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Plasticity of Fitness and Diversification Process During an Experimental Molecular Evolution

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Abstract. A simplified experimental evolution encompassing the essence of natural one was designed in an attempt to understand the involved mechanism. In our system, molecular evolution was observed through three serial cycles of consecutive random mutagenesis of the glutamine synthetase gene and chemostat culture of the transformed *Escherichia coli* cells containing the mutated genes. Selection pressure was imposed solely on the glutamine synthetase gene when varieties of mutant genes compete in an unstructured environment of the chemostat. The molecular phylogeny and population dynamics were deduced from the nucleotide sequences of the genes isolated from each of the chemostat runs. An initial mutant population in each cycle, comprised of diversified closely-related genes, ended up with several varieties of mutants in a state of coexistence. Competition between two mutant genes in the final population of the first cycle ascertained that the observed coexisting state is not an incidental event and that cellular interaction via environmental nutrients is a possible mechanism of coexistence. In addition, the mutant gene once extinct in the previous passage was found to have the capacity to reinvade and constitute the gene pool of the later cycle of molecular evolution. These results, including the kinetic characteristics of the purified wild-type and mutant glutamine synthetases in the phylogenetic tree, revealed that the enzyme activity had diverged, rather than optimized, to a fittest value during the course of evolution. Here, we proposed that the plasticity of gene fitness in consequence of cellular interaction via the environment is an essential mechanism governing molecular evolution.

Key words: Experimental molecular evolution — Interaction — Coexistence of closely related genes — Plasticity of fitness — Diversification — Glutamine synthetase gene

Introduction

Biological molecules, such as an enzyme and its gene, are products of natural evolution brought forth by many factors, each of which is impetus to the complexity of molecular evolution. Experimental evolution in a simplified system, encompassing the essence of natural evolution, has gained its ground in the prospect of biological evolution as a method of comprehending the mechanism of molecular evolution (Lenski and Travisano 1994; Dykhuizen and Dean 1990). Experimental evolution studies on *Drosophilae* (Fontdevila et al. 1975) revealed that the population of several generations in a single environment maintained protein polymorphism on some genetic sites. In addition, stable coexistence of some mutant flies isolated from the population was observed (Ayala 1971), a phenomenon which seems to be a consequence of more than a balance between the supplement

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of accidental mutations and their depletion by selection, and/or genetic drift, i.e., the standard interpretation for molecular polymorphism (Nei 1987).

Recently, some phenotypes from experimentally evolved *Escherichia coli* populations in a simple unstructured environment were reported to diverge, of which diversity was maintained (Helling et al. 1987). Although physiological features of these phenotypes were so similar that severe competition was guaranteed, the phenotypes did coexist within the environment. It was suggested that mutations occurring at different genetic sites during the experimental evolution developed the phenotypes to acquire a complementary relationship for coexistence in the same environment (Helling et al. 1987). While a small number of mutations at different genetic sites are sufficient to allow coexistence, are mutations on multiple genetic sites really necessary for maintaining the diversity of a single gene during the evolution?

In pursuance of the question, we have previously established a simple chemostat culture involving *E. coli* strains differing only in glutamine synthetase gene. Under selection based on the differences in enzyme activity or its function, the *E. coli* strains achieved stable coexistence indifferent to mutations, if any, other than those on the glutamine synthetase gene (Xu et al. 1996; Kashiwagi et al. 1998). In this paper, molecular evolution of glutamine synthetase gene was observed through three serial cycles of consecutive random mutagenesis of the gene, and competition of its products in a simple unstructured environment of the chemostat, as stated above. Results suggest that the outcome of evolution tends not to be an existence of one fittest gene, but of several closely related genes with appropriate fitness for coexistence. Here we proposed that the essential mechanism of molecular evolution is the plasticity of the fitness of a gene towards cellular interaction via the environment. Changes in fitness temporarily suppress the optimization process, allow the coexisting state, and direct diversification.

