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Antibiotic-mediated antagonism leads to a bacterial game of rock–paper–scissors *in vivo*

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Colicins are narrow-spectrum antibiotics produced by and active against *Escherichia coli* and its close relatives. Colicin-producing strains cannot coexist with sensitive or resistant strains in a well-mixed culture, yet all three phenotypes are recovered in natural populations[1]. Recent *in vitro* results conclude that strain diversity can be promoted by colicin production in a spatially structured, non-transitive interaction[2], as in the classic non-transitive model rock–paper–scissors (RPS). In the colicin version of the RPS model, strains that produce colicins (C) kill sensitive (S) strains, which outcompete resistant (R) strains, which outcompete C strains. Pairwise *in vitro* competitions between these three strains are resolved in a predictable order (C beats S, S beats R, and R beats C), but the complete system of three strains presents the opportunity for dynamic equilibrium[2]. Here we provide conclusive evidence of an *in vivo* antagonistic role for colicins and show that colicins (and potentially other bacteriocins) may promote, rather than eliminate, microbial diversity in the environment.

Colicins are high-molecular-mass bacteriocins produced by *E. coli*[3,4]. *In vitro* experiments show that coexistence of a colicin producer and a sensitive strain in a well-mixed culture is not possible[5–7]. In natural populations a further phenotype, colicin-resistant, is always present, and more recent *in vitro* and *in silico* work has incorporated this third phenotype[2,6,8,9]. Theoretical and empirical studies reveal that a combination of colicinogenic, sensitive and resistant provides a stabilizing factor in a mixed, spatially structured assemblage[2,6,8]. This complex of three strains has the same formal structure as the RPS game[2], with C beating S (by killing), S beating R (by growth-rate advantage, because the resistant strain has had to modify a receptor used in nutrient uptake), and R beating C (because colicin production involves bacterial suicide). Thus, these authors conclude that antagonistic factors, such as colicins, may promote biological diversity in natural microbial populations and communities[2].

Colicins have repeatedly been the subject of *in vivo* studies that failed to confirm expectations generated by laboratory and mathematical studies for potent antagonism[4,10]. However, whereas those studies generally regarded resistance as a nuisance phenomenon, the RPS model treats it as central to the establishment of a dynamic equilibrium. To validate this new model of colicin-related population dynamics, a new *in vivo* experimental model was applied. Controlled enteric bacterial populations were established in small populations of mice so that the bacterial population dynamics could be monitored as bacteria migrated from mouse to mouse.

This mouse model has proved to be highly effective in studies of enteric bacteria in the mammalian colon[11]. Streptomycin eliminates from the mouse colon the minority Gram-negative microbial community, which is replaced with the desired streptomycin-resistant Gram-negative test strains. The majority Gram-positive communities remain relatively undisturbed[12,13], and the mouse experiences normal colon development and function[11,14].

To study the impact of colicins on bacterial dynamics in this mouse model, four related *E. coli* strains were generated. The base strain, *E. coli* K12 (BZB1011), is naturally sensitive to most known colicins. A streptomycin-resistant mutant of BZB1011 was selected as the colicin-sensitive (S) strain. Two colicin-producing strains (C_{E1} and C_{E2}) were derived by introducing naturally occurring colicin plasmids into the S strain by electroporation. These two colicins (E1 and E2) were chosen because they represent colicins commonly found in mouse colon isolates, are encoded on easily manipulated, non-transformable, low-molecular-mass plasmids, and act in distinctly different ways. Colicin E2 is a DNase that enters the cell and nonspecifically cleaves DNA[15]. In contrast, colicin E1 produces voltage-gated pores in the cell membrane, depolarizing the cell[15]. To produce the fourth strain, S was exposed to colicin E2, and a resistant strain (R) was isolated. This strain was resistant to both colicin E1 and colicin E2. DNA sequencing of the *btuB* locus of the R strain suggests that the resistance phenotype was generated by an IS1 insertion (J. E. Wertz and C. Winkworth, personal communication).

