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Genomic evolution during a 10,000-generation experiment with bacteria

(experimental evolution/molecular evolution/morphological evolution/insertion sequence elements/*Escherichia coli*)

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ABSTRACT **Molecular methods are used widely to measure genetic diversity within populations and determine relationships among species. However, it is difficult to observe genomic evolution in action because these dynamics are too slow in most organisms. To overcome this limitation, we sampled genomes from populations of *Escherichia coli* evolving in the laboratory for 10,000 generations. We analyzed the genomes for restriction fragment length polymorphisms (RFLP) using seven insertion sequences (IS) as probes; most polymorphisms detected by this approach reflect rearrangements (including transpositions) rather than point mutations. The evolving genomes became increasingly different from their ancestor over time. Moreover, tremendous diversity accumulated within each population, such that almost every individual had a different genetic fingerprint after 10,000 generations. As has been often suggested, but not previously shown by experiment, the rates of phenotypic and genomic change were discordant, both across replicate populations and over time within a population. Certain pivotal mutations were shared by all descendants in a population, and these are candidates for beneficial mutations, which are rare and difficult to find. More generally, these data show that the genome is highly dynamic even over a time scale that is, from an evolutionary perspective, very brief.**

Our collaborative work builds on two previous studies. One examined genomic variation among cells recovered from populations of *Escherichia coli* that had been stored as a "stab" culture for ≈30 years without renewal of the medium and, hence, with little opportunity for growth (1, 2). A high level of diversity was found using restriction fragment length polymorphism (RFLP) analysis with eight insertion sequence (IS) elements as molecular probes. Clones differed from their putative ancestor by ~12 changes, on average. It was unclear, however, whether the prolonged starvation had an important role in promoting or maintaining this variability and whether the derived bacteria were any better adapted to the storage regime than was their ancestor.

The other study examined the dynamics of phenotypic evolution in populations of *E. coli* that were propagated by daily serial transfer for 1,500 days, yielding 10,000 generations of binary fission (3, 4). The fitness of the bacteria improved by ~50%, on average, relative to the ancestor, and other phenotypic properties, such as cell size, also underwent large changes. The rate of phenotypic evolution was very fast during the initial 2,000 generations, but much slower during the subsequent 8,000 generations. However, certain other issues were not addressed, including the extent of genomic change and whether the rate of molecular evolution decelerated in parallel with phenotypic evolution. At one extreme, the derived genotypes may differ from their ancestor by only a small number of point mutations, which would require very extensive DNA sequencing to discover. At the other extreme, the derived lines may have undergone many chromosomal rearrangements (transpositions, inversions, deletions, etc.), in which case genomic evolution should be detected easily by RFLP analysis using IS elements as probes.

The present paper combines the methods of the first study with the populations from the second study to address these issues.

MATERIALS AND METHODS

Bacterial Strains. Twelve populations of *E. coli* B were founded from a common ancestor and serially propagated for 10,000 generations (1,500 days) in a glucose-limited minimal medium (3, 4). Samples from each population were obtained at 500-generation intervals and stored at −80°C. The ancestral strain has no functional viruses or plasmids, and it therefore is strictly asexual (clonal). This study examines the common ancestor and 7–20 clones randomly chosen after 500, 1,000, 1,500, 2,000, 5,000, 8,000, and 10,000 generations from two populations, designated Ara−1 and Ara+1. Each of these two populations retained point-mutation rates similar to that of their ancestor, unlike some of the other populations (5). We also examined 9–10 clones randomly sampled from each of the 10 other populations after 10,000 generations only. Three of these populations had spontaneously acquired mutator phenotypes by becoming defective in their methyl-directed mismatch repair pathways (5).

