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# The emergence and maintenance of diversity: insights from experimental bacterial populations

Paul B. Rainey, Angus Buckling, Rees Kassen and Michael Travisano

A central problem in evolutionary biology concerns the nature of the forces that maintain variation among individuals within populations<sup>1–4</sup>. Three principal processes have been proposed: selection, mutation and genetic drift. Although drift undoubtedly has a role in maintaining molecular variation and mutation is essential for the origin of genetic variation, selection is the only evolutionary force capable of maintaining variation in fitness-related traits (Box 1).

Here, we focus attention on recent studies using microbial-based systems, which shed light not only on the forces and mechanisms responsible for the maintenance of ecologically significant variation (i.e. non-neutral), but also on the fundamental issue of how diversity evolves from the monomorphic state. Space restrictions prevent a comprehensive account and we refer the reader to Bell<sup>5</sup> for greater detail.

## Experimental bacterial populations and evolutionary ecology

‘Evolutionary change occurs within an ecological context, but the workings of that context can be infinitesimally complex<sup>6</sup>. Therefore, progress requires experimental systems that reduce complexity to the point where mechanistic processes can be observed and rigorously tested. In this

**Mechanisms maintaining genetic and phenotypic variation in natural populations are central issues in ecology and evolution. However, the long generation times of most organisms and the complexity of natural environments have made elucidation of ecological and evolutionary mechanisms difficult. Experiments using bacterial populations propagated in controlled environments reduce ecosystem complexity to the point where understanding simple processes in isolation becomes possible. Recent studies reveal the circumstances and mechanisms that promote the emergence of stable polymorphisms.**

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respect, bacteria are ideal<sup>7</sup>. They are easily propagated, have short generation times, have large population sizes and are amenable to genetic-level analyses. Environmental factors affecting population growth can be carefully controlled and reproduction by binary fission means that identical populations can be established from a single genotype<sup>8–13</sup>. From the perspective of evolutionary ecology, large population sizes and rapid generation times ensure the coincidence of ecological and evolutionary timescales. This means that the ecological dynamics of evolutionary change can be observed in real time. Unlike traditional ecological experiments, microbial populations founded from single genotypes allow the evolution (emergence) of diversity, from the milieu of variant genotypes produced by mutation, to be separated from the ecological forces that determine the patterns of diversity that persist in the long term.

## The niche-exclusion principle and evolution in asexual populations

Experiments initially performed during the 1930s, with mixed populations of either yeast or *Paramecium* propagated in simple homogeneous environments, led to formulation of the competitive-exclusion principle. This principle

**Box 1. Theories for the maintenance of diversity**

**Heterosis (overdominance):** genetic variation is maintained because of the selective advantage of the heterozygote<sup>2</sup>. This mechanism is not relevant to haploid organisms.

**Negative frequency-dependent selection (balancing selection):** genetic variation is maintained by frequency-dependent selection, where the fitness of a genotype is a negative function of its frequency. Selection maintains genetic variation because rare genotypes are protected from loss<sup>1</sup>.

**Recurrent beneficial mutations:** genetic variation is maintained by continual selective sweeps in which successively more fit genotypes replace less fit ones. This is the classic model of selection outlined by Muller<sup>16</sup>, elaborated by Atwood *et al.*<sup>17</sup>, and more recently elaborated by Gerrish and Lenski<sup>41</sup>.

**Recurrent deleterious mutations:** genetic variation is maintained by recurrent deleterious mutations, which give rise to a mutation–selection balance<sup>42</sup>.

states that the number of coexisting genotypes cannot exceed the number of limiting resources<sup>14,15</sup>. When extended to asexual populations, the competitive-exclusion principle predicts that evolution in an environment containing a single limiting resource will proceed by a series of clonal replacements. Newly arisen adaptive mutants will drive any less fit genotypes to extinction<sup>16</sup>. Indeed, precisely this stepwise mode of evolution has been observed in experimental bacterial populations<sup>17,18</sup>. However, in apparent contravention of the competitive-exclusion principle, stable polymorphisms can evolve in asexual populations.