Seventy-two mice were given streptomycin in their drinking water. The sensitive and resistant *E. coli* were inoculated by mouth into 24 mice each, and C_{E1} and C_{E2} were inoculated into 12 mice each. In all cases the new strains rapidly colonized their hosts, and all achieved final densities of about 10^6 colony-forming units per gram of faeces, similar to the frequency of enteric bacteria

generally present in the mouse colon[16]. These strains were stably maintained for over four weeks.

Experiments with each of the colicin-producing strains were conducted according to a parallel design. For each experiment, 12 cages of three mice each (36 mice per experiment, described above) were established. Each cage served as a model community, and each of the three mice in it initially carried one of three strains (C, S or R). The mice were permitted to interact freely with each other, and the bacterial populations recovered from their faecal pellets were monitored.

In the colicin E1 experiment, changes in the dominant bacterial populations occurred repeatedly in the majority of the mice (Fig. 1). Sampling over 12 weeks at half-weekly intervals showed over 98 putative transitions between the dominant strains. Only rarely was a mixed population recovered from individual faecal pellets; at any given time each mouse was dominated by a single strain. Detection limits for a second strain were 1% of the population. Sampling over 12 weeks (Fig. 1) revealed that strain C_{E1} tends to displace S, R displaces C_{E1}, and S displaces R, as predicted from earlier *in vitro* studies[2] and the RPS model[17,18]. Within certain cages S persisted, whereas other cages fixed on the R phenotype. No cages fixed on the C phenotype, which in all but two cages was eliminated by week 7.

An explicitly cooperative RPS model (for example, two R mice yielding a fourfold advantage to R invading the third mouse in that cage) can be tested by examining pairs of opposing hypotheses (for example, S replaces R versus the hypothesis that R replaces S). The RPS cycle comprises three hypotheses, and their individual inverses thus form the null hypothesis that such a cycle does not exist. A conservatively administered one-tailed Wald test allowed rejection (at the 95% confidence level) of all three null hypotheses: that C_{E1} did not displace S, R did not displace C_{E1}, and S did not displace R. The data thus support an RPS model of strain displacement *in vivo*.

The colicin E2 experiment also revealed cycling between the three phenotypes (S, C and R). Within a week, four of 36 mice had already experienced displacement of their dominant strain (Fig. 2). Overall, fewer strain transitions were observed in the colicin E2 experiment (43 versus 123), and the S strain, once eliminated, did not reappear, as it often did in the colicin E1 experiment. Using the above model, the hypothesis that C_{E2} did not displace S was rejected at 98.7%, and the hypothesis that R did not displace C_{E2} was rejected at 97.7%. However, in the colicin E2 experiment, there was insufficient evidence to accept or reject the hypothesis that R replaced S.

In the colicin E2 experiment, the relatively short resident time of S in any of the cages appears to preclude adequate investigation of the R-to-S transition. To investigate this, after all of the mice in the colicin E2 experiment had been dominated by an R strain, a single new S-equilibrated mouse was swapped into each cage in exchange for one of the three R-equilibrated mice. In the absence of C_{E2}, S invaded and displaced the R strains in 11 of 16 mice (Fig. 3). In this case, a one-tailed Fisher's exact test suggests that S displaces R, with a P value of 0.055 (marginally non-significant). Alternatively, if invasions are non-cooperative, on a per cage basis one might assume fixation at S to occur one-third of the time, yet five out of seven fix on R. This is almost significant, and if invasions are presumed to be cooperative, then S becomes a highly significant invader.

The generation of an RPS game in normal form from the data sets is desirable but difficult. Generating models with 6–12 parameters has been pursued, and heuristic maximum-likelihood estimation does find local minima conforming to the expectations of a generalized RPS model. However, at one extreme overfitting is a problem, and at the other the assumptions become questionable. Estimating the six key parameters of an RPS game from the data requires assumptions about cooperativity and the reappearance of strains that had disappeared from a cage. Cooperativity can be included as three additional parameters in a model. However, the reappearance of strains is more difficult to address explicitly. It would require at least three parameters, and the form of these parameters depends on the presumed mechanism by which the strains reappear. One assumption is that these strains are re-acquired from environmental refugia (faeces, bedding). Another is that they fall below the detection threshold while still in the mouse itself, or thrive in a part of the gastrointestinal tract that does not encourage their detection in faeces. A third hypothesis is that they arise by mutation. This is most likely in the cases of resistance (which reappears nine times), and possible for sensitivity (nine times), but highly unlikely for colicin production (which reappears 14 times and whose detection was the least subject to error). These data and the apparent stability of the individual strains in the mice suggest that mutation is not the regenerative method. Furthermore, the low survivorship of strains in dry faeces (data not shown) suggests that undetected minority populations in the mice may stage unexpected reprisals.

