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Evolution

Evidence for multiple adaptive peaks from populations of bacteria evolving in a structured habitat

(habitat structure/adaptation/divergence/fitness/*Comamonas* sp.)

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ABSTRACT **Natural selection tends to promote the divergence of populations living in different environments. Even in identical environments, however, replicate populations may diverge if they find alternative adaptive solutions. We describe the evolution of 18 bacterial populations (*Comamonas* sp.) founded from a single progenitor genotype and propagated separately for 1000 generations in two distinct environments, one physically unstructured (mass-action liquid) and the other structured (agar surfaces). Phenotypic diversity, as reflected in colony morphology, was greater in the structured habitat than in the unstructured habitat. More importantly, the trajectories for mean fitness, as measured by competition against the common ancestor, were more divergent for populations in the structured habitat than those in the unstructured habitat. Structured environments may accelerate evolutionary diversification by promoting genetic polymorphisms within populations, thereby increasing the complexity of genetic constraints that allow divergence among replicate populations.**

Adaptation by natural selection results from the systematic relationships between genotype and phenotype and between phenotype and reproductive success in a given environment. Natural selection thus promotes parallel adaptations for populations living in similar environments and divergent adaptations to dissimilar environments. However, even in identical environments, initially identical populations may diverge in traits that influence reproductive success if, through the effects of historical contingencies and random processes (mutation and drift), the populations find alternative adaptive solutions to the environment (1–5).

From a theoretical perspective, a key factor in determining the reproducibility of adaptive evolution is the complexity of the fitness surface, or adaptive landscape. This landscape provides a graphical representation of a population's mean fitness (in a particular environment) as a function of its genetic composition (1, 3, 5). If this surface is rugged, with numerous fitness peaks (local optima, separated by less-fit intermediate states) in the vicinity of a population's initial genetic state, then chance events are more important in determining the course of adaptive evolution than if the surface has only a single accessible peak. Theoreticians have paid considerable attention to factors governing the likelihood that populations may shift from one adaptive peak to another, including random genetic drift and founder effects, population structure and migration, and additive versus nonadditive genetic effects on fitness (1, 5, 6–9).

Despite the heuristic appeal of this framework and its importance for such fundamental evolutionary processes as adaptation and speciation (10, 11), empiricists know very little about the structure of real adaptive surfaces, and it is not even clear how these structures can be elucidated (12, 13). One approach that is feasible with certain rapidly reproducing organisms is to found initially identical replicate populations, propagate them in identical laboratory environments, and analyze the extent of their adaptation and divergence (14–20). In one such experiment, 12 populations of the bacterium *Escherichia coli* were propagated for thousands of generations in a glucose-limited liquid medium, during which time they underwent similar (but not identical) changes in mean fitness and in the life-history traits responsible for their higher fitness (19, 21). The similar responses to selection in this experiment could not be attributed to selection for alleles identical by descent, because the only genetic variation in the replicate populations came from new mutations. A possible limitation of this study is that the founding bacterium had already been in the laboratory for several decades, perhaps constraining the extent of its further adaptation. Another possible limitation is that the experimental environment was so simple as to restrict the range of potential adaptations, thereby favoring the outcome of evolutionary parallelism.

In this study, we sought to extend this work by addressing those limitations. As the experimental organism, we used a freshly isolated soil bacterium capable of degrading certain chlorinated aromatic hydrocarbons. By propagating a freshly isolated strain on an unusual substrate, we sought to create the opportunity for more rapid and extensive adaptation. We also employed two distinct selection regimes, one relatively simple and the other more complex. The simpler environment consists of liquid medium, containing a chlorinated aromatic hydrocarbon as the sole carbon source. The more complex environment offers the same nutrients, but on a surface that may develop various physical and chemical gradients. The number of generations and the population size were the same in both environments. We refer henceforth to these two environments as mass-action and structured, respectively (22–24).

MATERIALS AND METHODS

Bacterial Strain. The progenitor bacterium used in this study was isolated from soil in central Michigan and tentatively identified as *Comamonas* sp. strain TFD41 (N. Tonso, personal communication). This strain harbors a large (≈160 kb) catabolic plasmid that hybridizes with probes for all six genes on plasmid pJP4 (25, 26) encoding enzymes required for degradation of 2,4-dichlorophenoxyacetic acid (2,4-D).

