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EVOLUTIONARY ADAPTATION TO TEMPERATURE. IX. PREADAPTATION TO NOVEL STRESSFUL ENVIRONMENTS OF *ESCHERICHIA COLI* ADAPTED TO HIGH TEMPERATURE

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Abstract.—Stressful environments may be considered as those that reduce fitness, sometimes due in part to the increased metabolic expenditure required to sustain life. Direct adaptation to a stressor is expected to increase fitness and reduce maintenance metabolism, with the latter leading to increased biomass production. In this study, we test the general hypothesis that such adaptation to one stressor can preadapt organisms to novel stressful environments. Six lines of *Escherichia coli* propagated for 2000 generations at 41–42°C (42 group), a stressful temperature, were compared to six control lines propagated for 2000 generations at 37°C (37 group) and to the common ancestor of both groups. We assayed biovolume yield (a measure of growth efficiency) and competitive fitness in the 42 group's selective high temperature environment as well as five novel stressful environments—acid, alkali, ethanol, high osmolarity and peroxide. As previously reported, at high temperature the 42 group had both higher yield and fitness than the 37 group and ancestor. In the novel environments, the 42 group generally produced yields higher than the 37 group (and marginally higher than the ancestor), but we found no differences in competitive fitness among the 37 and 42 groups and the ancestor. We also found that the performance of lines within groups was not correlated across stressful environments for either yield or relative fitness. Because previous adaptation to one stressor did not improve our measure of Darwinian fitness in novel stressful environments, we conclude that the 42 group shows no useful preadaptation, or cross-tolerance, to these types of environments.

Key words.—Cross-tolerance, Darwinian fitness, metabolic efficiency, preadaptation, stress resistance.

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Does evolutionary adaptation to one stressful environment preadapt (''exapt,'' Gould and Vrba 1982) a population to other stressful environments? Stressful environmental conditions are those that, by definition, reduce the fitness of individuals or populations (Levitt 1980; Sibly and Calow 1989; Hoffmann and Parsons 1991). Stressful environments may also be characterized as those that demand an increased metabolic expenditure, and thus give lower yield, relative to more favorable environments (Lenski and Bennett 1993). It has been proposed (Hoffmann and Parsons 1991) that one means of adaptation to stressful environments may be a reduction in the cost of maintenance metabolism and a consequent increase in the energy available for growth and reproduction. Such increments in the efficiency of the conversion of resources into biomass are presumed to provide a fitness advantage relative to less efficient phenotypes. An additional hypothesis of Hoffmann and Parsons (1991), and the focus of the study presented here, is that the mechanisms underlying adaptations to stress may be general rather than specific; that is, they may provide cross-tolerance to a variety of environmental stresses. Thus, we might expect that evolutionary adaptation to one stressor would also result in increased efficiency and fitness in novel stressful environments, and would preadapt them to the occupation of these environments.

Despite the relatively straightforward nature of this hypothesis, direct tests can be difficult in natural populations because the evolutionary history of stress exposure is typically not known. Studies involving laboratory selection, in which the evolutionary exposure to a stressful environment has been controlled by an investigator for multiple generations, are particularly useful in evaluating this hypothesis. A few such studies have already been undertaken to investigate some aspects of cross-resistance to novel stressful environments. For example, fruit flies selected for increased resistance to desiccation also have increased tolerance to ethanol, acetic acid, heat shock, and starvation resistance (Hoffmann and Parsons 1989a,b).

This study was designed to test these cross-resistance hypotheses by using populations of bacteria adapted to high temperature stress. Microorganisms such as bacteria offer several advantages for such an investigation. First, multiple test and control populations can be propagated for thousands of generations, so recent evolutionary histories are completely known and controlled. Moreover, as multiple populations are founded from clones, there is no shared ancestral genetic variation, and therefore each population's adaptive solution is independent of all others. Second, different populations can be competed directly against one another to assay relative fitness. Third, mechanisms by which such species might evolve general stress-resistance adaptations are already well understood. In many microorganisms, phenotypic acclimation to one type of environmental stress often provides increased tolerance to a number of other stressors in addition to the one inducing the response. For example, an initial exposure to heat stress can increase both heat and acid tolerance (Humphrey et al. 1993; Wang and Doyle 1998), and acid exposure can increase tolerance of heat, salt, and oxi-

dizing agents (Leyer 1993) as well as acid (Foster and Hall 1990). The physiological basis for cross-tolerance of physiological stressors apparently lies at least in part in the functional overlap of a number of stress response proteins (VanBogelen et al. 1987; Watson 1990). Additional evidence for the general utility of these proteins in tolerating environmental stress is provided by bacterial genotypes with mutations in the genes encoding or regulating the proteins; these lines frequently show changed sensitivity to several stressors (e.g., Li et al. 1994; Cheville et al. 1996). If evolutionary adaptation in microorganisms involves mechanisms similar to those seen in phenotypic acclimation and genetic knockouts, then cross-resistance to novel stressors could evolve in populations adapted to a single stress.