**Ecological mechanisms**

*Resource partitioning and cross-feeding*

In the mid-1980s, Julian Adams *et al.* began a series of selection experiments in which *Escherichia coli* populations were propagated for hundreds of generations in spatially homogeneous, glucose-limited, chemostat cultures. Populations were founded from a single ancestral *E. coli* clone and samples were regularly plated onto agar media to check for contamination. Initially, colonies appeared identical in size; however, as time progressed samples from all evolving populations began to show variation in colony diameter. By comparing genetic markers from aberrant colonies against the ancestral genotype, Helling *et al.*<sup>10</sup> were able to dismiss the possibility that the variant colonies were contaminants and concluded that polymorphisms had emerged in all populations. To test the stability of these polymorphisms, four dominant genotypes from one population were mixed together at equal frequency. Three of the four genotypes persisted, while one rapidly became extinct<sup>10</sup>. Further reconstruction experiments examined the ability of each persisting genotype to compete with every other genotype and in all pairwise combinations; these experiments showed that in every instance stable equilibria were obtained – no single genotype was able to drive any other to extinction.

To determine the physiological factors responsible for the maintenance of this polymorphism each genotype was subjected to an analysis of growth rate, glucose-uptake kinetics, equilibrium glucose concentrations and global patterns of gene expression<sup>10,19,20</sup>. The ability of genotypes to use metabolites excreted by other genotypes (cross-feeding) was also examined by growing each in culture filtrates obtained from competing genotypes<sup>10</sup>. Genotype CV101 was able to grow in the culture filtrate of CV103, but the reverse was not possible, thus suggesting that genotype CV103 secreted a metabolite that could be used by CV101. Further study revealed that the polymorphism could indeed be explained by cross-feeding coupled with a

difference in glucose-uptake kinetics. The numerically dominant genotype (CV103) appeared to have evolved a superior glucose-uptake system, but also excreted acetate. This newly available resource was used by one of the two less abundant genotypes, CV101, which had evolved an enhanced ability to metabolize acetate. The third genotype, CV116, also showed evidence of an enhanced glucose-uptake system, as well as an improved capacity to recover glycerol, which was excreted by all genotypes<sup>20</sup>.

The emergence of a stable polymorphism involving cross-feeding has also been observed in *E. coli* populations propagated for many thousands of generations in glucose-limited batch culture<sup>21,22</sup>. In one case subjected to detailed analysis, a two-genotype polymorphism was shown to have existed for more than 12 000 generations. Analysis of the dynamics revealed it to be highly complex and maintained not only by cross-feeding but also by a mixture of allelopathy and trade-offs in growth-phase parameters<sup>22</sup>.

*Temporal variation*

The serial transfer regime used to propagate bacterial populations in batch culture is analogous to a seasonal environment in which resources are abundant early in the season but become scarce as the population approaches its saturation density. Such an environment can, in theory, promote the stable coexistence of two genotypes on a single resource with no cross-feeding, provided one genotype has a growth rate advantage when the resource is abundant and the other has an opposing advantage when the resource is scarce<sup>21,23,24</sup>.

In a recent study of a polymorphism stably maintained under a serial transfer regime, Turner *et al.*<sup>21</sup> examined the relative importance of both demographic trade-offs and cross-feeding as explanations for coexistence. Trade-offs were observed between growth rates at high and low concentrations of glucose; however, the magnitude of the trade-off alone was insufficient to explain the coexistence of genotypes (subsequent experiments revealed the polymorphism to be maintained by cross-feeding). This outcome is consistent with theoretical work, which shows that the conditions for maintenance of polymorphisms under temporal variation are stringent and unlikely to be encountered even in simple bacterial populations<sup>25,26</sup>.

*Spatial structure*

Recently, increasing attention has been focused on the role that spatial heterogeneity plays in the maintenance of diversity<sup>27–29</sup>. Rainey and Travisano<sup>13</sup> explicitly examined the effect of spatial structure on the emergence of diversity, by allowing replicate populations of the plant-colonizing bacterium *Pseudomonas fluorescens* to evolve in two kinds of microcosm that differed solely in the degree of spatial structure they afforded. A spatially homogeneous environment was obtained by incubating broth cultures in an orbital shaker, whereas a spatially heterogeneous environment was obtained by incubating cultures without shaking. After seven days, populations propagated in the heterogeneous environment had diversified, whereas populations propagated in the homogeneous environment remained genetically uniform.

Spatial structure was implicated as a key factor for the emergence of this polymorphism. However, in order to ascertain the significance of spatial structure for the long-term maintenance of the polymorphism, *P. fluorescens* populations initially propagated in the spatially heterogeneous environment were switched to the homogeneous environment. Within one week of switching diversity began to fall, whereas diversity of control populations

remained high. Therefore, spatial structure appears to be essential for both the emergence and maintenance of these polymorphic populations.