DNA Preparation and Hybridization. Molecular methods were described previously (1, 2). Briefly, clones were grown in LB medium and their genomic DNA was harvested by using standard methods. DNA was digested with *Eco*RV, and the resulting fragments (10^3-10^4) were separated by electrophoresis. The DNA fragments then were transferred to a nylon membrane, and Southern blot hybridizations were performed using internal pieces of the IS elements as probes; these internal pieces all lacked *Eco*RV restriction sites. Every clone was scored for the presence or absence of each fragment that hybridized with a particular IS probe. Ambiguous fragments of similar size usually were resolved by running the relevant clones in parallel and, otherwise, were scored conservatively.

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Abbreviations: IS, insertion sequence; RFLP, restriction fragment length polymorphism.
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Phylogenetic Methods. Phylogenies were constructed by using a parsimony method in which the roots of the trees were forced to be the actual common ancestor (6). The purpose of the phylogenies is to illustrate the divergence of the clones from their ancestor and from one another, rather than to make probabilistic statements about the validity of specific groupings. Hence, we did not weight losses and gains of IS elements differently, nor did we attempt to adjust for the fact that certain genetic events may cause the simultaneous loss of one band and gain of another. All genetic distances are calculated as the total number of differences between any pair of clones (including the ancestor).

RESULTS

Phylogenetic Relationships Among Clones Based on RFLP. The ancestral genome contains one or more copies of seven IS elements: IS*1*, IS*2*, IS*3*, IS*4*, IS*30*, IS*150*, and IS*186* (IS*5* is absent from the strain used in this study). Numerous clones were chosen randomly from frozen samples of two evolving populations, designated Ara−1 and Ara+1, obtained at several time points. Each clone's genomic DNA was hybridized successively with probes for each IS element. The RFLP is a genetic "fingerprint" that consists of the presence or absence of each fragment that hybridizes with an IS probe. These data were used to compute genetic distances among clones and reconstruct clonal phylogenies by parsimony methods (6).

It is important to bear in mind that the bacteria in this experiment were strictly asexual (3). Therefore, a mutation may have reached high frequency either because it conferred a selective advantage or because it "hitchhiked" with another mutation that was beneficial (7–9). However, any mutation was very unlikely to have reached high frequency solely by genetic drift; the expected number of generations for a new neutral mutation to drift to fixation is of the same magnitude as the population size (10), which was $>10^6$ even at the bottleneck during transfer (3, 4). Another possibility is that some mutations might have become common by recurrent insertions into "hot spots" for such events. If that were the case, then one would expect extensive convergence between the Ara−1 and Ara+1 populations. Using the 10,000-generation samples, we calculated an index of divergence as the observed genetic distance between the populations divided by the distance expected if all their mutations were unique. We obtain an index of 0.88, which indicates that only 12% of their evolutionary changes are convergent. (The actual convergence may be even less, because apparent convergence can arise from imprecision in distinguishing between fragments of similar size. Further molecular analysis will be needed to resolve these cases.)

Fig. 1 shows the inferred phylogenies for the two focal populations. Several features are noteworthy. First, we saw many clones that had the same IS fingerprint as the ancestor until generation 2,000 in both populations, but these were never seen in any of the later samples. Yet, on the basis of heritable and persistent changes in fitness and cell size, we know that several beneficial mutations swept through these populations during this period (3, 4, 11). Evidently, the earliest beneficial mutations to be substituted in these populations did not involve IS elements and went undetected by this approach.

Second, despite the continued persistence of the ancestral fingerprint, many genomic rearrangements also were observed during this initial phase. Moreover, some of these were quite successful, at least transiently. For example, in Ara+1 (Fig. 1*A*), a clade comprising several related genotypes represented ~15% of the population between generations 1,000 and 1,500, but the members of this clade left no descendants among the clones subsequently isolated. Given the large population, it is very unlikely that a clade of this size would arise, or be lost, so quickly by random drift. Instead, this clade may have increased because it carried a beneficial mutation and then declined because another clade acquired an even more beneficial mutation (12). A similar effect, but even more pronounced, is seen in this same population among the clones sampled in generations 5,000 and later. A clade comprising ~70% of the clones at generation 5,000 was no longer seen in generations 8,000 and 10,000. This phenomenon, in which the dominant clade at some later time emerges from outside (rather than within) an earlier majority type, indicates a "leapfrog" event; such events often can occur in large asexual populations that produce two or more competing beneficial mutations (12). These data therefore also suggest that many beneficial mutations appeared and achieved temporary success, but were not retained by natural selection over the long term in the face of other, even better mutations.

