Evolution of Diversity in Spatially Structured Escherichia coli Populations

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The stochastic Ricker population model was used to investigate the generation and maintenance of genetic diversity in a bacterial population grown in a spatially structured environment. In particular, we showed that Escherichia coli undergoes dramatic genetic diversification when grown as a biofilm. Using a novel biofilm entrapment method, we retrieved 64 clones from each of six different depths of a mature biofilm, and after subculturing for ~30 generations, we measured their growth kinetics in three different media. We fit a stochastic Ricker population growth model to the recorded growth curves. The growth kinetics of clonal lineages descendant from cells sampled at different biofilm depths varied as a function of both the depth in the biofilm and the growth medium used. We concluded that differences in the growth dynamics of clones were heritable and arose during adaptive evolution under local conditions in a spatially heterogeneous environment. We postulate that under nutrient-limited conditions, selective sweeps would be protracted and would be insufficient to purge less-fit variants, a phenomenon that would allow the coexistence of genetically distinct clones. These findings contribute to the current understanding of biofilm ecology and complement current hypotheses for the maintenance and generation of microbial diversity in spatially structured environments.

The mechanisms that lead to the genesis and maintenance of diversity in communities have intrigued geneticists and ecologists alike for decades (6, 17, 27, 33, 39, 49). This is particularly challenging for microbial communities, in which ecological and evolutionary processes occur on roughly the same time scale (3, 16, 38) and where the outcome of these processes may be affected by the spatial structure in which these communities grow.

Bacterial biofilms are examples of spatially structured communities that have been the subject of intense research in medical and engineering contexts in recent years (3, 8, 20, 48, 56). Previous work has shown that the phenotypic characteristics of bacterial populations in biofilms are distinct from those of their free-swimming counterparts (8). These bacterial assemblages form physically and chemically heterogeneous structures (20) whose complex architecture strongly influences mass transfer (56). This results in the formation of steep gradients of nutrients, waste products, pH, redox potential, and electron acceptors, which results in the creation of distinct and perhaps unique niches on a microscale. This places selective pressure on variants that have enhanced fitness and are well adapted to local conditions. From a theoretical perspective, this would be expected to increase genetic diversity within a population by precluding competitive exclusion, yet this has not previously been demonstrated empirically.

The degree of diversification that occurs within populations growing in biofilms is not well understood, nor are the spatial and temporal dynamics of bacterial species succession in biofilms. However, it is known that the physical and chemical heterogeneity of microbial biofilms has profound effects on microbial growth and activity. Most bacterial cells in biofilms are not highly active and grow slowly if at all. For example, active protein synthesis occurs only in the uppermost zone (32 ± 3 μm) of Pseudomonas aeruginosa biofilms (4). Likewise, in Klebsiella pneumoniae biofilms, fast growth occurs near the interface of the biofilm and bulk fluid, and cells inside the biofilm show little growth (55). The near absence of growth in interior regions of biofilms may lead to an increased tempo of diversification, since numerous studies have shown that mutation frequencies are elevated in slowly growing cells (28). If this occurs within a biofilm, then clones might exhibit a high genotypic variability that could have significant practical implications in terms of yielding spontaneous mutants that are resistant to antimicrobial agents.

Experimental evolution has contributed greatly to our understanding of the causes and consequences of genetic diversity in populations (reviewed in references 23, 29, and 42). Initially, research focused on characterizing diversity within populations that evolved in spatially homogenous environments (e.g., chemostat and batch systems) (11, 13, 15, 19, 30–32, 45, 47, 50–53). Several studies have highlighted a role for spatial heterogeneity in the emergence and maintenance of genetic diversity (25, 26, 43). Korona and colleagues (25, 26) compared populations that evolved in batch cultures to populations that evolved with a spatial structure and demonstrated that phenotypic diversity was greatest with spatial structure. In other work, Rainey and Travisano (43) showed that populations of Pseudomonas grown in static broth microcosms diversified so that some ecotypes occupied a floating biofilm on the surface of the broth while others occupied the liquid phase or glass surface of the culture. Boles et al. (2, 3) investigated the extent of diversification of Pseudomonas using biofilms that evolved in flow-cell systems. They reported that genetic changes produced by a recA-dependent mechanism affected
multiple traits, with some biofilm-derived variants being better able to disseminate while others were better able to form biofilms (3). Further study showed that in some cells, endogenous oxidative stress caused double-stranded DNA breaks that when repaired by recombinatorial DNA repair genes gave rise to mutations (2). These previous studies demonstrate the pivotal role of spatial structure in the generation and maintenance of diversity in evolving bacterial populations.

