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ORIGINAL ARTICLE

Competitive interactions in *Escherichia coli* populations: the role of bacteriocins

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Explaining the coexistence of competing species is a major challenge in community ecology. In bacterial systems, competition is often driven by the production of bacteriocins, which are narrow-spectrum proteinaceous toxins that serve to kill closely related species, providing the producer better access to limited resources. Bacteriocin producers have been shown to competitively exclude sensitive, nonproducing strains. However, the dynamics between bacteriocin producers, each lethal to its competitor, are largely unknown. In this study, we used *in vitro*, *in vivo* and *in silico* models to study competitive interactions between bacteriocin producers. Two *Escherichia coli* strains were generated, each carrying a DNA-degrading bacteriocin (colicins E2 and E7). Using reporter-gene assays, we showed that each DNase bacteriocin is not only lethal to its opponent but, at lower doses, can also induce the expression of its opponent's toxin. In a well-mixed habitat, the E2 producer outcompeted its adversary; however, in structured environments (on plates or in mice colons), the two producers coexisted in a spatially 'frozen' pattern. Coexistence occurred when the producers were initiated with a clumped spatial distribution. This suggests that a 'clump' of each producer can block invasion of the other producer. Agent-based simulation of bacteriocin-mediated competition further showed that mutual exclusion in a structured environment is a relatively robust result. These models imply that colicin-mediated colicin induction enables producers to successfully compete and defend their niche against invaders. This suggests that localized interactions between producers of DNA-degrading toxins can lead to stable coexistence of heterogeneously distributed strains within the bacterial community and to the maintenance of diversity.
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Introduction

Without question, bacteriocins serve some function in microbial communities. This statement is a result of the detection of bacteriocin production in all surveyed lineages of prokaryotes (Klaenhammer, 1988). The determination of that function is precisely what remains to be elucidated. Bacteriocins may serve as anticompetitors, enabling the invasion of a strain into an established microbial community (Gordon and Riley, 1999; Lenski and Riley, 2002). They may also prevent the invasion of other strains or species into an occupied niche or limit the advance of neighboring cells (Kerr *et al.*, 2002). Additional roles have recently been proposed for Gram-positive bacteriocins, as mediators in quorum sensing (Gobbetti *et al.*, 2007) and as communication signals in bacterial consortia, for example, in biofilms (Gillor, 2007; Hibbing *et al.*, 2010).

Escherichia coli produce its own species-specific bacteriocin, given the name colicin to identify the producing species (Cascales *et al.*, 2007). These high-molecular-weight toxic proteins specifically target close relatives and kill through one of a variety of mechanisms, including pore formation and nuclease activity (targeting either DNA or RNA). Colicins are the most extensively studied bacteriocins produced by Gram-negative bacteria. They serve as a model system for investigating the mechanisms of bacteriocins structure and function, genetic organization, ecology and evolution (Cascales *et al.*, 2007). Early experimental studies investigating the ecological role of colicins were inconclusive and contradictory (Ikari *et al.*, 1969). More recently, a theoretical and empirical base has been established defining the conditions that favor maintenance of toxin-producing bacteria in both population and community settings. Chao and Levin (1981) showed that the conditions for invasion of a colicin-producing

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strain are much broader in a spatially structured environment than in an unstructured one. In an unstructured environment with mass action, a small population of producers cannot invade an established population of sensitive cells (Durrett and Levin, 1997). This failure occurs because producers pay a price for toxin production (energetic costs of plasmid carriage and lethality of production), whereas the benefits, the resources made available by killing sensitive organisms, are distributed at random. In a physically structured environment, such as on the surface of an agar plate, the strains grow as separate colonies and toxin diffuses out from a colony of producers, killing sensitive neighbors. The access to resources that would have otherwise been consumed by sensitive cells, as well as nutrients from the killed individuals, are made available to the producing colony because of its proximity (Chao and Levin, 1981; Kerr *et al.*, 2002; Kerr, 2007); therefore, killers can increase in frequency even when initially rare, resulting in the displacement of the sensitive strain.

Modeling efforts have incorporated additional biological realities introducing a third species, one that is resistant to the toxin but cannot itself produce toxins (Durrett and Levin, 1997; Nakamaru and Iwasa, 2000). Resistance might be conferred through mutations in either the binding or the translocation site that is required for a colicin to enter the target cell (Cascales *et al.*, 2007). It is assumed that there is a cost to resistance, but that this cost is less than the cost of toxin production borne by the killer strain (Kerr *et al.*, 2002); hence, the interactions among the strains have the nontransitive structure of a child's game of rock-paper-scissors (Kerr *et al.*, 2002; Karolyi *et al.*, 2005; Neumann and Schuster, 2007): the producer strain beats the sensitive strain, owing to the toxin's effects on the latter. The sensitive strain beats the resistant strain because only the latter suffers the cost of resistance. And the resistant strain outcompetes the producer because the latter bears the highest cost of toxin production and release, whereas the former pays only the cost of resistance. Such nontransitive competitive dynamics have been shown for the colicin E2 producer with its isogenic-sensitive and -resistant strains, both *in vitro* (flasks and plates; Kerr *et al.*, 2002) and *in vivo* (the mouse colon; Kirkup and Riley, 2004). These three strains can coexist when interacting in a spatially structured habitat, but not in a well-mixed one (Durrett and Levin, 1997; Kerr *et al.*, 2002). Here, we see that the added complexity of a third species (resistant clones) enables the maintenance of diversity in a structured environment. In this paper, we consider the community-level effects of a different type of added complexity: multiple producers.

