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EVOLUTIONARY ADAPTATION TO ENVIRONMENTAL pH IN EXPERIMENTAL LINEAGES OF *ESCHERICHIA COLI*

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This study uses the enteric bacterium *Escherichia coli* as an experimental system to examine evolutionary responses of bacteria to an environmental acidic-alkaline range between pH 5.3 and 7.8 (15–5000 nM [H^+]). Our goal was both to test general hypotheses about adaptation to abiotic variables and to provide insights into how coliform organisms might respond to changing conditions inside and outside of hosts. Six replicate lines of *E. coli* evolved for 2000 generations at one of four different constant pH conditions: pH 5.3, 6.3, 7.0, or 7.8. Direct adaptation to the evolutionary environment, as well as correlated changes in other environments, was measured as a change in fitness relative to the ancestor in direct competition experiments. The pH 5.3 group had the highest fitness gains, with a highly significant increase of 20%. The pH 7.8 group had far less significant gains and much higher variance among its lines. Analysis of individual lines within these two groups revealed complex patterns of adaptation: all of the pH 5.3 lines exhibited trade-offs (reduced fitness in another environment), but only 33% of the pH 7.8 lines showed such trade-offs and one of the pH 7.8 lines demonstrated exaptation by improving fitness in the pH 5.3 environment. Although there was also prevalent exaptation in other groups to the acidic environment, there were no such cases of exaptation to alkalinity. Comparison across the entire experimental pH range revealed that the most acidic lines, the pH 5.3 group, were all specialists, in contrast to the pH 6.3 lines, which were almost all generalists. That is, although none of the pH 5.3 lines showed any correlated fitness gains, all of the pH 6.3 lines did.

KEY WORDS: **Bacteria, environmental stress, *Escherichia coli*, evolution, experimental evolution, pH adaptation, trade-offs.**

Experimental evolution is particularly well suited for effectively examining adaptation to abiotic environments. Such environments are easy to regulate and control, and many replicate populations can be exposed simultaneously to identical conditions. However, most experimental evolutionary studies on environmental adaptation have focused on temperature (Huey et al 1991; Bennett et al. 1992), and it remains to expand the scope of this work to include other environmental variables. The study described here is the first to examine differential adaptation to environmental acidity.

Many types of organisms may not experience environmental acidity and alkalinity at stressful levels, but pH is a biologically significant environmental stress for enteric bacteria, such as *Escherichia coli*. *Escherichia coli* are often considered neutrophiles that grow best at neutral pH, although they can also grow in moderate acid or base. Growth under these conditions can be challenging; however, *E. coli* K-12 growing at pH 5.0 have cytoplasmic pH lowered by 0.6 units (Hickey and Hirshfield 1990), which can alter catalytic properties of regulatory enzymes and membrane functions (Somero 1986). Tolerance of even more extreme pH

conditions is fundamental to the survival of enteric bacteria, as the mammalian intestinal tract subjects them to extreme acidity in the stomach before reaching the more alkaline intestine. With tolerance to pH as low as 2.0, some *E. coli* are able to colonize within a host with as few as 10 bacterial cells, whereas *Salmonella*, with an acid tolerance limited to a pH of only 3.0, requires over 10,000 cells to produce a similar infection (Audia et al. 2001). Tolerance of changes in pH is also important in other contexts. Recently, due to mishandling of sewage, the ocean is becoming an environment in which enteric bacteria experience an alkaline pH near 8.0. The health implications of bacterial survival and evolution in these conditions have led to broadening interest in the patterns of adaptation to pH of *E. coli* exposed to novel environments.

While in the stationary phase, *E. coli* may be capable of survival through brief exposure to low and high pH conditions. For example, in stationary phase *E. coli* can survive at a pH as low as 2 (Gorden and Small 1993), and as high as 10 (Small et al. 1994) for several hours. However, the range of pH values under which growth is possible is significantly smaller (Zilberstein et al. 1984). In the culture conditions used for our experiments, the ancestral bacterium is able to persist consistently only between pH 4.8 and pH 8.4 (A.g. Cullum, unpubl. ms.). Beyond this range the bacteria were unable to maintain the population growth necessary to sustain their numbers under daily 100-fold serial dilution in our minimal medium. Given these limits, we confined our evolutionary environments to those between pH values of 5.3 and 7.8, providing a significant range of acidic exposures (over 300× $[H^+]$) that still permit adequate growth for persistence in serial dilution culture.

Our long-term selection experiments investigating adaptation to pH address several questions concerning adaptation patterns and trade-offs. The work described here involves evolution in constant environments. Simultaneous experiments were undertaken in environments of variable pH and these will be described and analyzed separately (Hughes et al. 2007).