In this study, we extended this work by using novel techniques to characterize diversity in Escherichia coli biofilms that allowed us to recover clones from specific depths within a biofilm. The growth kinetics of clones from six different biofilm depths were measured and modeled using an analysis-of-variance formulation of the stochastic Ricker model of population dynamics with environmental noise (11, 40). Rigorous statistical methods were used to show that after 1 month of cultivation, the extant diversity in E. coli biofilms was extraordinarily high and varied with depth.

**Materials and Methods**

Biofilm cultivation. *E. coli* LF1010 was used in these studies. This strain is a derivative of *E. coli* ATCC 47076 (MG1655), in which a gfp-kan cassette has been introduced downstream of the recA gene by using the method of Datsenko and Wanner (7a). The biofilm cultivation system consisted of seven parts: (i) medium reservoir, (ii) multichannel pump (Watson Marlow 205S, United Kingdom), (iii) bubbler trap (BioSurface Technologies Co., Bozeman, MT), (iv) flow cell, (v) outflow trap, (vi) air pump (DrsFosterSmith, Rhinelander, WI), and (vii) flow meter (Gilmont, BC Group, St. Louis, MO). The flow cell was made of acrylic plastic. It was constructed from two rectangular plates that were 104 by 48 mm in size. Sidewalls (62 by 26 by 5 mm) were glued to the top plate to form an elongated hexagonal growth chamber. There were 56- by 20-mm square openings in the top and bottom of the rectangular plates that were sealed with 60- by 24-mm glass slides (Fisher, Pittsburgh, PA). The upper and lower plates were assembled using screws and sealed using a microseal film (MJ Research, Waltham, MA). The volume of the flow cell was about 10.4 ml, the medium flow rate was 5.2 ml/h, and the hydraulic retention time was 2 h. Under these conditions, the linear surface velocity was about 40 mm/h at the center of the flow cell.

The growth medium (BMG1 medium) consisted of 11.3 g/liter of Na₂HPO₄, 5.0 g/liter of KH₂PO₄, 0.8 g/liter of NaCl, 1.7 g/liter of NH₄Cl, 0.05% of glucose, 4 mg/liter of CaCl₂, 80 mg/liter of MgSO₄, and 20 mg/liter of kanamycin. At the concentrations used, CaCl₂ and MgSO₄ did not precipitate during the experiment. To prepare the inoculum, *E. coli* LF1010 was grown at 37°C in BMG1 to mid-exponential phase. Cells were harvested by centrifugation and resuspended in a 1/10 dilution of the growth medium. The cells were then entrapped in calcium alginate. Alginate was dissolved in distilled water to yield a final concentration of 2.5 to 3.0% (wt/vol) that had a viscosity of 200 to 400 cP (Aldrich, Milwaukee, WI). Using a multichannel pump (205U; Watson-Marlow, Falmouth, United Kingdom), the alginate solution was introduced into the biofilm growth chamber at a rate so that surface velocity was 100 mm/h. The total amount of alginate solution supplied to the flow cell was about 50 ml, which is about five times the flow cell volume. The flow cell was inoculated for 2 h at room temperature, and then 30 ml of a CaCl₂ solution (60 mM) was pumped into the flow cell at a surface velocity of 500 mm/h. To entrap the biofilm, the flow cell was incubated for 2 h at room temperature and then the growth chamber was disassembled. A 5-mm by 5-mm section of the entrapped biofilm was cut using an autoclaved razor blade and immersed upside-down in a molten 3%-agar solution. After the agar hardened, 100-μm sections were prepared using a microtome (Vibratome 3000; Ted Pella, Redding, CA). The locations of the six layers relative to the bottom of the biofilm were as follows: B1, 0 to 100 μm; B2, 100 to 200 μm; M1, 1,050 to 1,200 μm; M2, 1,200 to 1,350 μm; T1, 2,100 to 2,250 μm; and T2, 2,250 to 2,400 μm. The biofilm sections were transferred to 1 ml of 2.5 mM phosphate-buffered saline, pH 7.0. Cells in the biofilm sections were dispersed by incubation for 1 h in a shaking incubator (Innova 44R model; New Brunswick Scientific, Edison, NJ). The cell suspensions were divided into 1 ml of 20% glycerol solution and stored at −72°C.