Materials and Methods

Materials

E. coli YMC21 (Δ(*glnA-glnG*)2000 Δ*lacU169 endA1 hsdR17 thi-1 SupE44*) lacking glutamine synthetase gene (Chen et al. 1982) was kindly provided by Dr. Boris Magasanik (Massachusetts Institute of Technology). A hybrid plasmid pKGN containing *E. coli* glutamine synthetase gene was prepared previously (Xu et al. 1994). pKP1500 (Miki et al. 1987) was a generous gift from Dr. Takeyoshi Miki (Kyushu University). Glutaminase was a generous gift from Daiwa Kasei Co. Ltd. (Osaka). DNA manipulation and transformation of *E. coli* cells were carried out as described by Maniatis et al. (1982).

Random Mutagenesis

Error-prone PCR using Δ*Tth* polymerase (Arakawa et al. 1996) and primers 5′-GGGCCAGAACCTGAATTCTTCC-3′ and 5′-GTTTTGGCATAAAGGTCGCGGTT-3′ was employed for mutating G466 to C709 region of glutamine synthetase gene on plasmid pKGN. PCR fragments were digested with *Eco*RV and *Bgl*II and ligated to pKGN previously digested with the same restriction enzymes. *E. coli* YMC21 was then transformed with the hybrid plasmids. An aliquot of 0.1 ml from a total of 0.51 ml transformation mixture was plated on an LB agar plate (Maniatis et al. 1982) containing 50 μg/ml ampicillin. Mutation rate was determined based on the mutated gene harbored in the colonies isolated from the plate.

Pre-Culture of Transformants

A 0.2-ml aliquot of the transformation mixture (0.51 ml) was inoculated in 5 ml of medium C (0.1 M L-glutamate, 4 g/l glucose, 10.5 g/l K_2HPO_4, 4.5 g/l KH_2PO_4, 50 mg/l $MgSO_47H_2O$, 5 mg/l thiamine HCl, and 50 mg/l ampicillin), and the transformant mixture were grown at 37°C for 12 h. An aliquot of the 12-h culture (OD_{600} × volume = 1.0) was then transferred into fresh medium (100 ml), and cultivated further for 48 h at 37°C before dividing the culture into two equal volume (40 ml) and centrifuged. Cells collected were used as inocula for two concurrent runs of chemostat culture.

Chemostat Culture

Culture conditions were the same as those described previously (Xu et al. 1996; Kashiwagi et al. 1998) except for the glutamate concentration and the value of dilution rate, which were fixed at 0.1 M (medium C) and 0.075/h, respectively. Glutamate serves as the sole nitrogen source. By the established chemostat cultivation conditions, wall growth is ensured not to occur both in the chemostat and on the sampling tube.

Characterization of Mutant Enzymes

Genes encoding mutant enzymes to be characterized were subcloned to modified pKP1500 having an additional *Xba*I site. *E. coli* YMC21 cells transformed with the hybrid plasmids were cultivated at 37°C on medium C. The enzymes produced were purified as reported previously (Xu et al. 1994) except that a Q Sepharose Fast Flow column was used instead of a DEAE-Sepharose CL-6B column. Glutamine synthetase activity of the purified enzymes was measured as described previously (Xu et al. 1994).

Assay of Glutamine Concentration in Chemostat Culture

A 65 ml of culture effluent was collected, centrifuged, and the supernatant was consecutively passed through on three molecular weight cut-off filters, 100,000, 30,000, and 5,000 (Millipore-UFC4THK, UFC4LTK, and UFC4LCC, respectively) to remove contaminating biopolymers. Glutamine in solution was separated from glutamate by charging the filtrate onto an AG1-X8-acetate column (Bio-Rad), which adsorbs glutamate. The pass-through fractions were collected, concentrated, and the volume of the concentrated filtrate measured. Glutamine concentration was measured either by enzymatic method using glutaminase and glutamate dehydrogenase (Lund 1985) or by chromatographic method as follows. Glutaminase was added to a 2-ml aliquot of the concentrated filtrate, and the reaction mixture was incubated at

37°C for 120 min. The reaction mixture was then loaded onto an AG1-X8-acetate column, and the adsorbed glutamate was eluted with 1 M acetic acid. The amount of glutamate in the eluate was measured by the PICO-TAG method (Bidlingmeyer et al. 1984). Glutamate concentration in another 2-ml aliquot of the concentrated filtrate without the glutaminase treatment was measured as well. The glutamine concentration in the chemostat culture was estimated from the difference of two glutamate concentrations measured with and without glutaminase treatment.