Surveys of colicin production in natural populations suggest that 10–50% of isolated *E. coli* produce colicins (Riley and Gordon, 1992; Gordon and Riley, 1999; Gordon and O'Brien, 2006; Barnes *et al.*, 2007). In any one community, multiple producing strains coexist (along with resistant and sensitive strains). What are the important factors favoring such coexistence? Here we show that colicin from one producer can induce colicin production in a second producer and *vice versa*. We explore the community-level consequences of such cross-induction for coexistence of multiple producers. We focus on colicins E2 and E7 (Table 1). We have selected these colicins because of their similarity, as both are encoded on easily manipulated, nontransformable, low-molecular-mass plasmids (pColE2 and pColE7) (Pugsley, 1985); and because they kill in a similar way by nonspecific DNA cleavage (James *et al.*, 2002). We outcompeted the strains producing these colicins in unstructured and structured environments and used computer simulations to further explore the dynamics of communities with multiple producers.

Materials and methods

Bacterial strains and plasmids

Table 1 lists the strains and plasmids used in this study. Two colicin-producing strains were created.

Table 1 Bacterial strains and plasmids used in this study

Strain	*Identification*	*Description*	*Reference*
Bacteria			
BZB1011	BZB1011	W3110; *gyrA*; Str^r	(Kirkup and Riley, 2004)
BZB1011 with pDEW201	pDEW201	W3110; *gyrA*; Str^r; promoterless plx, *luxCDABE*(−)Amp^r	This study
BZB1011 with pDEW-E2	PE2lum	W3110; *gyrA*; Str^r; pDEW201P*ce2a*::*luxCDABE*) Amp^r	This study
BZB1011 with pDEW-E7	PE7lum	W3110; *gyrA*; Str^r; pDEW201P*ce7a*::*luxCDABE*) Amp^r	This study
BZB1011 with pColE2	ColE2	W3110; *gyrA*; Str^r; pColE2-P9	This study
BZB1011 with pColE7	ColE7	W3110; *gyrA*; Str^r; pColE7-K317	This study
Plasmids			
pDEW201	pDEW201	Promoterless plx, *luxCDABE*(−)Amp^r	(Van Dyk and Rosson, 1998)
pDEW-E2	pDEW-E2	pDEW201P*ce2a*::*luxCDABE*) Amp^r	This study
pDEW-E7	pDEW-E7	pDEW201P*ce7a*::*luxCDABE*) Amp^r	This study
pColE2-P9	pColE2	Colicin E2 plasmid	(Pugsley, 1985)
pColE7-K317	pColE7	Colicin E7 plasmid	(Pugsley, 1985)

P, promoter region; p, plasmid; Str, streptomycin.

A streptomycin-resistant mutant of *E. coli* strain BZB1011 (Pugsley, 1985; Kirkup and Riley, 2004) was chemically transformed with plasmids encoding colicins E2 and E7, resulting in strains ColE2 and ColE7. The colicin promoter regions of pColE2 and pColE7 were PCR-amplified, restricted with *Eco*RI and *Bam*HI (Fermentas, Vilnius, Lithuania) and cloned into the multiple cloning site of pDEW201 (Van Dyk *et al.*, 2001), resulting in strains PE2lum (with pDEW-E2) and PE7lum (with pDEW-E7). Promoter inserts were confirmed by sequencing.

Growth conditions
Luria–Bertani (LB) broth and agar (Difco, Lawrence, KS, USA) and MacConkey agar (Sigma, St Louis, MO, USA) were prepared according to the manufacturer's instructions. The media were supplemented with either ampicillin or streptomycin sulfate (Sigma), as required. Cultures were grown at 37 °C with shaking at 200 r.p.m. Mouse inocula were prepared from overnight LB cultures produced from a single colony on LB agar plates. The cultures were pelleted, washed and resuspended in phosphate-buffered saline (PBS; Sigma) to a final concentration of 10^9 bacteria per ml.

Reporter assay
Strains PE2lum and PE7lum (Table 1) were grown overnight in LB broth supplemented with 100 μg ml^{-1} ampicillin. The cultures were diluted in LB (1:100 (v v^{-1})) and grown to a density of $\sim 2 \times 10^8$ cells per ml. The cultures were treated with crude lysates of (i) the ancestral control strain (for PE2lum and PE7lum); (ii) ColE7 (for PE2lum); and (iii) ColE2 (for PE7lum)-producing strains. All lysates were prepared according to the procedure described in Suit *et al.* (1983) and stored at −80 °C until use. A twofold dilution series of the appropriate lysates was added to 96-well microtiter plates (Grainer, Frickenhausen, Germany) and equal volumes of appropriate cells were added. The plates were incubated in a temperature-controlled plate reader (Infinite M200 Tecan, Grödig, Austria) and the emitted light was measured at 5-min intervals. All experiments were run in duplicate and were repeated at least three times. Luminescence values are presented as the ratio of luminescence of the induced sample to that of the uninduced control (response ratio) as described previously (Van Dyk *et al.*, 2001).

Flask assay
Flask competition assays were performed as described previously (Kerr *et al.*, 2002) with slight modifications. To initiate the competition, 150 μl of overnight cultures of strains ColE2 and ColE7 (Table 1) were transferred into a 50 ml flask with 15 ml of fresh LB broth placed in a shaking incubator (New Brunswick Scientific, Edison, NJ, USA) at 200 r.p.m. per min at 37 °C. Samples were retrieved from the flask at intervals and the bacterial concentration and phenotype were explored. Each experiment was performed in duplicate and repeated at least twice.

Static plate assay
Plate competition assays were performed as described previously (Kerr *et al.*, 2002) with slight modifications. To initiate the competition, 15 μl each of an overnight culture of strains ColE2 and ColE7 (Table 1) was spotted onto an LB plate in a 24-point lattice. The droplet pattern was generated by randomly assigning the identity of the strain at each lattice point at equal distribution. After 24 h of growth at 37 °C, cells were transferred by replica plating onto three fresh LB plates. The first plate was used for estimating strain densities by scraping the bacterial cells into 5 ml of PBS, vortexing and dilution plating. The second plate was used to transfer the cell matrix onto a fresh plate and the third plate was photographed using a Gel Documentation system (Bio-Rad, Hercules, CA, USA). Each experiment was performed in duplicate (with randomized lattice inoculation) and repeated twice.