The nature of the ecological factors maintaining diversity in the spatially structured environment is complex and not fully understood. However, interactions between morphs appear to depend on the density of genotypes within each niche. For example, the morph known as 'wrinkly spreader' (WS), which refers to the colonies it produces on agar plates, forms a self-supporting 'mat' at the surface of the broth culture. Membership in the mat community is strongly favoured by selection, because cells within the mat receive high amounts of both nutrients and oxygen. However, as the density of the WS morph becomes too great, the mat loses its ability to remain at the air–broth interface and, therefore, sinks. At this point, the fitness of genotypes with niche preferences for the broth phase increases owing to the greater availability of oxygen. The fact that the polymorphism is dynamic through time indicates that temporal, as well as spatial, factors play a role in maintaining diversity in this model system.

A similar relationship between population diversity and spatial structure has been indicated by both theoretical and empirical studies of colicin production by *E. coli*. Colicins are toxic substances produced by certain bacteria, which kill competing bacteria of different genotypes through what is known as allelopathy. In a homogeneous environment a population dominated by colicin-producing bacteria cannot be invaded by a colicin-sensitive genotype, because any sensitive cells will be killed quickly<sup>30</sup>. Similarly, a population dominated by the colicin-sensitive genotype cannot be invaded by a colicin-producing genotype, because the toxic effect of colicin is diluted and the cost of production outweighs any benefit. Thus, both colicin production and sensitivity are evolutionarily stable strategies and selection only favours genotypes when they are common – rare genotypes cannot invade and diversity cannot be maintained.

However, in an environment that allows for spatial structuring, such as the surface of an agar plate, it is both theoretically<sup>31,32</sup> and experimentally<sup>33</sup> (M.A. Riley, pers. commun.) possible to maintain not only these two genotypes, but also a third. In a spatially explicit environment a population of colicin-sensitive genotypes can be invaded by a colicin-producing genotype because the effects of colicin are no longer diluted – death of neighbouring cells directly benefits the producer and more than offsets the cost of colicin production. As the frequency of the colicin-producing genotype increases it becomes susceptible to invasion by a cheating genotype, which produces no colicin but retains resistance to colicin. The cheat then increases in frequency, but as it becomes common it too becomes susceptible to invasion, but this time by the sensitive genotype. The sensitive genotype, being free of the costs of colicin production (and now in an environment with little colicin), can increase in frequency to the point where it once again becomes susceptible to invasion by the colicin-producing genotype. In this example, the interactions between genotypes are nontransitive (i.e. colicin-producing > colicin-sensitive > colicin-resistant > colicin-producing), thus leading to an endless cycle of fluctuating genotype frequencies.

## Ecological factors and processes

### Opportunities

On the basis of the competitive-exclusion principle, ecological opportunity (or vacant niche space) is implicated as an

essential factor for the emergence of a stable polymorphism. Indeed, a causal relationship between environmental complexity and diversity is predicted by ecological theory<sup>5,29</sup>.

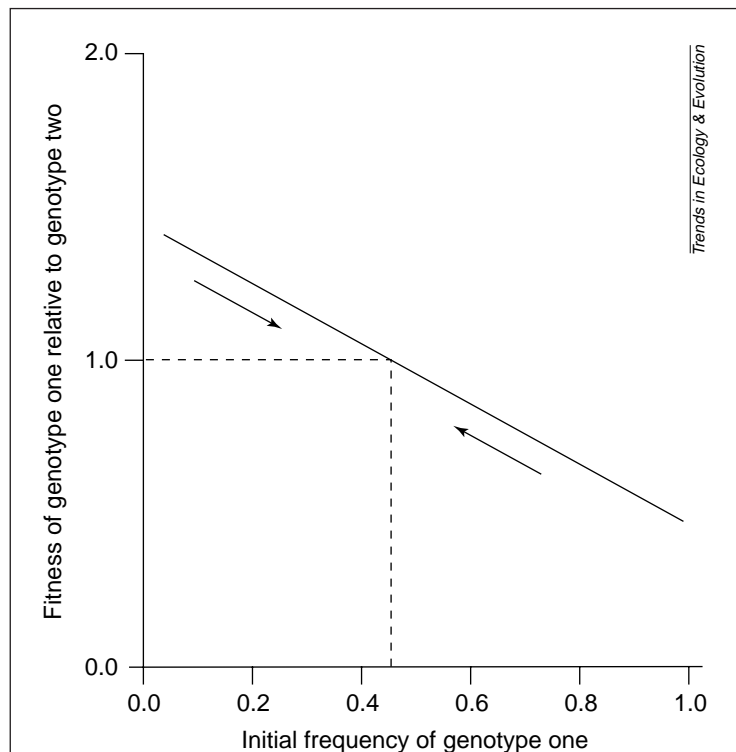
Niche-destruction experiments showed that ecological opportunity is essential for the emergence and maintenance of polymorphisms in both *P. fluorescens*<sup>13</sup> and colicinogenic *E. coli* populations<sup>30</sup>. Conceptually similar experiments have been used to demonstrate that polymorphisms based on cross-feeding are also dependent upon ecological opportunity. In both chemostat and batch-culture studies, coexistence of cross-feeding genotypes is predicted to depend upon the resource-partitioning activities of the dominant genotype. Therefore, it ought to be possible to destabilize these polymorphisms by reducing the concentration of the primary nutrient source (glucose) to the point where the metabolite available for cross-feeding becomes negligible. Both Rosenzweig *et al.*<sup>20</sup> and Turner *et al.*<sup>21</sup> observed a significant reduction in the density of the cross-feeding genotype when they reduced the concentration of glucose. Moreover, Turner *et al.* showed that there was a crucial glucose concentration, below which the cross-feeding genotype became extinct.

