Ohno's Model Revisited: Measuring the Frequency of Potentially Adaptive Mutations under Various Mutational Drifts

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The divergence of new gene functions is described by various scenarios that involve gene duplication, albeit, at fundamentally different stages. We performed experimental measurements and developed a subsequent model, aimed at predicting the rate of divergence under different scenarios. We used gene libraries of TEM-1 β-lactamase that were drifted under purifying selection toward the original penicillinase activity or under no selection at all. The frequency of genes conferring a new function (degradation of a cephalosporin antibiotic) was measured at various stages of the drift, and a model that accounts for the differences in the observed adaptation dynamics of the drifting TEM-1 populations was derived. The results indicate that rapid nonfunctionalization in the population relieved from selection (Ohno's model) afforded only a narrow window of adaptation to cefotaxime (neofunctionalization). The trade-off between TEM-1's original function and the new evolving function also disfavored the "gene sharing" model. The rate of adaptation was maximal when selection for the original function was partially relieved to enable the accumulation of potentially adaptive mutations, while still purging a large fraction of otherwise deleterious mutations. Altogether, scenarios of subfunctionalization seem more feasible, whereby sustaining the original function by two copies facilitates the accumulation potentially adaptive mutations while purging nonfunctionalization mutations.

Introduction

The mechanisms governing the divergence of new gene/protein functions comprise a central part of evolutionary theory. Ohno's model (Ohno 1970), expanded later by Kimura and Ota (1974), is currently the textbook paradigm. This model assumes that duplication is a neutral and frequent event. The redundant duplicated copy is free to accumulate mutations under no selection. If and when the need arises, mutations that endow a new function become under positive, adaptive selection, thus leading to neofunctionalization.

Although gene duplication and divergence are established mechanisms for expansion of protein repertoires (Chothia et al. 2003), the part of Ohno's model that surmises that gene duplication and the subsequent mutational drift occur under no selection is being questioned (Zhang 2003). First, most duplicated genes appear to drift under functional constraints. Namely, when both members of a pair are expressed, they appear to be subjected to purifying (or negative) selection that purges deleterious mutations (Hughes 2002; Lynch and Katju 2004; Scannell and Wolfe 2008). Second, expression of redundant copies carries substantial energetic costs (Wagner 2005), and there exists a selection pressure to inactivate their expression (Cooper and Lenski 2000; Dekel and Alon 2005). Third, gene duplication is often not a neutral event but rather positively selected under demands for higher protein doses (McLoughlin and Ollis 2004; Bergthorsson et al. 2007). Lastly, about a third of all possible random mutations in a given protein are deleterious (Bershtein et al. 2006; Camps et al. 2007; Tokuriki et al. 2007). Thus, when drifting in the absence of any selection, nonfunctionalization (loss of all functions primary due to mutations that diminish gene expression, or the ability to form a stable fold [Yue et al. 2005; Bershtein et al. 2008]) is much more likely than neofunctionalization.

Key words: Ohno's model, divergence, neofunctionalization, gene duplication, gene sharing, subfunctionalization.

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Why then does Ohno's model assume that relief from the "burden of selection" is a prerequisite for divergence? A key consideration imbedded in Ohno's model regards negative trade-offs, or antagonistic pleiotropy, between the evolving new function and the existing one. Indeed, beneficial mutations selected in one environment are often deleterious in other environments (Cooper and Lenski 2000; Kondrashov 2005). Hence, selection for the existing function is expected to purge mutations with adaptive potential.

Evidence indicating lack of trade-off, and thus the emergence of a new protein function prior to, or even without, gene duplication, led to the hypothesis of "gene sharing," by which, a gene with a given function is recruited for a very different function without significant changes in the coding region (Piatigorsky 2007). This concept was followed by several models that comprise alternatives to Ohno's (Hughes 1994; Lynch and Force 2000; Bergthorsson et al. 2007). These models surmise that 1) a certain degree of divergence can occur under selection for the original function, for example, the gene possesses a secondary, or promiscuous, activity with adaptive potential and 2) selection never ceases to operate on both duplicates. The second assumption provides a possible solution to the high frequency of nonfunctionalization under no selection. The feasibility of these alternative scenarios is also supported by the observation that promiscuous activities are prone to large increases by single, or a few mutations, that often have much smaller effects on the original function (Kondrashov 2005; Khersonsky et al. 2006). However, despite their obvious merits, the support for these alternative models is rather indirect. In addition, a major caveat of these alternative models (let alone gene sharing) is that mutations that endow new enzymatic functions often possess a measurable negative effect on the stability (and thus the levels of soluble active enzyme) (Wang et al. 2002; Tokuriki et al. 2008) and on the original enzymatic function. Even if these effects are minor relative to the potential innovation benefit (weak negative trade-offs), the acquisition of beneficial mutations can only continue as long as the existing function is reduced to an extent that does not compromise organismal fitness. However, by virtue of two genes carrying the

function of one gene, duplication can offer a margin that allows potentially beneficial mutations to accumulate.