Mouse assay
Fourteen female CD-1 mice, all 4 weeks of age, were obtained from Charles River Laboratories (Wilmington, MA, USA). Before bacterial inoculation and throughout the experiment, the mice were given 5 g l^{-1} streptomycin sulfate in their drinking water to eliminate any resident facultative Gram-negative bacteria. After 1 week of preliminary streptomycin treatment, the mice were screened for fecal enteric bacteria by plating fecal pellets on MacConkey agar plates. All mice were free of detectable enteric bacteria. The experimental bacterial strains (streptomycin resistant) were given *per os* to each mouse. The two colicinogenic strains, ColE2 and ColE7 (Table 1), were each established in seven mice. Three experimental and four control cages, each containing two mice, were then set up. Each of the experimental cages contained one mouse established with ColE2 or ColE7 (cages E2/E7). The four control cages included two cages with two mice, each harboring ColE2 (cages E2/E2), and two cages with two mice, each harboring ColE7 (cages E7/E7). The mice within each cage were permitted to interact freely with one another. Each strain's concentration and phenotype were monitored for 112 days. Fecal samples were taken in sterile plastic boxes, transferred onto PBS supplemented with 20% (v v^{-1}) glycerol (Sigma), and weighed. The samples were homogenized and subsamples were diluted in PBS for plating on selective medium – MacConkey agar supplemented with 100 mg l^{-1} streptomycin sulfate – whereas the remainder were

stored at −80 °C. Colony-forming units (CFUs) were monitored per gram of feces.

Phenotypic determination
LB plates supplemented with streptomycin were preinoculated with the ancestral colicin-sensitive strain BZB1011 (Table 1). Twenty colonies of streptomycin-resistant *E. coli* obtained from each mouse were assayed at 4-week intervals for the production of inhibition zones on sensitive lawns. Strains ColE2 and ColE7 (Table 1) were differentiated by subsequence plating on LB plates spread with a 100 µl crude extract of ColE2 or ColE7 (colicin-producing strains are immune to their killing effect of their own colicin).

Simulations
Agent-based simulations were performed to examine the interaction dynamics of bacteriocin producers. The virtual population of cells occupied a regular square lattice of size $L \times L$ with wrap-around boundaries (that is, no edges). The possible state of any lattice point was given by an ordered pair (*cell*, *bacteriocin*), in which the first entry indicates the presence or identity of the cell and the second entry indicates the presence or identity of the bacteriocin. Our simulations investigated the dynamics of two strains (labeled $\mathbf{P_1}$ and $\mathbf{P_2}$), wherein each strain produces its own bacteriocin (labeled $\mathbf{B_1}$ and $\mathbf{B_2}$, respectively). Both bacterial cells and their bacteriocins occupied lattice points, which had to be in one of the following states:

(0, 0) an empty lattice point;
($\mathbf{P_1}$, 0) a lattice point filled with a cell of strain $\mathbf{P_1}$;
($\mathbf{P_2}$, 0) a lattice point filled with a cell of strain $\mathbf{P_2}$;
(0, $\mathbf{B_1}$) a lattice point filled with the bacteriocin produced by strain $\mathbf{P_1}$;
(0, $\mathbf{B_2}$) a lattice point filled with the bacteriocin produced by strain $\mathbf{P_2}$;
($\mathbf{P_1}$, $\mathbf{B_1}$) a lattice point filled with $\mathbf{P_1}$'s bacteriocin and a $\mathbf{P_1}$ cell; and
($\mathbf{P_2}$, $\mathbf{B_2}$) a lattice point filled with $\mathbf{P_2}$'s bacteriocin and a $\mathbf{P_2}$ cell.

The rate at which any lattice point changes its state depends on its current state and on the states of points in its neighborhood. In this study, we focused on local interactions; hence, the neighborhood of a focal point was defined as the nearest eight points (a Moore neighborhood). Table 2A describes the rates at which the transitions occur. A cell of strain $\mathbf{P_i}$ is 'born' into a point not possessing a cell at a rate $\beta_i f_i$, where f_i is the fraction of the cell-free point's neighborhood occupied by $\mathbf{P_i}$, and β_i is the birth rate given a completely filled neighborhood. We note that a cell of a given strain cannot be 'born' into a site containing the other producer's toxin. A cell of strain $\mathbf{P_i}$ dies without producing its bacteriocin at a rate $\delta_i + \tau_{ij} g_j$, where g_j is the fraction of its neighborhood filled with the bacteriocin of the other producer, τ_{ij} measures the toxicity of the other producer's bacteriocin and δ_i is the base death rate. Thus, the death rate of each strain depends on the surrounding concentration of the other strain's bacteriocin (that is, as the concentration of extracellular toxin increases, so too does the probability of death). The same strain $\mathbf{P_i}$ dies and simultaneously produces bacteriocin at a rate $\pi_I + \gamma_{ij} g_j$, where γ_{ij} measures the induction of the focal strain's production by the other producer's bacteriocin, and π_i is the base production rate. Thus, the probability of bacteriocin production by one strain depends on the surrounding concentration of the other strain's bacteriocin. Finally, bacteriocin $\mathbf{B_i}$ decays at a rate μ_i. All of the parameters are defined in Table 2B. In the actual implementation of the simulation, every point carried a 'weight' equal to the sum of rates in its row in Table 2A. A point was chosen randomly on the basis of its weight and transition occurred on the basis of its relative fraction of the total weight. Thus, we use the term 'rate' loosely, as these quantities are actually components of weights in the simulation that determine the probability of different transitions. The C++ code for the model is available at: http://faculty.washington.edu/kerrb/code.shtml.