Third, as a consequence of selection and competition among beneficial mutations, the phylogeny appears not as a fat bush, comprising several roughly equal branches, but rather as a slender tree in which all of the side branches eventually end, leaving one main trunk (13). Along this trunk lie a succession of pivotal genotypes that—like mitochondrial "Eve" in human evolution (14, 15)—are ancestral to all individuals that subsequently were sampled. Indeed, several of these pivotal genotypes—these bacterial "Eves"—were actually present in our samples, for example, clones 1500.07 and 2000.09 in Ara+1 (Fig. 1*A*) and clones 2000.07 and 5000.08 in Ara−1 (Fig. 1*B*). Every IS-associated mutation that defines a pivotal genotype either hitchhiked with a beneficial mutation that arose in the same chromosomal background or was itself beneficial. Hence, these mutations are interesting candidates for further analysis.

Discrepancies Between Rates of Genomic and Phenotypic Evolution. Fig. 2 shows the time course of the average genetic distance between the derived clones and their ancestor. In both populations, this distance increased significantly with time, based on linear regression with intercept fixed at 0 (Ara−1, slope $= 9.09 \times 10^{-4}$ per generation, $F_{1,6} = 195.6$, $P < 0.0001$; Ara+1, slope $= 2.57 \times 10^{-3}$ per generation, $F_{1,6} = 456.0$, $P < 0.0001$). These data also indicate that population Ara+1 evolved significantly faster than did Ara−1 ($F_{1,12} = 147.2$, $P < 0.0001$). Yet, despite this almost 3-fold difference in their rates of genomic evolution, these two populations had similar gains in fitness relative to their common ancestor during the 10,000 generations (Ara−1, $\Delta W = 0.443$; Ara+1, $\Delta W = 0.477$; ref. 4). Thus, a comparison between populations that were initially identical and propagated under identical conditions indicates a conspicuous discrepancy between the rate of genomic evolution (based on IS fingerprints) and the extent of phenotypic improvement.

It is therefore also interesting to compare and contrast the temporal dynamics of genomic and phenotypic evolution within each of the populations. To that end, we calculated rates of evolutionary change, expressed per generation, for three traits over two periods of the experimental evolution. Table 1 shows that performance and morphological traits (fitness and cell size, respectively) changed much more rapidly during the first 2,000 generations than the subsequent 8,000 generations, in both focal populations. The same pattern for both of these traits was seen in all 12 replicate populations (4). By contrast, no deceleration was seen in the rate of genomic evolution in either focal population (Table 1). Given that the rates of phenotypic evolution decelerated in all 12 of the replicate populations, whereas the rate of IS-associated genomic evolution did not decelerate in either focal population, a Fisher's exact test indicates that this difference is significant (two-tailed $P = 0.0110$).

The continued persistence of the ancestral IS fingerprint for 2,000 generations (Fig. 1) may reflect the impact of several rapid sweeps of beneficial mutations (3, 4, 11). Each sweep, or "periodic selection" event, would have purged many other mutations (12, 16, 17). Over time, however, these selective

