Experimental Evolution of *Pseudomonas fluorescens* in Simple and Complex Environments

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ABSTRACT: In complex environments that contain several substitutable resources, lineages may become specialized to consume only one or a few of them. Here we investigate the importance of environmental complexity in determining the evolution of niche width over ~900 generations in a chemically defined experimental system. We propagated 120 replicate lines of the bacterium *Pseudomonas fluorescens* in environments of different complexity by using between one and eight carbon substrates in each environment. Genotypes from populations selected in complex environments evolved greater mean and variance in fitness than those from populations selected in simple environments. Thus, lineages were able to adapt to several substrates simultaneously without any appreciable loss of function with respect to other substrates present in the media. There was greater genetic and genotype-by-environment interaction variance for fitness within populations selected in complex environments. It is likely that genetic variance in populations grown on complex media was maintained because the identity of the fittest genotype varied among carbon substrates. Our results suggest that evolution in complex environments will result neither in narrow specialists nor in complete generalists but instead in overlapping imperfect generalists, each of which has become adapted to a certain range of substrates but not to all.

Keywords: ecological specialization, experimental evolution, genotype-by-environment (G × E) interaction, niche, resource complexity, adaptive radiation.

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All organisms require supplies of certain essential resources, the simplest of which are elements such as carbon and nitrogen, whose rate of supply limits the rate of growth. An ideal simple environment contains a single limiting substrate, providing one of these resources, that is freely available to all individuals. As this substrate is depleted, types able to grow on lower concentrations will be favored, until at last only the single most frugal type remains (Tilman 1977). Organisms may differ in the ratio of resources that they require, however, and two or more different kinds may then coexist within a certain range of intermediate resource supply rates (Tilman 1977; Gottschal et al. 1979, 1981; Laanbroek et al. 1979; Dykhuizen and Davies 1980; Legan et al. 1987; Brzezinski and Nelson 1988; Grover 1988). Resources of this kind cannot be substituted for one another: carbon, say, cannot be made to take the place of nitrogen. There is a wide range of substrates, however, that will supply either: for example, most microbes are able to use a variety of sugars, organic acids, and other compounds to provide energy or carbon skeletons. Such substrates are said to be substitutable, because growth can be supported by any one of them or any mixture of them and is not prevented by the absence of any one of them. Consequently, organisms may evolve as generalists that are able to consume a large proportion of the substitutable substrates in a given category or as specialists that are able to grow on only one substrate or a very few substrates. Communities might thus comprise a diversity of specialists at one extreme or a single universal generalist at the other.

The outcome of competition between generalists and specialists will depend in part on how the available substrates are distributed. If they occur patchily in time with only a single substrate being available in each period of time, the single generalist type with the greatest geometric mean fitness should exclude all others (Bennett et al. 1992; Bell and Reboud 1997; Kassen and Bell 1998; Weaver et al. 1999). If they are distributed patchily in space, then a

variety of specialists may coexist, depending on the relative extent of the different kinds of patch and the rate of migration between them (Silver and Mateles 1969; Dykhuizen and Davies 1980; Wasserman and Futuyma 1981; Verdonck 1987; Garcia-Dorado et al. 1991; Bell 1996; Joshi and Thompson 1997; Taplitz and Coffin 1997). If the patches are completely separated, adaptive radiation leads quickly to the evolution of a wide range of specialists (MacLean and Bell 2002). Although temporal and spatial structure are important attributes of natural environments, most communities grow under conditions such that several or many substitutable resources are simultaneously available. We may then ask whether specialists or generalists will tend to evolve in mixtures of substitutable resources.

A single type of microbe inoculated into growth medium containing a mixture of carbon substrates usually uses them in sequence, typically consuming glucose first and switching to other sugars only when glucose has been reduced to very low concentration (see Harder and Dijkhuizen 1976). When growing in mixtures of substrates, bacteria are capable of tuning their metabolism quite precisely through mechanisms such as catabolite suppression (Paigen and Williams 1970). If the growth medium is initially very dilute, however, all substrates may be utilized simultaneously; for example, Lindenmann et al. (1996) have shown that *Escherichia coli* is capable of utilizing at least six substrates simultaneously, without establishing any upper bound on the number that might be used in more complex mixtures. The response of organisms to mixtures of substrates thus depends on the conditions of culture: sequential utilization of substrates is more likely to occur in batch culture, and simultaneous utilization is more likely to occur in chemostats (see Harder and Dijkhuizen 1976). The behavior of mixed cultures of microbes on mixtures of substrates is considerably more complicated. In many cases, a generalist able to use both (or all) of the substrates present eliminated a specialist able to use only one (e.g., Gottschal et al. 1979), but more complex outcomes have also been observed, perhaps as the result of less direct interactions between the competing types. The field has been reviewed by Harder and Dijkhuizen (1982) and Gottschal (1986).