Results

Reporter assay
The promoter regions of the genes encoding colicins E2 and E7 were fused upstream of the *Photorahbdus*

Table 2A Rates of lattice point transitions

	(0,0)	($\mathbf{P_1}$,0)	($\mathbf{P_2}$,0)	(0,$\mathbf{B_1}$)	(0,$\mathbf{B_2}$)	($\mathbf{P_1}$,$\mathbf{B_1}$)	($\mathbf{P_2}$,$\mathbf{B_2}$)
(0,0)		$\beta_1 f_1$	$\beta_2 f_2$				
($\mathbf{P_1}$,0)	$\delta_1 + \tau_{12} g_2$			$\pi_1 + \gamma_{12} g_2$			
($\mathbf{P_2}$,0)	$\delta_2 + \tau_{21} g_1$				$\pi_2 + \gamma_{21} g_1$		
(0,$\mathbf{B_1}$)	μ_1					$\beta_1 f_1$	
(0,$\mathbf{B_2}$)	μ_2						$\beta_2 f_2$
($\mathbf{P_1}$,$\mathbf{B_1}$)		μ_1		$\delta_1 + \tau_{12} g_2 + \pi_1 + \gamma_{12} g_2$			
($\mathbf{P_2}$,$\mathbf{B_2}$)			μ_2		$\delta_2 + \tau_{21} g_1 + \pi_2 + \gamma_{21} g_1$		

The entries in this table give the rate at which a point in the row state transitions to the column state. The gray boxes correspond to transitions that never occur or the lack of a transition (see Table 2B for descriptions of the parameters).

Table 2B Descriptions of simulation parameters and variables

Parameter or variable	*Description*
β_i	The rate at which a cell of strain *i* is 'born' into an empty lattice point given that the entire neighborhood of the empty point is filled with cells of strain *i*.
δ_i	The rate at which a cell of strain *i* dies given that there is no bacteriocin from the other strain in its neighborhood.
τ_{ij}	The increase in the rate at which a cell of strain *i* dies given that its neighborhood is filled with bacteriocin from strain *j*.
π_i	The rate of lethal bacteriocin production by a cell of strain *i* when there is no bacteriocin from the other strain in its neighborhood.
γ_{ij}	The increase in the rate of lethal bacteriocin production by a cell of strain *i* when its neighborhood is filled with bacteriocin from strain *j*.
μ_i	The rate of decay of the bacteriocin from strain *i*.
f_i	The fraction of a neighborhood occupied by cells of strain *i*.
g_i	The fraction of a neighborhood occupied by bacteriocin from strain *i*.

luminescence luxCDABE reporter operon (Table 1) and transformed into *E. coli* strain BZB1011 (Pugsley, 1985). *E. coli* strain BZB1011 and its derivatives harboring colicin E2- or E7-encoding plasmids were induced with mitomycin C for 5 h and their proteins were extracted. Colicin E2 and E7 lysates were diluted by six and three orders of magnitude, respectively, with PBS.

Figure 1 shows the bioluminescence resulting from colicin induction of colicin E2 and E7 promoters. The patterns of light emission by PE2lum, induced by colicin E7 lysate, and PE7lum induced by colicin E2 lysate were similar. A 30-min lag phase was followed by a rapid increase in light emission for 1 h, reaching a plateau at a response ratio of ~120. The control lysate (BZB1011) did not induce bioluminescence in either reporter strain.

Flask assay

To explore the outcome of competition in a mass action environment, ColE2 and ColE7 strains were grown together in shaken flasks containing liquid media. The results for the flask environment are shown in Figure 2. The concentration of ColE7 dropped below our detection limits following the introduction of the two strains to the fresh medium. In a sample taken half an hour after the initiation of the competition experiment, we could no longer detect the ColE7 strain, whereas the concentration of the ColE2 strain steadily increased.

Static plate assay

The interactions between colicin-producing strains (ColE2 and ColE7) were examined using a 'static' environment in which dispersal and interaction were mostly local. The bacteria were grown on the surface of LB plates for 7 days. Every 24 h, cells were replica plated onto a fresh plate taking care to preserve the population structure that developed on the previous plate. Figure 3a provides images of the resulting strain interactions, which suggest that each producer patch prevents the advance of the other, such that a 'frozen' spatial pattern results. Figure 3b

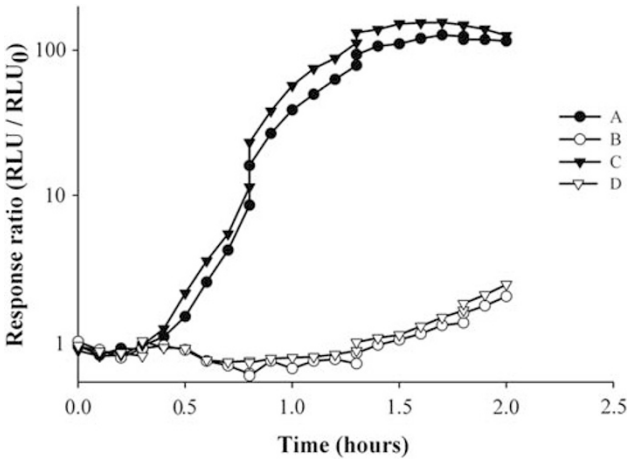

Figure 1 Mutual colicin induction. The proteins of isogenic strains carrying colicin E2 or E7 plasmids and a colicin-free control strain were crudely extracted and used to induce reporter strains carrying *ce2a* and *ce7a* promoters fused to the *Photorhabdus luminescence luxCDABE* reporter operon (Table 1). Colicin E2 crude protein extract was used to induce the pDEW-E7 reporter vector (A; filled circle), whereas colicin E7 extract was used to induce the pDEW-E2 reporter vector (B; filled triangle); the colicin-free strain was tested with the pDEW-E7 (C; open circle) and pDEW-E2 (D; open triangle) reporter vectors.