These results provide the first conclusive demonstration that colicins are effective antagonistic agents within *E. coli* populations in an animal host. Previous *in vivo* experiments failed to detect any strain transition effect that could be attributed solely to the action of colicin-producing strains[4,19]. The present set of experiments suggests that colicins E1 and E2 have a significant role in mediating strain dynamics in the mouse colon. The data also suggest the existence of an RPS relationship between C, S and R strains of *E. coli*—as predicted *in vitro* and *in silico*[2,6,20]. In this study, the pairwise relationships predicted from the RPS model were clearly observed in the mouse colons; that is, on average, C strains displaced S, R displaced C, and S displaced R. However, overall strain diversity

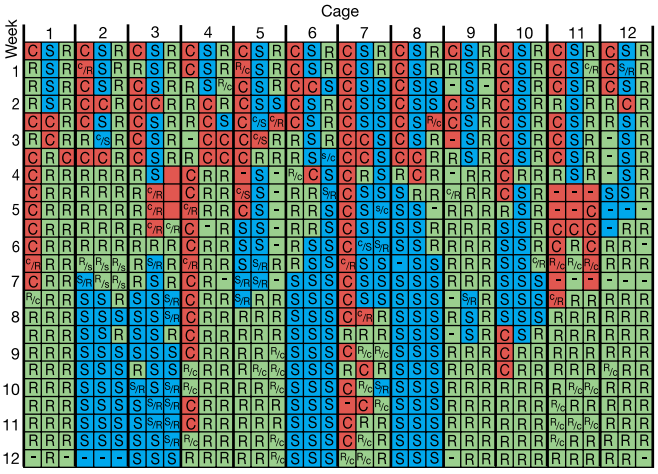

Figure 1 Occupation of co-caged mice by dominant strain: colicin E1. The boxes are coloured to show the dominant strain occupying each mouse. Red represents colicinogenic (C_{E1}); green, resistant; blue, sensitive.

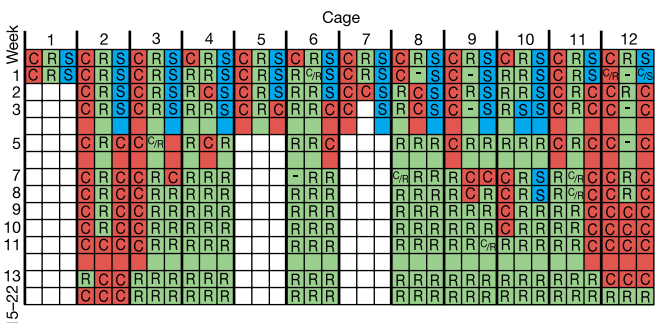

Figure 2 Occupation of co-caged mice by dominant strain: colicin E2. The boxes are coloured to show the dominant strain occupying each mouse. Red represents colicinogenic (C_{E2}); green, resistant; blue, sensitive. Cages 1, 5 and 7 were discontinued because of mice fighting.

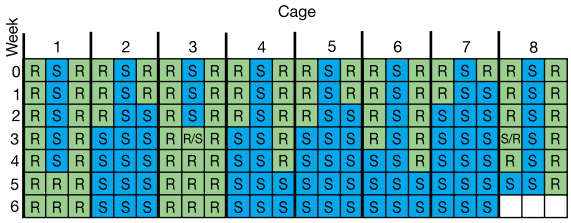

Figure 3 Occupation of co-caged mice by dominant strain after S is reintroduced. The boxes are coloured to show the dominant strain occupying each mouse. Green represents resistant; blue, sensitive. Cage 8 was terminated during week 6 because of a mouse death (natural causes).

was rapidly lost from individual mice, more slowly lost from a cage of mice, and even more slowly lost from all cages.