Experimental Overview

The ancestral bacterium, which served as the progenitor for our experimental lines, was taken from a line that was previously evolved for 2000 generations under similar serial dilution conditions at a pH of 7.0 (Lenski et al. 1991). We used this ancestor to found a total of 24 lines, with six replicate lines grouped in each of four constant pH buffered environments: pH 5.3, 6.3, 7.0, and 7.8. (Although not analyzed in this study, 12 additional lines were concurrently derived in variable pH environments with half evolved in a regularly cycling regime switching daily between pH 5.3 and 7.8 and the other half evolved in a randomly changing regime shifting daily among pH 5.3, 6.3, 7.0 and 7.8 [Hughes et al. 2007]). The four constant-pH evolved groups are identified simply by their environmental pH; specifically they are named the 5.3 (pH 5.3, equal to 5000 nM $[H^+]$), 6.3 (pH 6.3, 500 nM $[H^+]$), 7.0 (pH 7.0, 100 nM $[H^+]$), and 7.8 (pH 7.8, 15 nM $[H^+]$) groups. (Note that the $[OH^-]$ concentration varied in the above groups as well, with pH 5.3 equal to 2 nM $[OH^-]$, pH 6.3 to 20 nM $[OH^-]$, pH 7.0 to 100 nM $[OH^-]$, and pH 7.8 to 630 nM $[OH^-]$.) The pH 7.0 condition served as the control environment, having the same pH as experienced by the ancestral lineage. With conditions near the limit for the initial lineage, the pH 5.3 and 7.8 environments represent novel and potentially stressful extremes of acid and base, whereas the pH 6.3 environment provides a novel but only moderately acidic environment. All pH groups were propagated in Davis minimal media (see Lenski et al. 1991) that varied only in the relative concentrations of the buffering ingredients: acid-shifting potassium phosphate monobasic (KH_2PO_4), and alkaline-shifting potassium phosphate dibasic (K_2HPO_4).

We measured the extent of evolutionary adaptation in each line as the change in fitness relative to the common ancestor. This measurement was achieved through use of a neutral marker that allowed enumeration of the outcomes of direct competitions between lines (see Lenski et al. 1991). We measured both direct fitness (the fitness of a line in its evolutionary environment) and correlated fitness (the fitness of a line in the other environments) (Falconer 1989) for each line at 2000 generations.

The work presented here addressed three questions about evolutionary responses to changes in the abiotic environment, with regard to both adaptation and trade-off.

(1) With respect to adaptation (positive direct fitness responses), were fitness responses greater under the novel pH conditions than under the ancestral regime? The groups evolved in the novel pH 5.3, 6.3, and 7.8 environments were predicted to have a greater increase in fitness than the group evolved in pH 7.0, because the ancestral genotype for this study had already been propagated for 2000 generations in this environment and experienced a mean fitness gain of nearly 35% (Lenski et al. 1991). The 7.0 group was considered as the control group for comparison with the novel pH groups and was expected to account for any further adaptation to non-pH factors of the general laboratory environment. For the extreme pH environments, we predicted that specific adaptations to the challenges of acidic or basic conditions would exceed any general adaptations to the medium.

(2) Were there fitness trade-offs in other environments that accompanied adaptation to these pH environments? Evolutionary trade-offs have been a common expectation in theory, but previous thermal adaptation studies in *E. coli* show a surprising absence of trade-offs at high temperature (Bennett et al. 1992; Bennett and Lenski 1993), suggesting that adaptation may proceed without them. However, further experimentation with this system revealed that ther-

mal adaptation to 20°C does involve significant trade-offs (Mongold et al. 1996; Portner et al. 2006) with fitness at high temperatures, suggesting that the patterns of trade-offs may be complex.

(3) How does the magnitude of pH change affect the patterns of adaptation? Here we compared the evolutionary responses to two acidity levels, pH 5.3 and 6.3, which are nearer and further, respectively, from the niche edge. We also analyzed differences in patterns of adaptation to acid and base conditions by comparing responses to the pH 5.3 and 7.8 environments.

Materials and Methods

BACTERIA

The ancestral strain of *E. coli* B used to found the lines of our study is asexual, prototrophic (but incapable of growing on L-arabinose [Ara−]), has no plasmids or functional bacteriophages, is T5 sensitive and T6 resistant, and was isolated as a clone from one of 12 populations that were part of an earlier evolution study (Lenski et al. 1991). These 12 populations were allowed to adapt for 2000 generations (300 days) to a standard laboratory environment of pH 7.0 and a temperature of 37°C. This propagation followed a standard regimen of daily serial dilution of 1:100 (0.1 ml in 9.9 ml) in Davis minimal broth (Carlton and Brown 1981) supplemented with 25 μg glucose per ml (henceforth referred to as "DM"). During these 2000 generations, the mean fitness of these populations had increased by nearly 35% relative to their progenitor. Because most of this fitness increase occurred during the first 1000 generations, we inferred that our ancestor was already relatively well adapted to the basic culture conditions of our evolutionary regimes, except for the differences in pH levels.

Prior to use in our experiments, two forms of the ancestral genotype were isolated, which differ only in a neutral marker, which is characterized by the ability (+) or the inability (−) to use the sugar arabinose. These two marker states allow visual differentiation, via plating, between two lineages competing directly against each other in the same flask when they are oppositely marked (Lenski et al. 1991). We verified the neutrality of this marker gene over the range of pH values used in this study (see below). The two ancestral genotypes, as well as the lineages we evolved in this study, are stored at −80°C and could be revived at any time for use in experiments.

