Experimental Adaptive Radiation in Pseudomonas

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ABSTRACT: We studied the importance of selection and constraint in determining the limits of adaptive radiation and the consequences of adaptive radiation in an experimental system. We propagated four replicate lines of the bacterium Pseudomonas fluorescens derived from a single ancestral clone in 95 environments, where growth was limited by the availability of a single carbon source for 1,000 generations. We then assayed the growth of the ancestral clone and the evolved lines in all 95 environments. Evolved lines increased their performance in almost every selection environment and invaded 70% of the novel environments as a direct response to selection. Direct responses tended to be larger in environments where growth was initially poor. Although evolved lines lost the ability to grow on about three substrates that their ancestor could readily grow on, the correlated response to selection was, on average, positive. The correlated response allowed all of our evolved populations to expand their niches and to occupy collectively the remaining novel habitats. This is inconsistent with classical theories of niche evolution. In the most extreme cases, adaptation occurred through "roundabout selection": lineages became adapted to an environment through selection in another environment but not through selection in the environment itself. Our results indicate that mutation accumulation by neutral drift was responsible for generating the majority of costs of adaptation.

Keywords: adaptive radiation, selection, mutation accumulation, antagonistic pleiotropy, BIOLOG, *Pseudomonas*.

Adaptive radiation is the process by which ecological and genetic diversity evolves in a single lineage. Adaptive radiation occurred on a grand scale during the Cambrian diversification of metazoans, or the Devonian emergence of land plants and animals, or the early Cenozoic radiation of mammals; but it also occurs every day on a smaller

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scale whenever a fungal spore finds a discarded sandwich. When a new range of environments becomes ecologically accessible, or when a new range of body plans becomes developmentally accessible, a burst of diversification can rapidly create a new community of specialized types. The great radiations of the past have been extensively studied, but to understand them, we are limited to comparative studies that analyze the patterns of change in the few instances that time has provided us with. The processes that are involved can be determined only through experiment under controlled conditions. Experimental studies using microorganisms have contributed greatly to our understanding of adaptation to single environments (see reviews in Dykhuizen 1990; Lenski and Travisano 1994; Travisano et al. 1995), but adaptive radiation is a question that these studies have only recently begun to address (Rainey and Travisano 1998; see review in Travisano and Rainey 2000). We have carried out an extensive study involving selection in nearly 100 different environments in order to establish with more confidence the causes and consequences of adaptive radiation.

Adaptive radiation begins when a population invades a range of novel habitats. To begin with, the colonists have only a rudimentary ability to survive and persist in these novel habitats, but selection will favor any genotypes that improve their fitness in their new environment, and over time the operation of different selective pressures in different novel habitats should give rise to a range of ecologically and genetically distinct subpopulations (Schluter 2000). We want to address two features of this process. The first concerns the extent of the radiation. If selection is free to produce adaptation in more or less any circumstances, then the radiation will end with the occupation of all novel habitats. However, if adaptation is often constrained by the physiological and genetic properties of organisms, then only a part, perhaps a small part, of the novel habitats will be occupied.

The second feature is the consequences of the radiation. One of the most important theories in evolutionary ecology is that no single genotype can simultaneously maximize its fitness in all environments; specializing on a single habitat should be accompanied by a cost. According to classical theories of niche evolution, strict trade-offs exist in the ability of genotypes to survive and reproduce under different conditions; the jack-of-all-trades is master of none (Levins 1968; Roughgarden 1972; Lynch and Gabriel 1987; Wilson and Yoshimura 1994; see reviews in Futuyma and Moreno 1988; Kassen 2002). Implicit in this theory of niche evolution is the assumption that mutations display antagonistic pleiotropy, that is, that alleles that are favorable in one environment have consistently deleterious effects on fitness when expressed in other environments. The alternative theory of niche evolution, the drift theory, assumes that antagonistic pleiotropy is not a common phenomenon. This theory argues that specialists pay a cost because they cannot prevent the stochastic accumulation of mutations that are neutral in the environment the specialist inhabits but that reduce fitness in environments that no longer make a significant contribution to the reproduction of the specialist (Fry 1996; Whitlock 1996; Kawecki et al. 1997). In summary, costs of adaptation can be generated by two distinct population genetic processes: mutation accumulation and antagonistic pleiotropy. If costs are predominantly generated by pleiotropy, adaptation and cost should be tightly coupled. If costs arise as a consequence of mutation accumulation, costs should arise in a stochastic manner that is not coupled to adaptation.

The contribution of drift and pleiotropy to generating costs is an issue that has only begun to be explored (see review in Kassen 2002). Antagonistic pleiotropy has been argued to cause costs of adaptation when Escherichia coli is allowed to adapt to novel culture environments (Cooper and Lenski 2000), when Trichogramma is selected at different temperatures (Carrière and Boivin 2001), and when viruses are selected on cell culture lines (reviewed in Ebert 1998). Adaptation to autotrophic and heterotrophic metabolism in Chlamydomonas incurs a cost that is primarily due to mutation accumulation (Reboud and Bell 1987). We propose that the contribution of antagonistic pleiotropy and mutation accumulation to the overall cost of adaptation can be assessed through the relationship between direct responses to selection (i.e., adaptation) and the costs of adaptation expressed by replicate selection lines of bacteria propagated in a common environment. If antagonistic pleiotropy contributes to the cost of adaptation, there would be a positive correlation between the direct response to selection and the cost of adaptation. If mutation accumulation contributes to the cost of adaptation, the magnitude of costs of adaptation would be independent of the direct response to selection. Thus, the regression of the cost of adaptation on the direct response to selection partitions the two sources of cost (fig. 1). The intercept is a measure of the contribution of mutation accumulation: an intercept that is >0 indicates that mutation accumulation is responsible for part of the overall