Results and Discussion

Experimental Molecular Evolution of Glutamine Synthetase Gene

A mixture of transformants harboring glutamine synthetase genes diversified by in vitro random mutagenesis were subjected to continuous chemostat cultivation with ensured well-stirred environment to avoid any spatial bias in the culture of approximately 10^{10} cells. Glutamine synthetase genes were extracted from all the cells in the chemostat at the end of the first cycle of molecular evolution (270 h), pooled, and subjected to another in vitro random mutagenesis. For the second cycle of molecular evolution, the consecutive processes were the same as stated above except that cultivation in the chemostat was extended to 552 h. Genes from the end of the second chemostat culture were once again subjected to in vitro random mutagenesis as the preparatory step for the third chemostat run for 552 h. Mutation rate due to the in vitro random mutagenesis, estimated from the nucleotide sequences of the genes isolated from randomly chosen 58 clones after each mutagenesis, was 0.77 substitutions per gene with a high preference on A to G and T to C mutations as reported previously (Arakawa et al. 1996; Cadwell and Joyce 1992). Among the 55 substitutions detected, 24 were synonymous, and 31 were non-synonymous substitutions.

Molecular phylogeny and population dynamics were deduced from the nucleotide sequences of the glutamine synthetase genes isolated from each of the three chemostat runs. Twenty clones were randomly chosen at 270 h of the first chemostat culture, while 50 clones each were likewise chosen at 270 and 552 h of the second, and 552 h of the third culture (Fig. 1). From a diversified first mutant population, only the wild-type (W1) and two mutant genes (A1 and W2) were found, with A1 gene being major at the end of the first chemostat run. The other genes present during the initial stage may have been extinct or decreased in frequency below the sampling probability (5%). Final population of the second cycle of molecular evolution showed the presence of W1 and A1 genes, however, dominating the population was a new mutant C1 gene, which seems to be a derivative of the W1. A decrease in the variety of the mutant populations during the chemostat cultures were evidently shown when the second chemostat run was extended to 552 h (Fig. 1). Only 3 types of genes were detected at 552 h out of the 9 varieties found at 270 h, A1, A2, B1, C1, W1, W2, W3, W4, and W5. The A2 gene seems to be derived from A1, while W3, B1, and C1 from W1, and both W4 and W5 from W2. The frequency of the six mutants not detected at 552 h may have decreased in a level below the detection limit (2%). At the end of the third chemostat run, the A1 gene regained its standing as the major gene in the population with the existence of C1 in addition to the 7 other new mutant genes found (Fig. 1). The A3 appears to be a derivative of A1, while C2, C3, D1, E1, F1, and G1 are derivative of C1.

Reproducibility of the results was attested by sequence analysis of the glutamine synthetase genes at 270 h from randomly chosen clones in duplicate runs of the first chemostat culture. Both final population structures comprised a majority of the A1 gene with the W1 gene as one of the minority (see Fig. 1). Though genes of minority differ in each run, the chance of picking different genes with low frequency is feasible on random sampling. Hence, the resulting population structure was reproducible and not incidental.