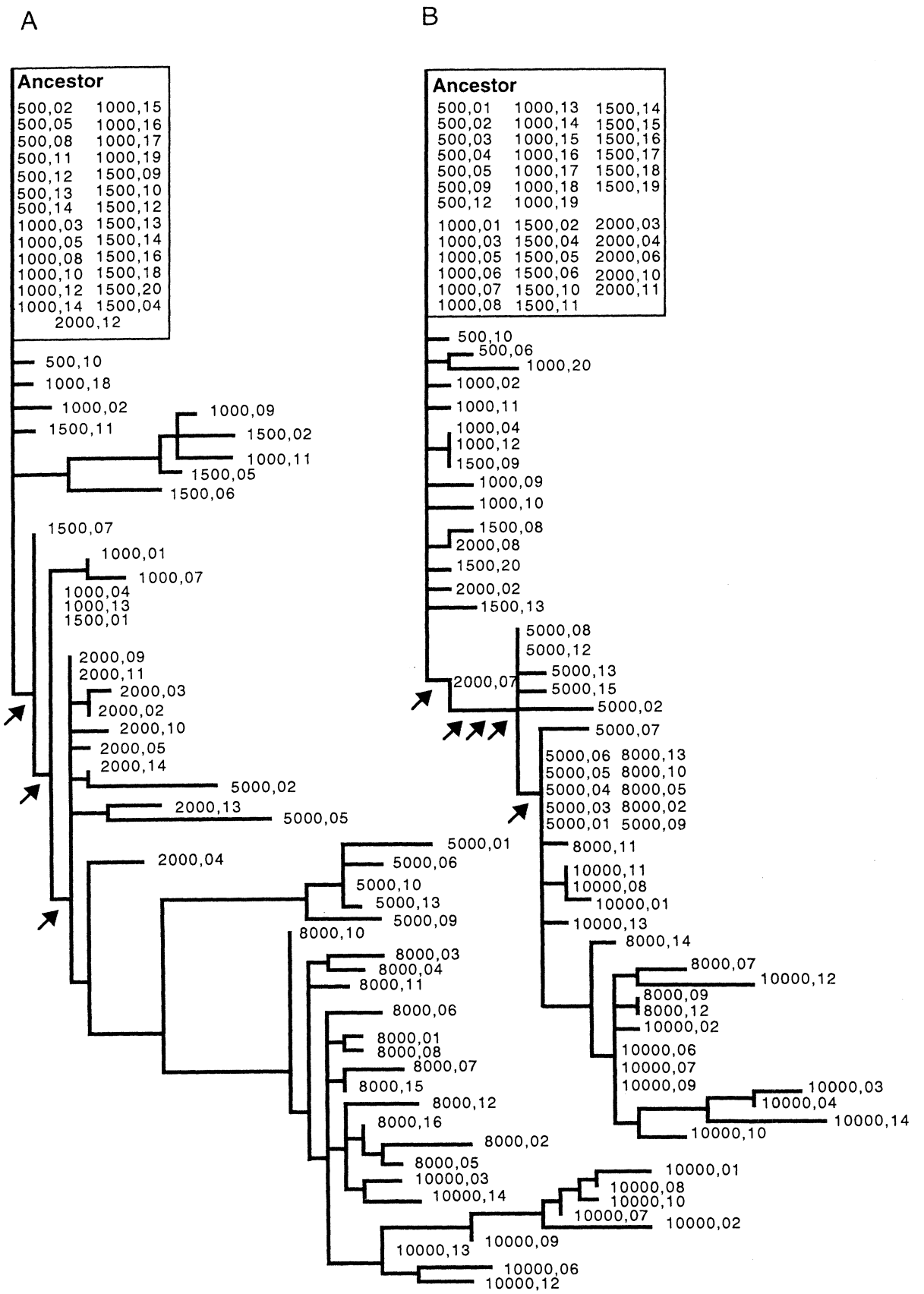

FIG. 1. Phylogenies for clones from two evolving populations of *E. coli*, rooted by using the actual ancestral genotype. Phylogenies were inferred by parsimony (6) from RFLP data obtained using IS elements as probes. Notation indicates the generation at which each clone was sampled, followed by an arbitrary number to distinguish clones from the same sample. Clones in the box with the ancestor were identical to the ancestor on the basis of their IS fingerprints. Arrows mark some of the pivotal mutations that were shared by all clones in every later sample. (*A*) Population Ara+1. (*B*) Population Ara−1.