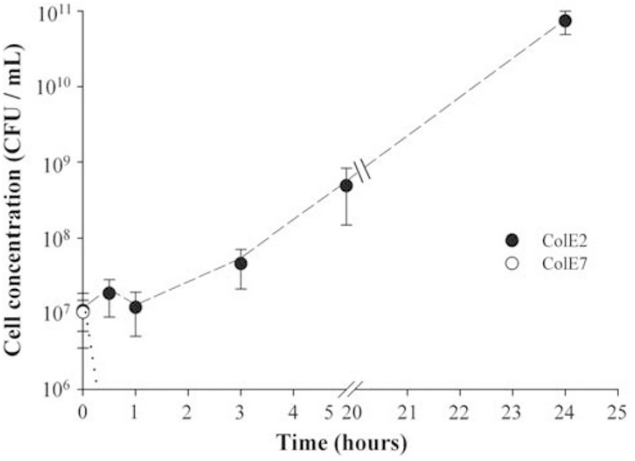

Figure 2 Community dynamics in an unstructured environment. Flask environment was initiated by introducing *E. coli* strain ColE2 (closed circles) and ColE7 (open circles) simultaneously into a flask and monitoring their concentrations over time. The dashed line indicates that the abundance of the ColE7 strain has decreased below its detection limit. Data points are the mean of two independent experiments, each performed in duplicate. Bars represent the standard deviation of the average cell concentration.

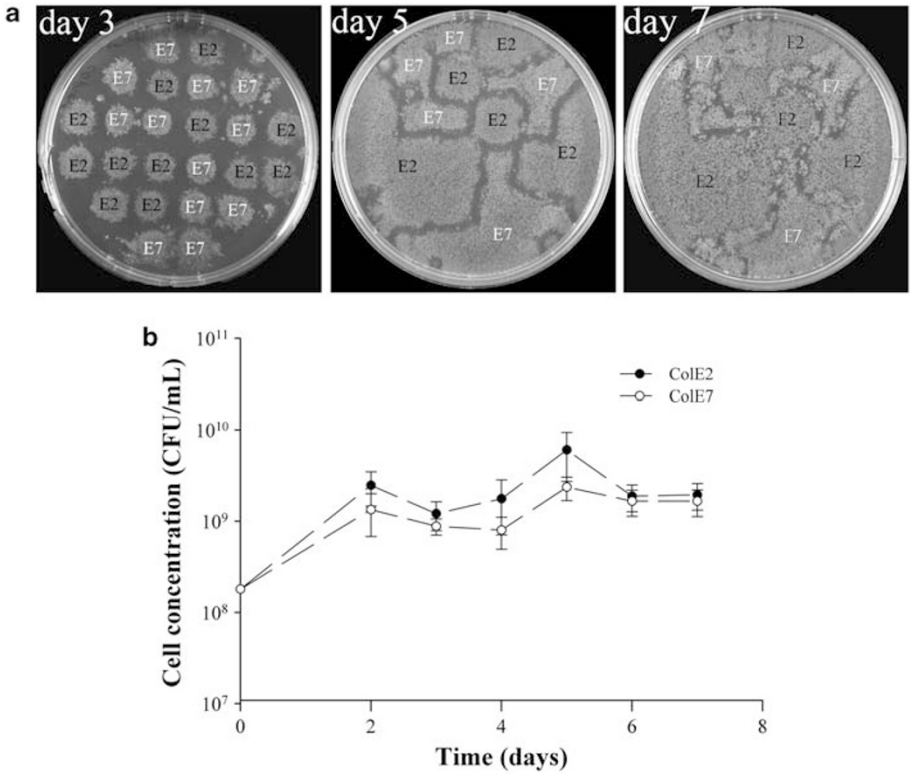

Figure 3 Community dynamics in a structured environment. A static plate environment was initiated by randomly depositing 24 droplets from pure culture of strains ColE2 and ColE7. The changing spatial pattern of the community is photographed over time (**a**) showing the spread of the strains droplets (day 3) to lawns bordered by a clearing zone (day 5) that was later colonized by strains resistant to both colicins (day 7). On analysis of the cells' concentration (**b**), the abundance of *E. coli* harboring colicin E2 (filled circles) and E7 (open circles) encoding plasmids was shown to remain invariable throughout the experiment. Data points are the mean of two independent experiments, each performed in duplicate. Bars represent the standard deviation of the average cell concentration.

further shows that the density of both strains remained constant throughout the experiment, each consisting of about half of the community throughout the experiment. The emergence of resistant strains on the sixth and seventh day of the experiment led to its termination.

Mouse assay

The competitive interactions between colicin-producing strains in the colons of co-caged mice were examined over a period of 112 days. Over time, the strain concentrations in the colon of mice occupying the control cages (E2/E2 and E7/E7) fluctuated, ranging from $\sim 10^7$ to $\sim 10^3$ CFU per g feces (Figure 4a). In contrast, the cell density monitored in the three experimental cages (E2/E7) was less variable, ranging from $\sim 10^5$ to $\sim 10^3$ CFU per g feces (Figure 4b). Furthermore, in the mixed cages, each mouse retained its original strain, suggesting that no strain replacement occurred between mice. This is in sharp contrast to the dynamics observed when a mouse with sensitive bacteria was caged with a mouse with ColE2 producer. In this case, the producer strain invaded the colon of the mouse originally with the sensitive strain (Kirkup and Riley, 2004). We note that the E2-producing bacterial strain and the mouse cage setup from this previous experiment were identical to this study.

Simulations

Figure 5 illustrates the results of simulation runs on a 100×100 lattice in which diffusion of the bacteriocin and growth of bacterial cells are spatially restricted. As a base case, we start with a simulation in which there is no bacteriocin-mediated induction. This is a case in which both strains $\mathbf{P_1}$ and $\mathbf{P_2}$ are toxic to one another (in Tables 2A and B; $\tau_{12} > 0$ and $\tau_{21} > 0$); however, neither strain releases bacteriocin at a higher rate when in the presence of the other strain's bacteriocin (in Tables 2A and B; $\gamma_{12} = \gamma_{21} = 0$). The parameters of each strain are equal to one another, except that strain $\mathbf{P_2}$ has a higher replication rate than strain $\mathbf{P_1}$ (in Tables 2A and B; $\beta_2 > \beta_1$). This gives strain $\mathbf{P_2}$ a distinct advantage over strain $\mathbf{P_1}$. In this case, strain $\mathbf{P_2}$ can invade from low density and displace strain $\mathbf{P_1}$ (five replicates are shown in Figure 5a). If strain $\mathbf{P_1}$ starts at a low frequency, it cannot invade strain $\mathbf{P_2}$ (five replicates are shown in Figure 5b). Thus, strain $\mathbf{P_2}$ replaces strain $\mathbf{P_1}$ in this case.