Here, we conclude that the glutamine synthetase gene has evolved under the selection pressure imposed solely on the gene itself, as based on the facts and results of the experimental molecular evolution described above: (1) The glutamine synthetase gene is critical for the survival of the host strain, *E. coli* YMC21 deficient of the gene, under the culture conditions employed (Xu et al. 1996). (2) The only difference among the transformants in the chemostat lies on their glutamine synthetase gene carried on the plasmid. (3) The presence of selection pressure on the gene was evidently shown by the elimination of many of the varieties of genes present at the initial stage of each chemostat run. (4) While a total of 24 synonymous and 31 non-synonymous substitutions were found from randomly chosen clones in the initial mutant population of the three chemostat cultures, a total of 15 synonymous and 8 non-synonymous were detected at the end of the three cultures (Fig. 1A). High frequency of synonymous mutations ($P < 0.001$) inferred the existence of the selection pressure on the gene. (5) With the large population (10^{10}) used in observing the molecular evolution, it is unlikely that the perceived changes in the gene frequency (Fig. 1B) were attributed solely to genetic drift (Kimura 1983). For instance, the gene frequency of the C1 gene in the second chemostat culture increased from 70% at 270 h to 88% on 552 h. Average rate of frequency change per generation is more than 10^{-2} order $[(0.88 - 0.70)/\{(552 - 270) \times (0.075)\}]$, where 0.075 represents the generation per hour based on the dilution rate. This value is much bigger than the expected 10^{-5} order if genetic drift is assumed for the frequency change in such large population size. (6) Although genomic mutation may occur during the chemostat culture even for the *Rec*$^-$ host strain, the effects of genomic