In this study, we utilize laboratory-selected populations of the bacterium *Escherichia coli* adapted to a high-temperature environment (Bennett et al. 1992) by propagation at 41–42°C. This is a stressful temperature for the ancestral strain, as judged by depression of biovolume yield (see Methods), in comparison to that in its ancestral environment of 37°C (Lenski and Bennett 1993; Bennett and Lenski 1997). During 2000 generations, both the efficiency of energy utilization in these lines (as indicated by biovolume yield) and their competitive fitness relative to the ancestor have increased by over 30% in their native high-temperature environment (Bennett and Lenski 1996), indicating substantial adaptation to heat stress. At least some of these fitness improvements may be due to changes in the number and expression of stress-protein genes, including *rpoS* and *surE* (Riehle et al. 2001), and to the increased accumulation of heat-stress proteins (Riehle and Bennett 1998) at high temperatures. The cross-tolerance hypothesis predicts that these high-temperature adapted lines should have both increased yield and higher Darwinian fitness in novel stressful environments. In addition, it predicts that relative performance among the lines should be correlated across stressful environments, with each line tending to have a similar yield or fitness rank in each stressful environment.

We tested these hypotheses using the ancestral lines, the six independently adapted lines in our high-temperature group (42 group), and a control group of six lines propagated at the ancestral temperature of 37°C (37 group) for 2000 generations under conditions otherwise identical to the high-temperature environment. All lines were tested in six stressful environments: the selective environment of the 42 group (high temperature) and five novel environments known to be stressful to this bacterium (acid, alkali, ethanol, high osmolarity, peroxide; VanBogelen et al. 1987; Ingraham and Marr 1996). None of these experimental groups had any exposure in their recent evolutionary history (at least 4000 generations) to any of these novel stressors. For each line in each stressful environment, we measured both the volume of bacteria formed from a limited amount of nutrient (''biovolume yield''), and the competitive fitness of each 42 line and 37 line relative to their common ancestor (''relative fitness''). Because phenotypic acclimation is known to influence cross-tolerance, all populations were exposed to the test stressor for several generations prior to these examinations of yield and fitness. If the high-temperature adapted lines became evolutionarily preadapted to tolerate other stressors, then they should tend to have higher average yield and relative fitness than the control lines (i.e., the 37 group) in the novel stressful environments, and these fitness differentials should be correlated across environments.

Materials and Methods

History of Experimental Lines

Information on the bacterial strains and culture conditions used in these experiments is presented in brief here. Additional details are available in Bennett et al. (1992) and Lenski et al. (1991). The ancestral bacterium used in these experiments was derived from a line of *Escherichia coli* B propagated for 2000 generations (300 days) at 37°C by 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 ''standard DM''). To allow direct competition of derived and ancestral bacteria in later experiments (see below), a spontaneous arabinose-utilizing mutant, designated ''Ara+,'' was derived from the ancestral genotype, which is incapable of utilizing the sugar L(+) arabinose and is designated ''Ara−.'' Six replicate populations (three from each arabinose state) were then founded using the ancestral genotype for each of two thermal treatments: a control group of six lines maintained under the ancestral conditions at 37°C (designated ''37 group'') and a high-temperature group of six lines maintained under the ancestral conditions, except for an increase of incubation temperature to 41–42°C (designated ''42 group''). Each group of six lines was propagated under these experimental conditions for 2000 generations, whereupon clones (single-colony isolates) were preserved from each population for further study. All genotypes are preserved at −80°C and can be revived for fitness assays.

Biovolume Yield and Relative Fitness Assays

Experimental assays of yield in different stressful environments were made by allowing bacteria first to grow in and acclimate to the environment for 24 h. They were then transferred into fresh media and grown under the same stressful conditions for an additional 24 h. Yield was measured as the total biovolume yield (nl cells/ml medium) of bacteria produced during this latter 24-h period from the limited amount of nutrient (25 μg glucose/ml) in the medium (Lenski and Bennett 1993). Relative fitness was measured as differential reproduction in direct competition experiments between the common ancestor and a line of either the 42 or 37 derived groups. Derived lines and the ancestor separately grew in and acclimated to a stressful test environment for 24 h. Each derived line was then combined with the ancestor and allowed to compete in this environment for 24 h, by which time all nutrients were exhausted from the medium. Relative fitness was then calculated as the ratio of the number of doublings achieved by the derived line and its ancestor during this direct competition (Lenski et al. 1991).