**Growth kinetics of clones from biofilms.** The growth kinetics of clones from six different layers in the biofilm were determined as follows. Cell suspensions from each layer were first spread on solidified BMG1 media and incubated for 72 h at 37°C. Afterwards, 64 clones from each layer of the biofilm were randomly chosen and suspended in 1 ml of 0.85% NaCl solution. These cell suspensions were used to inoculate three different kinds of media: BMG1, BMG1 supplemented with 5 mg/ml streptomycin (BMG1 + Str), and BMG1 supplemented with 5 mg/ml ampicillin (BMG1 + Amp). The inclusion of sublethal concentrations of either streptomycin or ampicillin in the growth medium accentuated differences among clones from a given layer. The 384-well microtiter plates (BioTek Instruments, Woburn, MA) contained 76 μl of media per well, and each was inoculated with 4 μl of a cell suspension prepared from a single clone. The plates were incubated at 37°C, and the optical density at 600 nm was measured every 15 min for a total of 25 h using a Bio-Kinetics microplate reader (BioTek Instruments, Woburn, MA). The plates were vigorously shaken prior to each reading. The data from the plate reader were imported into a spreadsheet, and for every culture (well), measurements at each time point of the optical density at 600 nm were adjusted by subtracting the initial optical density.

As a control, the growth kinetics of the parental strain in BMG1, BMG1 + Str, and BMG1 + Amp were determined in 64 replicate cultures for each growth medium. The inoculum for these cultures was prepared in the same way as that for the biofilm clones except that 64 clones were randomly selected from a mid-exponential batch culture.

**Statistical analysis of clonal growth dynamics**. The purpose of this analysis was to determine whether the growth dynamics of clones varied within and between depths in the biofilm. Data from 1,152 time series (6 depths × 6 media × 64 clones) were analyzed and then compared to the 64 × 3 time series of clones from the parental population using the stochastic Ricker model of population dynamics (11, 40).

The stochastic Ricker model, which accounts for departures from the deterministic predictions that occur in a given time series of population growth (11), was used to fit the time series data from the growth of each clone. The model used incorporated environmental stochasticity (11, 40), which accounts for variability in the population growth rate that arises from external factors (such as pH and temperature) that equally affect all the individuals in the population. It expresses the predicted population size as a function of the current population size and accounts for density dependence. The model equation is

\[
N_{t+1} = N_t e^{bN_t (1 - bN_t)}
\]

where \(N_t\) is the population size at time \(t\), \(a\) is the growth rate, and \(b\) is the density-dependent effect of population size on the growth rate function. \(\sigma^2\) is a normal noise with mean zero and variance \(\sigma^2\) and represents environmental stochasticity. Setting \(a\) equal to zero and \(b\) equal to zero defines a discrete-time Brownian motion process (random walk) with no stochastic drift and without a population density feedback typical of ecological processes. When \(a\) and \(b\) are not equal to zero, the model includes density dependence (9). In particular, in the model in which \(a\) is either positive or negative and \(b\) is negative represents a stochastic logistic growth. Under this model, the population no longer attains a single deterministic equilibrium located at \(K = \frac{a}{b}\) as in the deterministic Ricker model but instead approaches a “cloud of points.” Thus, the traditional carrying capacity \(K\) is no longer a point prediction but constitutes a probability distribution (11).