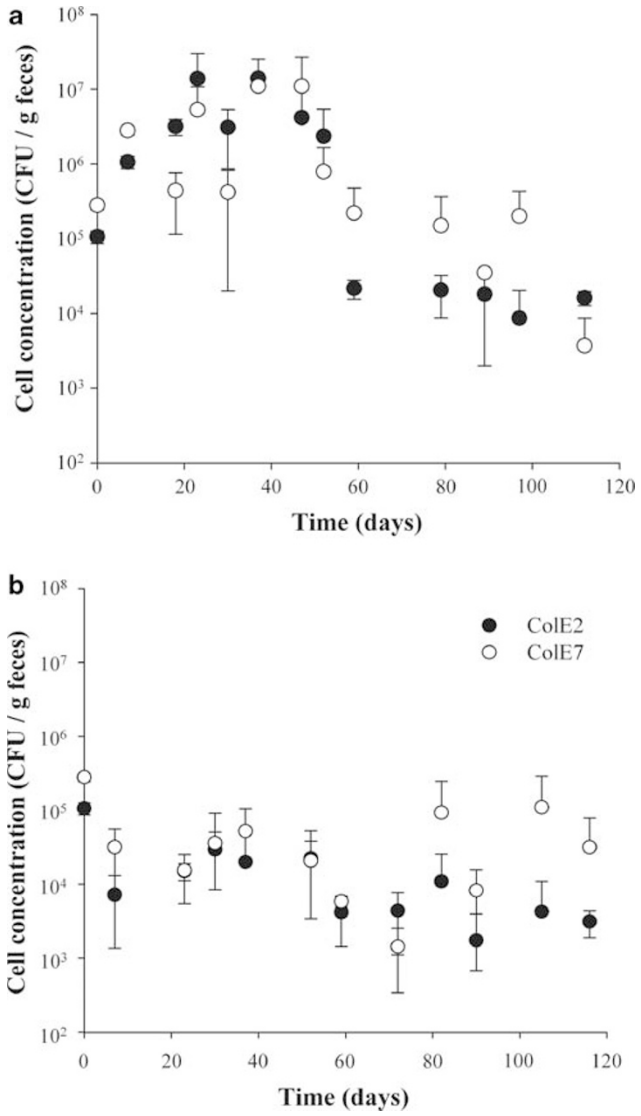

Figure 4 Effect of competition on bacterial population size in mice. Bacterial density (CFU per g fecal matter) monitored over time in mice in control (**a**) or experimental (**b**) cages. The control cages hosted mice harboring either *E. coli* strain BZB1011 bearing pDEW-E2 or mice harboring *E. coli* strain BZB1011 bearing pDEW-E7. The experimental cages contained one mouse established with the *E. coli* strain BZB1011 bearing pDEW-E2 and one mouse with *E. coli* strain BZB1011 bearing pDEW-E7. Each point represents the mean CFU per g feces averaged for strains bearing pDEW-E2 (filled circles) or pDEW-E7 (open circle) recovered from the mice. Bars represent the standard error for each point.

Now, we introduce bacteriocin-mediated induction. Each strain is induced to produce its bacteriocin at a higher rate when in the presence of the other strain's bacteriocin (in Tables 2A and B; $\gamma_{12} > 0$ and $\gamma_{21} > 0$). Across five replicates, we see that strain $\mathbf{P_2}$ (the better grower) can now no longer invade from low density (Figure 5c). This is because its bacteriocin ($\mathbf{B_2}$) triggers $\mathbf{P_1}$'s bacteriocin ($\mathbf{B_1}$), which raises its death rate (and further bacteriocin release, feeding the cycle). However, if we start $\mathbf{P_1}$ at low density, it does not invade (five replicates are shown in Figure 5d). Therefore, $\mathbf{P_1}$ cannot invade $\mathbf{P_2}$ and $\mathbf{P_2}$ cannot invade $\mathbf{P_1}$. Thus, we have a situation in which each strain is protected from invasion by the other. A survey through parameter space (varying growth rate and the rate of bacteriocin-mediated induction) shows that mutual exclusion is a relatively robust result when cross-induction occurs (Figure 6).

Discussion

Despite the pervasive role of toxin production in the microbial world, little is known about the ecology and evolution of this form of competition (Hibbing *et al.*, 2010). Previous theoretical and empirical studies have suggested that toxin production serves as a strategy to obtain access to nutrients (Chao and Levin, 1981; Ivanovska and Hardwick, 2005). However, a recent study testing competitive interactions between toxin producers and sensitive yeast strains under low and high nutrient conditions concluded that toxin producers can only outcompete sensitive cells in high nutrient environments, whereas they were outcompeted when grown under low nutrient conditions (Wloch-Salamon *et al.*, 2008). This observation supports the theoretical prediction that toxin production has evolved to occur as a competitive strategy under conditions of abundant resources (Frank, 1994). Both studies suggest that toxin production may be more important in the invasion of niches than in obtaining nutrients (Brown *et al.*, 2009). Here, we offer an additional explanation to the competitive role of toxin production; we suggest that this allelopathic behavior might also be a result of interactions between the producers of toxin. We hypothesize that an established community of certain bacteriocin producers challenged by an invading colicinogenic population will result in the enhanced production of both toxins. In this scenario, each producer is induced to generate its toxin, leading to chemical warfare that will favor the numerically dominant resident producer and lead to the elimination of the invading producer. Alternatively, different producers occupying the same space may be able to 'hold their ground' through mutual exclusion, resulting in stable coexistence among the bacteriocinogenic populations.