Yield and relative fitness were measured in six stressful environments, summarized in Table 1. The heat stress assay took place in the selective environment of the 42 group. Hydrogen ion concentration (pH) was altered for the acid and alkali environments by adjusting the ratio of mono- and di-

TABLE 1. Test environments for stress resistance.

Environment	Description
Standard[1]	37°C at pH 7.2 in Davis minimal broth
High temperature[2]	Standard except 41.5°C
Acid	Standard except pH 5.4
Alkali	Standard except pH 7.9
Ethanol	Standard plus 3% EtOH by volume
High osmolarity	Standard plus 0.4 m NaCl
Peroxide	Standard plus 90 μM H_2O_2

[1] 37 group's selective environment.
[2] 42 group's selective environment.

basic phosphate buffers in the DM. The other novel stressful environments were created by adding ethanol, NaCl, or hydrogen peroxide to DM. In these experiments, the 37 group serves as a control for the 42 group with respect to adaptation to other aspects of culture condition than temperature stress.

The protocol for biovolume yield and relative fitness assays was as follows. Lines of the 42 group, 37 group, and ancestor were inoculated from frozen samples into Luria broth and incubated for 24 h at 37°C. They were then diluted 1:10,000 into standard DM and incubated at 37°C for another 24 h, followed by 1:100 dilution into ''stress'' DM under test conditions (see Table 1) and incubation for a further 24 h. This period served to acclimate the experimental populations to the appropriate stressful conditions prior to competition. At this point, the procedures for the yield and relative fitness assays diverged.

Biovolume yield.—Populations were diluted 1:100 into stress DM under test conditions and incubated at the appropriate temperature for 24 h. At the end of this period, fixed-volume samples of the populations were collected and analyzed using a Coulter counter, Model ZM (Beckman, Miami, FL), which measures the volume of each cell in a liquid sample. These volumes are summed to give the total biovolume yield (expressed in nl/ml). Yield assays included an additional set of ancestral lines (Ara+ and Ara−) acclimated and tested under ancestral culture conditions (standard DM at 37°C) as a control. Six replicate yield assays were conducted for each line in each environment.

Relative fitness.—For each derived line, a population was transferred into fresh stress DM at 1:200, simultaneously with a transfer of an equal volume of the ancestral isolate with the opposite marker state. No fitness differences have been associated with the marker state of the ancestral genotype at different temperatures (Bennett et al. 1992), and no difference in fitness associated with marker state was found in our experiments. A sample of the mixed population was plated immediately after transfer to determine relative population size at time zero. Populations were then incubated for 24 h at 37°C (41.5°C in the case of the heat stress assay) at which time a final sample was plated from the medium. Six replicate fitness assays were conducted for each derived line in each environment. Plate counts for each replicate were used to determine the number of doublings during the 24-h-test period by both the derived line and the competing ancestral line. Relative fitness was then calculated as the ratio of the derived to ancestral values.

Statistical Analyses

In a few assays of relative fitness, derived lines had population sizes so low that no colonies appeared in our samples in some replicates. When this occurred in the initial sample, the replicate was discarded. When the zero count was in the final population sample, we used a cell count of 0.5 instead of zero when calculating mean relative fitness. This approach resulted in an estimate of fitness that reflects the maximum possible value for population growth below the threshold of detection in our sampling protocol. In no case did this conservative procedure influence the acceptance or rejection of the hypotheses being tested.

In preliminary analyses, we tested for possible effects of the ancestral marker state for each variable in each of the stress environments. We found no difference between the marker states in any case (analyses not shown), and therefore pooled data for the two marker states (Ara+ and Ara−) for the main analyses.

Means and 95% confidence intervals of both yield and relative fitness values of the derived lines were calculated for individual lines using the six replicate trials and for evolutionary groups using the means of the six derived lines. For yield, deviation of the individual evolved lines from the ancestral value was tested by one-factor analysis of variance (ANOVA), with replicate measures used to compare each line to the ancestor via Dunnett's posthoc tests. Overall group effects were tested using line means (for the derived groups) and replicate measures (for the ancestor) in one-factor ANOVAs, with the 37 and 42 groups compared to both the ancestral values and each other using Scheffe's posthoc test. For relative fitness, deviation of the relative fitness of a line or group from a null value of 1.0 was tested by one-sample *t*-test, again using replicate trials for lines and the means of the six lines for groups. Differences among fitness values of lines within groups, and between fitness values of the two groups, were tested using ANOVA. In addition, to test for overall group trends across the five novel stressful environments, we performed repeated-measures ANOVAs comparing the 37 and 42 groups for biovolume and relative fitness.