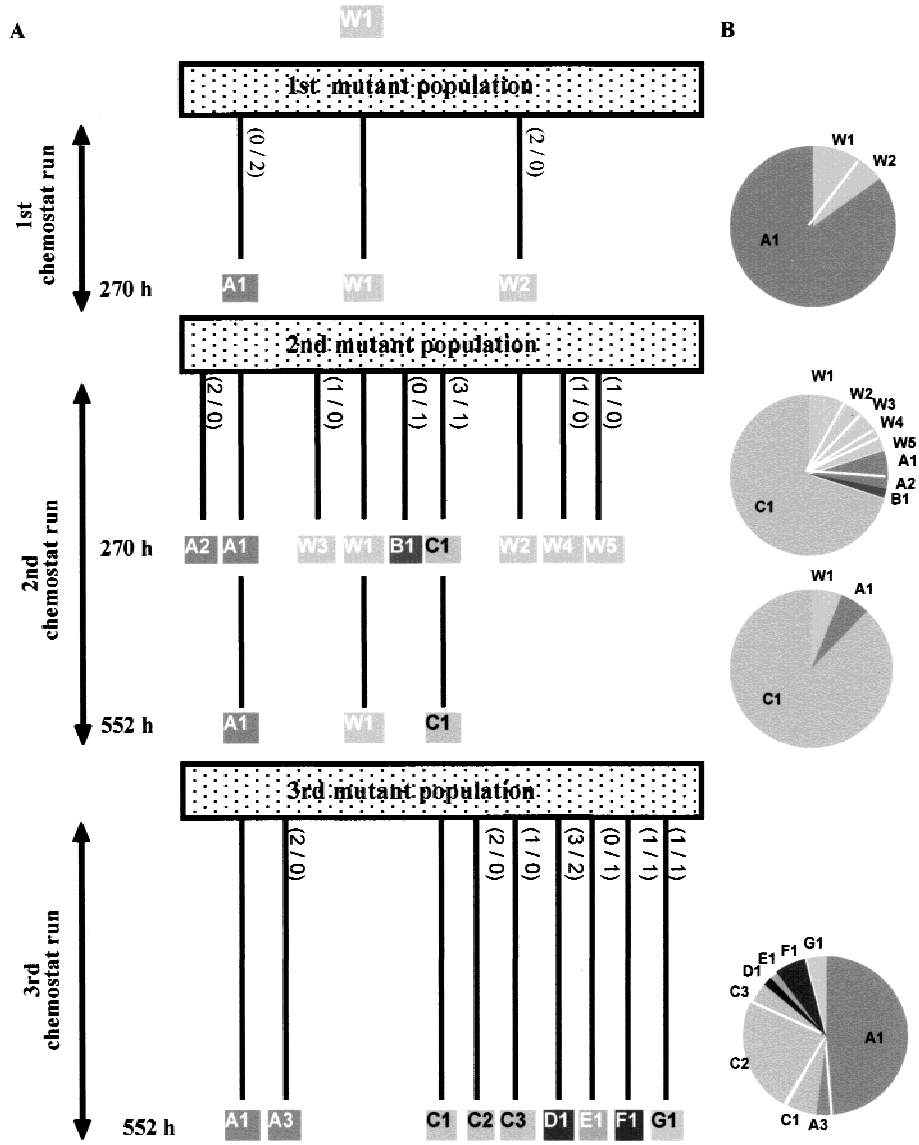

Fig. 1. Molecular evolution of glutamate synthetase. (A) Phylogenetic tree of glutamine synthetase gene. W1 denotes the wild-type gene. Paired capital letter and numerical number represents the type of mutant gene. Each letter denotes a different deduced amino acid sequence. Genes with the same letter but different number are synonymous mutants. The number of synonymous/non-synonymous mutations appears in parentheses. Double-headed vertical arrows represent the time span of cultivation. (B) Population structures at the indicated time of each chemostat run.

mutation on the dynamics of the molecular evolution of the understudied gene were confirmed to be very small by the reproducibility of the experimental results.

Mechanism of Coexistence as Means of Diversification

Clearly, as described above, different types of glutamine synthetase gene coexisted under a selective environment regardless of the long span of each evolution cycle. Moreover, effect of mutations, if any, on genetic sites other than the glutamine synthetase gene was very small and hence, not necessary for maintaining the diversity of the gene during the evolution. In our previous work, closely related competitors of *E. coli* strains differing only in the glutamine synthetase gene were shown to reach a stable coexistence (Xu et al. 1996; Kashiwagi et al. 1998). Consecutively, a mathematical model revealed that the essential mechanisms of coexistence involve the interplay between the internal metabolic network of competitors and the external variables of the environment, thereby allowing competitors to reach the same growth rate and coexist in the same environment (Yomo et al. 1996).

In view of the interplay between the internal metabolic network of the competitors and the environment, glutamine, a product of the glutamine synthetase reaction, may be one of the important external variables. To evaluate the role of glutamine, competition experiments between the A1 and W2 genes found at the end of the first chemostat run was performed (Fig. 1). Duplicate runs under the same conditions as in the molecular evolution resulted in the same population structure although the transient behavior is different (Fig. 2). The results indicate that the observed coexisting state is not an incidental event but may be governed by an underlying mechanism as presented in the mathematical model (Yomo et al. 1996). Indeed, glutamine was detected at an average value of 1.5 μM from the sampled culture at