Direct response to selection

Figure 1: The three possible outcomes of the regression of the cost of adaptation on the direct response for replicate populations of bacteria propagated in a common environment. A positive slope and intercept (A) indicate that both mutation accumulation and antagonistic pleiotropy contribute to the cost of adaptation. A positive intercept and negative slope (B) indicate that only mutation accumulation contributes to the cost. A negative intercept and positive slope (C) indicate that only antagonistic pleiotropy contributes to cost.

cost. The slope is a measure of the contribution of antagonistic pleiotropy: a slope >0 indicates that antagonistic pleiotropy is responsible for part of the cost. It is quite conceivable that both slope and intercept will be positive; in that case, the proportion of the overall cost attributable to mutation accumulation and antagonistic pleiotropy can be calculated for any given direct response. It is also quite possible that this proportion will vary among the environments in which selection is practiced and among the environments in which selection lines are assayed; no published study is sufficiently extensive for these possibilities to be evaluated.

The main objectives of our experiment were thus to determine the importance of selection and constraint in setting limits to the extent of adaptive radiation, to determine the extent of costs of adaptation, and to determine the contribution of mutation accumulation and antagonistic pleiotropy to generating these costs. These objectives demanded the construction of a large environment containing many novel habitats, inoculated with a population able to undergo an extensive adaptive radiation within a short space of time, whose subsequent performance in all habitats could be monitored rapidly and reliably. We found that these requirements were met by the growth of the common aerobic bacterium *Pseudomonas fluorescens* on BIOLOG plates, commercially available 96-well microplates in which each well contains a single carbon source and an indicator dye of carbon metabolism. Inoculating a bacterial strain onto a BIOLOG plate allows two types of measurement to be made concerning the strain: a qualitative score, whether or not the strain can grow on a given compound, and a quantitative score, the extent of growth on a given substrate. The 95 carbon-limited environments of the BIOLOG plate provide the ecological opportunity that is a prerequisite for adaptive radiation; the propagation of cultures of *Pseudomonas* on BIOLOG plates thus enables us to study both the extent and consequences of a simulated large-scale adaptive radiation.

Material and Methods

Ancestral Strain

A single "smooth" morph clone of strain SBW25 of *Pseudomonas fluorescens* was used to found the four replicate selection lines used in this experiment. The ancestral clone was kept frozen at -80° C during the experiment in a mixture of 50% glycerol : 50% water (v : v).

Transfer Protocol

Populations of bacteria were grown on commercially available 96-well BIOLOG (Hayward, Calif.) GN2 microplates supplemented with growth medium. Each well on a BIOLOG plate contains a simple agar medium and a tetrazolium dye that acts as an indicator of oxidative carbon metabolism. Ninety-five wells on the plate contain a unique single carbon compound, and one well acts as a carbon-free control. All of the 95 BIOLOG environments can be classified according to the following scheme on the basis of chemical structures (Garland and Mills 1991): polymers (5), carbohydrates (28), esters (2), carboxylic acids (24), brominated chemicals (1), amides (3), amino acids (24), amines (3), alcohols (2), and phosphorylated chemicals (3).

Daily transfers were carried out under sterile conditions in a laminar flow hood using autoclaved materials and septic techniques. A 96-pin replicator (Nalge, Aarhus, Denmark) was used to "print" the populations grown on a selection plate onto a fresh selection plate. The 96-pin replicators are specifically designed to replicate the contents of one microplate to another without cross-well contamination, and in preliminary experiments to test the transfer protocol, we failed to detect cross-well contamination. Our replicator transfers 0.06–0.07 μ L of fluid on each pin to give a dilution factor of approximately 2,500fold per transfer, representing 11 doublings. The experiment was continued for 100 transfers, or about 1,100 bacterial generations.

In order to sustain bacterial cultures on BIOLOG plates over long periods of time, it is necessary to supplement the BIOLOG growth medium with a source of nitrogen. We used KB (King's medium B: glycerol 10 g/L, proteose peptone 20 g/L, K₂HPO₄ 1.5 g/L, MgSO₄ 1.5 g/L) as a source of nitrogen and as an additional source of carbon for our Pseudomonas cultures during this experiment. Before each transfer, 150 μ L (100 μ L for the first eight transfers) of liquid medium was added to each well. For the first four transfers, the added medium contained 34 μ L of KB in sterile distilled water; this quantity was reduced by 2 µL per transfer until transfer 20. Two microliters of KB continued to be added to each well until transfer 47, after which glycerol was removed from the KB. For the rest of the experiment, the wells received only 2 μ L of depleted KB, which provided a source of 4 \times 10⁻⁵ g/well of peptides consisting of a mixture of amino acids that our cultures likely used primarily as biosynthetic precursors.