CULTURE CONDITIONS

The basic culture conditions of our evolution experiments were identical to those previously described (Bennett et al. 1992), except for the modification of pH in the DM (Carlton and Brown 1981; Lenski 1988a). Lines revived from the freezer were initially inoculated into Luria broth (LB), but otherwise propagation was in DM, modified by varying proportions of the mono- and dibasic potassium phosphate present in the buffer system. Our DM mixtures were buffered at pH 5.3, 6.3, 7.0, and 7.8, all ± 0.1 pH units, to achieve the media of our four different experimental regimes. Because pH is temperature dependent, media pH was measured at 37°C with a Fisher Accumet Model 15 (Fisher Scientific, Pittsburgh, PA) pH meter at the time of mixing and after storage. The appropriate pH levels of the cultures were verified by direct measurement throughout the selection experiment to assure constant environmental acidity within 0.1 pH unit. Although in nonbuffered media at high cell densities bacterial metabolism will shift pH significantly, the low densities and buffered media of our experiments restricted such pH shifts to no more than 0.1 pH unit. The culture temperature was maintained at a constant 37 ± 1°C by shaking incubators (New Brunswick Models G25 and G25-KC; New Brunswick Scientific Co., Inc., Edison, NG) at 120 rpm. Propagation of lineages, during both the evolutionary period and experimental assays, was conducted through daily serial transfer of 0.1 ml of each culture into 9.9 ml of fresh medium. These daily transfers effectively diluted population counts by 100-fold, so that the bacterial population grew by 100-fold (∼6.64 generations) to regain its stationary phase density. At a pH of 7.0, stationary phase culture had a population density of $\sim 4 \times 10^7$ cells per ml, at 5.3 pH the density was slightly higher, whereas at 7.8 pH this value was only $\sim 2 \times 10^7$ cells per ml. These different yields indicate that variable amounts of energy may be devoted to nongrowth processes (e.g., metabolism, respiration, excretion) at different pH levels, while cell size may also affect final densities. Although growth density differences were unavoidable without confounding nutrient levels, these density differences did not affect the number of generations, because the starting and ending cell counts were both proportionally lowered.

EVOLVING LINES

Six lines were founded within each of the four evolutionary conditions of pH 5.3, 6.3, 7.0, and 7.8, for a total of 24 lines altogether. Half of the lines in each group originated from the Ara+ form of the ancestor and the other half came from the Ara− form (Lenski et al. 1991). Initially, all lineages within our experiment were genetically identical, except for the neutral arabinose-utilization marker. Individual lines were identified by the group name (the pH value in which they evolved), a "+" or "−" to identify the marker state, and a replicate number. As an example of the terminology we consistently employ in our descriptions, the 5.3 group (evolved at 5.3 pH) comprised the 5.3 + 1, 5.3 + 2, 5.3 + 3, 5.3 − 1, 5.3 − 2, and 5.3 − 3 lines. Evolving populations were propagated by the methods indicated above; Lenski et al. (1991) provide additional details. Given the initial homogeneity of the lines, controls against migration, and the asexuality of the bacterium, the evolutionary responses to our pH regimes must have been the result of mutations that occurred de novo within the lines.

RELATIVE FITNESS

Using the isolates obtained after 2000 generations, measurements of relative fitness for each of the 24 evolved lines were made in every pH regime using the methods of Bennett et al. (1992). While samples of both the mixed evolving populations and a single colony isolate were frozen, the single colony isolates were used in the fitness assays reported here. (In an investigation of two lines, no significant differences in relative fitness were evident between the single colony isolates and their source mixed populations [data not shown].) Fitness was determined through a direct competition between each evolved line and the reciprocally marked ancestral genotype in a single flask. The two competing strains were revived from the −80°C frozen storage and separately inoculated into a standard nutrient-rich LB culture medium, with a pH of 7.0 and temperature of 37°C, for 24 h of growth. The lines were then each transferred into the ancestral pH 7.0 DM for the second day's growth, and on the third day the competitors were each transferred separately into the pH environment in which they would eventually compete to provide the opportunity for phenotypic acclimation to the test regime prior to competition. On the fourth day competitions were initiated by transfer of 50 μl of each line mixed into 9.9 ml of the same test pH medium, which produced the standard 100-fold dilution for the combined population, and were then incubated under standard conditions for 24 h. Initial and final population density of each competitor was estimated by plating diluted samples on tetrazolium arabinose (TA) agar, on which the two competitors could be distinguished and enumerated through the differential colony color afforded by the reciprocal neutral marker. After 24 h, the competition medium was spread on TA agar plates, and incubated at 37°C in a Fisher Isotemp 200 series Model 255D Incubator. The relative fitness of the evolved line was calculated as the ratio of the number of its doublings compared to the doublings of the ancestor during the 100-fold combined population growth of the competition period (Lenski 1988b; Bennett et al. 1990). Six simultaneous replicates were run for each line in each pH environment. Measures of direct fitness for each line were made in the pH environment in which the lines evolved, whereas measures of correlated fitness were made in the other three pH environments.

STATISTICAL ANALYSIS

We compared the fitness measures between and among both individual lines and pH groups using several approaches. In all tests, we used $\alpha = 0.05$ to delineate significance. Neutrality of the arabinose marker was assessed by conducting 10 replicate competitions between the Ara+ and Ara− ancestral strains. Mean fitness values of evolved lines are based on six replicates for each assay, and group means are calculated from the six line means. Significance of fitness means was analyzed by *t*-distributions compared to a null hypothesis value of 1, representing fitness equal to the ancestor. One-tailed probabilities were used with the expected fitness gains in direct responses, whereas two-tailed probabilities were used for the unpredicted correlated responses. We also made comparisons between groups using two-tailed, two-sample *t*-tests. Because some of our hypotheses used the same experimental datasets, we took special care to account for the nonorthogonal aspects of our hypothesis testing and we tested only a priori hypotheses. We also made some comparisons among the lines within groups by ANOVA, followed by the Tukey–Kramer test to identify significantly different subgroups within each group.