The relative performance of lines within each group across all six stressful environments was tested using Kendall's coefficient of concordance, which is capable of examining correlations among three or more variables simultaneously (Sokal and Rohlf 1994). Values of the reported statistic, *W*, can range from zero (no concordance) to 1 (perfect concordance).

RESULTS

Direct adaptation to a stressful environment (high temperature)

The assays at 41.5°C test the evolutionary adaptation of the 42 lines to their native stressful environment.

Biovolume yield.—The 42 group showed significantly improved yield in comparison to both the ancestral genotype and the 37 group (Table 2). In fact, yield of the 42 group at 41.5°C was no different than that of the ancestor at 37°C (Scheffe's $P = 0.83$). That is, the greater than 35% depression in yield at high temperature in the ancestor was completely compensated during adaptation to this environment. The 37

TABLE 2. Biovolume yield (nl/ml) in stressful environments (means ± 95% CI).

Line	Environment						
	High temperature	Acid	Alkali	Ethanol	High osmolarity	Peroxide	All novel environments
Ancestor[1]	15.92 ± 0.70†	25.12 ± 0.73	17.32 ± 1.03†	10.34 ± 2.00†	16.99 ± 0.61†	19.51 ± 0.94†	17.86 ± 1.32
42 + 1	26.68 ± 2.76*	24.44 ± 2.32	22.68 ± 1.60*	0.14 ± 0.12*	17.66 ± 1.44	20.35 ± 1.94	
42 + 2	21.16 ± 2.09*	26.71 ± 1.01	21.37 ± 0.99*	14.49 ± 2.86*	23.04 ± 1.07*	24.72 ± 0.58*	
42 + 3	24.39 ± 0.97*	24.34 ± 1.64	18.50 ± 2.23	12.52 ± 4.62	22.16 ± 0.91*	23.94 ± 1.51*	
42 − 1	25.59 ± 2.11*	20.18 ± 1.42*	22.32 ± 1.19*	18.08 ± 4.89*	20.76 ± 3.05*	25.86 ± 1.70*	
42 − 2	22.51 ± 0.55*	25.16 ± 0.61	21.17 ± 1.41*	11.99 ± 4.41	22.88 ± 2.87*	23.04 ± 2.18*	
42 − 3	23.66 ± 2.89*	23.65 ± 1.33	21.72 ± 1.29*	0.08 ± 0.12*	16.84 ± 1.02	22.48 ± 1.74*	
42 Mean	24.00 ± 2.11	24.08 ± 2.29	21.29 ± 1.56	9.55 ± 7.99	20.56 ± 2.83	23.40 ± 2.01	19.42 ± 2.41
42 versus Ancestor	$P < 0.001$	$P = 0.89$	$P = 0.013$	$P = 0.98$	$P = 0.064$	$P < 0.001$	$P = 0.065$
37 + 1	15.46 ± 2.48	25.38 ± 0.80	10.29 ± 0.61*	13.49 ± 3.14	18.97 ± 1.62	20.70 ± 1.39	
37 + 2	16.91 ± 0.54	25.04 ± 0.90	13.11 ± 1.48*	11.56 ± 2.29	17.32 + 0.79	20.28 ± 0.84	
37 + 3	13.38 ± 0.70	25.16 ± 0.53	16.33 ± 4.68	5.92 ± 2.75*	18.80 ± 1.37	21.75 ± 1.74*	
37 − 1	15.92 ± 2.48	24.10 ± 1.13	16.38 ± 1.04	8.77 ± 2.14	16.30 ± 1.33	22.82 ± 2.21*	
37 − 2	0.05 ± 0.12*	9.79 ± 1.41*	22.39 ± 0.44*	0.00 ± 0.07*	16.82 ± 1.14	22.41 ± 1.83*	
37 − 3	17.30 ± 4.98	18.00 ± 1.16*	21.04 ± 0.83*	11.39 ± 3.80	4.78 ± 2.72*	21.12 ± 1.83	
37 Mean	13.17 ± 6.90	21.24 ± 6.58	16.59 ± 4.82	8.52 ± 5.17	15.50 ± 5.62	21.51 ± 1.04	16.62 ± 2.45
37 versus Ancestor	$P = 0.36$	$P = 0.074$	$P = 0.90$	$P = 0.88$	$P = 0.64$	$P = 0.037$	$P = 0.27$
42 versus 37	$P < 0.001$	$P = 0.41$	$P = 0.008$	$P = 0.98$	$P = 0.012$	$P = 0.12$	$P = 0.006$

Note: Six replicate assays were performed to measure the biovolume yield of each line in each environment. These replicate assays were used to test for heterogeneity among the lines within each derived group by running separate ANOVAs for each group and environment. Among-line heterogeneity was significant ($P < 0.001$) in every case, except for the 37 group in the peroxide environment ($P = 0.075$). Line means were used to test for overall differences among the 37 group, 42 group, and ancestor by running one-factor ANOVAs for each environment, and by running a repeated-measures ANOVA to test for overall effects across all five novel environments (Scheffe's posthoc test *P*-values shown; see Methods).