Assay

At the end of the experiment, all of our evolved lines were frozen at -80°C in a mixture of 50% glycerol : 50% water (v:v). Before our assay, cultures were reconditioned in 96-well microplates containing KB medium at 28°C for 1 d. Reconditioned cultures were then transferred to fresh microplates containing KB medium and were incubated at 28°C for 2 d before the assay to give stationary phase cultures. Our cultures were then diluted 150-fold in M9 salt solution and were starved for at least an hour and a half before the assay began. One hundred fifty microliters of starved cells ($\approx 1 \times 10^5$ viable cells) from each culture were added to each well on BIOLOG GN2 microplates. The absorbance of the microplate was read immediately at 660 nm using an EL_x800 narrow beam plate reader (Bio-Tek Instruments, Winooski, Vt.) and again after 24 h \pm 5 min of incubation at 28°C. The assay was carried out on four different days, with one replicate selection line being assayed on each day along with two replicates of the ancestral clone.

The absorbance score of any given well reflects both the absorbance of light by the tetrazolium dye and the scattering of light by bacterial cells. To minimize the absorbance of light due to tetrazolium, we measured our cultures 70 nm away from the absorbance peak of tetrazolium (590 nm). Under our assay conditions, the absorbance scores for the 95 wells on otherwise identical BIOLOG plates with tetrazolium (GN2) and without tetrazolium (SNF2) were well correlated with each other (1 strain × 95 wells = 95 scores, r = 0.7).

Data Collection

The raw absorbance score for each well, ΔA , was calculated as the increase in absorbance of the well over the 24-h incubation period, corrected by subtracting the reading of the blank control. We suspect that physical gradients in temperature exist in our incubator within stacks of BIOLOG plates and that minute differences in temperature exist within the incubator between assay days. The evidence for this is the significant differences in the average well color development of the base population measured on different days. We compensated for this variation by using a model in which the change in absorbance ΔA increases asymptotically over time *t* toward the maximum possible absorbance reading A_{max} :

$$\Delta A = A_{\max}(1 - e^{-kt}),$$

so that the slope parameter k can be estimated for each well from

$$k = -\frac{1}{t} \ln\left(1 - \frac{\Delta A}{A_{\max}}\right).$$

This value differs among plates, presumably because of microenvironmental variance. We calculated the mean value of *k* for each of the four plates on which the four replicate lines selected on a given substrate were scored and used the largest of these means to recalculate the value of ΔA , ΔA_{corr} , for each well on each plate.

In order to estimate the error inherent in this technique, we calculated the correlation between replicate measures of the same strain on the same day and the correlation among replicate measures of the same strain on different assay days. The average correlation among the $\Delta A_{\rm corr}$ values for replicate measurements of the ancestral clone on the same day (1 line × 95 wells = 95 scores) was 0.979; measuring the same strain twice on the same day yielded essentially identical results. The correlation among two measurements of replicate selection line 1 (95 lines × 95 wells = 9,025 scores) on different days was 0.87; measuring the same strain on different days yielded somewhat different, but highly correlated, scores.

A more straightforward means of interpreting BIOLOG scores is to determine the ability of a given strain to either grow or not grow in each BIOLOG well. On the basis of preliminary data and the visual inspection of plates, those wells with a corrected absorbance >0.025 were considered positive for growth while wells with absorbance <0.025 were considered negative for bacterial growth. The eight replicate measurements of the ancestral clone always produced visible growth (i.e., $\Delta A_{\rm corr} > 0.025$) in 46 wells; these wells represent substrates to which the ancestral clone is

preadapted. They can be thought of as the BIOLOG niche of the ancestral clone. Numerous other experiments that we have carried out involving >20 measurements of the ancestral clone on BIOLOG plates with and without tetrazolium also identified positive growth in these wells. In five wells, the ancestral clone yielded average values of $\Delta A_{corr} = 0.025$ -0.08 that were scored as positive on some occasions and negative on others. In the remaining 44 wells, the ancestral clone consistently yielded negative growth results.

Results

The Direct Response to Selection

The direct response to selection for each replicate of a selection line was calculated as the difference in corrected absorbance between the evolved line and the ancestral clone for the substrate on which the line was selected. Replicate selection lines were always compared with the mean of two replicates of the ancestral clone that were measured on the same day as the evolved line. The mean direct response for the 95 selection lines, 0.236, was significantly greater than 0 (t = 9.74, df = 94, $P \ll .001$), showing that average growth was increased by selection. The response varied widely among lines (fig. 2), however, and in many cases, the direct response was close to 0. These involved both lines that failed to evolve the ability to grow on their novel selective substrate and lines selected in the ancestral niche whose performance did not improve. There were 13 substrates on which the ancestral clone



Figure 2: Frequency distribution of direct responses to selection for evolved lines. The direct response to selection was calculated as the mean difference in corrected absorbance between the four replicates of a selection line and the ancestral clone population for the substrate on which the line was selected. The mean direct response for all 95 lines was 0.236 (SD = 0.334).

never grew and on which at least one replicate selection line failed to grow (table 1).

Variation of the Direct Response

ANOVA shows that the direct response to selection varied among chemical categories of substrate and among selection lines within these chemical categories (table 2). This result continues to hold when substrates on which one or more replicate lines failed to grow are excluded. For the three principal classes of carbon compounds, the direct response to selection was greater among carbohydrates than among amino acids and carboxylic acids.