Results

EFFECTIVE NEUTRALITY OF THE GENETIC MARKER

The arabinose-utilization marker that allowed differential enumeration in our competitions is known to be neutral with respect to fitness in many test environments (Bennett et al. 1992), which include the common ancestor at pH 7.0. We further confirmed neutrality of the marker for our pH studies by measuring the relative fitness of the two marker states across all the pH levels of our experiment, as shown in Table 1. Neutrality is a nondeparture from a relative fitness measurement of 1.0, when the Ancestor A+ is competed against the Ancestor A−. We found no significant differences between the marker states, based on one-sample *t*-tests with df = 9, as reported in Table 1. These tests had a power of greater than 80% to detect fitness differences as small as 3% in fitness.

DIRECT FITNESS RESPONSES

The direct fitness responses are summarized in Table 2 and are also included in Figure 1. Shown for each group is mean fitness relative to the ancestor when tested at the group's evolutionary pH. Also reported in Table 2 are the patterns of direct fitness responses for the six lines in each group. (Note: Rather than present descriptive statistics on every combination of line and environment in this section, we have chosen to provide a simplified overview of variation

Table 1. Effective neutrality of the arabinose-utilization marker in the ancestral strain under each experimental pH regime.

Experimental regime	Fitness of Ara^+ relative to Ara^-	
	Mean (±95%CI)[1]	Significance[2]
5.3 pH	0.977 (±0.024)	0.059
6.3 pH	1.019 (±0.023)	0.095
7.0 pH	0.988 (±0.039)	0.502
7.8 pH	0.995 (±0.025)	0.678

[1] Means (and 95% confidence intervals) are calculated from 10 replicate assays for each experimental pH.

[2] Significance values are based on two-tailed *t*-test with $\mu_{null} = 1$ and df = 9.

Table 2. Direct fitness responses for each group after 2000 generations with patterns of adaptation.

Selective pH	Mean fitness (±95%CI)[1]	*P* values[2]	Line responses[3]	Subgroups[4]
5.3	1.200 (±0.062)	0.0002	6+ 0≈ 0−	2
6.3	1.050 (±0.009)	<0.0001	4+ 2≈ 0−	1
7.0	1.037 (±0.074)	0.1284	3+ 2≈ 1−	3
7.8	1.076 (±0.105)	0.0615	4+ 2≈ 0−	3

[1]The mean relative fitness (and 95% confidence interval) for each group is calculated from six line means, each of which is based on six replicates.
[2]Significance values are based on one-tailed *t*-distribution with $\mu_{null} = 1$ and df = 5.
[3] Each number followed by +, ≈, or −, indicates the number of lines in the group with significant gains, no significant changes, or a significant losses, respectively, in mean direct fitness. See Table 4 and the Appendix for mean fitness and confidence intervals of each line.
[4]Shown is the minimum number of statistically distinguishable groupings for fitness responses (i.e., fitness level subgroups) based on the Tukey–Kramer test following ANOVA.

within groups in most cases. However, line means and confidence intervals not presented here appear in the Appendix.) Although all four environments produced significant direct fitness gains among at least half of the lines propagated, only the acidic pH 5.3 and 6.3 environments produced significant increments when analyzed by group. The 5.3 group was the only regime that produced significant direct fitness gains among all six of its lines when tested within the selection regime, with two statistically distinguishable fitness level subgroups, suggesting at least two adaptive pathways in the genetic changes within the group. The 6.3 group had significant gains in four of its lines and no significant change of direct fitness in its other two lines, but showed no distinguishable fitness level subgroups. Although the 7.8 group fitness gain of 8% was not quite statistically significant ($P = 0.06$), it had the same fitness pattern as the 6.3 group, with four of its six individual lines showing significant gains and the other two lines showing no significant change in fitness, but in this case three distinguishable fitness subgroups were present. The 7.0 group had a fitness increase of less than 4% that was not statistically significant. Three of the individual 7.0 lines did show significant fitness gains, two showed no significant change, and one line actually had a significant loss of fitness; this pattern resulted in three fitness levels within the group. Although the finding of a loss in fitness in the 7.0 − 1 line was surprising, such an occurrence is not without precedent. There is some limited support for the probability of an occasional loss in fitness (Paquin and Adams 1983; Chao 1990), but also possible is that a measurement of fitness loss may occur as the result of experimental or type-I error (Lenski et al. 1991). We also noted that the mean fitness of the 7.0 group did not differ statistically ($P = 0.63$, *t*-test) from that of the 37°C group of Bennett and Lenski (1996), which consists of six lines previously propagated independently under the same pH and temperature conditions as our 7.0 group.

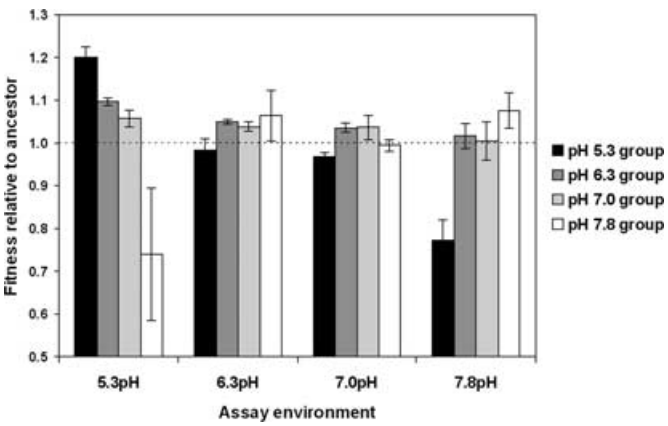

Figure 1. Direct and correlated fitness responses of the experimental groups after 2000 generations of evolution at constant pH of 5.3, 6.3, 7.0, or 7.8. Values shown are group means with SEM error bars, based on six independent lines.