[1] Mean biovolume yield (± 95% CI) for the ancestor in the standard, non-stressful environment was 25.21 (± 0.64).

† Ancestor measured under test conditions differs ($P < 0.05$) from the ancestor measured under standard conditions. The ancestor's biovolume yield in the acid environment did not differ from that measured in the standard environment, but its growth rate in the acid environment was reduced by 23% (A. J. Cullum, unpubl. data).

* Individual line differs ($P < 0.05$) from the ancestor measured under the same test conditions.

group in comparison to the ancestor showed lower mean yield, but this difference was not significant. Both evolved groups showed significant heterogeneity among their respective lines.

Relative fitness.—The relative fitness assays at 41.5°C (Table 3) produced very similar results to those seen for the measures of yield. The 42 group as a whole had a mean relative fitness of 1.33 at this temperature (Table 3), a value that is significantly greater than 1.0. An examination of the individual lines reveals that each one has significantly improved fitness relative to the ancestral genotype, although there are differences among the lines in the degree of fitness improvement. The test of the 37 group at 41.5°C produced very different results, with the relative fitness of the six lines averaging 0.66 (Table 3) with extreme heterogeneity among lines. The mean value does not quite differ significantly from 1.0 owing to this heterogeneity, but it is significantly less than the 42 group fitness. The lack of fitness improvement in the 37 group at 41.5°C indicates that the fitness gains in the 42 group at this temperature do not simply reflect adaptation to general aspects of the culture conditions, but rather indicate adaptation specifically to high temperature. These results accord with previous observations on these groups (Bennett and Lenski 1993).

Preadaptation to novel stressful environments

The assays in the stressful acid, alkali, ethanol, high osmolarity, and peroxide environments test for possible preadaptation of the 42 group to environments not previously encountered by these lines (i.e., during their derivation from the ancestral strain and divergence from the 37 group). The stressful nature of these novel environments, relative to the standard environment, was evidenced by the significantly reduced biovolume yield of the ancestral line in every case except the acid environment (Table 2); in that environment, a 23% reduction in growth rate was taken as an indication of stressful conditions (A. J. Cullum, unpubl. data; maximal growth rates of the ancestor, measured spectrophotometrically using a Bioscreen C incubator/reader [Labsystems Oy, Helsinki, Finland] were 1.04 doublings/hr in the acid environment versus 1.35 doublings/hr in ancestral environment [$P < 0.001$ by *t*-test]).

Biovolume yield.—The 42 group shows significantly higher yields than the ancestor in two of the five novel environments (alkali and peroxide), and a trend in this direction for a third (high osmolarity) (Table 2). No difference in yield between the 42 group and ancestor was seen for the acid and ethanol environments. When compared to the 37 group, roughly similar results were obtained, except that the higher biovolume yield of the 42 group at high osmolarity was significant, whereas the higher yield in peroxide became nonsignificant (Table 2). The repeated-measures ANOVA for the two derived groups and the ancestor across all five novel stressful environments indicated significant differences among the three ($F_{2,21} = 6.64$, $P = 0.006$), with the 42 group showing significantly better yield than the 37 group (Table 2) and marginally better yield than the ancestor. The 37 group and the ancestor showed no difference in biovolume when the novel environments were viewed as repeated measures.

Relative fitness.—The results of these assays, shown in Table 3, indicate that the lines in the 42 group tend to have lower fitness relative to the ancestor in the acid, ethanol and high osmolarity stress environments, but higher fitness in the alkali and peroxide environments; however, the differences are significant only for the high osmolarity and peroxide environments. The fitness tests of the 37 lines in these same environments produced qualitative results remarkably similar to those of the 42 lines, but with only the peroxide environment showing a significant group departure from a value of 1.0 (Table 3). The means of relative fitness were not significantly different between the 37 and 42 group in any individual environment or when tested across all five novel stressful environments by repeated-measures ANOVA (Table 3).

Relative performance across environments

For both biovolume yield and relative fitness, the rank of each line within its group was determined for each environment using the data in Tables 2 and 3. These fitness ranks are summarized graphically in Figure 1. Kendall's coefficients of concordance resulting from these ranks are shown in Table 4 for each variable (biovolume and fitness) and group (42 and 37). In no case was Kendall's *W* significant, indicating that the relative performance of a line in one stressful environment is not a good predictor of its relative performance in other stressful environments for either biovolume yield or relative fitness.