It has been suggested that adaptation should occur more rapidly in stressful environments closer to the edge of the niche (Bennett et al. 1992). More generally, it might be expected that the advance under selection on a given substrate will be negatively correlated with the performance of the ancestral clone on that substrate. This relationship was found in our lines (fig. 3; F = 12.75, df = 1,93, $P < .001, r^2 = 0.12$). The alternative explanation for this result is that it simply reflects the inability of our assay to detect changes in growth beyond a certain point. If this were the case, we would expect that the growth scores of the evolved lines should cluster around a maximal threshold, irrespective of the growth of the ancestral clone in the environment in which the lines were selected. Our results clearly show that this pattern did not occur (fig. 4) in our lines.

 Table 2: Nested ANOVA for the direct response to selection testing effects of selection environment type and selection line nested within environment type

Source	df	SS	MS	F	Р
Selection environment type ^a	10	9.03	.903	3.71	<.001
Selection line (selection					
environment type)	84	20.45	.243	5.34	<.0001
Error	285	12.98	.045		

^a Carbohydrate, carboxylic acid, amino acid, polymer, esters, amines, alcohols, brominated chemicals, phosphorylated chemicals, and amides.

Qualitative Correlated Responses to Selection

Qualitative correlated responses to selection occurred when evolved lines lost the ability to grow on substrates in the ancestral niche or gained the ability to grow on novel substrates. Most (311/380) of the evolved populations lost the ability to grow on at least one of the 46 substrates in the ancestral niche, the average loss being 2.58 substrates/population (fig. 5). The distribution of losses of function per substrate in the ancestral niche was roughly bimodal (fig. 6); few (i.e., <50) evolved populations lost the ability to grow on most substrates, while a large number of lines (i.e., >50) lost the ability to grow on a relatively small subset of substrates in the ancestral niche. If the losses of function that we measured represent statistical noise that arises as a consequence of measurement error in substrates where growth is weak, we would expect that losses of function should have occurred most often in substrates where the ancestral clone grew poorly. The correlation between the average growth of the ances-

Table 1: Constraints on adaptation to novel substrates

	No. of lii on s	nes that grow substrate	No. of lines that show "strong" growth		
Substrate	As a direct response (4)	As a correlated response (376)	As a direct response (4)	As a correlated response (376)	
α-cyclodextrin	0	26	0	0	
Glycogen	2	188	1	43	
i-erythritol	2	49	0	5	
α-D-lactose	2	288	1	184	
Lactulose	2	270	1	131	
D-melibiose	1	40	1	20	
γ -hydroxybutyric acid	3	105	2	51	
L-ornithine	2	175	0	15	
Glycyl-L-glutamic acid	2	156	1	17	
D-serine	1	67	0	27	
L-threonine	2	224	0	45	
Phenyl ethylamine	2	99	0	70	
2.3 butanediol	1	43	0	2	

Note: Lines were defined as showing growth on a substrate when $\Delta A_{corr} > 0.025$ and strong growth when $\Delta A_{corr} > 0.1$. None of the eight replicate measurements of the ancestral clone gave positive growth scores on these substrates.



Figure 3: The direct response to selection as a function of ancestral performance for all selection environments. Plotted points are the mean direct response to selection of a selection line ± 1 SE and the mean growth of the ancestral clone on the substrate on which the line was selected. The dashed line shows the *X*-axis, and the solid line shows the linear regression.

tral clone on a substrate and the number of times that evolved lines lost the ability to grow on a substrate was weak and only marginally different from what we would expect because of chance alone (r = -.311, t = 2.17, df = (2), 44, P = .035).

Qualitative correlated responses to selection in novel habitats occurred when evolved populations gained the ability to grow in novel substrates that the ancestral clone could not grow in as a correlated response to selection. Remarkably, evolved populations gained the ability to grow on every substrate as a correlated response to selection. In the most extreme cases, evolved populations gained the ability to grow on substrates as a correlated response to selection where the direct response to selection was not effective (table 1). Given that only one measurement was made on each evolved population, some of these positive responses undoubtedly represent false-positive growth scores. As a conservative estimate of the strength of the correlated response to selection, we calculated the number of evolved lines that showed "strong" growth in novel substrates (i.e., $\Delta A_{corr} > 0.1$). The rationale for this cutoff is that any substrates in which the mean growth of the ancestral clone was >0.1 always gave positive growth scores. Using this conservative estimate of the presence or absence of bacterial growth, the result that the correlated response to selection is effective at providing lines adapted to growth on novel substrates continues to hold with the exception of one substrate, α -cyclodextrin (table 1).

Niche Breadth of Evolved Lines

We estimated the BIOLOG niche breadth of selection lines as the mean number of substrates on which the line tested positive for growth. Replicate measurements of the ancestral clone yielded an average niche breadth score of 51 substrates (range = 46–56, SD = 2.73). All 95 selection lines had niche breadths that were >51 substrates, with an average of 78.15 substrates/line (SD = 3.74), and none of the evolved lines had an average niche breadth that was in the same range as the ancestral clone (fig. 7; range = 69.5-85.5).