To connect time series data to our population growth model, a likelihood function was specified that quantified how likely it would be to have recorded the observed growth data. The likelihood function was

\[
L(a,b,\sigma^2) = \prod_{i=1}^{n} p(x_i | a, b, \sigma^2) = \prod_{i=1}^{n} \left[ \frac{1}{2\pi\sigma^2} \right]^{1/2} \int_{-\infty}^{\infty} \left| \frac{1}{\sigma^2} \right| e^{-\left( x_i - \frac{1}{2\sigma^2} \right)^2} dx_i
\]

The MLEs for a single time series were obtained through a routine maximization of the likelihood function in equation (2) (11). For a group of \(m\) independent time series groups under the same condition, the overall likelihood function is the product of the individual likelihoods (equation 2), and there are only three
model parameters to estimate. If $X$ is a $(q + 1 \times m)$ matrix with $m$ log-transformed time series on its columns, then the overall likelihood function needed to find the model parameters (40) is

$$L(a, b, \sigma^2 | X_{q+1:m}) = \prod_{i=1}^{m} L(a, b, \sigma^2 | x_{i})$$

where $X_{i}$ is the value from row $t$ and column $i$ of matrix $X$. The MLEs were found by minimization of the negative log-likelihood function using the Nelder-Mead simplex algorithm (37) in MATLAB (The MathWorks, Natick, MA). As with a two-way analysis of variance, the sources of variation were divided into two factors: biofilm depth and growth media (BMG1, BMG1 + Str, and BMG1 + Amp) (40). The 64 time series obtained for each of these 6 experimental conditions were used as replicates to assess the variability between clones. Finally, the patterns of variation in the time series data from biofilm clones were also compared to those obtained from clones of the parental population.

**RESULTS**

Variation among clones from different depths. The purpose of this study was to determine the extent of divergent evolution that occurs in a population of *E. coli* when grown in a biofilm. The approach was to retrieve individual clones from six discrete regions of a biofilm and determine the extent of variation in their growth kinetics when cultured in different media. Stark differences in the growth kinetics of clones were readily apparent, and the variability in kinetic parameters was magnified in the presence of subinhibitory concentrations of antibiotics (Fig. 1 and 2). These phenotypic differences appeared to beheritable, because the clones from each layer of the biofilm were grown from single cells on an agar medium for 72 h (~30

**FIG. 1.** Clonal and spatial growth patterns of clones from three of the six depths sampled from biofilms grown in different media. Sixty-four randomly chosen clones from the top layer (2,250 to 2,400 μm), middle layer (1,050 to 1,200 μm), and bottom layer (0 to 100 μm) of the biofilm were selected. Each clone was grown in BMG1 (panels A, B, and C), BMG1 + Str (panels D, E, and F), and BMG1 + Amp (panels G, H, and I). The top panels (A, D, and G), middle panels (B, E, and H), and bottom panels (C, F, and I) correspond to the top, middle, and bottom layers of the biofilm, respectively. OD, optical density.

**FIG. 2.** Mean optical density (OD) values at four different time points (300, 600, 900, and 1,200 min) for cultures grown in three different media: BMG1 (A), BMG1 + Str (B), or BMG1 + Amp (C). Each bar is the average final optical density of 64 cultures. The error bars represent the standard deviations of the means. The colors distinguish between clones sampled in the following biofilm layers: bottom (black), middle (light gray), and top (dark gray).
generations) prior to measurement of their growth in liquid medium. While the data shown are from clones derived from a single biofilm, the degree of variation among clones was reproducible in independent experiments (data not shown).