### Niche specialization and fitness trade-offs

In an asexual population ecological opportunity is necessary for evolution of a stable polymorphism, but it alone is not sufficient. The evolution of genotypes that have traded-off their ability to compete effectively in all niches against enhanced fitness in a smaller number of specific niches is also required. Without specialization and concomitant fitness trade-offs, stable coexistence of genotypes is generally not possible.

Existence of fitness trade-offs is usually determined by examining the fitness of genotypes in different environments<sup>21</sup>, but crossdiscipline studies are offering new opportunities for unravelling the complex connections between niche specialization, fitness trade-offs and population dynamics. Studies of the polymorphic *P. fluorescens* population showed that each genotype occupied a distinct niche within spatially structured microcosms. For example, the WS morph colonized only the air–broth interface, whereas the 'fuzzy spreader' (FS) genotype displayed a preference for the base of the microcosm. These niche preferences differed markedly from the ancestral 'smooth' (SM) genotype, which colonized the broth phase. Subsequent genetic and biochemical analyses of the WS morph showed that niche specialization is determined by semi-constitutive expression of the *wss* locus (encoding a cellulose-based polymer), which causes cell clumping and mat formation. When this locus was inactivated (by insertional mutagenesis) niche specialization was abolished along with previously detected fitness trade-offs. Moreover, competitive evolution experiments, between the SM genotype and an isogenic derivative (SM13) carrying a defective *wss* locus, showed that the two genotypes were equally fit when propagated in a homogeneous environment; however, in the heterogeneous environment SM13 was substantially less fit. This was attributable solely to the fact that SM13 was unable to generate the WS phenotype during the course of evolution and therefore was unable to compete effectively for occupation of the air–broth interface (S.G. Kahn, D. Phil. thesis, University of Oxford, 1998; A.J. Spiers *et al.*, unpublished).

Studies of the genetics of acetate cross-feeding in *E. coli* also provide new insights into the evolution of niche specialization. Previous work had established that the acetate cross-feeding genotype was a result of semiconstitutive



**Fig. 1.** Negative frequency-dependent selection operates whenever one genotype has a selective advantage when rare, which disappears when that genotype becomes common. Turner *et al.* mixed two *Escherichia coli* genotypes suspected of stable coexistence at initial frequencies ranging from 0.01 to 0.99 and determined the genotype frequencies after 24 h of competition. Fitness was found to be a negative function of genotype frequency. The dashed line indicates the point at which genotypes are of equivalent fitness (relative fitness of 1.0), which corresponds to the equilibrium frequency in the population. Modified, with permission, from Ref. 21.

**Box 2. Future directions of study**

Possibilities for the use of microbial populations in ecology and evolution are limited only by imagination. Therefore, the following list is far from exhaustive, but reflects a few areas where explicit tests would serve to dispel controversy and clarify issues:

- The effect of productivity on diversity.
- The effect of environmental disturbance on diversity.
- Environmental conditions promoting the evolution of specialists versus generalists.
- Conditions favouring the evolution of phenotypic versus genotypic plasticity.
- The relationship between random variation, natural selection and adaptation.
- Wright, Fisher and the nature of the adaptive landscape.
- Genetic causes of adaptive evolution; the search for common molecular rules; and the nature of epistasis, pleiotropy and its causes.
- The nature of the phenotype-to-genotype map.
- The importance of modularity in evolution.

expression of the enzyme acetyl CoA synthetase. Although, in principle, mutations in several different genes can generate the acetyl CoA synthetase overproducing phenotype, analysis of acetate cross-feeders from six independent polymorphic populations showed that the phenotype was caused by mutations in the gene encoding acetyl CoA synthetase (*acs*). Two different mutagenic events were detected (with subtly different effects), but both were located at a single genetic target. One involved insertion of a transposable element and the other a single nucleotide substitution – in both instances the mutations occurred in the regulatory region of *acs* and each caused semiconstitutive

expression of *acs* (Ref. 34). This information, combined with detailed knowledge of the regulation of metabolic pathways in *E. coli*, provides the basis for a comprehensive understanding of the relationship between fitness trade-offs and the pleiotropic effects of niche specialization.