Almost all the experiments reported in the literature have been ecological, in the sense that the types used to inoculate the system are assumed to retain their properties unchanged. In this study, we ask whether an initially isogenic population will adapt to a defined mixture of substrates and, if so, whether the evolved population will be dominated by generalists or by specialists. We transferred replicate lines of *Pseudomonas fluorescens* in 15 environments containing between one and eight carbon substrates for ~900 generations in batch culture. All lines were founded from clones so that all variance and covariance expressed by evolved genotypes arose through novel mutations during the selection experiment. At the end of the selection experiment, we measured the fitness of genotypes from each line on every single substrate in the experiment. This allowed us to test whether populations had adapted to the substrates in which they had been cultured and also whether they had adapted to other substrates. In particular, we tested whether specialists or generalists evolve in complex mixtures of substitutable resources. We expected that the outcome would depend on whether there were tradeoffs involved in utilizing different substrates. This experiment follows previous work in which we have documented adaptive radiation in undefined mixtures of substrates (Maclean et al. 2005) and in spatially structured environments (MacLean and Bell 2002).

Material and Methods

Ancestral Strain

We used clonal isolates of *Pseudomonas fluorescens* strains SBW25 and SBW25ΔpanB to found 15 selection lines, each replicated eight times. Strain SBW25 was isolated from the leaf of a sugar beet plant at the University Farm, Wytham, Oxford, in 1989 (Rainey and Bailey 1996). Strain SBW25ΔpanB is an isogenic strain of SBW25 containing a complete deletion of the panB gene. The panB gene is used to synthesize the vitamin pantothenate, and when plated on indicator plates with a low concentration of pantothenate (2.4×10^{-6}%), SBW25ΔpanB grows noticeably smaller colonies than SBW25. This marker is selectively neutral and has no effect when pantothenate is present in high concentrations (Rainey 1999; MacLean et al. 2004). The two strains were mixed in equal proportions to form a common pool to start the experimental populations (fig. 1 in the online edition of the *American Naturalist*). The ancestral clones were kept frozen at −80°C during the experiment in a mixture of 50% glycerol : 50% water (v : v).

Selection Experiment

We chose eight carbon substrates involved in different pathways of *Pseudomonas* metabolism. These were used to set up eight culture media, each with a single carbon substrate, four with two substrates, two with four substrates, and one with all eight substrates (table A1 in the online edition of the *American Naturalist*). Lines selected in single carbon environments will hereafter be referred to as simple lines, and lines selected in two-, four-, or eight-carbon environments will be referred to as complex lines. This is a somewhat artificial division but is necessary to simplify the analysis. We grew populations in 96-well plates, with

each well containing an M9 salt solution (NH_4Cl 1 g/L, Na_2HPO_4 6 g/L, KH_2PO_4 3 g/L, NaCl 0.5 g/L) supplemented with a high concentration of pantothenate (2.4×10^{-3}%) and a mixture of carbon substrates. We maintained a constant concentration for each substrate (0.3 g/L per substrate) rather than maintaining a constant total concentration for each environment. This decision was made so that the rewards of specializing on a specific substrate were equal at all levels of complexity. Every 24 h, we transferred selection lines by using a 96-pin replicator to "print" the populations grown on a selection plate onto a fresh selection plate. The replicator transfers 0.06–0.07 μL of culture ($\sim 10^5$ cells) on each pin to give a dilution factor of approximately 3,000-fold per transfer. After each transfer, we diluted and plated one replicate line from each selection environment on indicator plates containing a low concentration of pantothenate. For each of these lines, we counted the ratio of colonies from each marker state using a ProtoCOL SR/HR counting system (Synoptics, Cambridge, UK). A sudden deviation in marker frequency would indicate that either a marked or unmarked genotype was linked to a beneficial mutation on its way to fixation. Monitoring marker frequencies allowed us to confirm that adaptation was occurring in the selection experiment. Because of the large population sizes and large number of colonies counted ($\sim$100), it is very unlikely that deviations would be caused by drift. We continued the selection experiment for 80 transfers, which is equal to $\sim$900 generations. After the final transfer, we plated $\sim$100 colonies from all replicate lines to record the proportion of lines from each selection environment that had fixed for one of the marker states. Fixation was declared when one of the marker states reached a proportion >98%.