Quantitative Correlated Responses to Selection

Quantitative correlated responses to selection for an evolved line were calculated as the change in growth, relative to the ancestor, in environments other than that in which the line was selected. Replicate selection lines were always compared with the mean of two replicates of the ancestral clone that were measured on the same day as the evolved line. The frequency distribution of the 94 responses/line \times 95 lines \times 4 replicates/line = 35,720 correlated responses to selection is given in figure 8. The mean correlated response to selection for the 95 selection lines in all 95 environments, 0.176, was significantly >0 (t =58, df = (2), 8, 834, $P \ll .001$) but varied quite broadly (SD = 0.284). The mean quantitative correlated response to selection expressed by evolved lines on substrates in the ancestral niche, 0.114, was smaller than that expressed on substrates outside the ancestral niche, 0.236 (t = 2.31,



Figure 4: Growth of selection lines as a function of ancestral performance. Plotted points are the mean growth of a selection line ± 1 SD and the mean growth of the ancestral clone on the substrate on which the line was selected.



Figure 5: Frequency distribution of negative qualitative correlated responses to selection. For each replicate of a selection line, we calculated the number of substrates in the ancestral niche on which the evolved line lost the ability to grow (i.e., $\Delta A_{corr} < 0.025$). On average, evolved populations lost the ability to grow on 2.58 substrates (SD = 2.30).

df = (2), 93, P = .023), perhaps because correlated responses in novel habitats cannot be negative.

Antagonistic Pleiotropy and Mutation Accumulation

We chose to use the number of substrates in the ancestral niche on which evolved lines lost the ability to grow as our measure of the cost of adaptation. Regression analyses were carried out on the relationship between the direct response to selection and the cost of adaptation for the four replicates of each selection line. Our null hypothesis was that half of the slopes of this regression should be positive due to chance alone. Because negative slopes of this regression are automatically associated with positive intercepts (fig. 1), our null hypothesis was that all of the regressions with positive intercepts, as well as half of the regressions with positive slopes, should be negative due to chance alone. One selection line was excluded from this analysis because all four replicates of the selection line lost the ability to grow on the same number of substrates and a meaningful regression could therefore not be calculated for this line. The number of positive slopes (37/94) was somewhat less than our null expectation of 94/2 = 47positive slopes ($\chi^2 = 4.25$, df = 1, P = .039), while the number of positive intercepts (83/94) was marginally greater than the 37/2 + (94 - 37) = 75.5 positive intercepts that we would have expected due to chance alone $(\chi^2 = 3.78, df = 1, P = .051).$

Local Adaptation

For each of the 95 environments, we calculated a mean performance of residents (the line selected on the substrate) and nonresidents (all other lines). The performance of residents was greater than that of nonresidents in 66 environments (fig. 9), and the average difference between the mean performance of residents and nonresidents, 48%, was highly significant under a paired-sample *t*-test (t = 4.74, df = (2), 94, P < .0001). Figure 9 also shows that there is a highly significant correlation between the performance of residents and nonresidents across all 95 environments (F = 486, df = (1), 1, 94, P < .0001, $r^2 = 0.84$). This correlation is apparently generated by a very strong correlation between the direct and correlated responses to selection expressed in a given environment (F = 354, df = (1), 1, 94, P < .0001, $r^2 = 0.79$).

Discussion

The Direct Response to Selection

Our experiment introduced an isogenic ancestral strain that had some ability to grow on 51 BIOLOG substrates to the new environmental conditions represented by 44 novel substrates. The four replicate lines provided 44 opportunities to acquire novel adaptation, of which 31 led to success. Thus, natural selection during about 1,000 generations caused the occupation of about 70% of the newly available niche space as a direct response to selection.

Increase in performance was more marked for completely novel substrates than for those that were already a



Figure 6: Frequency distribution of qualitative correlated responses to selection in the ancestral niche for different substrates. For each substrate in the ancestral niche, we calculated the number of evolved populations that lost the ability to grow the substrate (i.e., $\Delta A_{\rm corr} < 0.025$) as a correlated response to selection.



Figure 7: Frequency distribution of BIOLOG niche breadths for selection lines. For each selection line, we calculated the mean number of substrates on which replicates of a selection line tested positive for growth (i.e., $\Delta A_{\text{corr}} > 0.025$). All lines had a mean niche breadth that was greater than that of replicate measures of the ancestral clone (mean = 78.1, SD = 3.74).

part of the niche of the ancestral strain. The cause of rapid adaptation at the edge of the niche can be easily understood in the framework of Fisher's (1930) geometrical analogy of adaptation. At the outset of the experiment, populations that were selected in poor habitats (i.e., where initial mean fitness is far from the optimum) could adapt by fixing mutations of large and small effect. However, populations that were selected in good habitats (i.e., where initial mean fitness is close to the optimum) could adapt only by fixing a small number of slightly beneficial mutations. If the optimum is the same across all selection environments, populations selected in poor environments should rapidly increase their performance until they "catch up" with populations selected in good environments. Although several authors have proposed that adaptive evolution may occur more rapidly at the edge of the niche (Bennett et al. 1992; Mongold et al. 1996), the only experimental test of this hypothesis, which involved adaptation at the upper bound of the thermal niche of E. coli, showed no evidence to support this hypothesis (Mongold et al. 1996). Our results show conclusively that there is a negative correlation between the direct response to selection in a given environment and the performance of the ancestral clone in that environment.