Genetic markers were necessary to distinguish replicate evolving populations from one another and from their common ancestor. Marker variants were selected on plates containing either streptomycin or nalidixic acid. Both streptomycin-resistance (Str^r) and nalidixic acid-resistance (Nal^r)

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Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; Str^r, streptomycin resistance/resistant; Nal^r, nalidixic acid resistance/resistant.

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markers had small but statistically significant effects on fitness in competition experiments with the unmarked progenitor. Therefore, the results of competition experiments between derived and ancestral genotypes will be presented separately for each marker and corrected for these effects.

Selection Experiment. Eighteen populations were founded from single cells of the progenitor bacterium and subsequently maintained in the same incubator at a constant temperature (25°C). Of these populations, 12 (6 Str^r and 6 Nal^r) were serially propagated in the mass-action environment, and 6 (3 Str^r and 3 Nal^r) were serially propagated in the structured environment. However, the 6 Nal^r populations in the mass-action environment reverted to nalidixic acid sensitivity and were excluded from analyses of fitness, which required a marker (see below).

The mass-action environment consisted of a liquid minimal salts medium, MMO (27), supplemented with 0.5 mg/ml of 2,4-D as the sole carbon source; this concentration supported a stationary phase density of $\approx 2.6 \times 10^8$ cells per ml. Every 48 hr, a sample of 0.039 ml ($\approx 10^7$ cells) from each population was diluted into 10 ml of fresh medium held in a 50 ml Erlenmeyer flask, which was set in a shaking incubator (120 rpm). This regime permitted eight generations of binary fission [$\log_2(10/0.039)$] every 2 days.

The structured environment consisted of agar surfaces. In this environment, bacterial cultures were also held in flasks containing 10 ml of the same medium described above, except that the medium was solidified by the addition of 16 mg of agar per ml. Four sterile glass beads (4-mm diameter) were added to each flask containing hardened agar. Cells added to the agar surface were spread evenly by gently shaking the glass beads and then were left to grow into a dense lawn. After 48 hr, the cell layer was easily diluted into 10 ml of saline, and as for liquid cultures, 0.039 ml of the resulting mixture was transferred to an agar surface in a new flask, resulting in very similar initial and final population sizes and, hence, the same number of generations as in the mass-action environment.

Every 24 or 26 days ($\approx$100 generations), each population was tested for contaminants on selective plates, and a sample of each population was mixed with glycerol and stored at −80°C. Serial transfers of populations possessing each marker were strictly alternated, but no cross-contamination was detected. The absence of external contaminants was confirmed by DNA-fingerprinting several clones from each final population using REP (repetitive extragenic palindromic) and ERIC (enterobacterial repetitive intergenic consensus) sequences for PCR amplification (28, 29).

Colony Morphology. We also scored the evolving populations on the basis of colony morphology. Briefly, we observed three distinct morphs: wild type (that of the progenitor), small, and translucent. The small morph had a diameter less than one-third that of the wild type on uncrowded plates; these two morphs were easily distinguished because of the absence of colonies of intermediate size. The translucent morph appeared noticeably different from the wild type in dim diffused light. The three colony morphs were even more distinctive on agar medium containing very dilute nutrients (5 mg of tryptone, 5 mg of peptone, 10 mg of yeast extract, 10 mg of fructose, and 16 g of agar per liter).

Several additional points are important. (*i*) The three colony morphs could be reliably scored. We performed blind trials and observed perfect concordance in classification. (*ii*) The variation in colony morphology was heritable. Numerous clones of the various morphs were stored at −80°C, propagated for many generations, and replated. The distinctive colony morphs persisted, indicating a genetic basis to the differences. (*iii*) The morphs were phenotypically stable over the course of the evolution experiment itself. Blind tests could not distinguish between colony morphs isolated at generations 200 and 1000.