Assay

At the end of the selection experiment, we froze all of our evolved lines at -80°C in a mixture of 50% glycerol : 50% water (v:v). Before our assay, we reconditioned cultures in 96-well microplates containing dilute M9KB medium (NH_4Cl 0.1 g/L, Na_2HPO_4 0.6 g/L, KH_2PO_4 0.3 g/L, NaCl 0.05 g/L, glycerol 1 g/L, proteose peptone 2 g/L) at 28°C for 2 days. Two replicates of the ancestral clones of SBW25 and SBW25ΔpanB were also reconditioned with the evolved lines. We serially diluted and plated out cultures on indicator plates containing a very low concentration of pantothenate. We then randomly picked two (for one- and two-carbon-selected lines), four (for four-carbon-selected lines), or seven (for the eight-carbon-selected line) colonies from two lines of each selection environment. We only used lines that showed marker fixation to ensure that adaptation had occurred within the populations. A greater number of colonies were picked from environments with more carbon sources to facilitate finding genotypes that had specialized on different carbon substrates. We grew the selected colonies in dilute M9KB medium at 28°C for 2 days. We diluted and starved cultures in M9 salt solution for at least 2 h before the assay began. We then added 20 μL of starved cells (10^6 viable cells) from each culture to 96-well plates. Each well on each plate contained 180 μL M9 solution plus 0.3 g/L of one of the carbon substrates used in the selection experiment. We scored three replicates of each genotype in every assay environment (3 replicates × 78 genotypes × 8 assay environments).

We measured optical density at 600 nm using a Synergy HT narrow beam plate reader (Biotek Instruments, Winooski, VT) at 24 h $\pm$ 15 min of incubation at 28°C so that the assay conditions were identical to those experienced by each population during one transfer in the selection experiment. The optical density score of any given well reflects the scattering of light by bacterial cells. Optical density increases asymptotically with cell density, becoming saturated at very high cell density (R. C. MacLean, unpublished data). We have used optical density as a measure of growth because it can be measured much more rapidly and accurately than cell density. We corrected optical densities by subtracting control well scores from each absolute score. We calculated the direct response to selection of each replicate of a genotype as the difference in corrected optical density between the evolved genotype and the ancestral clone, on the substrates that the genotype was selected on. We measured the correlated response to selection as the difference between the evolved genotype and the ancestral clone, on substrates that the genotype was not selected on. The responses to selection for each line were calculated as the mean of the replicates from all genotypes in a line.

Statistical Analysis

The variance of the selection response within and between replicate lines was calculated using ANOVA with JMP 4.0 software (SAS Institute, Cary, NC). In forming expected mean squares, lines and genotypes have been taken as random while assay and selection environments have been taken as fixed. The justification is that the specific lines used in the assay were chosen randomly from the subset of lines that had fixed for one marker state. The likelihood that one replicate line will achieve marker fixation before another from the same selection environment is assumed to be random. Therefore, each can be regarded as a representative *Pseudomonas* population. Similarly, the genotypes used from each line were picked randomly from the populations growing on agar plates and can be considered a representative sample from the population. In contrast,

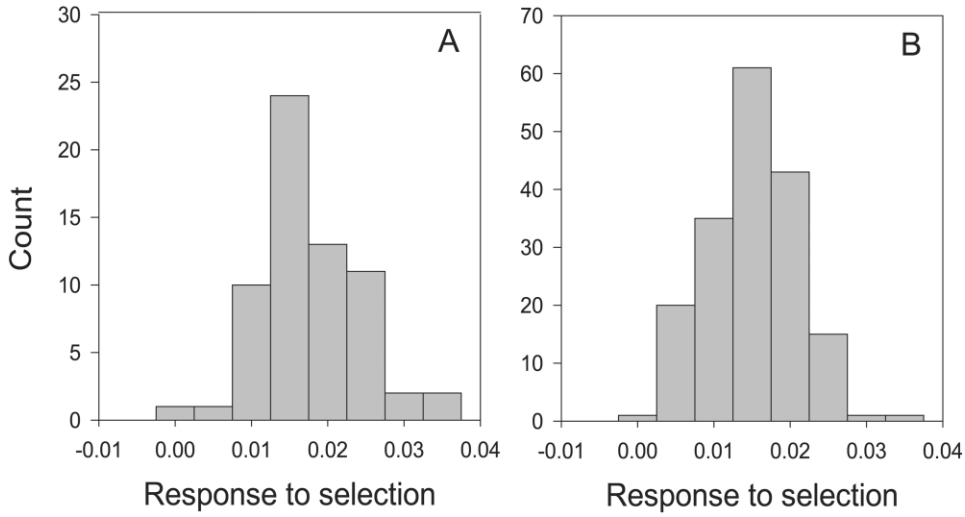

Figure 3: Frequency distribution of direct (*A*) and correlated (*B*) responses to selection for evolved lines. Almost all responses were >0. The mean direct response was 0.015 ($n = 64$, SE = 0.002), and the mean correlated response was 0.012 ($n = 177$, SE = 0.001).

the selection and assay environments were predefined and cannot be taken to be representative of natural habitats.