CORRELATED FITNESS RESPONSES

The correlated fitness responses are summarized in Figure 1 and Table 3. Shown for each group is mean fitness relative to the ancestor when tested at a different pH than the one in which the group evolved. Of the 12 correlated fitness responses measured, five suggested an increase in fitness and the other seven suggested a decrease in fitness. However, only six of these 12 changes in fitness were statistically significant. Because most of the statistically significant trade-offs were found in the 5.3 and 7.8 groups, we examined variation among individual lines within these groups (Table 4). (A trade-off is defined here as a significant loss in correlated fitness accompanying a significant gain in direct fitness.)

While the 5.3 group had a significant direct fitness gain, the group also experienced a highly significant fitness decrease at pH 7.8 (Table 3). Notably, all six lines individually showed a significant loss in fitness, suggesting that trade-offs to higher pH environments consistently accompany adaptation to pH 5.3 (Table 4). The fitness values of the 5.3 lines in pH 7.8 fell into two statistically distinguishable subgroups; they also fell into two subgroups when tested at pH 6.3 (with one case of a fitness trade-off), but no subgroups or trade-offs were distinguishable at pH 7.0 (Table 3).

Like the 5.3 group tested in pH 7.8, the 7.8 group in pH 5.3 also showed a large mean decrease in fitness, exceeding even the significant trade-off of the 5.3 group in pH 7.8 (Table 3). In this case, however, there was much more variability in fitness changes among the individual lines (Tables 3 and 4). This high variance reflects a more complex correlated response pattern in

Table 3. Correlated fitness responses of each group after 2000 generations with patterns of adaptation.

Evolutionary pH	Assay pH	Mean fitness (±95%CI)[1]	*P* values[2]	Line responses[3]	Subgroups[4]	Trade-off patterns[5]	Overall patterns[6]
5.3	6.3	0.982 (±0.073)	0.5505	0+, 5≈, 1−	2	1T, 5N, 0X	6S, 0G
	7.0	0.968 (±0.024)	0.0178	0+, 6≈, 0−	1	0T, 6N, 0X	
	7.8	0.773 (±0.122)	0.0049	0+, 0≈, 6−	2	6T, 0N, 0X	
6.3	5.3	1.097 (±0.021)	<0.0001	6+, 0≈, 0−	1	0T, 4N, 2X	1S, 5G
	7.0	1.036 (±0.030)	0.0266	2+, 4≈, 0−	1	0T, 4N, 2X	
	7.8	1.016 (±0.072)	0.5862	0+, 5≈, 1−	1	1T, 3N, 2X	
7.0	5.3	1.058 (±0.052)	0.0348	4+, 2≈, 0−	2	0T, 3N, 2X	1S, 3G
	6.3	1.039 (±0.030)	0.0215	1+, 5≈, 0−	1	0T, 3N, 2X	
	7.8	1.005 (±0.115)	0.9158	0+, 5≈, 1−	2	1T, 2N, 2X	
7.8	5.3	0.740 (±0.396)	0.1519	1+, 2≈, 3−	3	2T, 2N, 2X	3S, 2G
	6.3	1.064 (±0.153)	0.3311	2+, 4≈, 0−	2	0T, 4N, 2X	
	7.0	0.994 (±0.036)	0.6813	0+, 4≈, 2−	2	2T, 2N, 2X	

[1] Group mean and 95% confidence interval based on six line means of six replicates.

[2] Two-tailed *t*-distribution with $\mu_{null} = 1$ and df = 5.

[3] Each number followed by +, ≈, or -, indicates the number of lines in the group with significant gains (exaptations), no significant changes, or a significant losses, respectively, in mean direct fitness. See Table 4 and the Appendix for mean fitness and confidence intervals of each line.

[4] Shown is the minimum number of statistically distinguishable groupings for fitness responses (i.e., fitness level subgroups) based on the Tukey–Kramer test following ANOVA.

[5] Shown is the number of lines that fall into each of the following categories:

N (No Trade-off): direct fitness gain with no correlated fitness loss.

T (Trade-off): direct fitness gain but with correlated fitness loss.

X (Not evaluated): no direct fitness gain.

[6] Shown is the number of lines that fall into each of the following general categories for each evolutionary pH:

G (Generalist): exhibits higher average fitness than the ancestor in at least one regime with no trade-offs in any of the other regimes tested.

S (Specialist): exhibits higher average fitness than the ancestor in its direct response but has trade-offs in one or more of the other regimes.

the 7.8 group, with three statistically distinct subgroups at pH 5.3 and two subgroups at both pH 6.3 and 7.0. With regard to the identification of significant fitness trade-offs at pH 5.3, only two of the six 7.8 lines met the criteria for this classification. Although an additional line, 7.8 + 1, also had a significant loss of fitness in this environment, it was not strictly considered to show a trade-off because it did not show a significant increment in direct fitness. Two of the other three 7.8 lines showed no significant fitness change in pH 5.3 (and hence no trade-off), whereas the last actually showed exaptation to this environment, which is defined here as a significant gain in correlated fitness. Interestingly, all three of the 7.8 lines that exhibited fitness decrease in pH 5.3 showed a loss that was more extreme than those of the 5.3 lines in pH 7.8. The 7.8 lines also showed variable responses to the other two environments, with two cases of trade-offs in pH 7.0 and two more cases of exaptation in pH 6.3. Evidently, there was more heterogeneity in the correlated responses of the 7.8 lines than in those of the 5.3 lines.