In a few environments within the ancestral niche, evolved lines actually performed worse than their common ancestor did. The obvious explanation—fixation of slightly deleterious mutations through drift—is unlikely because of the large number of cells transferred for lines selected in the ancestral niche. A second possibility is that these populations evolved tetrazolium resistance. Tetrazolium, the indicator dye for carbon metabolism, acts as an artificial electron receptor and is deposited intracellularly in its reduced form. Tetrazolium-resistant mutants would be able to prevent the dye from being reduced intracellularly and would, consequently, have a low turbidity/cell density ratio that could be confounded with a loss of fitness. We argue that the likelihood of this having occurred in our selection lines is low given that tetrazolium is nontoxic in *Pseudomonas* (Bochner and Savageau 1977).

The Cost of Adaptation

Both trade-off- and drift-based theories of niche evolution make the common prediction that becoming specialized on a narrow range of environmental variation should carry a cost of adaptation outside of this range of environmental variation. In terms of our experiment, this predicts that selection in a single BIOLOG environment should have created a cost of adaptation in terms of reduced fitness in the ancestral niche of Pseudomonas. In principle, we could have measured the cost of adaptation in terms of either a qualitative or quantitative correlated response to selection. A qualitative measure of the cost of adaptation has several advantages. First, we have a high degree of statistical confidence in our measurement of the number of substrates in the ancestral niche on which evolved lines grew. Second, this measure of cost has a clear ecological interpretation-the number of habitats in the ancestral niche in which evolved lines lost the ability to persist. Finally, experiments in E. coli show that the inability to grow on



Figure 8: Frequency distribution of quantitative correlated responses to selection. Quantitative correlated responses for an evolved line were calculated as the change in growth, relative to the ancestor, in substrates other than that in which the line was selected. The mean of the 94 responses/line \times 95 lines = 8,930 correlated responses was 0.176 (SD = 0.284).



Figure 9: Local adaptation. Plotted points show the mean growth of the line selected on a substrate (residents) and the mean growth of all other lines (nonresidents) on the same substrate. The dashed line is a plot of the equation y = x. Points above the line represent environments where the residents are locally adapted. Points below the line represent environments where the residents are locally maladapted.

specific BIOLOG substrates can arise as a consequence of either antagonistic pleiotropy, when common insertion mutations that knock out the ribose operon become fixed because they confer a selective advantage in a glucoselimited environment (Cooper et al. 2001), or mutation accumulation, when lines that are passaged through extreme bottlenecks fix mutations that cause lines to lose the ability to grow on many BIOLOG substrates (Funchain et al. 2000). The most common mutation in the experiment of Funchain and colleagues was that lines often lost the ability to grow on xylose. At a molecular level, this results from nucleotide additions or deletions to a long string of GC residues upstream of the xylose operon that cause a reading frame shift in the xylose operon.

We found that, on average, evolved lines lost the ability to grow on about 2.5 substrates, or about 5% of the niche breadth of the ancestral clone. It must be noted, however, that this figure is a mean with a substantial variance and that many (69/380) populations did not lose the ability to grow on a single compound in the ancestral niche. Two population genetic processes could have been responsible for generating the cost of adaptation that we observed: mutation accumulation and antagonistic pleiotropy. Mutation accumulation occurs when conditionally deleterious mutations accumulate in a specialist population by drift. It is important to note that these mutations do not impact fitness in the environment in which they occur and are therefore expected to behave in a neutral manner. According to the neutral theory of population genetics, conditionally deleterious mutations become fixed by drift in

an asexual haploid population at a rate equal to the genomic rate of appearance of conditionally deleterious mutations (Kimura 1983). This implies that the contribution of mutation accumulation to generating costs of adaptation is independent of variation in effective population size between selection lines and of variation in the direct response to selection between replicates of a selection line. We have proposed that the relationship between the direct and correlated responses to selection for replicate populations propagated in a common environment may therefore be used to partition the contribution of antagonistic pleiotropy and mutation accumulation to creating costs of adaptation. This analysis implies that mutation accumulation by neutral drift was responsible for the majority of the cost of adaptation for two reasons: there is no tendency for slopes of the regression of the cost of adaptation on the direct response to selection to be positive, and there is a weak tendency for the intercepts of this regression to be positive.

If costs of adaptation were primarily generated by second-site deleterious mutations that hitchhiked to fixation with adaptive mutations, replicate lines that fixed the greatest number of adaptive mutations should, on average, have also fixed the greatest number of second-site deleterious mutations. In other words, hitchhiking of deleterious mutations would generate a positive correlation between the direct response to selection and the cost of adaptation for replicate selection lines. Given that we did not observe more positive correlations between the direct response to selection and the cost of adaptation than we expected due to chance alone, we can rule out the possibility that the hitchhiking of conditionally deleterious mutations was a common occurrence in our lines. This result agrees with long-term selection experiments in E. coli that show that hitchhiking does not contribute to the cost of adaptation, even in selection lines with mutation rates' orders of magnitude above the "wild-type" mutation rate of E. coli (Cooper and Lenski 2000).