The variance within populations was further analyzed by decomposition into genetic, environmental, and genotype-by-environment (G × E) interaction components. Finally, G × E variance was partitioned into "inconsistency" and "responsiveness" components. Robertson (1959) showed that the G × E variance of a collection of genotypes tested in two environments can be expressed as

$$\sigma^2_{GE} = \frac{(\sigma_{G1} - \sigma_{G2})^2}{2} + \sigma_{G1}\sigma_{G2}(1 - \rho_{G1G2}), \qquad (1)$$

where σ_{G1} and σ_{G2} are the genetic standard deviations of a character expressed in environments 1 and 2, respectively, and ρ_{G1G2} is the genetic correlation of that character across environments 1 and 2. This equation can be rearranged to interpret G × E for a slightly different scenario (Cockerham 1963; Bell 1990; Cooper and Delacy 1994; Wu and Stettler 1997). If we instead test two genotypes over a range of environments, then Robertson's equation becomes

$$\sigma^2_{GE} = \frac{(\sigma_{E1} - \sigma_{E2})^2}{2} + \sigma_{E1}\sigma_{E2}(1 - \rho_{E1E2}). \qquad (2)$$

In this case, σ_{E1} and σ_{E2} are the environmental standard deviations of a character expressed by genotypes 1 and 2, respectively, and ρ_{E1E2} is the environmental correlation of that character across the two genotypes. Thus G × E can be partitioned into two components. The first is responsiveness due to differences between environmental variances among genotypes, $(1/2)(\sigma_{E1} - \sigma_{E2})^2$. The second is inconsistency due to lack of complete correlation between genotypes over environments, $\sigma_{E1}\sigma_{E2}(1 - \rho_{E1E2})$. In order to estimate G × E for a population comprising several genotypes over a range of environments, we must take the means of these components over all pairwise combinations of genotypes:

$$\sigma^2_{GE} = \sum \frac{(\sigma_{Ei} - \sigma_{Ej})^2}{2G(G-1)} + \sum \frac{\sigma_{Ei}\sigma_{Ej}(1 - \rho_{EiEj})}{G(G-1)}, \qquad (3)$$

where G is the number of genotypes chosen from the population. We calculated the proportion of responsiveness versus inconsistency in the G × E of each population and then compared populations selected in simple versus complex environments.

Results

Experimental Evolution

We found evidence for periodic selection: after 900 generations of selection, 68% of replicate lines had fixed for one of the two marker states (fig. 2 in the online edition of the *American Naturalist*). Fixation was equally likely in simple and complex media ($\chi^2 = 0.04$, df = 1, $P = .84$). However, fixation was more likely to occur in simple or complex media containing serine than in other media ($\chi^2 = 10.00$, df = 1, $P = .002$), showing that some of the

Table 1: ANOVA for the response to selection testing effects of assay substrate (A), selection environment (S), line (L), and genotype (G)

Source	df	df denominator	Mean square ($\times 10^{-4}$)	F	P
S	14	15	.404	3.78	<.01
A	7	105	116.500	108.97	<.001
S × A	98	105	.752	7.03	<.001
L(S)[a]	15	48	.000	<1	NS
L(S) × A[a]	105	336	.005	<1	NS
G(L(S))[a]	48	336	1.061	9.92	<.001
G(L(S)) × A[a]	336	1243	.148	1.39	<.001
Replicate, r	1,243		.110		

Note: All simple and complex selection lines are included in the analysis. NS = not significant.

[a] Random effects tested with restricted maximum likelihood.

beneficial mutations that became fixed had effects specific to a particular substrate.

Adaptation in Simple Media

The ancestor showed relatively poor growth in all environments (table A2 in the online edition of the *American Naturalist*). The lines cultured in simple media became adapted to them: the mean direct response was 0.015 (SE = 0.002), representing an increase of 150% over the ancestor. The response varied substantially over lines (SD = 0.008; fig. 3), with an observed range of −0.005 to 0.047. The direct response to selection on a given substrate was negatively correlated with the performance of the ancestor on that substrate ($P < .001$, $r^2 = 0.22$) so that there was more progress in more stressful environments.

The correlated response to selection was positive (mean = 0.012, SE = 0.001): lines cultured on simple media grew better than the ancestor on substrates neither had previously encountered. The direct response exceeded the correlated response (paired $t = 2.26$, df = 27, $P = .032$), so adaptation was to some extent specific to the substrate on which a line was cultured. This is reflected in the significant selection × assay environment interaction in the overall analysis of variance (table 1). The difference between the direct and correlated responses was quite small, however (mean difference across lines = 0.003, SD = 0.006), equivalent to 16% of the mean growth of the lines. There was no significant correlation between direct and correlated responses ($P > .05$, $r^2 = 0.13$).