Fitness Assays. Estimates of relative fitness were obtained in pairwise competition experiments between evolved populations and the unmarked ancestral strain (except when noted otherwise). These assays were performed in the same environment (mass-action or structured) in which the evolved population was propagated. Samples of both competitors were thawed and then serially propagated three times to ensure comparable acclimation to the competition medium. In the fourth transfer, both competitors were mixed together. The densities of the unmarked ancestor and the genetically marked evolved population were determined at the beginning and end of this fourth transfer by diluting the culture on nonselective agar and the relevant selective agar (containing either streptomycin or nalidixic acid). From these plate counts, the number of doublings for each competitor was calculated. Relative fitness is defined as the ratio of the number of doublings achieved by the evolved and ancestral competitors, which is equivalent to the ratio of their Malthusian parameters over the course of the competition experiment (18, 19).

In the competition experiments, the initial density of the ancestor was 10^3–10^4 times higher than the evolved competitor. The advantages of this design are 2-fold. First, the time course of resource depletion and other density-dependent changes in the habitat were largely determined by the same majority population, ensuring that the environment was similar in all cases. Second, relative fitnesses of the replicate populations are directly comparable because the denominator (the number of doublings achieved by the ancestral competitor) is always $\approx$8.

We considered the possibility that adaptation to growth on nutritional contaminants of agar might be a significant component of the observed fitness increase of the populations propagated in the structured habitat. To test this, we washed Difco agar in liquid MMO medium supplemented with 0.5 mg of 2,4-D per ml. We then grew the common ancestor (six replicates) and 12 clones from the populations that evolved in the structured habitat for 1000 generations (two clones per population) in this agar-enriched medium and, as a paired control, in pure liquid MMO containing 0.5 mg of 2,4-D per ml. Using a Coulter Counter, we estimated the growth rates of the progenitor and the derived clones in each medium over an 11-hr period, when densities went from $\approx 10^6$ to $>10^7$ cells per ml. Both the ancestral and derived genotypes grew somewhat faster on the agar-enriched medium; however, there was no difference between them in the ratio of their growth rates on media with and without agar enrichment [$t = 0.680$, $6 + 12 - 2 = 16$ df (degrees of freedom), $P = 0.506$]. Also, agar enrichment provided resources sufficient to account for only 2–3% of the final yield of bacterial cultures; again, this effect was not significantly different for the ancestral and derived genotypes. We conclude that contaminants in the agar have little or no effect on relative fitness in the structured environment.

RESULTS

Diversity of Colony Morphology in Mass-Action and Structured Habitats. Heritable, discrete polymorphisms in colony appearance were observed in cells that were plated between generations 200 and 1000. As a measure of morphotypic diversity, we applied the Shannon index [$H = -\Sigma\,(p_i \ln p_i)$], where p_i is the frequency of a particular colony morph. The solid lines in Fig. 1 show the changes over time in the total diversity (over all replicate populations) of colony morphs for both environments. Based on the jackknife procedure (see the legend to Fig. 1), the populations evolving in structured habitat had significantly higher total diversity of colony

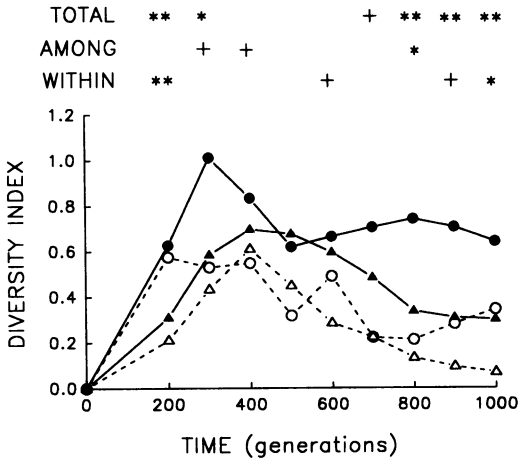

FIG. 1. Diversity of colony morphology in the mass-action (△, ▲) and structured (○, ●) environments. Solid lines (and symbols) indicate the total diversity over all replicate populations. Dashed lines (and open symbols) indicate the average within-population diversity. The difference between the solid and dashed lines indicates the among-population diversity. Along the top margin are shown statistical analyses for generations 200 to 1000 of the difference between mass-action and structured environments in each of the diversity components. For the within-population component, a Mann–Whitney test was performed on the separate estimates from each of the independent populations (6 structured and 12 mass-action). For the total and among-population components, Mann–Whitney tests were performed by using pseudo-values obtained by the jackknife procedure (30), with the independent populations as the unit of replication. Tests were one-tailed, with the expectation that diversity would be greater in the structured environment. **, $P < 0.01$; *, $0.01 < P < 0.05$; +, $0.05 < P < 0.1$.