Previous studies have shown that, when antagonistic pleiotropy creates costs of adaptation in experimental populations of bacteria, replicates of a selection line often lose the ability to grow on the same BIOLOG substrates (Cooper and Lenski 2000; Cooper et al. 2001). A detailed analysis of the phenotypic similarity among 23 of our selection lines has revealed that replicates of a selection line are not, on average, more similar to each other than they are to lines selected in some other environment and that the divergence among replicates of a selection line is almost entirely attributable to differences in the correlated response to selection (R. C. MacLean and G. Bell, unpublished manuscript). This analysis further suggests that mutation accumulation, and not antagonistic pleiotropy, was responsible for generating costs of adaptation in this experiment.

Given that the mean genomic mutation rate for DNAbased microbes, μ , is estimated to be 3.3 × 10⁻³ mutations per replication (Drake 1991), it might be argued that our experiment was not carried out for a long enough period of time to detect mutation accumulation. It is important to remember, however, that this mean has an associated variance and that mutation rates vary ≈2.5-fold around this mean, suggesting that μ lies between 1 × 10⁻³ and 1×10^{-2} mutations per replication. We argue that this conventional estimate of μ should be treated with caution because it is based on cultures of microorganisms and viruses well adapted to the laboratory environment that were propagated under benign conditions. In the unicellular alga Chlamydomonas, mild sources of environmental stress, such as increased temperature and osmolarity, have been shown to lead to at least a 10-fold increase in the genomic rate of mutations affecting fitness (Goho and Bell 2000) in a strain well adapted to the laboratory environment. The rather severe stress of the novel culture conditions that we imposed on our selection lines, which were founded from an ancestral clone isolated from the wild, could have potentially increased the mutation rate well beyond the order of magnitude increase in experiments with Chlamydomonas. Drake's estimate also excludes the mutation rates of hypermutable loci that, as a consequence of DNA structure, have mutation rates that are deemed to be unrepresentative of the rest of the genome. In at least two cases, xylose (Funchain et al. 2000) and ribose (Cooper et al. 2001), it is known that the genes responsible for the catabolism of BIOLOG substrates are hypermutable in E. coli. These considerations suggest that mutation rates estimated from the frequency of well-defined mutations in specific genes in a handful of model organisms cannot be extrapolated to this experiment. Lines of E. coli with genetically elevated mutation rates that are protected from selection, but not drift, lose the ability to grow on many BIOLOG substrates on a timescale comparable with that of this experiment (Funchain et al. 2000). Provided that we accept that mutation rates in our cultures were likely considerably greater than Drake's estimate, mutation accumulation becomes a plausible explanation for the cost of adaptation that we observed.

In summary, although our analysis of the cost of adaptation does not allow us to rule out the possibility that particular instances of antagonistic pleiotropy and hitchhiking may have been responsible for creating some of the decay in niche breadth that we observed during this experiment, numerous lines of evidence clearly demonstrate that mutation accumulation through neutral drift was responsible for generating the vast majority of costs of adaptation in this extensive adaptive radiation.

The Correlated Response to Selection

According to the Hutchinson-Levins concept of the niche (Hutchinson 1957; Levins 1968), the niche of a population may be represented as a series of *n*-axes in an *n*-dimensional space, with each axis representing an element (i.e., thermal tolerance, food source, habitat type, etc.) of the population's "fundamental niche." Performance can then be plotted against each of these axes to yield tolerance curves (Lynch and Gabriel 1987) for the population. The area under any one of these curves was assumed to remain fixed by Levins (1968, p. 15). This implies that evolution on a particular niche axis is dominated by constraint and trade-offs; advances made in one portion of a niche axis are necessarily coupled to regress at other points along the axis.

We found that all of our evolved lines could grow on a greater range of BIOLOG substrates than the ancestral clone. This change in niche breadth between the evolved populations and the ancestral clone was driven by two opposing forces: the tendency to lose the ability to grow on about three substrates in the ancestral niche as a cost of adaptation and gaining the ability to grow on about 30 novel substrates as a positive correlated response to selection. Quantitatively, we found that, although many negative correlated responses to selection occurred within the ancestral niche, the average quantitative correlated response to selection was positive. In summary, adaptation to single carbon-source environments did produce both qualitative and quantitative costs of adaptation, but these costs were smaller than the indirect benefits associated with adaptation. This result does not agree with classical theories of niche evolution but is consistent with the findings of previous studies of experimental evolution (Bennett et al. 1992; Jaenike 1993; Velicer 1999). It is not surprising that the BIOLOG niche of our evolved lines did not evolve according to Levins's theory; this theory is built on the assumption of antagonistic pleiotropy, and pleiotropy clearly did not make a large contribution to the cost of adaptation in our lines.

How did the premium on adaptation that we detected arise? The most obvious cause of this premium is that it represents adaptation to the general conditions of growth on BIOLOG plates, for example, chronically high temperature and humidity. The ancestral clone was isolated from a plant leaf, and there must be widespread side effects of domestication under laboratory conditions. At the genetic level, the initial stages of adaptation by bacteria to novel substrates generally involve gene duplication (Hartley 1984*a*, 1984*b*) and the constitutive expression of normally inducible systems (Clarke 1984; Hartley 1984*a*; Mortlock 1984*a*, 1984*b*; Kurlandzka et al. 1991). The physiological result of these mutations is that adaptive mutants increase their ability to uptake the novel carbon compound (Brown et al. 1998) that can then be metabolized, albeit inefficiently, by weakly exapted catabolic enzymes. The genome of *Pseudomonas fluorescens* SBW25 is certainly quite large (6.7 MBp; Rainey and Bailey 1996) and exceptionally rich in regulatory regions (Spiers et al. 2000). A general elevation of gene expression and breakdown of gene regulation would explain the increase of the niche caused by selection. This is our preferred explanation for the dramatic response to selection, but other possibilities exist and the issue can only be resolved by further experimentation.