According to the *in silico* model of colicin-mediated dynamics explored by Kerr *et al.*[2], strain diversity maintained in the colicin-based RPS system relies not only on the existence of an equilibrium point in the game between the three strains, but also on the existence of numerous local populations. This reduces the probability that a local surge in one of the strains will eliminate any competitor entirely. Kerr *et al.*[2] were attempting to explain the difference in results obtained from batch culture experiments, in which strain diversity was rapidly lost (S within ~10 generations/one day, C within ~50 generations/five days), and results obtained from agar plate experiments, in which strain diversity was maintained (>70 generations/seven days). They proposed that the local interactions allowed by the structure introduced on the plates stabilized the strain diversity, and they demonstrated that a partial disruption of the structure also destabilized the diversity.

Kerr *et al.* used a model system much more favourable to diversity than the mouse system. The *E. coli* populations in the mouse colon are relatively small (~10^7 cells per mouse), and the timescales studied in this mouse experiment were an order of magnitude longer (>10 weeks). Kerr *et al.* modelled at least 32 local populations on each plate, and hundreds in each *in silico* world. In contrast, the mice were not populated for extended periods with mixed populations. Thus, each mouse was an 'effective neighbourhood', and each cage contained only three neighbourhoods. As a system becomes discrete, an RPS system maintains diversity under more narrow parameters and for a shorter time[18]. Thus, both the length of the experiment and the small size of the populations predisposed the *in vivo* experimental system to resolution rather than diversity. However, in natural mouse populations, such local extinction is not predicted or observed[1]. Mice in the wild are considered to have high population structure, in territorial demes of 5–100 individuals[21,22], with relatively little interdemic migration.

The mouse model used here has allowed a significant advance in our understanding of the natural role of microbial toxins in mediating strain and, potentially, species dynamics in natural populations and communities of microbes. We present here the first experimental evidence for an *in vivo* role for colicins, which suggests that this abundant and diverse family of antibiotics serves to promote microbial diversity in one of the dominant niches exploited by enteric bacteria, the mammalian colon. The model does suffer from one significant limitation: the number of mice that can be experimentally monitored precludes adequate modelling of the normal population structure of the mice. This limitation may have precluded the long-term maintenance of strain diversity that has been shown clearly *in vitro*, *in silico* and in natural populations of mice. With the advent of fluorescently labelled strains, it may now be possible to move the experimental venue from the laboratory and animal facilities to a more natural setting by following strain dynamics in wild-caught mouse populations. □

Methods

Strain construction

Strain BZB1011 (ref. 23) was selected for resistance to 100 μg ml^{-1} streptomycin sulphate (Sigma) to become strain S. Strain C_{E1} was derived from S by electroporation of colicin E1 plasmid, pColE1-K53 (ref. 23), according to standard methods[24]. Strain C_{E2} was derived from S by electroporation of colicin E2 plasmid, pColE2-p9 (ref. 23), according to standard methods[24]. Colicin E1 and Colicin E2 lysates were prepared following standard procedures[25]. Strain R was derived from S by selection for resistance to colicin E2 lysate (800 μl per plate during the initial selection).

Mouse model

Adult CD-1 male mice were obtained from Charles River Laboratories. They were given 5 g per litre streptomycin sulphate for two days before the first inoculation[10]. The mice were screened for faecal enteric bacteria by plating faecal pellets on MacConkey agar (Sigma) with streptomycin sulphate. Bacterial inoculations of 10^9 cells per ml in phosphate-buffered saline (PBS; Sigma) were given by mouth. Faecal samples were taken in sterile plastic boxes, transferred into PBS, massed, homogenized, and then diluted for plating onto MacConkey plates with 100 μg ml^{-1} streptomycin. Strain phenotypes were determined by velvet transfer of colonies onto LB plates containing 100 μg ml^{-1} streptomycin and 100 μl colicin lysate, which allows only R and C_{E1} or C_{E2} cells to grow, or onto LB plates containing a strain S cell lawn, which reveals C strains by their killing phenotype.

Wald test

The one-tailed Wald test[26] was applied to transition matrices tabulated from the raw data. Some information was lost because the matrices were tabulated as a 10 × 10 matrix, with individual mouse identity homogenized between sample points. The Wald test was conducted with a presumed exponential cooperativity among mice sharing a single cage.

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