Adaptation in Complex Media

Lines cultured on complex media had greater average growth, when assayed on each substrate separately, than lines cultured on simple media (fig. 4 in the online edition of the *American Naturalist*). This superiority was shown by the growth of the lines themselves ($t = 2.07$, df = 27.5, $P = .048$) and by the mean growth of genotypes isolated from the lines and assayed separately (pooled $t = 2.53$, df = 76, $P = .014$). Moreover, lines cultured on a complex medium (e.g., with composition ABCD) had on average the same growth on any of its constituents (e.g., A) as the lines cultured on simple media containing this substrate alone (paired $t = -0.69$, df = 7, $P = .51$; fig. 4).

The Fitness Rank Curve

For any given genotype, substrates can be ranked in order of growth from most to least. We call this the fitness rank curve. Combining these curves for several genotypes gives a useful visual summary of the differences between lines or treatments (fig. 5). Note that the identity of the substrate corresponding to a particular rank may, and usually will, differ from genotype to genotype. The curve for each genotype, and the band of curves from a group of genotypes, necessarily slopes downward from the highest-ranking to the lowest-ranking substrate. The slope of the curve or band of curves represents environmental variance: a steeper slope reflects a greater difference between the highest-ranked and the lowest-ranked substrates and thus greater environmental variance. The width of the band of curves reflects genetic variance among genotypes.

Plasticity and Genetic Diversity

One possible outcome of this experiment was that lines cultured on complex media would evolve greater plasticity (similar growth on all or most substrates) than lines cultured on simple media (good growth on the substrate of selection but poor growth on others). If this were the case, it would be reflected by the fitness rank curves, which

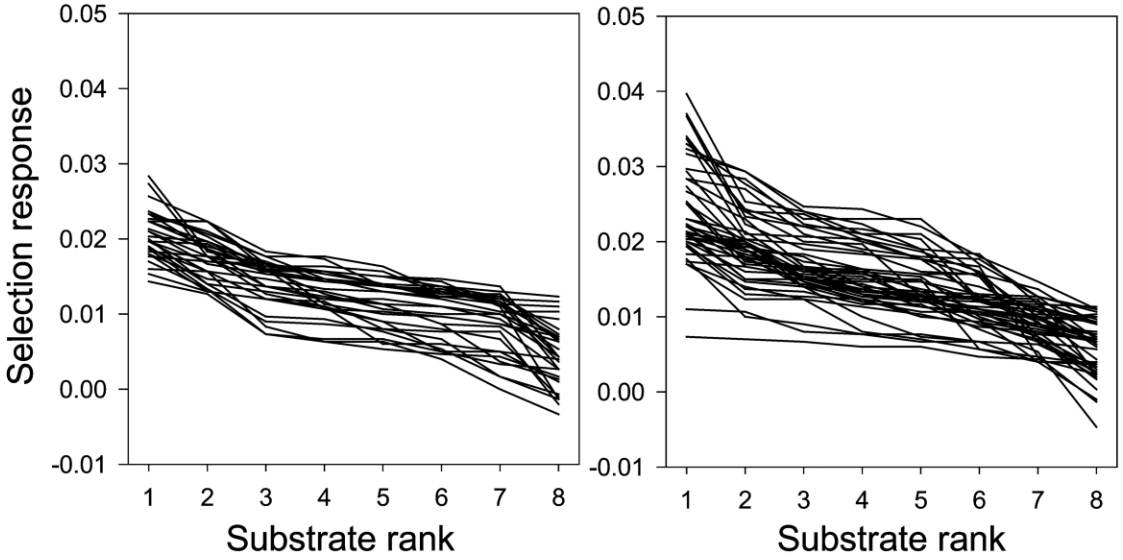

Figure 5: Ranked substrate by fitness. Each line represents the fitness of a genotype across different substrates. Substrates are ranked by decreasing fitness of each individual genotype, so the substrate at each rank may be different for different genotypes. Increased separation between responses indicates greater genetic variance among genotypes. Increased slope indicates greater environmental variance in response. Simple-selected genotypes (*right*; $n = 32$) had lower mean ($t = 2.56$, df = 69.4, $P = .01$) and variance ($t = 2.14$, df = 65.9, $P = .04$) than complex-selected genotypes (*left*; $n = 48$).

would slope down more steeply for simple than for complex environments. This is not supported by our results (fig. 5), which show that environmental variance was marginally greater among genotypes isolated from lines cultured in complex media than among those from simple media ($t = 2.14$, df = 65.9, $P = .036$). There is substantial genetic variance within the lines, however, even though they had been fixed for a single genotype through a selective sweep in the recent past (table 1). Estimates of the genetic variance component are greater for lines selected in complex media than for those selected in simple media ($t = 2.37$, df = 28, $P = .025$; fig. 6 in the online edition of the *American Naturalist*). This is reflected by the greater thickness of the band of fitness rank curves for the complex environments (fig. 5). There is a modest but significant G × E (table 1). This is much larger in lines cultured on complex media; it is almost absent from lines cultured on simple media (fig. 6). The estimate of inconsistency was greater than zero in 14/14 lines from complex media but in only 5/16 lines from simple media. Consequently, the fraction of G × E attributable to inconsistency was much greater in lines from complex media (65%) than in lines from simple media (24%; Mann-Whitney $U = 156$, $P = .02$; fig. 7).