morphs for most of the experiment, including at generation 1000. We also calculated diversity separately for each population to estimate the mean within-population diversity; the among-population diversity was obtained by subtracting the mean within-population diversity from the total diversity (31). Both the within- and among-population components of diversity were usually greater in the structured than in the mass-action environment (in six and seven, respectively, of the nine samples between generations 200 and 1000), although most of these differences were not statistically significant. Nonetheless, the total diversity with respect to colony morphologies is clearly greater in the structured than in the mass-action habitat, and it appears that this difference may reflect greater diversity both within and among replicate populations.

Fitness Trajectories in Mass-action and Structured Habitats. All six Nal^r populations propagated in the mass-action habitat lost the Nal^r marker between generations 200 and 400. (However, they did not acquire the Str^r marker, thus excluding cross-contamination as an explanation.) No marker losses were detected in any of the populations propagated in the structured habitat. Consequently, we were able to estimate fitnesses for all six surface populations (three Nal^r and three Str^r) but for only the six Str^r liquid populations.

Fig. 2 shows the trajectories for grand mean fitness (mean fitness averaged over the replicate populations) in the mass-action and structured environments. In both environments, there was an initial period of little or no change, followed by a period of rapid improvement, and ending with a period of somewhat slower improvement. The initial delay reflects the time required for favored mutants to increase to a frequency where they begin to appreciably affect the mean properties of a population (19). However, the dispersion (SD) of the fitness estimates was quite different in the two environments. In the mass-action environment, the dispersion initially increased and then decreased. This conclusion is supported by statistical tests when correlations between dispersion and time are calculated over two arbitrary intervals: from generation 0 to 500 ($r = 0.989$, $n = 6$, $P < 0.001$) and from generation 500 to 1000 ($r = -0.887$, $n = 6$, $P = 0.019$). Replicated fitness assays (five blocks) confirmed that there was no significant variation in mean fitness among the six populations in the mass-action habitat at generation 1000 ($F = 1.339$, 5 and 20 df, $P = 0.289$). In the structured habitat, the dispersion of fitness estimates increased continuously. There were positive correlations between the standard deviation of mean fitness and generation number for both Str^r populations ($r = 0.814$, $n = 11$, $P = 0.002$) and Nal^r populations ($r = 0.877$, $n = 11$, $P < 0.001$). This sustained divergence in fitness among replicate populations in the structured habitat was particularly intriguing and stimulated us to perform additional experiments.

Genetic Divergence for Mean Fitness Among Populations in the Structured Habitat. In the preceding analyses, the evolved competitor comprised a sample of the whole population, and hence was a mixture of whatever clones were present in that population. Such experiments do not allow one to determine how much of the total genetic variance can be attributed to variation among populations and how much to variation among clones (within populations). To address this issue, four clones from each population propagated in the structured habitat were chosen at random at generations 500 and 1000, and two competition experiments against the ancestor were conducted for each clone. Analyses of variance point to the among-population component as the major source of variation in fitness, especially at the end of the experiment (Table 1). The among-population variation was significant for the Str^r block at 500 generations and for both the Str^r and Nal^r blocks after 1000 generations. For both blocks, the among-population genetic variance component was larger after 1000 generations than at 500 generations.