Some of these more complex patterns were also observed in the other two groups (see Table 3). In its correlated responses across three pH environments, the 7.0 group exhibited only one trade-off (line 7.0 + 3 at pH 7.8) but five cases of exaptation, with four of these at pH 5.3. Similarly, the 6.3 group had a trade-off occurring in only one line at pH 7.8, but eight examples of exaptation, with all six lines showing improved fitness at pH 5.3.

SPECIFICITY OF ADAPTATION

Specificity of adaptation between acidity and alkalinity was examined by comparing patterns of adaptation between the acidic 5.3 lines and alkaline 7.8 lines, the groups at the extremes of our evolutionary range. A comparison of the mean direct fitness values of these two groups (i.e., the 5.3 group tested at pH 5.3 and 7.8 group tested at pH 7.8) revealed a significant difference ($P = 0.03$, two-sample $t-$test assuming unequal variance, df = 10), with the 5.3 group showing a fitness increase almost three times that of the 7.8 group. A similar comparison of the mean correlated fitness values of the two groups (i.e., the 5.3 group tested at pH 7.8 and 7.8 group tested at pH 5.3) was insignificant; however, this group-level comparison obscures a more complex pattern of trade-offs and exaptation seen at the level of individual lines (Table 4). Notable were the relatively homogeneous direct and correlated (pH 7.8) fitness responses of the 5.3 lines, whereas the 7.8 lines showed a great deal of heterogeneity in direct and particularly their correlated (pH 5.3) fitness changes. Analysis across the entire set

Table 4. Acid versus alkaline patterns of adaptation in the lines of the 5.3 and 7.8 groups. Values shown are means ±95% confidence limits of relative fitness based on six replicate assays (line means) or the six line means (group means).

Evolutionary pH	5.3 acid group (±95%CI)			7.8 alkaline group (±95%CI)		
Assay pH	Direct: 5.3	Correlated: 7.8	Pattern[1]	Direct: 7.8	Correlated: 5.3	Pattern[1]
+1 Line	1.239 (±0.048)	0.718 (±0.143)	T	0.897 (±0.107)	0.469 (±0.033)	–
+2 Line	1.179 (±0.064)	0.863 (±0.079)	T	1.102 (±0.097)	0.236 (±0.071)	T
+3 Line	1.089 (±0.043)	0.844 (±0.062)	T	1.171 (±0.086)	1.035 (±0.100)	N
−1 Line	1.242 (±0.025)	0.560 (±0.089)	T	1.029 (±0.078)	1.130 (±0.078)	E
−2 Line	1.224 (±0.080)	0.832 (±0.110)	T	1.150 (±0.053)	0.520 (±0.089)	T
−3 Line	1.228 (±0.060)	0.820 (±0.059)	T	1.105 (±0.045)	1.050 (±0.070)	N
Group mean	1.200 (±0.062)	0.773 (±0.122)		1.076 (±0.105)	0.740 (±0.396)	
Fitness subgroups[2]	2	2		3	3	

[1] Shown for each line is the pattern of evolutionary change across the two environments.
E (Exaptation): correlated fitness gain.
N (No Trade-off): direct fitness gain with no correlated fitness loss.
T (Trade-off): direct fitness gain but with correlated fitness loss.
[2] Shown is the minimum number of statistically distinguishable groupings for fitness responses (i.e., fitness level subgroups) based on the Tukey–Kramer test following ANOVA.

of experimental regimes, summarized in Tables 2 and 3, further reveals the more variable pattern of 7.8 alkaline adaptation in contrast with the homogeneity of the 5.3 acid lines. The observation that each 5.3 line lost fitness in pH 7.8 relative to the ancestral genotype led us to characterize all six of these lines as specialists. A specialist is here defined as a phenotype exhibiting a significant increase in direct fitness, accompanied by trade-offs in one or more of the other regimes. Three of the 7.8 lines also met this definition and were identified as specialists. We also recognize a generalist classification, defined as a phenotype exhibiting higher fitness than the ancestor in at least one regime, with no significant trade-offs in any of the other regimes tested. There were two such generalists among the 7.8 lines.

Specificity of adaptation as a consequence of the intensity of the stressor was also examined, in this case by comparison of the two acid-adapted groups, which evolved at pH 5.3 and 6.3. Comparisons between the 5.3 and 6.3 groups revealed a highly significant difference in the direct fitness between the groups, with the stronger acid environment resulting in 15% higher direct fitness than the weaker acid ($P < 0.001$, two-sample $t-$test assuming unequal variance, df = 10). In contrast, the weaker acid environment produced significantly higher correlated fitness responses than the stronger acid, both at pH 7.0 (7% greater, $P < 0.001$) and pH 7.8 (24% greater, $P < 0.002$). As seen in Table 3, the gradation in evolutionary pH between 5.3 and 6.3 produced a number of contrasting outcomes, but perhaps the most compelling is the overall pattern of adaptation assessed across all regimes: all six of 5.3 pH-adapted lines were specialists, whereas all but one of the 6.3 pH-adapted lines were generalists (Table 3, last column).