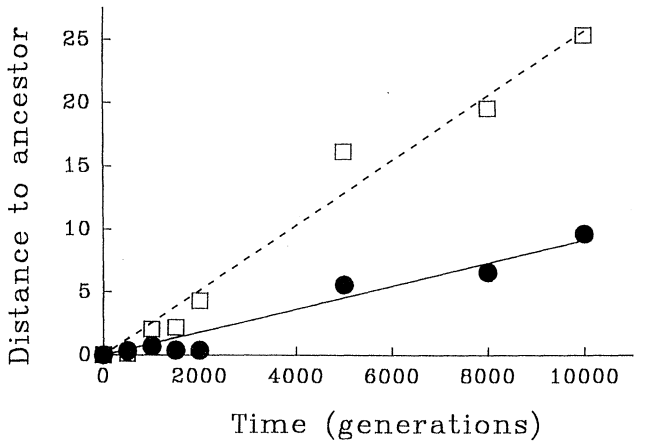

FIG. 2. Trajectory of the average genetic distance between two evolving populations and their common ancestor, calculated as the number of genomic changes detected using IS elements as probes. Solid circles, solid line: Population Ara−1. Open squares, dashed line: Population Ara+1.

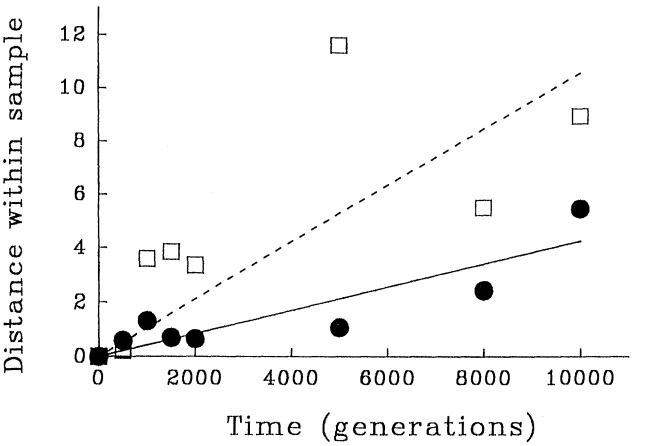

FIG. 3. Trajectory of the average genetic diversity within two evolving populations, as calculated from all pairwise genetic distances. Solid circles, solid line: Population Ara−1. Open squares, dashed line: Population Ara+1.

sweeps became much less frequent as the bacteria became better adapted to the experimental regime (4). This slower rate of adaptive evolution would allow many other mutations—including mildly deleterious as well as neutral—to reach higher frequency, where they then would be more likely to hitchhike with any beneficial mutation that subsequently arose (18).

In summary, there are conspicuous and significant discrepancies between the rates of genomic and phenotypic evolution, both across the replicate populations and over time within each population.

Variation Among Clones Within Each Population. Fig. 3 shows the extent of genetic variation among clones sampled from the same population as a function of time. In both focal populations, the average genetic distance between clone pairs from the same sample increased significantly over time, using linear regression with intercept fixed at 0 (Ara−1, slope = 4.24×10^{-4} per generation, $F_{1,6} = 47.78$, $P = 0.0005$; Ara+1, slope = 2.34×10^{-4} per generation, $F_{1,6} = 20.39$, $P = 0.0040$). At generation 10,000, the diversity was so great that all 11 clones from Ara+1 had distinct IS fingerprints, whereas 13 clones sampled from Ara−1 included 10 distinct genotypes.

Yet, the increase in diversity was not monotonic. The variation in Ara−1 declined by half from generation 1,000 to 1,500, and it fell even more sharply in Ara+1 between 5,000 and 8,000 generations. To test the statistical significance of these declines, we first calculated each individual clone's average pairwise difference from all of the individuals within the sample, so that the degrees of freedom correspond to the number of independent observations. We then compared these difference scores between consecutive samples. Both declines were highly significant based on two-tailed Mann–Whitney U tests ($P = 0.0015$ and $P < 0.0001$, respectively); both remain significant ($P < 0.01$) even after performing a Bonferroni correction (19) to adjust for the fact that each time series includes seven points that would allow six such comparisons between consecutive samples. These temporary reversals presumably reflect the variation-purging effect of the substitution of beneficial mutations in asexual populations (7, 12, 16, 17). In the absence of selective sweeps, genetic diversity in a population founded from a single clone should increase monotonically to a quasi-equilibrium that reflects the joint balance between mutation, selection against deleterious mutations, and random genetic drift (20–23).