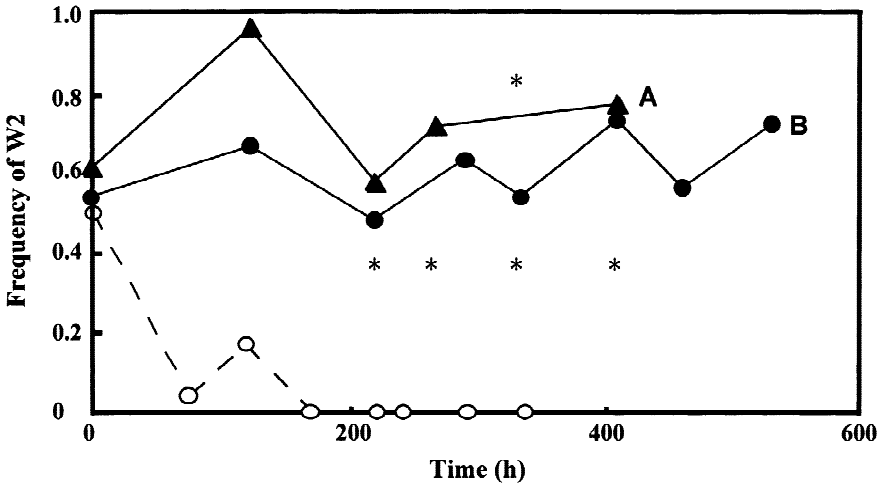

Fig. 2. Competition between the W2 and A1 genes. Each of the plasmid containing W2 gene or A1 gene of the same concentration was used to transform fresh host cells independently. The total resultant transformants from each transformation were mixed and an aliquot was used as inoculant for the chemostat run as described under Materials and Methods. Frequency of W2 was determined as follows: An aliquot of the chemostat culture was sampled at the indicated time and plated on 2xTY agar plate (12) containing 50 μg/ml ampicillin. The plasmids from randomly picked 26 clones per sampling were analyzed by double restriction endonuclease digestion. W2 has only the *Pst*I site, while A1 has both the *Pst*I and *Bst*EII sites. (▲) and (●) are parallel runs, A and B, of the competition experiments; (○), glutaminase (final 0.47 unit/ml) was added in the feeding medium. Glutamine concentration in the culture medium were measured at the indicated time (*) either by the PICO-TAG (for the A run) or enzymatic method (for the B run) as described under Materials and Methods. At 336 h of the A run, 1.2 μM glutamine was detected in the culture, while at 221 h, 268 h, 336 h, and 410 h of the B run, glutamine concentrations in the chemostat culture were 0.8 μM, 1.9 μM, 2.2 μM, and 1.3 μM, respectively.

indicated time of each chemostat runs (asterisks in Fig. 2). As glutamine was not added in the fresh medium fed to the chemostat, there could be a leakage of glutamine from the dead or live cells of both or either of the strains with the A1 or W2 gene. When glutaminase was added in the feeding medium to hydrolyze glutamine leaked in the medium, coexistence of the strains no longer prevails and the W2 gene was excluded (Fig. 2). These results indicate that glutamine is one of the important factors for the observed coexistence. Hence, cellular interaction among competitors through their major nutrients such as glutamine allows the maintenance of mutant genes within a population. Cross-feeding of a nutrient has been demonstrated to be a possible mechanism for the maintenance of coexistence of *E. coli* strains with different genotypes in a simple environment (Turner et al. 1996; Treves et al. 1998). Here we showed that even competitors differing only in a single gene were capable of reaching a state of coexistence through cellular interaction mediated by nutrients. The mechanisms underlying the coexistence of closely related competitors in a minimal model system can be the provision for bio-diversification occurring in nature.

Diversification and Not Optimization in Molecular Evolution

The experimental molecular evolution showed the exclusion of many of the varieties of the closely related genes produced by random mutagenesis through the selection pressure imposed on the gene, yet some remained and reached the state of coexistence. The possibility of such coexistence of the mutants in the same physical environment is low or nil if Darwinian selection is followed, especially when the competitors are closely related as used in this study (Futuyma 1986). Competitors could have adjusted their fitness by means of the interplay between the internal states of the competitors and the environment. Hence, the environmental conditions, as well as the internal state of a competitor, vary depending on the population structure. Therefore, when a gene type exists with different groups of competitors, its fitness differs in each case in accordance to the change in the environmental conditions. In fact, the A1 gene dominant in the first cycle of molecular evolution was replaced by the C1 gene on the second cycle, and was back to its dominant status on the third cycle (Fig. 1B). Could it then be that only gene types with higher fitness have been selected during the continuous progression of molecular evolution? To clarify the issue, the W2 gene, which was extinct, or may have decreased its frequency below the detection limit (2%) during the second chemostat run, was subjected to the following competition experiments. The W2 gene was mixed with the pooled genes isolated from the final population of the third chemostat run before introducing to fresh host cells, and the total resultant transformants were cultivated in the chemostat under the same conditions used for the molecular evolution experiments. Interestingly, the W2 gene was able to coexist with the new population structure regardless of the initial frequency of the W2 gene (Fig. 3). If the selection in the evolutionary system is based on gene