Finally, although trade-offs are necessary for stable coexistence of genotypes, establishing whether the magnitude of the detected trade-off is sufficient to explain the observed dynamics is important. Turner *et al.*<sup>21</sup> detected a demographic trade-off among two coexisting *E. coli* genotypes, but showed that the magnitude of the trade-off was insufficient to explain the observed polymorphism.

*The importance of frequency-dependent selection*

In an environment that affords ecological opportunity and where selection has favoured the evolution of niche specialists, the maintenance of coexisting genotypes is assured through the operation of density-dependent processes<sup>25</sup>. Assuming a constant primary resource, the fitness of a niche-specialist genotype will be a function of the availability of the primary resource<sup>24</sup>. Therefore, selection will operate in a negative frequency-dependent manner, favouring genotypes when they are rare (because resources will be most abundant) but not when they are common (because resources will be scarce and competition intense)<sup>1,35</sup>.

In each of the polymorphic populations considered here, negative frequency-dependent selection has been shown to maintain the variation. Evidence has come primarily from experiments that test the ability of rare genotypes to invade an established population of another type<sup>13,21,22,36</sup> (Fig. 1), but also from knowledge of the ecological mechanisms<sup>20</sup>.

Overwhelming evidence for the operation of negative frequency-dependent selection does not exclude the possibility that different population genetic mechanisms (recurrent beneficial and deleterious mutations; Box 1) might also contribute to the maintenance of variation. Elena and Lenski performed a systematic analysis of six long-term *E. coli* populations and found evidence of a minor role for recurrent deleterious mutations in one population, but found no evidence for recurrent beneficial mutants in any of the populations. By contrast, negative frequency-dependent selection was found to operate in all six populations<sup>36</sup>.

Negative frequency-dependent selection is a powerful force maintaining variation in these experimental bacterial populations. It is also possible that it plays a significant role in maintaining the considerable genetic diversity observed in most bacterial species<sup>37,38</sup>. But, where relations among genotypes are nontransitive this same mechanism can prevent the mean fitness of a population from increasing and might even cause it to decline<sup>5,39</sup>. Where intransitivity exists, the outcome of competition between two genotypes depends less upon the fitness of each genotype in a single environment than on the presence of a third genotype (this was evident in the interaction among colicinogenic *E. coli* genotypes). Under these circumstances, each successful genotype is the cause of its own demise, because it creates an environment in which another genotype will be the superior competitor. Typically, the negative effects of increasing frequency fail to feedback until the genotype has overrun its optimum frequency, which causes a time delay that leads to an endless fluctuation in genotype frequency<sup>5</sup>. This cycling is seen among colicin genotypes and *P. fluorescens* genotypes that evolve in spatially heterogeneous environments. Just how widespread intransitivity is among populations is not known, but its detection in several experimental microbial populations suggests that it might be common in complex natural environments.

## Conclusions

Simplification is essential for an understanding of the fundamental features that underlie complex processes. The studies discussed here demonstrate two essential causes of polymorphism – ecological opportunity and competitive trade-offs. Neither factor is a cause of the other and neither factor alone is sufficient to promote emergence of the polymorphic state. Nevertheless, if vacant niche space exists and selection is capable of generating specialist types that trade-off fitness in different environments, then polymorphisms are likely. The polymorphism will be stable if population regulation is density dependent and this will cause selection to operate in a frequency-dependent manner. Thus, in the microbial populations surveyed, both ecological opportunity and competitive trade-offs are necessary to maintain ecologically significant variation. Whether these are the only factors that maintain diversity in other systems awaits further experimentation. Box 2 lists some of the most promising opportunities for using bacterial systems to answer fundamental questions regarding the fate of variation in heterogenous environments and the study of adaptation.

The enormous diversity of life has long fascinated biologists and there has been much discussion regarding its causes and factors affecting its maintenance<sup>40</sup>. In the simplest of all possible environments, these studies show how the activities of one life form can promote the emergence and stable coexistence of a competing genotype. Moreover they reveal how easily disturbed (or destroyed) these interactions can be by environmental changes that affect either resource availability or physical structure. The precision and delicate balance of these associations mirrors those believed to exist within natural ecosystems.

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