Discussion

The theory of coexistence in mixtures of substitutable substrates has been exhaustively analyzed in the ecological literature, especially from the point of view of how many species can be maintained on a given number of substrates (MacArthur and Levins 1964; MacArthur 1969; Stewart and Levin 1973). The most general result is an extension of Gause's exclusion principle that the number of species stably coexisting cannot exceed the number of distinct substrates. This holds for competition in chemostats (Taylor and Williams 1975), for nonsubstitutable resources (Tilman 1977), and for spatially structured environments (Strobeck 1975). Thus, when a single genotype is cultured in a complex medium, we might expect to observe either the evolution of a single broadly adapted generalist or an adaptive radiation involving a diversity of specialized types. What we actually found lay between these two extremes.

The fixation of the neutral marker in most of the lines bore witness to the passage of at least one beneficial mutation. Despite the fact that the substrates we used support populations of different sizes, we do not believe that fixation was in any instance caused by genetic drift, because mean population size was $\sim 3 \times 10^8$, the population size in the poorest substrate (serine) was $\sim 3 \times 10^7$, and the number of cells directly transferred was $\sim 10^5$. Once the marker had become fixed, it was not possible to identify further change, and the number of substitutions occurring in any given line is not known. It is clear, nonetheless, that evolution in many of the lines proceeded through a series of selective sweeps (periodic selection) and that increased fitness is therefore attributable to adaptive genetic

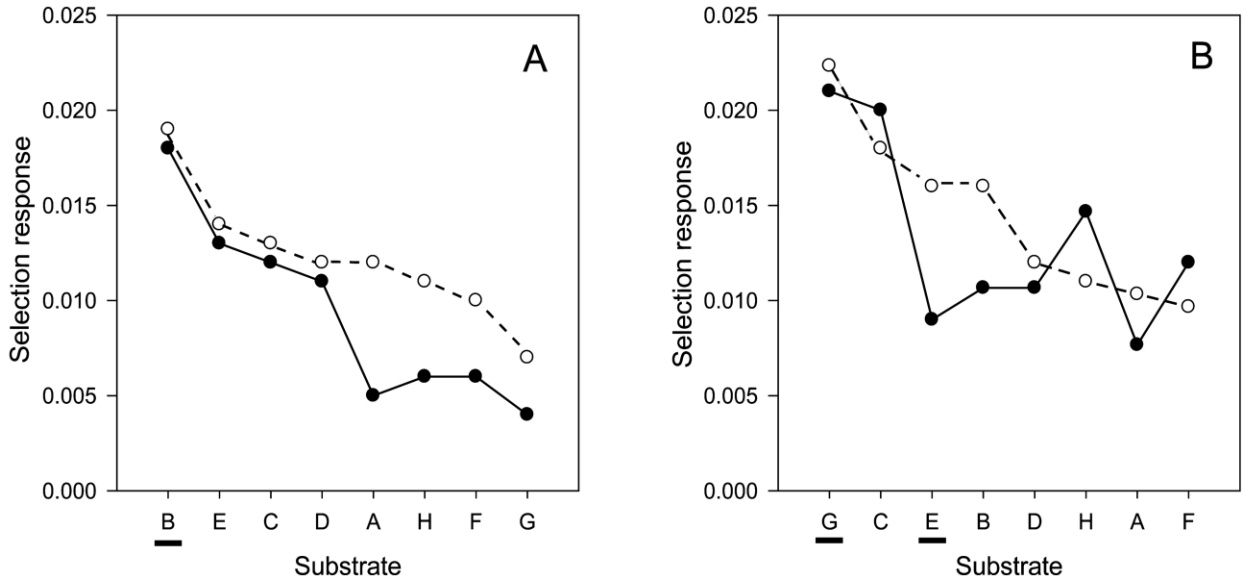

Figure 7: Inconsistency versus responsiveness in genotype-by-environment variance. Each line represents the fitness of a genotype across different substrates. A difference in slope between genotypes indicates that genotypes have unequal variances on different substrates (responsiveness). Intersecting responses indicate a lack of correlation between genotypes on different substrates (inconsistency). *A*, Two simple-selected genotypes from the same population. *B*, Two complex-selected genotypes from the same population. Substrates are ranked by decreasing fitness of the genotype with the highest mean out of the pair. The substrates present in the selection environments are underlined. In complex-selected populations, 65% (SE = 9%) of G × E variance is generated by changes in the ranking of genotypes with respect to fitness among environments. In simple-selected populations, 24% (SE = 10%) of G × E is attributable to this component.