Statistical analysis of the data was done to assess whether these differences in the growth kinetics of clones could be explained by two factors either individually or in combination: biofilm depth and culturing media. Standard statistical methods like analysis of variance were not appropriate because they assume that the data are independent and normally distributed, and the variables are related through a simple linear regression model. Here we are dealing with highly nonlinear and dependent patterns of population growth reflected in time series data of population sizes with a complex error structure. The variability in the growth of each population was explained by fitting the data from each clone to the stochastic Ricker model (11). All the information needed for the statistical inferences made lies in the sequential observations of population growth, and the statistical power of such inferences directly depends on the length of the time series. That is, the number of observations is dependent on the length of each time series, which in this study is unusually large. Throughout this article, we have assessed the variability at every level using a technique called the parametric bootstrap, which is the most reliable method for assessing the variability of model parameter estimates when standard statistical error formats are not appropriate (14). The parameter estimates of the stochastic Ricker model \((a, b, \text{ and } \sigma^2)\) for the growth of each clone were calculated. These estimates summarize all the information contained in the time series of clone growth data while providing a means to formally identify the main sources of variation. We used the Bayesian information criterion (BIC) (46), which is a well-established method for scoring probability models according to how well the model fits the data while favoring models that can explain the data with the fewest parameters (10, 41, 46). BIC is an extension of the likelihood ratio test commonly used to distinguish between competing hypotheses and is particularly useful when multiple comparisons are being made. The best model is deemed to be the one with the lowest BIC value (46).

To distinguish between various observed growth dynamics, three hypotheses were formulated. The first hypothesis was a “null model” that assumed variability across the entire set of 1,152 growth curves (64 clones from six depths grown in three media) was not more than would be expected under 1,152 occurrences of a single growth pattern described by a single set of growth parameters. This hypothesis asserts that the phenotypic characteristics of the individual clones reflected in their growth dynamics did not vary significantly between the different biofilm depths or among all the clones sampled. To test this hypothesis, a single set of the stochastic Ricker model parameters was estimated using equations 1 and 3 with \(m\) equal to 1,152 and \(q\) equal to 99. The second hypothesis stated that differences in the growth of clones from a given depth in the biofilm were not significantly different from one another, but there were meaningful differences between groups of clones from different depths in the biofilm. This hypothesis, which was the basis for a “depth alone model,” was tested by estimating six sets of the stochastic Ricker model parameters, with \(m\) equal to 192 (64 clones grown in three different media). A “depth and media model” was based on a third hypothesis in which each clone from each depth had a distinct growth dynamic and each one grew differently in the three media used. Thus, 18 different sets of model parameters corresponding to all the combinations of depths and media were estimated. The parametric bootstrap method with 2,000 replicates was used to compute confidence intervals for these parameters. The confidence intervals obtained from the estimated sampling distribution of each model parameter were used to assess differences between the values of the estimated model parameters under any two different conditions (44). These analyses led to several conclusions. First, the BIC value of the null model was \(-585,347.66\), which was higher than the BIC values for the depth-alone model \((-585,974.23)\) or the depth and media model \((-607,437.64)\). Thus, there was strong evidence to reject the hypothesis that all the growth dynamics reflected a single stochastic growth process. Raftery (41) has shown that a difference in the BIC of about two points is equivalent to a significant difference at the 5% level. In our case, the best model differed from the next best by 21,463.41 points. This would be equivalent to an effectively null \(P\) value. Because the BIC for the depth and media model was smaller than that for the depth-alone model, the former is more consistent with the data. While hypothesis testing and our estimation procedures rigorously establish the differences in parameters, the box plots in Fig. 3 graphically represent these differences. The box plots can be interpreted as follows: if two or more box plots overlap, then there is enough uncertainty surrounding the corresponding estimates to suggest that the parameter values are the same and any observed differences can be explained by the stochastic variance. If the box plots do not overlap, then the observed differences in the parameter estimates are too large to be explained by chance fluctuations. For example, the data show that in medium without antibiotics, the maximum growth rate \((a)\) of clones from the parental population differed from those of clones from the biofilm, while there was no difference in the density-dependent effect of population size, \(b\) (Fig. 3A and B).