Discussion

In this study, we examined in a variety of stressful environments the biovolume yield and relative fitness of a group of *E. coli* lines evolved at and genetically adapted to high temperature (42°C), another group of control lines evolved at and adapted to a benign temperature (37°C), and the common ancestor of both groups. Our results provide equivocal support for the hypothesis that adaptation to one type of environmental stress produces a general stress-resistant genotype (Hoffmann and Parsons 1991).

In novel stressful environments, such as alkali and peroxide, the total biovolume of cells produced from a limited energy supply tended to be higher in the lines previously adapted to a high-temperature stress (the 42 group) than in the control lines propagated at the benign temperature (the 37 group). This result suggests that the heat-stress adapted lines developed a more generally efficient metabolism under stressful conditions. For example, less energy may be wasted in repairing DNA, replacing proteins, or maintaining the chemical composition of the cytoplasm (Visick and Clarke 1995; Ingraham and Marr 1996). It is also possible that greater biovolume could be achieved by a reduction in stress-induced deaths that have underlying causes unrelated to metabolic efficiency; for example, more bacteria could survive if mechanisms evolved to reduce lethal damage to DNA under stressful conditions (Humayun 1998). In any case, the results of the biovolume measurements are in accordance with the cross-tolerance hypothesis.

However, the greater biovolume production by the 42 group did not generally translate into higher relative fitness in comparison to the 37 group. The 42 group outperformed

TABLE 3. Relative fitness in stressful environments (means ± 95% CI).

Line	Environment						All novel environments
	High temperature	Acid	Alkali	Ethanol	High osmolarity	Peroxide	
42 + 1	1.469 ± 0.193*	1.062 ± 0.057*	1.421 ± 0.145*	0.341 ± 0.074*†	0.859 ± 0.037*	1.710 ± 0.247*	
42 + 2	1.164 ± 0.110*	0.923 ± 0.053*	1.061 ± 0.139	1.118 ± 0.119	0.869 ± 0.065*	1.901 ± 0.343*	
42 + 3	1.414 ± 0.306*	0.868 ± 0.069*	0.815 ± 0.088*	1.118 ± 0.100*	0.941 ± 0.055*	1.681 ± 0.419*	
42 − 1	1.573 ± 0.209*	0.453 ± 0.117*	1.004 ± 0.122	0.594 ± 0.106*	0.613 ± 0.110*	2.692 ± 0.266*	
42 − 2	1.202 ± 0.128*	0.810 ± 0.056*	1.114 ± 0.056*	1.021 ± 0.194	0.583 ± 0.102*	2.635 ± 0.216*	
42 − 3	1.126 ± 0.094*	0.853 ± 0.053*	1.199 ± 0.114*	0.321 ± 0.043*†	0.909 ± 0.070*	1.174 ± 0.077*	
42 Mean	1.325 ± 0.194	0.828 ± 0.214	1.102 ± 0.212	0.752 ± 0.398	0.796 ± 0.164	1.965 ± 0.621	1.089 ± 0.211
42 versus 1.0	$P = 0.008$	$P = 0.093$	$P = 0.27$	$P = 0.17$	$P = 0.024$	$P = 0.010$	$P > 0.05$
37 + 1	0.565 ± 0.254*†	1.018 ± 0.037	1.036 ± 0.081	1.267 ± 0.052*	0.990 ± 0.077	1.792 ± 0.127*	
37 + 2	0.624 ± 0.264*†	1.015 ± 0.059	1.019 ± 0.138	1.123 ± 0.102*	0.974 ± 0.040	2.056 ± 0.688*	
37 + 3	0.995 ± 0.028	1.022 ± 0.039	1.131 ± 0.102	1.025 ± 0.064	0.898 ± 0.069*	1.245 ± 0.095*	
37 − 1	1.075 ± 0.092	1.069 ± 0.048*	0.916 ± 0.043	0.796 ± 0.058*	0.958 ± 0.031*	2.144 ± 0.298*	
37 − 2	0.598 ± 0.062*†	0.353 ± 0.078*	0.985 ± 0.135	0.252 ± 0.071*†	1.015 ± 0.081	1.672 ± 0.272*	
37 − 3	0.109 ± 0.106*†	0.739 ± 0.076*	1.108 ± 0.098	1.287 ± 0.174*	0.657 ± 0.274*†	1.658 ± 0.226*	
37 Mean	0.661 ± 0.364	0.869 ± 0.293	1.033 ± 0.083	0.958 ± 0.409	0.915 ± 0.139	1.761 ± 0.338	1.107 ± 0.156
37 versus 1.0	$P = 0.062$	$P = 0.30$	$P = 0.36$	$P = 0.80$	$P = 0.18$	$P = 0.002$	$P > 0.05$
42 versus 37	$P = 0.002$	$P = 0.78$	$P = 0.45$	$P = 0.38$	$P = 0.18$	$P = 0.47$	$P = 0.81$