change. Although this is not unexpected, it has seldom been documented in complex media. Dykhuizen and Davies (1980) used two genotypes of *Escherichia coli*, one of which was deleted for *lac*, to study competition in mixtures of lactose and maltose. During the course of the experiment, a novel mutation to constitutive expression occurred in the lac^+ generalist, conferring the ability to grow at low levels of lactose. This was able to coexist with the ancestral strain apparently because of functional interference between the mechanisms responsible for uptake of the two substrates. In very complex undefined mixtures, Maclean et al. (2005) found that metabolic diversity evolved within experimental lines of *Pseudomonas* and was maintained by negative frequency-dependent selection, but they observed no selective sweeps. Thus, adaptation to mixtures of substrates may or may not be accompanied by the periodic fixation of a single genotype. In many cases, the passage of a beneficial mutation may be halted at intermediate frequencies, causing irregular fluctuations in marker frequency.

Lines cultured on simple media became specifically adapted to a single carbon substrate. Responses tended to be greater in stressful environments to which the ancestor was poorly adapted. This finding is consistent with Fisher's geometric analogy of adaptation (Fisher 1930; Orr 2005), in which the expected fitness effect of beneficial mutations is greater in populations further from the optimal phenotype. The correlated response was also positive, on average, so that growth tended to increase on substrates that had not previously been encountered. This constitutes synclinal selection (Bell 1997; Bell and Reboud 1997), which occurs when the response is positive in both selection and novel environments (fig. 8). It is important to recognize that it is still possible to have specific adaptation when selection is synclinal. In this case, although the responses in selection and novel environments are both positive, the response was greater in the environment of selection. Synclinal selection must be fueled by beneficial mutations that are capable of enhancing growth on several substrates at the same time or to more general conditions of growth. It is not difficult to imagine that genes affecting transport or regulation might be responsible, although we have not attempted to identify them. In any case, we can distinguish between two categories of beneficial mutation: one confers specific adaptation to a single substrate, whereas the other confers broader adaptation to a range of substrates. Adaptation to simple media is in part attributable to mutations of specific effect, but the positive correlated response shows that it is also in part attributable to mutations of broad effect. The magnitude of the correlated

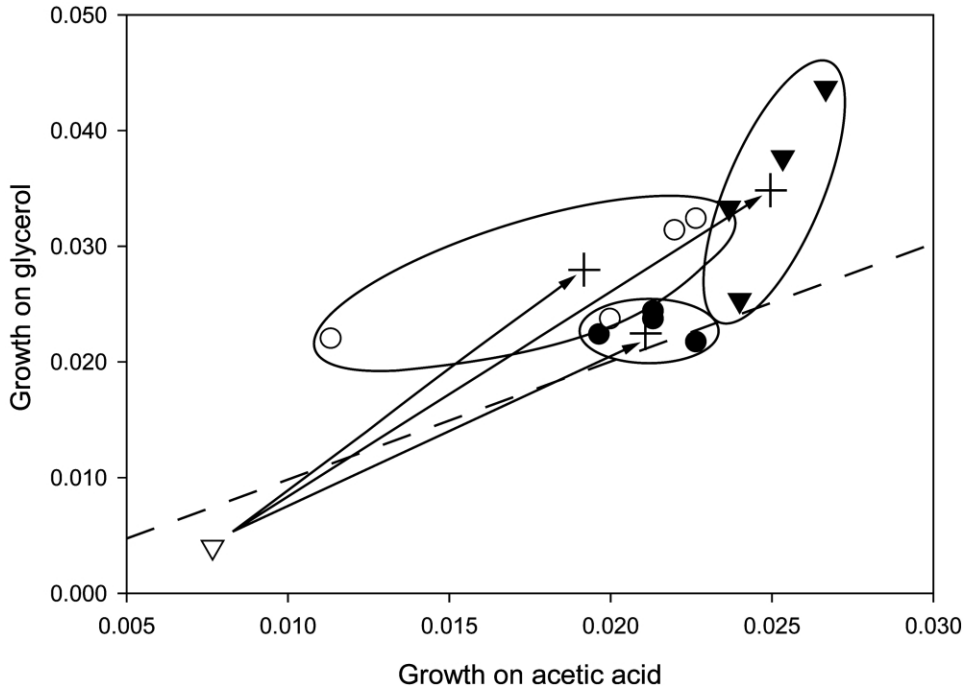

Figure 8: Example of synclinal selection. Each point represents the growth score of a genotype on glycerol and acetic acid. Solid circles represent acetic acid–selected genotypes, open circles represent glycerol-selected genotypes, solid triangles represent glycerol/acetic acid mixture–selected genotypes, and the open triangle represents the ancestor. Crosses represent mean growth score for genotypes from each selection treatment. Arrows reflect the mean response to selection for genotypes from each selection treatment. The dashed line is a plot of the equation $y = x$.

response relative to the direct response suggests that mutations of broad effect have been responsible for most of the observed response to selection. These results are consistent with previous work in single carbon environments (MacLean and Bell 2002).