Overall, the data showed there were significant differences in the growth dynamics of clones from different biofilm layers when clones from a given layer were grown in different media. Moreover, there was a dramatic difference between the growth dynamics of the parental and biofilm clones (Fig. 3C, F, and I). However, there were differences between maximum growth rates \(a\) and density-dependent effects \(b\) throughout the data.

Variation among clones from a single depth. To assess patterns of variation within the growth kinetics of clones from a single depth in the biofilm, we performed a hierarchical cluster analysis of the stochastic Ricker model parameters \((a, b, \text{ and } \sigma^2)\) for clones grown in BMG1+Str. The optimum number of clusters was chosen according to the cubic clustering criterion (34), and the identities of the clones in each cluster were recorded. This was done for clones from each of the six layers sampled. To rigorously test the statistical significance of each cluster, a parametric bootstrap likelihood ratio test was done. This analysis suggested that for any particular depth, there were distinct clusters that were statistically significant. The number of distinct clusters ranged from a low of 7 in depth B1 to a high of 15 in depth T1 (Table 1). To exclude the possibility that the resulting clusters were a statistical anomaly and that there was actually only a single growth pattern, three models
were formally specified and compared via their BIC statistics. The first was a stochastic exponential model in which the growth dynamics of clones from a given depth could be described by a single stochastic exponential equation (obtained by setting $b/H_{1005}$ in equation 1). In this case, each of the 64 growth curves would be an independent observation of a simple exponential growth process. The second model (stochastic Ricker) posits that the growth dynamics could be described by a single stochastic Ricker equation, and each of the 64 growth curves was an independent observation of that particular growth process. The last was a clusters model that is based on the supposition that growth of the 64 clones from a particular depth could not be described by a single equation but instead requires multiple equations, each of which has different growth rates and different carrying capacities. The number of equations needed would be reflected in the number of clusters identified by the cubic clustering criterion. The stochastic exponential model was an adequate explanation for only one of the six biofilm depths (B2), while the stochastic Ricker model best described the growth patterns in two layers, T1 and T2. The growth patterns in the remaining three depths were best explained by the clusters model (Table 1).

**DISCUSSION**

Here we showed that *E. coli* undergoes extensive diversification when grown as a biofilm and that differences between clones are manifest in their growth kinetics. This conclusion was based on rigorous statistical analysis of data obtained for 384 clones that had been isolated from six distinct depths in a biofilm and grown in three kinds of media. It is important to note that the growth kinetics of clones from the biofilm dif-
ferred from that of the wild-type strain, as well as within and between layers of the biofilm. By fitting the stochastic Ricker model to the 1,152 growth curves, we showed that differences in the growth kinetics of bacterial clones coming from different biofilm depths and three cultivating media were not due merely to random variation of a unique growth process. Instead, we showed that the cultivated clones displayed characteristics that varied according to the location of their ancestor clone in a spatial gradient in the biofilm. Thus, we concluded that the observed phenotypic differences were heritable, suggesting that the clones had one or more mutations that in some cases had profound effects on cellular physiology. These findings are important because they clearly demonstrate the importance of the spatial dimension as a critical factor in the generation and maintenance of a high bacterial diversity.

We argue that the persistence of phenotypic variants within a biofilm is a predictable and direct outcome of nutrient limitation within biofilms, which is in turn a manifestation of spatial structure. Previous studies have clearly shown that the diffusion of nutrients into a biofilm is limited, and so with the exception of those cells near the surface, bacteria in biofilms grow slowly or are inactive (55, 57). Under conditions that do not favor the growth (or death) of any subpopulation, it is difficult for even extremely beneficial genes to sweep through the entire population. This may account for the persistence of genetic variants in biofilms that would otherwise be competitively excluded in a well-mixed homogenous environment. The propensity to increase genetic diversity may also be compounded by an elevated mutation frequency, as suggested by Boles et al. (2, 3). This too seems plausible, since studies have shown that when E. coli is grown under nutrient-limited conditions in batch or continuous culture, the expression of stress response genes is elevated and this is associated with an increased mutation frequency (1). Bacteria deep within biofilm matrices do experience nutrient limitation (20), and there is evidence that the expression of stress response genes in biofilms is elevated (57). Consequently, an elevated mutation rate might well be expected to occur, although empirical data to support this are lacking.