Discussion

In this study, we examined the direct and correlated evolutionary responses to constant pH environments by *E. coli*. We now address the questions that guided our investigation.

DIRECT FITNESS RESPONSES OF ADAPTATION WERE POSITIVE IN ALL OF THE REGIMES

Many previous experimental evolution studies have demonstrated a rapid genetic adaptation, measurable by fitness assays relative to the ancestor within the evolution regime (e.g., Elena and Lenski 2003). As a basic premise of our study, we predicted that such an adaptation would evolve in *E. coli* exposed for 2000 generations to each of our experimental environments, thus allowing us to examine our other hypotheses. Our results generally indicate adaptation to each regime, with the largest fitness gain of 20% occurring in the stronger acid environment, pH 5.3. Although the 6.3 group also showed highly significant adaptation, the change was only a 5% increase. The 7.0 and 7.8 groups had lesser fitness improvements that were only significant for some of the individual lines (Table 2, Fig. 1). The hypothesis that adaptation should be most pronounced near niche boundaries with a sharp shift from rapid growth to marked death (Mongold et al. 1996) was not fully supported by our results. Both the upper and lower pH niche edges for the ancestral genotype appear to be relatively steep (A.g. Cullum, unpubl. ms.), but proximity to the upper (alkaline) niche edge produced far less fitness improvement than was produced by proximity to the lower (acid) niche edge. However, in agreement with the observations of a previous study of this system in thermal evolution (Bennett et al. 1992), the hypothesis that adaptation in the novel pH 5.3, 6.3, and 7.8 environments would be greater

than the fitness increase evolved at the ancestral pH of 7.0 was supported by the evolutionary response of the 5.3 group and to a lesser extent by the 6.3 and 7.8 group fitness responses.

TRADE-OFF PATTERNS OF ACID AND ALKALINE ADAPTATION DIVERGED

The high frequency of trade-offs observed in the present study suggests that trade-offs may be more prevalent than indicated by previous, temperature-based studies with the same *E. coli* system. These earlier thermal evolution experiments found a surprising lack of trade-offs resulting from adaptation to high temperatures (Bennett et al. 1992; Bennett and Lenski 1993), and detected trade-offs in less than two-thirds of the population adapted to cold temperatures (Mongold et al. 1996; A.F. Bennett and R.E. Lenski, unpubl. ms.). In contrast, we observed trade-offs in 100% of the 5.3 lines (Table 3). Trade-off patterns were heterogeneous among the other groups, but were still seen in half the 7.8 lines, whereas the 6.3 and 7.0 lines each had only one line exhibiting a trade-off. Acid and alkaline evolution differs further, with the pattern of the acidic 5.3 group exhibiting all specialists with 100% trade-offs, compared to the alkaline 7.8 group showing only 33% trade-offs with a mixture of generalists and specialists (Tables 3 and 4). The fact that adaptation to the alkaline environment did not always result in trade-offs, whereas acid adaptation did, might add to the growing evidence detailing the acidophilic nature of *E. coli*. It could be inferred that any further increase in acid resistance by an acid-resistant bacterium that has already exhausted nonantagonistic pleiotropic adaptation may only come at a cost. However, a competing pattern may be noted in observing that while exaptation to the alkaline environment never occurred, such correlated fitness increases were found frequently in the acidic conditions. Such evidence may help to support the recent description of *E. coli* as an amateur acidophile (Foster 2004), with a mix of both neutrophilic and acidophilic characteristics.

ADAPTATION PATTERNS WERE HIGHLY DEPENDENT ON ACID STRENGTH

Although we expected to find some pH-specific differences in adaptation between the stronger and weaker acid environments, we were surprised to find that such dramatic distinctions arose with such pH-specific sensitivity. There was a highly significant, 15% greater direct fitness response to the more intense stress level at pH 5.3 compared to pH 6.3. This result provides evidence to support the hypothesis that as a stress (in this case, acidity) becomes more extreme, adaptation becomes more rapid and extensive. Others previously testing this hypothesis with thermal experiments using *E. coli* found the same result at 42°C but not at 20°C (Bennett and Lenski 1993; Mongold et al. 1996). Trade-off patterns were also different between the 5.3 and 6.3 groups, as the 5.3 group had significant trade-offs across all six of its lines when tested in pH 7.8, whereas the 6.3 group only had one line with trade-offs when tested in pH 7.8 (Table 3). Two statistically distinguishable fitness groups were found in the 5.3 group, as compared to the less variable 6.3 group, which had only one. Apparently the more extreme acid environment produced more genetic variability among lines, but it did not produce any correlated fitness gains, whereas the lines evolved at pH 6.3 exhibited correlated adaptation and less variation in fitness. When the patterns of adaptation were analyzed at the broadest level, across all regimes, the 5.3 lines were all identified as specialists whereas almost all of the 6.3 lines were generalists. Such contrasting patterns of adaptation that were found within this acidic range revealed a high degree of adaptive specificity to pH gradients in bacterial evolution.