Table 1. Rates of evolutionary change in performance, morphological, and genomic traits during initial and later phases of the experimental evolution

	Initial phase (0–2,000 generations)	Later phase (2,000–10,000 generations)	Ratio of initial/later
Fitness			
Ara − 1	1.16×10^{-4}	2.65×10^{-5}	4.38
Ara + 1	1.10×10^{-4}	3.20×10^{-5}	3.44
Cell size			
Ara − 1	1.15×10^{-4}	8.75×10^{-6}	13.14
Ara + 1	1.03×10^{-4}	1.56×10^{-5}	6.56
IS fingerprint			
Ara − 1	1.88×10^{-4}	1.16×10^{-3}	0.16
Ara + 1	2.15×10^{-3}	2.63×10^{-3}	0.82

Rates are calculated simply as the difference in average trait values at the indicated time points divided by the elapsed time. All rates therefore are expressed *per generation*. A value >1 in the last column thus indicates that evolutionary change decelerated as the experiment proceeded. (Data used in calculations for fitness and cell size are from ref. 4.)

Dynamical Behaviors of the Various IS Elements. Table 2 shows that the seven IS elements had very different dynamical behaviors. Moreover, the same element sometimes behaved quite differently in the two focal populations. The three IS elements with single copies—IS*2*, IS*4*, and IS*30*—were completely stable, retaining the same copy number and physical location in every derived clone as in the ancestor. Interestingly, based on the distribution of IS elements among natural isolates of *E. coli*, Sawyer *et al.* (24) suggested that IS*2*, IS*4*, and IS*30* may have mechanisms that repress transposition. Such mechanisms could contribute to their observed stability in this study. IS*3* was only slightly less stable, with occasional clones from each population yielding slightly different fingerprints

Table 2. Changes in copy number of seven different IS elements from the ancestor to 10,000 generations in two experimental populations of *E. coli*

	Ancestor	Ara − 1	Ara + 1
IS*1*	20	19.77 (±0.12)	17.36 (±0.31)
IS*2*	1	1.00 (±0.00)	1.00 (±0.00)
IS*3*	5	5.00 (±0.00)	5.00 (±0.00)
IS*4*	1	1.00 (±0.00)	1.00 (±0.00)
IS*30*	1	1.00 (±0.00)	1.00 (±0.00)
IS*150*	5	6.54 (±0.18)	16.45 (±0.41)
IS*186*	5	6.23 (±0.28)	6.64 (±0.15)

Values are means (±SE) based on 13 and 11 clones for populations Ara − 1 and Ara + 1, respectively.

(including, in Ara+1, slight variation in copy number in certain generations).

By contrast, IS*1*, IS*150*, and IS*186* underwent many changes in each focal population. The average copy number after 10,000 generations for IS*1* was close to the ancestral value of 20 in Ara−1, but it declined to ~17.5 in Ara+1. IS*186* experienced a small increase in copy number in both populations, from 5 in the ancestor to ~6.5 at generation 10,000.

IS*150* behaved similarly to IS*186* in Ara−1, showing a slight increase in copy number from 5 to a final average of ~6.5. By contrast, IS*150* was much more active in Ara+1, where its average copy number more than tripled to ~16.5 after 10,000 generations. This difference in the dynamics of IS*150* between the two focal populations had nothing to do with the Ara marker. A survey of its copy number in clones sampled at 10,000 generations from all 12 replicate populations (six Ara^- and six Ara^+) reveals substantial variability, with the average copy number ranging from ~4.8 to ~16.5, but there is no statistical association with the Ara marker state (t = 0.547, two-tailed P = 0.5965).