To test our hypothesis, we used two producers, both DNA-degrading colicins found in environmental samples (Gordon *et al.*, 1998). First, we explored whether the presence of one of these bacteriocins would result in the induction of another. To that end, the expression of colicin E2 promoter was subjected to the extracted colicin E7 protein and *vice versa*; as a control both were subjected to the crude extract of the colicin-free isogenic strain. We showed that each DNase colicin has the ability to induce its counterpart's production (Figure 1). Over 50 years ago, it was established that the inducers

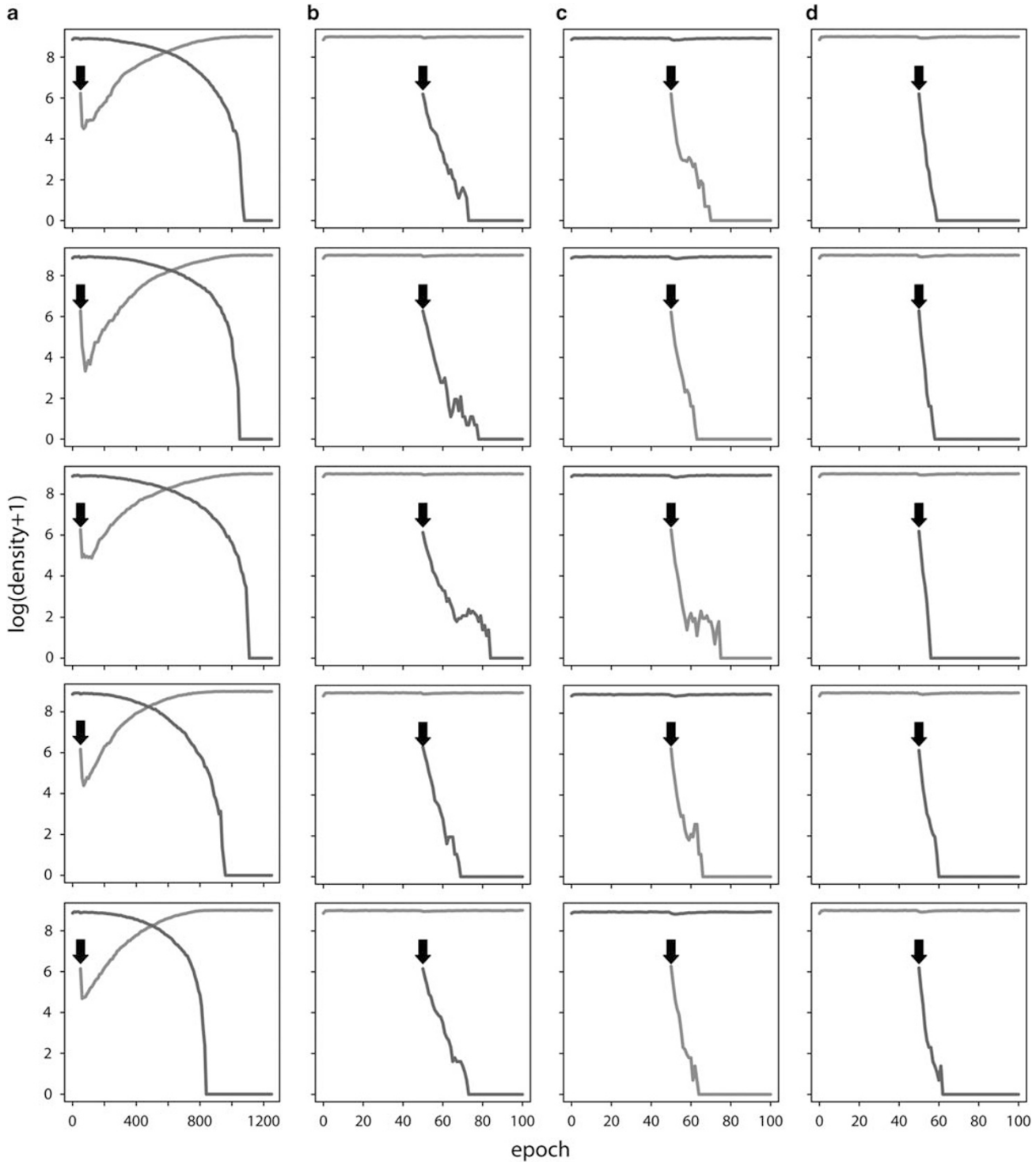

Figure 5 An agent-based simulation of two bacteriocin-producing strains. The interactions between strains $\mathbf{P_1}$ (blue line) and $\mathbf{P_2}$ (red line) with $\beta_1 = 7.0, \beta_2 = 9.0$, $\delta_1 = \delta_2 = 1.0$, $\mu_1 = \mu_2 = 0.5$, $\pi_1 = \pi_2 = 0.5$ and $\tau_{12} = \tau_{21} = 0.5$ (see Table 2B for a description of these parameters) were simulated. (**a**) The 'base case' without bacteriocin-mediated induction (that is, $\gamma_{12} = \gamma_{21} = 0$; see Table 2B) showing that the better grower, $\mathbf{P_2}$, invades from low density and displaces its competitor $\mathbf{P_1}$. Strain $\mathbf{P_2}$ was introduced from low density after 50 epochs (marked by the arrows), such that $\mathbf{P_1}$ can first reach its equilibrium. Five replicates are shown. (**b**) If the better grower $\mathbf{P_2}$ starts at high density, it prevents invasion by $\mathbf{P_1}$ (five replicates are shown, introduction occurs after 50 epochs in each case). (**c**) The case of bacteriocin-mediated induction ($\gamma_{12} = \gamma_{21} = 5.0$) showing that the better grower $\mathbf{P_2}$ is now excluded by a resident population of $\mathbf{P_1}$ (five replicates are shown). (**d**) When commonly present at higher density, $\mathbf{P_2}$ still excludes $\mathbf{P_1}$ (across five replicates). Thus, bacteriocin-mediated induction can produce a case of mutual exclusion where it would otherwise not be expected. The color reproduction of this figure is available on the html full text version of the paper.