MECHANISMS AND BROADER IMPLICATIONS

Although empirically testing evolutionary theory, such as the patterns of fitness improvements and trade-offs or specificities of adaptation to stressor intensity, was the major focus of this study, our findings are also of interest to other areas of biology. Acid resistance and the consequent ability to infect mammalian guts is now a major health issue due to the emergence of pathogenic strains of enteric bacteria. Pathogenic and nonpathogenic strains of *E. coli* have similar resistance to acidity (Lin et al. 1996). *Escherichia coli* are often challenged to endure exposure to high acidity during colonization in the human gastrointestinal tract, and their complex mechanisms for acid resistance have suggested that they may be partly acidophilic and partly neutrophilic (Foster 2004). In addition, *E. coli* are also exposed to some alkaline conditions at the pancreatic duct (Evans et al. 1988) and increasingly in the oceans, as a result of the release of sewage into ocean waters. Although little was known previously about evolutionary responses to environmental pH, understanding of the underlying mechanisms responsible for *E. coli* pH stress resistance is progressing rapidly. Three different mechanisms of acid resistance in *E. coli* have been described. The first mechanism is an RpoS-dependent oxidative or glucose-repressed system (Small et al. 1994); the second mechanism is a GAD system that involves glutamate decarboxylase (Lin et al. 1995; Hersh et al. 1996; De Biase et al. 1999); and the third mechanism is an ARG system that requires arginine decarboxylase activity (Lin et al. 1995; Castanie-Cornet et al. 1999). In the presence of glutamate or arginine, *E. coli* can reverse the electrical membrane potential to make the inside of the cell positively charged. This is the same strategy that is used by various acidophiles that grow in extremely low pH environments (Richard and Foster 2004). Alkaline stress resistance may involve different mechanisms, with some evidence suggesting that a protein repair mechanism of L-isoaspartyl protein carboxyl methyltransferase (PCM) becomes important only in high pH conditions (Hicks et al. 2005). Further genetic analysis of our 5.3 and 7.8 pH-evolved lines would be of particular interest

to investigators considering the observations in 5.5 pH and 8.0 pH of topoisomerase I influence on RpoS-dependent and GadA and GadB systems (Stewart et al. 2005), and the decarboxylase activity noted at pH 5.6 linked to chloride ion exchange activities of ClC-ec1 (Gut et al. 2006), or the identification of alkaline resistance activity through ATP synthase and NhaA/MdfA antiporters (Padan et al. 2005). The need for a molecular-to-ecological integration (Portner et al. 2006) in addressing the issue of pathogenic bacteria in the environment may have benefited from this study of experimental evolutionary adaptation to environmental pH.

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Appendix. Relative fitness values of the 24 evolutionary lines in each pH environment. Shown are mean fitness values ±95% confidence limits based on six replicate competitions with the ancestor.

Evolutionary pH	Line	Assay pH			
		5.3	6.3	7.0	7.8
5.3	+1	1.239 (±0.048)	0.962 (±0.067)	0.938 (±0.154)	0.718 (±0.143)
	+2	1.179 (±0.064)	0.947 (±0.073)	0.954 (±0.078)	0.863 (±0.079)
	+3	1.089 (±0.043)	0.868 (±0.037)	0.953 (±0.054)	0.844 (±0.062)
	−1	1.242 (±0.025)	1.053 (±0.073)	0.993 (±0.029)	0.560 (±0.089)
	−2	1.224 (±0.080)	1.033 (±0.051)	0.991 (±0.044)	0.832 (±0.110)
	−3	1.228 (±0.060)	1.027 (±0.036)	0.978 (±0.029)	0.820 (±0.059)
6.3	+1	1.089 (±0.072)	1.052 (±0.036)	1.075 (±0.062)	0.921 (±0.054)
	+2	1.078 (±0.036)	1.056 (±0.043)	1.045 (±0.049)	1.041 (±0.061)
	+3	1.125 (±0.038)	1.064 (±0.044)	1.052 (±0.061)	1.065 (±0.124)
	−1	1.104 (±0.019)	1.048 (±0.029)	1.035 (±0.031)	1.084 (±0.200)
	−2	1.074 (±0.023)	1.040 (±0.063)	0.994 (±0.053)	0.939 (±0.099)
	−3	1.108 (±0.047)	1.042 (±0.055)	1.015 (±0.034)	1.047 (±0.113)
7.0	+1	1.043 (±0.023)	1.079 (±0.088)	1.016 (±0.071)	1.066 (±0.079)
	+2	1.078 (±0.045)	1.035 (±0.065)	1.145 (±0.073)	1.035 (±0.104)
	+3	1.122 (±0.037)	1.066 (±0.067)	1.041 (±0.032)	0.803 (±0.063)
	−1	1.096 (±0.077)	1.013 (±0.079)	0.928 (±0.047)	1.057 (±0.123)
	−2	1.019 (±0.066)	1.006 (±0.038)	1.030 (±0.059)	1.106 (±0.121)
	−3	0.990 (±0.039)	1.034 (±0.016)	1.062 (±0.046)	0.962 (±0.062)
7.8	+1	0.469 (±0.033)	0.968 (±0.052)	1.016 (±0.028)	0.897 (±0.107)
	+2	0.236 (±0.071)	1.017 (±0.036)	1.022 (±0.067)	1.102 (±0.097)
	+3	1.035 (±0.100)	0.994 (±0.025)	0.980 (±0.018)	1.171 (±0.086)
	−1	1.129 (±0.078)	0.985 (±0.057)	0.960 (±0.049)	1.029 (±0.078)
	−2	0.520 (±0.089)	1.353 (±0.141)	0.952 (±0.031)	1.151 (±0.053)
	−3	1.050 (±0.070)	1.067 (±0.031)	1.034 (±0.057)	1.106 (±0.045)