theoretical predictions to be highly relevant for future experimental studies. Moreover, our simulations revealed that cyclic dominance is not a necessary prerequisite for biodiversity. For range expansion ecologies, biological diversity can even be maintained if the toxin-producing C strain grows fastest. This result seems paradoxical at first sight, but it demonstrates that both the initial ratios and the growth rates of competing strains are equally important ecological parameters that influence biodiversity.
parameters. During the initial phase of expansion after inoculation, the combined effect of the two parameters determines which strain is more likely to establish sector-like domains. In order to avoid being overgrown by the other two strains, the C strain must compensate for its lower initial ratio by growing at a faster rate. A phase diagram that resembled the one in figure 5b was computed for range expansions of selectively neutral, non-interacting strains at equal initial strain ratios. The biodiversity window of this null model disappeared in the presence of toxin interaction, but was recovered upon reducing the initial C strain ratio. The changes to the null model were crucial for predicting the survival strains in our experiments. It would be highly interesting to study how other kinds of bacterial interactions affect the coexistence diagram of the null model.

Finally, to understand the role of colicin in maintaining biodiversity during range expansions, we analysed the importance of the toxin’s effective range (see the electronic supplementary material, appendix S1). Our in silico studies revealed that maintenance of biodiversity required a strong inverse correlation between the initial ratio of the toxic strain and the length scale of colicin toxicity: \( r_C \approx 1 / \lambda^{2/3} \) (figure 5c). A long-range toxin interaction (length scale of \( \lambda \approx 125 \mu m \)) was, therefore, optimal for species coexistence around the initial strain ratios S : R : C = 1 : 1 : 0.1. However, our simulations suggested that a more circumscribed radius of toxin action (\( \lambda \approx 50 \mu m \)) would be necessary to sustain coexistence at equal strain ratios 1 : 1 : 1. The reduction in colicin range weakened the allelopathic effect of C on the fast-growing S strain to a level at which all strains could coexist along the expanding front, despite equal initial ratios in the inoculum. In conclusion, the coexistence diagram in figure 5c revealed that changes in the range of colicin toxicity have a strong impact on biodiversity. The maintenance of coexistence relied on the fine-tuning of the interference competition via colicin between the strains. In more general terms, the biodiversity law encodes how coexistence depends on the balance between the amount of the producers of an interaction agent and the range of the agent. We expect that the inverse correlation between the two can also be observed in other systems in which an inhibiting interaction is mediated by an agent. Future studies should explore how the law changes for other kinds of interactions.

4. Conclusion
Range expansion experiments provide a new perspective on the significance of competition between species in spatially extended ecological systems. Neither strength of numbers, nor growth rate differences, nor choice of competition strategy alone determines success of their dispersal. The right balance between these factors must be struck. We identified this balance for range expansions of a bacterial model system of three Escherichia coli strains and experimentally validated theoretical predictions on strain coexistence. We used the model to extrapolate in parameter space and described the regimes of maximal coexistence in terms of phenomenological ‘biodiversity laws’. The laws showed how changes in the interaction between bacterial strains can have subtle but lasting effects on the eventual composition of a microbial ecosystem. Our approach may help to understand more complex ecosystems whose dynamics cannot be formulated in terms of simplistic rules.

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