In a recent paper, Nelson et al. (38) documented how internally generated stage-structured cycles in the population dynamics of Daphnia pulex can lead to fluctuations in resources. This in turn translates to fluctuations in the selection coefficients for subpopulations and an overall decrease in these values across all genotypes. The authors showed that the decrease in selection leads to increased extant diversity. Here, arrested growth and reproduction due to the depletion of resources on a spatial scale can be seen as an extreme case of suppressing the effect of selection due to asymmetric competitive abilities. Indeed, microbial biofilms may represent an ecosystem in which individuals compete only on a very local scale and can be thought of as not competing at all on a more global scale. In areas of the biofilm where nutrients are not too scarce, such as those positioned more closely to the bulk medium, spatial heterogeneity could also be playing an important role in the maintenance of diversity according to Huffaker’s model of spatial refuges of ecological interactions (22). According to this model, physical and structural impediments to ecological interactions decrease their strength and ultimately lead to a more diverse system. Finally, in theoretical ecology it is well known that intraspecific competition tends to increase the width of the ecological niche sensu Hutchinson (27). Thus, individuals that lie at the extremes of the niche would be favored in the long run. In macroecological systems, this has been demonstrated to occur in rocky intertidal regions on the coast of Chile (35, 36), where the survivability of young herbivore recruits is clearly favored when they metamorphose into sessile phases in less-crowded refuges and ponds. The relative importance of these possible mechanisms might be resolved only by experimentally exploring the interplay between ecological and evolutionary processes under conditions where environmental variability may allow for the fluctuation in selection coefficients and thereby promote coexistence of genetic variants (12).

Our results can also be explained using the concept of “r-K selection” strategies (5). The data in Fig. 3 show that when grown in the same medium, clones derived from the bottom layers of a biofilm grew faster but had a lower carrying capacity than clones from the upper layers of the biofilm. This phenomenon reflects an “r-K selection” strategy wherein there is a trade-off between the evolution of the maximum growth rate and the carrying capacity. This trade-off occurs when higher birth rates are accompanied by a disproportionate increase in the mortality rate. The estimates of stochastic Ricker model parameters suggest that the mortality rates of clones from the bottom of biofilms increased disproportionately as their maximum growth rates increased (Fig. 4). This interpretation of the Ricker model parameters, however, follows more naturally from the stochastic version of the model, where both demographic and environmental stochastistics are specified (7). Fitting demographic and environmental noise to these data remains a topic for further research.

We would like to stress that the stochastic Ricker model is not the only model that could be fitted. For instance, we could
specify a spatial population dynamics model to account for cowardices among clones coming from different biofilm depths. It would be expected, for example, that clones that are closer in the depth gradient would exhibit more-similar dynamics. In the experimental context, it remains to be demonstrated with genetic data whether or not this significant increase in mortality of clones from the bottom layer of a biofilm is due to the accumulation of mutations.

Finally, we note that the use of alginate and agar to entrap a biofilm is a new methodology that allows investigators to dissect biofilms and study clonal variation in bacterial populations on various spatial scales. Alginate has been widely used for the entrapment of various biological materials, such as enzymes and cells (24), and the process itself is not known to introduce artifacts. This method complements the use of microelectrodes, electron microscopy, confocal scanning laser and fluorescence microscopy, autoradiography, microfluorimetry, and various other methods used in studies of biofilm dynamics (18, 21, 54). It provides a means to examine the physiology and genetics of clones that have been isolated from discrete regions of biofilms and thereby study the effects of spatial structure on adaptive evolution in biofilms.

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