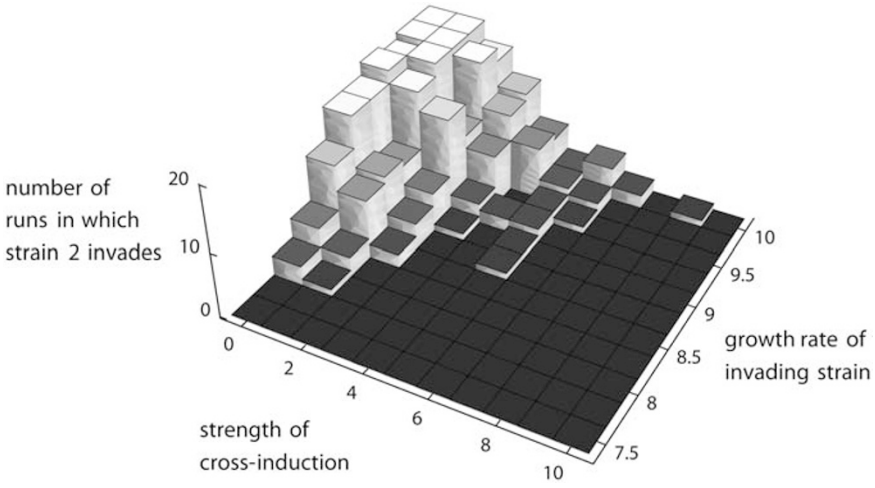

Figure 6 Exploration of parameter space. Here, we use the same parameters as in Figure 5, except that we vary the growth rate of strain 2 (β_2, ranging from 7.5 to 10) and the rate of cross-induction ($\gamma_{12} = \gamma_{21}$, ranging from 0 to 10). For each parameter combination, we run 20 replicates in which strain 2 is introduced at an initial frequency of about 5% after 50 epochs. We record the number of replicates in which strain 2 invades. The 'black floor' corresponds to runs in which strain 2 experiences uniform extinction. When strain 2 is common, it is able to exclude an invading strain 1 across the entire region of the parameter space shown. Thus, the black floor of this plot corresponds to regions of parameter space in which mutual exclusion occurs. We see that more equitable growth rates and higher rates of cross-induction promote mutual exclusion.

used to enhance colicin production were mutagenic agents, such as mitomycin C and UV light (Herschman and Helinski, 1967). Induction by DNA-damaging agents was later linked to SOS motifs, conserved in all the promoter regions of colicins (Gillor *et al.*, 2008). Interestingly, transcriptional response of an *E. coli* strain to damage induced by a DNA-degrading colicin (E9) showed strong induction of the LexA-regulated SOS system (Walker *et al.*, 2004). We thus suggest that the colicin mutual induction presented in this study results from the DNase toxins' induction of the SOS response that in turn enhances colicin production.

We then tested the colicin-mediated competitive interactions using *in vitro*, *in vivo* and *in silico* models: a flask experiment, imposing unstructured interactions between the two strains, was immediately resolved by the outcompeting of ColE7 by the ColE2 strain (Figure 2), although the growth rate of both strains is similar (Gillor *et al.*, 2009). Interestingly, the outcome of a similar competition between equal concentrations of sensitive and producer strains resulted in displacement of the sensitive strain (Chao and Levin, 1981). However, the static plate model, enforcing local interactions between the two populations, resulted in the coexistence of the strains in a spatially 'frozen' pattern (Figure 3a) and in the maintenance of diversity (Figure 3b). A previous study showed that similar competition between producer and sensitive strains resulted in the displacement of the sensitive strain by its competitor (Chao and Levin, 1981).

In the murine model, each of the co-caged mice was carrying a single colicinogenic strain in its colon for almost 4 months. Unlike previous reports in which a strain from one mouse competitively displaced the strain from another (Kirkup and Riley, 2004), in the current setting, no such displacement was observed (Figure 4). We hypothesize that Enterobactereaceae adhere to colon epithelial cells, and the bacteria form a stable biofilm (Everett *et al.*, 2004); thus, the colon provides a structured environment in which cell–cell interactions are localized in a manner similar to the static plate assay. We suggest that the established biofilm of ColE7 cells can successfully prevent the invasion of cells producing colicin E2 and *vice versa*, both competing in a structured environment at low dispersal. We further hypothesize that when interactions between populations are localized, it might be that a small part of the established population is induced by the invaders, just enough to prevent their advance (Figures 3 and 4). This hypothesis is supported by agent-based simulation, which shows that mutual exclusion is a robust result when bacteriocin producers interact locally and cross-induce one another (Figures 5 and 6).

Our experimental and theoretical work on the ecology of colicin-mediated allelopathy highlights the importance of cell–cell interactions and spatial structure in mediating the outcome of competition. We speculate that cross-induction in structured environments may control the invasion of susceptible DNase bacteriocin producers, as the established community is induced to increase the local concentration of the toxin, thus preventing invasion. The outcome of such an interaction pattern on the relative cost and benefit of investment in allelopathy has strong implications on an evolutionary scale. It has been shown that when toxin producers are

scarce, they are unable to generate sufficient toxins to compensate for the cost of production (Chao and Levin, 1981; Gardner *et al*., 2004). In contrast, if some toxin producers are induced by their competitors/invaders, then the produced DNA-degrading toxins confer that the gain from a given investment in killing is directed against an evident adversary. However, whether such interactions are limited to DNA-degrading bacteriocins or whether they apply to pore formers and RNA-degrading bacteriocin producers is an open question. It will also be interesting to gauge the effect of other strains (for example, sensitive and various resistant strains) on community dynamics in the presence of cross-induction. Further study will reveal how the intricate relations among antibiotic-producing bacteria and their neighbors affect the diversity within microbial communities.

Conflict of interest

The authors declare no conflict of interest.

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