In complex media, the lines were able to adapt simultaneously to several substrates to about the same extent that lines cultured in simple media adapt to each. This finding is consistent with recent work in viruses, which has shown that concurrent adaptation to several hosts does not limit ability to grow on each host separately (Novella et al. 1999; Weaver et al. 1999; Turner and Elena 2000). As with the positive correlated responses in simple media, the direct responses in complex media suggest selection of mutations of broad effect. The concentration of each substrate was the same in simple and complex media, creating the opportunity for the evolution of a community of specialists, each growing well only on one of the constituent substrates. Instead, the outcome of selection in complex media was the evolution of generalists. Despite this, the presence of substrate-specific effects, such as the increased likelihood of fixation occurring in environments containing serine, suggests that specific adaptation to a single substitutable resource is possible for certain substrates. Thus, as in simple media, adaptation in complex media is due in part to mutations of specific effect.

The mean fitness over all substrates was greater for genotypes isolated from the complex media. The average selection response for complex-selected lines is determined through a varying proportion of direct and correlated responses, depending on the level of complexity in the specific line. In contrast, the average selection response for simple-selected lines is largely due to correlated responses. Thus, the direct and correlated responses to selection on the complex media exceeded the correlated response to selection in the simple media. This might be attributable to the selection of mutations specific to each of a number of the substrates present in a complex medium. One objection to this interpretation is that such mutations, spreading through an asexual population, would interfere with one another as well as with any mutations of broad effect (Gerrish and Lenski 1998; Miralles et al. 1999; Wilke 2004). Alternatively, conditionally neutral mutations might accumulate more readily in simple media. These are mutations that obstruct the utilization of a particular substrate or substrates; they are neutral in media that lack the substrate but deleterious when the substrate is present (Kawecki 1994; Fry 1996; Whitlock 1996; Kawecki et al.

1997; Cooper and Lenski 2000). In complex media, they are more likely to be deleterious because they reduce growth below the level achieved when all available substrates are utilized. Hence, they will be more effectively eliminated by selection and will occur at lower frequency.

Despite the evolution of generalists in complex media, these populations display more genetic variance than those in simple media. A number of genotypes could be maintained, in principle, by negative frequency-dependent selection if the enhanced ability to utilize one substrate were accompanied by a loss of ability to utilize others (Haldane 1932; Dempster 1955; Ayala and Campbell 1976). If the loss were complete, indeed, the situation would differ little from divergent selection in allopatry. We did not find such clear-cut trade-offs, however, nor did the lines cultured in complex media come to consist of narrow specialists. We did find that lines from complex environments expressed substantial G × E and that much of this variance was attributable to inconsistency. Thus, they consisted neither of narrow specialists nor of complete generalists. They seem instead to be mixtures of overlapping imperfect generalists, each of which has become adapted to a certain range of substrates but not to all those available.

Most experiments with microbes use growth media with a single limiting carbon source, and in batch culture experiments, this substrate is initially present at high concentration. In our experiment, several substrates were available but again at high concentration. Such experiments are likely to favor the evolution of specialists either because no alternative substrate is available or because substrates will be used sequentially. In most natural environments, there will be a great variety of potential substrates for growth, but all will be present at low or very low concentration. Very few microbes are exclusive specialists on a single substrate because very few substrates will be available at concentrations capable of supporting growth. On the other hand, none can consume all the substrates they encounter. The metabolic profiles of bacteria and yeasts show that almost all species can consume a more or less broad range of substrates, whereas the identity of these varies among species. This is, indeed, the basis of identifying microbial taxa on the basis of their patterns of substrate utilization (Stanier et al. 1966; Victorio et al. 1996; Anderson et al. 2002; Dawson et al. 2002). Experiments using complex mixtures of many substrates, each present at low concentration, would be likely to select for broad generalization, although none have yet been reported. Our experimental design involving unrealistically high substrate concentrations was predisposed to select for the evolution of a diverse community of narrow specialists, each specifically adapted to a single substrate. The fact that we observed instead the evolution of overlapping imperfect generalists suggests that this may be the usual outcome of selection in complex environments.

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