These experiments also indicated that the rank order of the mean fitnesses for the populations evolved in the structured habitat was conserved between 500 and 1000 generations (data not shown). The probability of obtaining the same rank order for three populations by chance is 1/3!; the probability that this would happen in both the Str^r and Nal^r blocks is

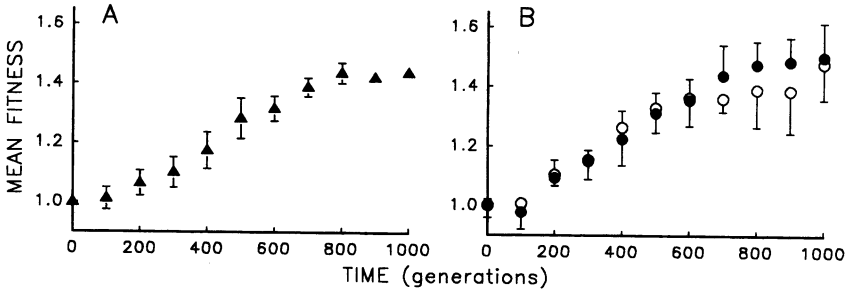

FIG. 2. Trajectory for mean fitness relative to the common ancestor in mass-action (*A*) and structured (*B*) environments. Vertical bars indicate SD (≤0.02 when not visible). Str^r and Nal^r populations are shown as filled and hollow symbols, respectively.

Table 1. Nested ANOVA (model II) of the clonal fitness assays for the populations in the structured habitat at generations 500 and 1000

Experimental block							
Marker	Generations	Source of variation	df	MS × 10^5	F	P	Variance component* × 10^5
Str^r	500	Among populations	2	2,808.1	47.456	<0.001	343.6
		Within populations	9	59.1	0.281	0.968	0
		Error	12	210.1			210.1
	1000	Among populations	2	10,581.0	47.434	<0.001	1294.7
		Within populations	9	223.1	1.276	0.370	24.1
		Error	12	174.8			174.8
Nal^r	500	Among populations	2	2,580.5	2.358	0.150	185.8
		Within populations	9	1,094.1	5.036	0.006	438.4
		Error	12	217.2			217.2
	1000	Among populations	2	3,542.9	5.728	0.025	365.5
		Within populations	9	618.5	1.438	0.274	94.2
		Error	12	430.1			430.1

MS, mean square.
*See ref. 30.

$(1/3!)^2 = 0.028$. Moreover, the ranks observed in these assays using clones were identical to the average ranks determined in the mixed-population assays (between generations 500 and 1000). These temporal correlations suggest that the variability among the populations evolving in the structured habitat was fixed during the early phase of rapid improvement and that subsequent evolution maintained the divergence, rather than diminishing it (as was observed for the populations evolving in the mass-action environment).

Thus, both the clonal and mixed-population assays indicate that the populations propagated in the structured habitat showed sustained divergence in their mean fitness relative to the common ancestor. However, this inference might depend on the fitness assays being performed in the ancestor-dominated environment; that is, fitnesses were estimated relative to the ancestor and with the ancestral genotype in the majority. For example, although clone *A* may be more fit than *B* when both are compared to their ancestor, this does not necessarily mean that *A* would prevail in direct competition with *B* (24, 32). Therefore, we performed additional fitness assays where both competitors came from generation 1000. In one set of experiments, a clone from each of the three Str^r populations competed (with 4-fold replication) against each of the three Nal^r populations, which formed the majority; in another set, the markers were reversed. If relative fitnesses were highly sensitive to the particular competitor used as a point of comparison, then we expect significant interactions (nonadditivity) in this experiment. However, no such interactions were observed for Str^r clones ($F = 0.786$, 4 and 27 df, $P = 0.545$) or for Nal^r clones ($F = 0.539$, 4 and 27 df, $P = 0.708$). Also, if there were strong interactions, then the ranks of a given clone in competition with different populations would be independent; but in fact the clones' ranks in competition with the ancestor and with the reciprocally marked evolved populations were significantly correlated (Str^r clones; $\tau = 0.576$, $n = 9$, $P < 0.05$; Nal^r clones: $\tau = 1.000$, $n = 9$, $P < 0.01$). These results corroborate the evidence for sustained divergence in mean fitness in the structured habitat.