Note: Six replicate assays were performed to measure the fitness of each line relative to the ancestor in each environment. These replicate assays were used to test for heterogeneity among the lines within each derived group by running separate ANOVAs for each group and environment. Among line heterogeneity was significant ($P < 0.001$ in every case, except the 42 group in the peroxide environment, where $P = 0.002$, and the 37 lines in the alkali environment, where $P = 0.009$). Line means were used to test for departures from the null hypothesis value of 1.0 by performing one-sample *t*-tests. Differences between the two derived groups were tested by running one-factor ANOVAs for each environment and by running a repeated-measures ANOVA to test for overall effects across all five novel environments.

* Individual line differs ($P < 0.05$) from fitness of 1.0 relative to the ancestor.

† These mean values include one or more assays in which a final plate-count of zero was obtained for the derived line, in which case a count of 0.5 was used to calculate its fitness for that assay. These means are therefore conservative, in that the true mean may be even lower (see Methods).

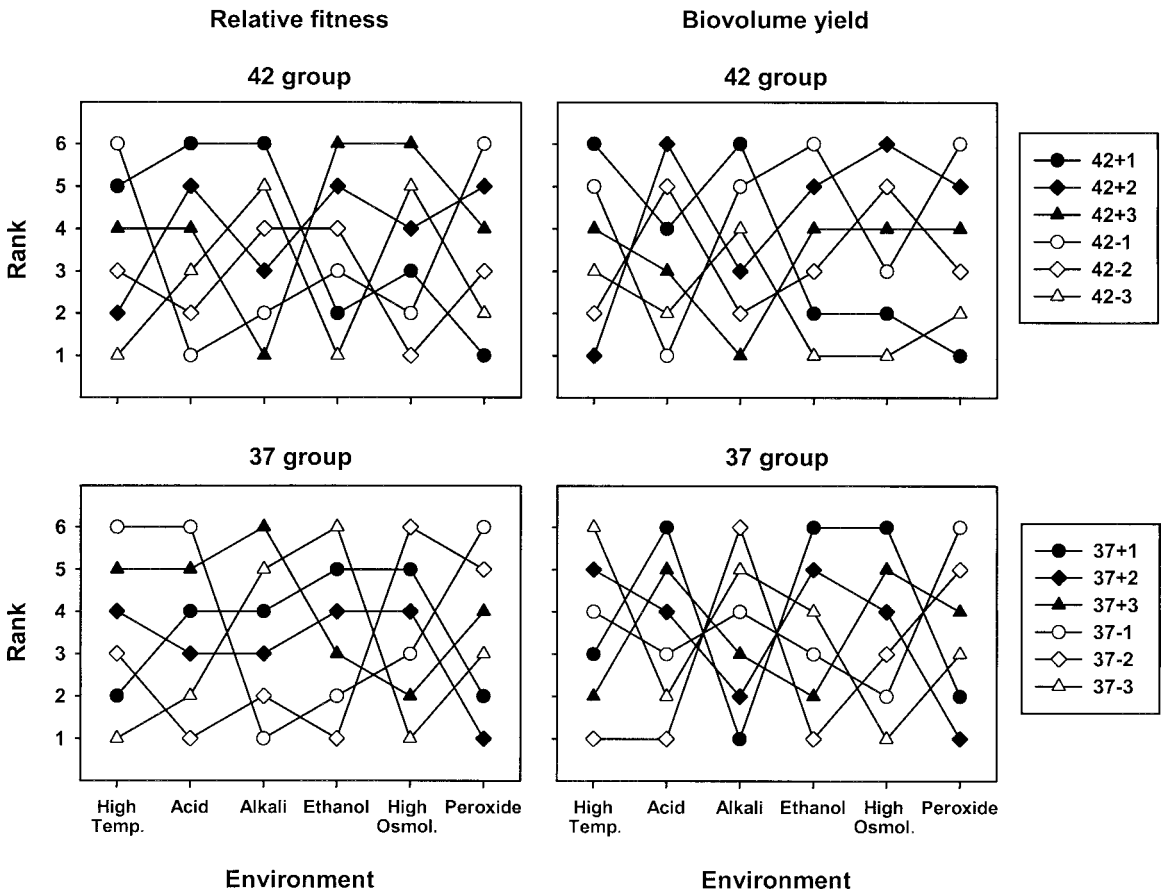

FIG. 1. Rank performance (relative fitness or biovolume yield) scores of each line within its group (selected at 42° or 37°C) across six stressful environments. Ranks are based on values shown in Tables 2 and 3, and each line within a group is indicated by a different symbol. The resulting Kendall's coefficients of concordance were nonsignificant in each case (see Table 4).

the 37 group for both biovolume yield and competitive fitness in the former group's native high-temperature environment, as expected. But there were no significant differences in fitness between the two groups in the novel stressful environments, whether the environments were considered individually (one-factor analyses) or as a whole (repeated-measures analysis). This lack of difference in relative fitness between the 42 and 37 groups was mirrored by cell counts collected as part of the biovolume measurements (A. J. Cullum, unpubl. data); that is, population densities (cells/ml) after 24 h were also generally indistinguishable between the two groups. Thus, with respect to both cell numbers and competitive fitness, the cross-tolerance hypothesis is not supported by our study.