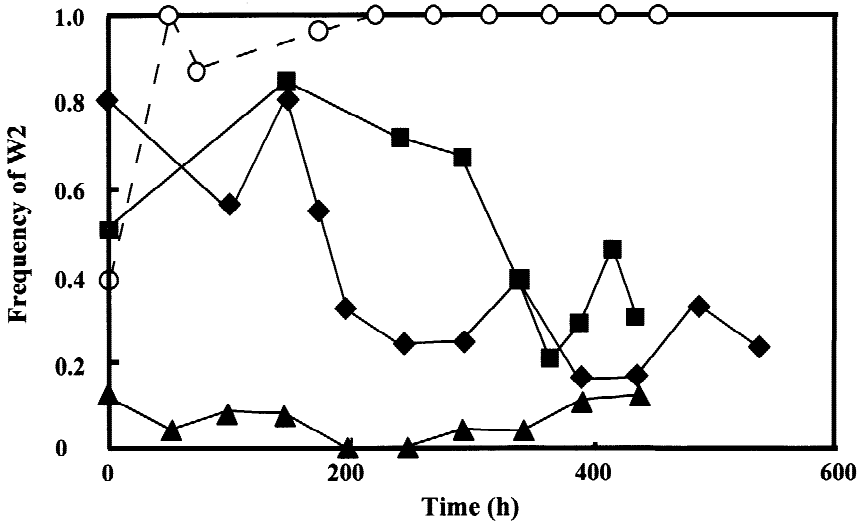

Fig. 3. Competition of the W2 gene with members of the final population of the third chemostat run. The once extinct W2 gene was mixed with the pooled genes isolated from the final population of the third chemostat run (see Fig. 1A). Frequency of the W2 gene was adjusted to 0.8 (♦), 0.5 (■), and 0.1 (▲) before mixing with the pooled genes. The three gene mixtures were introduced independently to fresh host cells and each of total resultant transformants were cultivated in three chemostat runs (♦, ■, and ▲) initiated with corresponding initial frequency of the W2 gene. Open circle (○) represents competition experiment conducted in the same manner as described above, with W2 gene frequency adjusted to 0.5 and glutamate concentration in the feeding medium reduced from 0.1 M to 10 μM. Frequency of W2 in the culture was determined as in Fig. 2.

fitness determined by its sequence and culture conditions, and the selection is independent of the presence of competitors, one would expect that genes pooled from the final population of the third chemostat have higher fitness than those of the second chemostat. In such a case, W2 gene could have no chance to survive when mixed with the population of the third chemostat. However, the results indicate that improvement of gene fitness is not always underway in the event of evolution. Although it may be natural for genes with higher fitness to be selected in a competitive chemostat, the emergence of new genes by mutation during the evolution process may set forth a change in the environment of the selected genes due to new cellular interaction. As a consequence, fitness of the genes changes. Accordingly, the fitness of the genes selected by an evolution cycle is not necessarily higher than that selected by the previous cycle. In addition, in most of the evolution cycles, several genes had reached the state of coexistence. Therefore, the outcome of the evolution tends not to be an existence of one fittest gene but of several genes with appropriate fitness for coexistence. Hence, the evolutionary stable strategies are not always the convergence stable strategies identified by maximization (Levin et al. 2000).

As cellular interaction among competitors temporarily relieves the optimization process in evolution, increase in gene fitness may be possible if interactions are weakened during competition, for instance, in low population density condition. When a very low concentration of glutamate was used in the feeding medium (10 μM), not only did the total population density decrease from 10^8 cells/ml to 10^5 cells/ml, the competing population also resulted to the dominance of the W2 gene (Fig. 3, open circle). Fig. 3 thus suggests that low population density promotes fitness increase while high population density drove diversification during evolution.

Although the fitness of the glutamine synthetase gene was shown as not optimized in the process of evolution, the function of the gene product, i.e., glutamine synthetase activity, may have done so. The wild-type and mutant enzymes in the phylogenetic tree in Fig. 1 were purified, and the kinetic property was analyzed with different glutamate concentrations. Results showed that enzymes coexisting in a population have different k_{cat} and k_{cat}/K_m values (Fig. 1 and Table 1). Saturation theory assumes that natural selection would cease to operate only if a function has reached a level that has no additional benefits to the fitness of a strain in a population (Hartl et al. 1985). Generally, it is presumed that a strain with mutant gene expressing a catalytic activity closer to the fittest value will dominate a heterogeneous population. Therefore, as the population propagates in several generations, the average enzyme activity over the population is expected to show a dynamics with a convergence towards the value of the fittest activity either on a monotonous increase or decrease. On the contrary, as shown in Fig. 4, fluctuation was observed in the average