The reason for the greater IS*150* activity in Ara+1 is presently unknown. It might indicate increased transposition rate because of changes in either the IS itself or the bacterial chromosome, or it might reflect reduced selection on copy number for this IS in certain genetic backgrounds (25). In any case, this "burst" of IS*150* activity in Ara+1 accounts for ~60% of the difference between the two focal populations in their genetic distances to the ancestor after 10,000 generations (Fig. 2).

Effect of Mutator Status on Genomic Divergence from the Ancestor. Both focal populations retained point-mutation rates similar to their ancestor. However, 3 of the 12 replicate populations acquired mutator phenotypes because of the fixation of mutations in genes in the methyl-directed mismatch repair pathway; these defects caused ~100-fold increases in the rate of point mutation (5). In two of the three populations that became mutators, the change occurred in the first 3,000 generations of the experimental evolution (5), thus providing ample time for any effect of the mutator phenotype on genomic divergence to be manifest.

Fig. 4 compares the average genetic distance from the common ancestor, based on the IS fingerprints, for the three mutator and nine nonmutator populations using clones sampled at 10,000 generations. Although the average distance is higher for the mutator populations, the difference is only 10% and does not even approach statistical significance (t = 0.351, one-tailed P = 0.3665). This result implies that point mutations in restriction sites are responsible for few, if any, of the changes detected by RFLP analysis. In conjunction with changes in the copy number of several IS elements (Table 2), it also indicates that rearrangements, including transpositions and deletions, were primarily responsible for the extensive genomic evolution that we have documented using this approach.

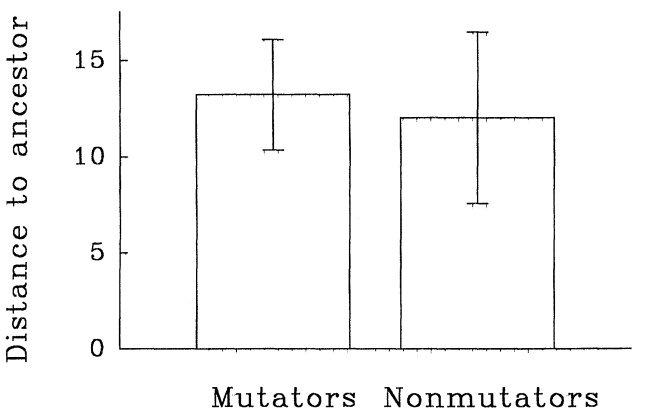

FIG. 4. Comparison between mutator (n = 3) and nonmutator (n = 9) populations of the average genetic distance from the common ancestor after 10,000 generations. Distances indicate the number of genomic changes detected using IS elements as probes. Error bars indicate 95% confidence intervals.

DISCUSSION

Our results demonstrate that these experimental populations of *E. coli* underwent rapid molecular evolution, leading to extensive changes in their genome structure, during ~4 years of adaptation to an environment in which they received nutrients every day. Divergence from the ancestor increased over time, as did genetic diversity within each population. The amounts of evolutionary divergence and genetic diversity were roughly similar to those seen after ~30 years of storage without any nutrient inputs (1, 2). Therefore, we conclude that constant, long-term starvation was not necessary to either substantially restructure the genome or to maintain a high level of genetic diversity, contrary to previous suggestions (1, 2, 26–28).

By contrast, selective sweeps of beneficial mutations evidently had important effects on the dynamics of genome evolution. Adaptive evolution was much faster in the initial phase of this experiment (Table 1), whereas the genetic diversity within populations reached its highest levels only after adaptive evolution had slowed significantly (Fig. 3). Selection has especially strong effects on the genomic evolution of asexual organisms; each beneficial mutation that sweeps to fixation eliminates diversity because the entire genome is one linkage unit (7, 12).