DISCUSSION

Wright (1, 5) developed the concept of an adaptive surface, wherein the mean fitness of a population is mapped onto its genetic composition. Such surfaces are typically portrayed as quite rugged, with multiple peaks of various heights separated along only a few genetic axes. This image, if correct, implies that stochastic processes (including drift and mutation) may often cause populations to become stuck on local fitness peaks that are not globally optimal, which has important consequences for understanding the dynamics of adaptation and divergence. In fact, however, very little evidence exists concerning the nature of real fitness surfaces. The concept of fitness surfaces has been sufficiently important in evolutionary theory that we believe empiricists should seek to gain further insight into their structure, and that was the primary goal of our study.

To that end, we propagated 18 populations of a freshly isolated soil bacterium, *Comamonas* sp., in two contrasting environments for 1000 generations. These environments were similar in their resource basis (2,4-D-limited minimal medium) and in other respects, except that one consisted of a mass-action (liquid) habitat, whereas the other was physically structured (surface). Population sizes and numbers of generations were equivalent.

The dynamics of adaptation and divergence in the mass-action environment are consistent with the inference that the replicate populations converged on similar solutions to the selective regime, at least with respect to mean fitness. In contrast, the populations that evolved in the structured habitat diverged substantially from one another in their mean fitnesses. This divergence does not appear to be transient, as might be explained by stochastic variation in the timing of equivalent favorable mutations; rather, it was sustained even as the rate of further adaptation substantially declined. The populations that achieved higher final fitness were those that had achieved higher fitness early in the experiment, suggesting that initial adaptations promoted or constrained subsequent adaptive potential. The replicate populations in the structured habitat were more heterogeneous not only in mean fitness but also in their colony morphology. Evidently, the replicate populations had more divergent evolutionary pathways in the structured habitat than in the mass-action environment.

What might account for this difference? Adaptive evolution is likely to be more diversified when qualitatively different adaptations to the environment can occur, especially if these adaptations interact adversely with one another (so that a single genotype cannot be well adapted in all respects). A spatially structured habitat may promote diverse adaptations, even relative to an otherwise identical but unstructured environment. In the serial transfer cycle, there is temporal variation in resource concentration in both mass-action and structured habitats, which may allow coexistence of genotypes adapted to high and low resource concentrations (33, 34). However, only in the physically structured habitat are populations of cells exposed to gradients in concentrations of carbon, oxygen, moisture, and metabolites (both useful and toxic) released by other cells (22, 23). This spatial heterogeneity, which is an important feature of many microbial

communities (23), may contribute to within-population variability by promoting frequency-dependent selection for genotypes that are adapted to unexploited niches (35–37). Although within-population variation does not necessarily imply divergence among replicate populations (which might converge on the same polymorphic state), it seems reasonable to suppose that replicate populations would be more likely to be driven towards different adaptive solutions in more complex environments, especially if there are conflicts (tradeoffs) between adaptations to different environmental factors.

Our results support the hypothesis that habitat structure affects the extent to which replicate populations diverge during adaptive evolution. Additional evidence may be obtained by performing similar experiments with other organisms to compare the extent of divergence in simple and complex environments. Alternatively, we hope to gain further insight by examining the ecophysiological bases of the adaptations observed in this study to determine if they are, in fact, qualitatively (as well as quantitatively) more diverse in the structured habitat. The following questions are among those that need to be addressed. How specific are the adaptations of the derived genotypes to a particular environment? For example, certain genotypes might be competitive only in the structured habitat because they exploit physical or chemical gradients that do not exist in the mass-action environment. And what genetic changes are responsible for the observed differences in fitness and in colony morphology? Preliminary investigations (C.H.N., unpublished data) have revealed at least two distinct classes of genetic changes: (*i*) large deletions in the catabolic plasmid, which do not impinge upon the structural genes involved in 2,4-D degradation; and (*ii*) loss of a particular chromosomal fragment, as revealed by REP-PCR fingerprinting. The former change appears to be more common in populations adapted to the structured habitat than in those adapted to the mass-action environment; the latter change arose independently in all 18 populations. We hope eventually to understand at the molecular, physiological, and ecological levels how these mutations have adapted the derived genotypes to the experimental environments.

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