Table 1. Kinetic constants of the wild-type and mutant glutamine synthetases in the phylogenetic tree of Fig. 1A. Glutamine synthetase activity was measured as described previously (Xu et al. 1994) with varying L-glutamate concentration from 1 mM to 100 mM. Results are expressed as ± S.D. (Cleland 1979). The specific activity of a mutant enzyme encoded by the D1 gene was less than the lower detection limit (10^{-2} s^{-1})

	Km (mM)	kcat (s^{-1})	kcat/Km ($mM^{-1}S^{-1}$)
W	12.1 ± 1.2	40.3 ± 1.6	3.3 ± 0.2
A	7.5 ± 0.4	20.7 ± 0.5	2.8 ± 0.1
B	1.1 ± 0.1	29.6 ± 0.8	26.1 ± 2.6
C	3.3 ± 0.2	28.9 ± 0.9	8.9 ± 0.4
E	23.2 ± 1.2	48.0 ± 1.1	2.1 ± 0.1
F	49.4 ± 14.8	6.4 ± 0.9	0.13 ± 0.02
G	9.5 ± 0.5	6.1 ± 0.1	0.64 ± 0.02

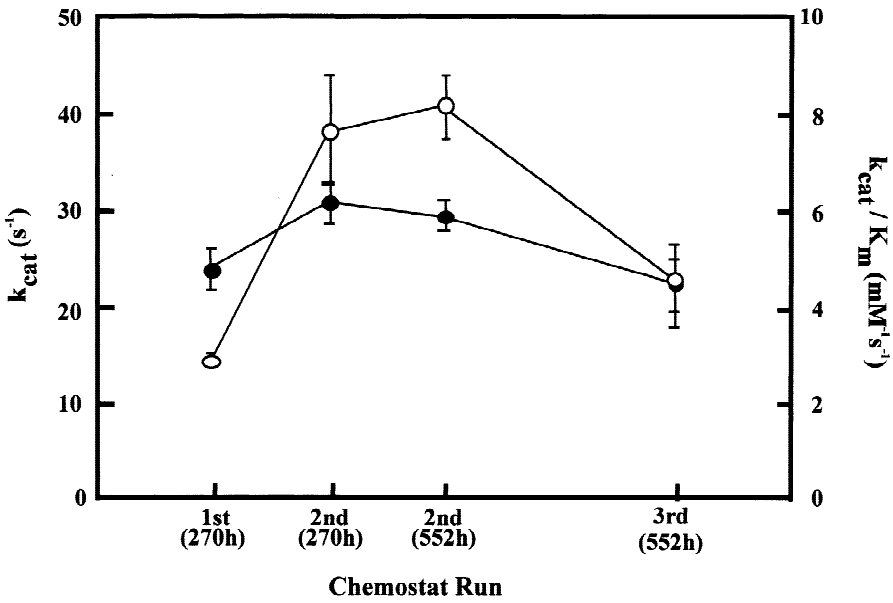

Fig. 4. Average values of the kinetic constants over the population at indicated time of the three chemostat runs. The average values were calculated from the data shown in Fig. 1B and Table 1.

value of glutamine synthetase activity. Accordingly, glutamine synthetase activity has diverged during the course of evolution rather than optimized to a finest value. We therefore proposed that an essential mechanism governing molecular evolution is the plasticity of the fitness of a gene towards cellular interaction via the environment. The changes in the fitness of genes temporarily suppress the optimization process, allow the coexisting state, and direct diversification. This study hence provides an example of balancing selection and could be an answer in some issues on neutralist-selectionist controversy (Gillespie 1991). The molecular diversification found in the experimental evolution so far described may serve as a basis in pursuance on issue regarding biodiversification and speciation.

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