TABLE 4. Concordance of performance across the six stressful environments.

Variable and group tested	Kendall's coefficient of concordance
Biovolume yield of 42 group	$W = 0.159$
	$P = 0.554$
Biovolume yield of 37 group	$W = 0.041$
	$P = 0.941$
Relative fitness of 42 group	$W = 0.150$
	$P = 0.587$
Relative fitness of 37 group	$W = 0.076$
	$P = 0.808$

Note: The six lines within each derived group were examined for the consistency of their performance across all environments, including the heat stress. Kendall's coefficient of concordance tests whether the lines within a group tend to rank in the same order across environments.

As was the case with the analysis of relative fitness, our examination of correlated performance across environments within groups failed to provide any real support for the existence of cross-tolerance. The rank concordances of performance of the derived lines were very low for both biovolume yield and relative fitness in both evolved groups, indicating that relatively good performance in one stressful environment was not associated with good performance in another.

In addition to these general trends, we found several specific aspects of our results that are worthy of note. The first of these is that, while the fitness of the 42 group relative to the ancestor did show a large and highly significant increase in one novel environment (peroxide), this increase was mirrored by a statistically indistinguishable increase in the relative fitness of the 37 group. Thus, the increased fitness under the peroxide stress was not indicative of any specific adaptations to an environmental stress, but instead must reflect general adaptation to some common aspect of the selective environment. These results highlight the importance of using appropriate controls when attempting to draw conclusions about patterns of adaptation.

The second notable aspect of our findings is the extensive variation among the lines in fitness values in the stressful environments. In the ethanol environment, for example, the range of yield and fitness values is quite large for both the 37 and 42 groups. It might be suspected that such variability is a consequence of the environmental stressors. However, lines evolved for 2000 generations at 37°C show similar divergence in fitness when competing for sugars other than glucose (Travisano et al. 1995). This variation in correlated responses thus appears to be the norm, rather than an exception. The temporal dynamics of this correlated variation are consistent with heterogeneous pleiotropic effects of different mutations that were selected in the several replicate lines, although genetic drift and hitchhiking of unselected mutations may also have played some role (Cooper and Lenski 2000; Cooper et al. 2001). In any case, such variation implies underlying genetic differences between the independently derived replicate lines, which have now been observed (Riehle et al. 2001).

One potential concern regarding the large variation within groups is that it may make detecting differences in stress resistance among lines relatively difficult. We believe, however, that such effects are not likely to have a major influence on our findings for several reasons. First, in our analyses of variance we found significant differences in biovolume between the 37 and 42 groups in novel environments, indicating that our tests can detect such differences even with considerable variation within groups. Second, the fact that the 37 group actually had higher average competitive fitness values than the 42 groups in three of the five novel environments suggests that there is truly no advantage associated with the 42 group for this trait. Finally, a large degree of variation among lines should actually make our other form of analysis, Kendall's test, more likely to detect any trends in performance across environments.

The final, and most surprising, aspect of our results relates to the different trend seen in biovolume yield and competitive fitness with regard to possible preadaptation to stressful environments. The finding that adaptation to one stressful environment tended to increase biovolume yield in novel stressful environments suggests that competitive fitness should also be increased in these environments, relative to nonstress adapted lines. However, this was not the case. It appears that, whereas biovolume yield provides a measure of the efficiency of growth and as such can be regarded as a fitness component, it is clearly not equivalent to Darwinian fitness when genotypes compete with one another for resources. Indeed, in a mass-action environment, there is no direct selection on growth efficiency, only on growth rate (Vasi et al. 1994). However, it is possible that increased biovolume yield might be advantageous under conditions other than those measured here. For example, given opportunities to colonize stressful but uninhabited environments, populations with greater total biovolume may be better able to establish new populations. Alternatively, greater biovolume may make populations better able to survive more extreme environmental challenges than those used in our experiments. However, these possibilities are merely speculative. What our results show is that the higher metabolic efficiency (as indicated by the increased yield) of the 42 group is not itself beneficial during direct competition under the particular stressful conditions used in this study.

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