The Evolution of Ecological Specialization

At the start of the experiment, before any adaptive mutations had been fixed in any of our selection lines, the mean performance of residents was equal to that of nonresidents in all 95 BIOLOG environments. Two fundamental changes occurred during this experiment: selection lines tended to increase their growth in their home substrate as a direct response to selection and, as a correlated response, tended to improve their quantitative performance across a range of substrates. After 1,000 generations of selection, the mean performance of residents was greater than that of nonresidents because the direct response to selection on a given substrate was, on average, 36% larger than the correlated response to selection on the same substrate. Even in the absence of the pleiotropic costs of adaptation that are often invoked to explain the evolution of ecological specialization, selection created locally adapted (i.e., specialized) types through what could be termed "opportunity costs" that arise from the general tendency of direct responses to selection to be larger than correlated responses.

Roundabout Selection

Direct selection may be ineffective if it is too severe, preventing the cumulation of genetic change by making it impossible for the population to persist at all. This is why Kings-B medium was administered for the first period of the experiment in order to allow selection lines to persist in novel environments. In some cases, however, these first stages apparently did not occur, and selection lines failed to consistently acquire the ability to grow on many novel substrates. In all of these cases, however, the ability to metabolize the substrate did evolve as a correlated response to selection on other substrates.

One potential explanation for this surprising result is that the ancestral clone requires multiple physiological changes in order to grow on some novel substrates. Adapting to such a substrate as a direct response to selection would be difficult; individual mutations that altered only one of the physiological components (i.e., membrane transporters or catabolic enzymes) necessary for growth would not confer any selective advantage. If mutations that alter the same, or roughly equivalent, physiological components are individually advantageous on other novel substrates, selection lines can acquire a rudimentary ability to metabolize a substrate as an indirect response to selection on a second substrate, even though the direct selection is ineffective. In this case, the direct response would be obstructed by epistatic interactions among beneficial mutations. The alternative explanation for roundabout selection is that it arises through demography. Consider a hypothetical mutation, x, that is beneficial in two selection lines. In one selection line, mutation x allows for the line to catabolize its novel substrate. In a second line, mutation x allows for a small, but significant, increase in growth on a substrate that can already be catabolized. Once mutation x has arisen, the probability that it will survive drift and go to fixation in an asexual haploid population that undergoes daily serial transfers is somewhat less than, but proportional to, $2s_{x,y}$ (Wahl and Gerrish 2001), where $s_{x,y}$ is the selection coefficient associated with the *x*th mutation in the yth line. The probability of mutation x becomes fixed in the yth line during t generations of selection is, therefore, $\approx \rho 2 s_{x,y} \times \mu_x \times N_{e(y)} \times t$, where μ_x is the genomic rate of appearance of mutation x and $N_{e(y)}$ is the effective population size of line y, and ρ is the reduction in the probability of fixation of mutation x due to the bottlenecking procedure (i.e., $\rho < 1$). Paradoxically, this approximation predicts that the probability of fixing mutation x could be greater in the line selected on the substrate in the ancestral niche (where s is small and N_e is large) than in the line selected on the novel substrate (where s is large and N_e is small), provided that the difference between the effective population size of the two lines is greater than about twice the difference in the selective coefficient associated with mutation x in the two lines.

Regardless of the basis of roundabout selection, it is clear that adaptation to a novel environment can sometimes be acquired unexpectedly in a roundabout fashion by selection in a completely different environment. If a line that has acquired a small amount of adaptation to a novel environment through roundabout selection then migrates into that environment, direct selection will be effective. It is not, perhaps, very surprising to find the occasional instance of roundabout selection. The surprising feature of our results is that it supplied adapted lines in every case where direct selection had been ineffective. Thus, roundabout selection increased the proportion of the new niche space that was occupied from 70% to 100%. The lack of any direct response in 13 novel environments might well have been interpreted as illustrating a constraint that prevented adaptation. The correlated response, however, makes it clear that no biochemical constraint existed; rather, a direct response was obstructed by the gradual nature of evolutionary change itself. Without an extensive assay in apparently unrelated environments, the failure of the direct response would have been misinterpreted.

Multifarious Consequences of Selection

In a more natural setting, one can imagine that new ecological opportunities will often appear successively, rather than simultaneously, and that populations will be able to disperse from one habitat to another. This might be thought of as a long and laborious process, with adaptation to one novel habitat having to be effaced in the process of adapting to another. The correlated responses that we have observed, however, suggest a completely different picture. Adaptation to one or a few novel substrates will greatly increase the potential for adaptation to others, through roundabout selection. There will, indeed, be positive feedback in the early stages of a radiation, in which each new substrate that is encountered generates a correlated response that makes several other substrates accessible. The result, for a population entering a region that offers a wide variety of untapped ecological opportunities, would be an explosive radiation of specialized types. The characteristic rapidity of large-scale radiations that is such an arresting feature of the history of life may reflect the multifarious consequences of selection in novel environments that our bacterial cultures show so clearly.

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