At the same time, linkage complicates the interpretation of the adaptive significance of any particular mutation. Were the IS-associated mutations that we detected merely passive "markers" or were they active "motors" in the adaptive evolution of these populations (25, 29)? The phylogenies shown in Fig. 1 will provide a useful tool for helping to answer this question because they identify pivotal mutations shared by all of the descendant population. Among the many mutations seen in our study, these pivotal mutations are the best candidates for having beneficial effects.

The derived populations became increasingly different from their common ancestor over time, both phenotypically and genetically; in that trivial sense, phenotypic and genomic evolution were concordant. However, we also showed that *rates* of phenotypic and genomic change were discordant in two important respects. First, populations that underwent similar fitness gains differed almost 3-fold in their rates of genomic evolution (Fig. 2) based on RFLP analysis using IS elements as probes. Second, the rates of change in both fitness and cell size decelerated sharply over time, whereas the rate of genomic change did not decline (Table 1). Such discrepancies in the rates of phenotypic and genomic evolution often have been suggested on the basis of historical and comparative evidence (30–34), but direct experimental evidence previously has been lacking.

The only other experiment that sought to examine directly the concordance between phenotypic and genomic change was the recent study by Bull *et al.* (35). They propagated several replicate lines of the bacteriophage ΦX174 for several weeks under novel growth conditions. They then measured the changes in viral growth rate, and they also sequenced the entire 5.4-kb genome of the ancestral and derived genotypes. The rate of fitness gain in the evolving virus populations decelerated significantly over time. They also saw a significant deceleration in the rate of nucleotide substitution over time. Thus, Bull *et al.* (35) did not observe the same *qualitative* discrepancy between rates of phenotypic and genomic change that we saw with *E. coli* (Table 1). However, it appears from their viral data that the deceleration in the rate of fitness improvement was

much more pronounced than was the deceleration in the rate of nucleotide substitution, which may indicate that the *quantitative* trend is similar in the two studies. Of course, there are important differences between these studies, including the organisms, duration of the experimental evolution, and methods to detect genomic changes.

The direct sequencing of entire *E. coli* genomes in a study such as ours is unfeasible. However, Michael Travisano (University of Houston, personal communication) sequenced more than 1,000 bp in clones sampled from each of the 12 populations after 2,000 generations. He found no mutations whatsoever from the ancestral sequence among the 15,552 total bp sequenced. The regions he sequenced include promoters for *ptsHI*, *crr*, *fruR*, and *cya*; they were chosen based on physiological evidence that regulatory changes in the phosphotransferase system might be responsible for some of the genetic adaptation to the glucose-limited selective environment (36).

We have shown that IS elements are efficient tools for monitoring genomic changes in these evolving populations, including both divergence from the ancestral state (Figs. 1 and 2) and diversity within a population (Figs. 1 and 3). We have not yet determined the molecular events responsible for the genomic changes detected in our study. However, three lines of evidence suggest that they are mostly due to IS transposition and other types of chromosomal rearrangement. First, as indicated above, point mutations are not abundant in these evolving populations. Second, the extent of genomic change detected by RFLP analysis, using the IS elements as probes, was similar among lines that had become genetic mutators and those that had wild-type point-mutation rates (Fig. 4). If many of the changes in IS fingerprints were due to point mutations in the restriction sites, then one would have expected a substantial increase in the rate of divergence from the common ancestor in the mutator lines, in which the point-mutation rate increased ~100-fold (5). Finally, we observed significant changes in the copy number of certain IS elements (Table 2); these changes are most easily explained by transposition and deletion events that produce gains and losses of copies, respectively.

Our future work will be directed toward identifying the molecular basis of the observed genomic changes, especially those pivotal mutations that are shared by all descendants within a population. We then will perform genetic manipulations to construct strains that are isogenic except for a pivotal mutation. By measuring the relative fitness of these constructs, we can determine which pivotal mutations simply hitchhiked with some (still unknown) beneficial mutation and which ones encode beneficial phenotypes that were selected during 10,000 generations of experimental evolution.

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