The initial steps of divergence are therefore curbed by two major constraints: deleterious mutations leading to nonfunctionalization (favoring gene sharing), on the one hand, and negative trade-offs, favoring Ohno's model, on the other. The likelihood of divergence under different models could therefore be predicted, provided that the following parameters are known: 1) the frequency of deleterious mutations (f_-); 2) the frequency of potentially beneficial mutations (f_+); and 3) the trade-off—that is, the dependency of f_+ on, whether, and at what level, selection to maintain the original function is operating. At present, however, these basic parameters are largely the subject of educated (and less educated) guesses. For example, simulations by Behe and Snoke (2004) and Lynch (2005) of the likelihood of neofunctionalization under Ohno's model gave dramatically different results. The differences are imbedded in the different values for the above-described parameters, and, in particular, whether potentially adaptive mutations can accumulate as neutral (i.e., whether f_+ decreases, or goes to zero, under deletion of the original function).

We performed experimental measurements aimed at deriving these parameters using TEM-1 β-lactamase as a model (Hughes 2002; Wang et al. 2002; Weinreich et al. 2006). We observed significantly different dynamics (changes in the frequency of library variants exhibiting cephalosporinase resistance), depending on the strength of purifying selection to maintain the penicillinase activity of the drifting TEM-1 population, and developed a model that accounts for the adaptation dynamics under various duplication scenarios. The results and model support the revision of Ohno's model in the spirit of previously proposed alternatives in which gene duplication promotes divergence by alleviating the selection pressure, rather than a total relief.

Materials and Methods

Library Construction and Purifying Selections

The TEM-1 β-lactamase gene under the control of its endogenous promoter was recloned into a modified pUC19 plasmid (NEB) carrying a chloramphenicol resistance gene, as previously described (Bershtein et al. 2006, 2008). Random mutagenesis using wobble base analogues was developed to create a library carrying an average of 2 mutations per gene and a typical pattern of nucleotide changes (Bershtein et al. 2006). The pUC19 TEM-1 libraries were selected on agar plates carrying 34 μg/ml chloramphenicol and 12.5 μg/ml (*Lib12.5*), 250 μg/ml ampicillin (*Lib250*), or no ampicillin (*Lib0*), as described (Bershtein et al. 2006, 2008). Surviving colonies were collected from the plates and their plasmid DNA extracted.

Selection on Cefotaxime

The drifted TEM-1 populations that have never been exposed to cefotaxime were transformed into XL1-Blue cells (Stratagene, La Jolla, CA) and plated (over 300,000 transformants per plate) on agar plates bearing 34 μg/ml chloramphenicol and the given concentrations of cefotaxime (0.2 or 0.5 μg/ml). The surviving colonies were counted after 24 h of incubation at 37 °C. Each transformation and plating experiment was repeated two–three times.

Fitness Measurements and Inhibitory Concentration Determination

A representative number of clones (300–400) were picked and grown individually in the 96-well plates that served as an inoculation source for replica plating on a set of chloramphenicol agar plates with varying ampicillin concentrations (0–2,500 μg/ml), as described (Bershtein et al. 2006). Fitness values (W) were derived from the fraction of clones that survived at a given ampicillin concentration. To determine the inhibitory concentrations (IC) of various TEM-1 clones, randomly picked colonies were individually grown in 96-well plates (in the presence of chloramphenicol only) and then replica plated on a set of chloramphenicol agar plates with varying concentrations of ampicillin (0–2,500 μg/ml) or cefotaxime (0.1–25 μg/ml). The last concentration of antibiotic on which the colony succeeded to grow was determined as its IC value.

Results

The Experimental System

TEM-1 is a proficient penicillinase ($k_{cat}/K_M \approx 4.2 \times 10^7\ s^{-1}M^{-1}$with ampicillin) that possesses negligible activity against "third-generation" cephalosporins such as cefotaxime ($k_{cat}/K_M \approx 2.1 \times 10^3\ s^{-1}M^{-1}$) (Wang et al. 2002). However, TEM-1 variants that efficiently degrade cefotaxime have been found in the clinic and laboratory. The most frequently found mutation, Gly238Ser, confers dramatically higher cefotaxime resistance: IC (the minimal concentration of cefotaxime that leads to inhibition of *Escherichia coli* growth) increases from nil in wild-type TEM-1 to 0.2 μg/ml (all IC values refer to the experimental conditions applied here). But this mutation also destabilizes the protein and reduces its penicillinase activity: IC toward ampicillin reduced from 3,000 μg/ml in wild type to 1,000 in the Gly238Ser mutant. The acquisition of cefotaxime degradation by TEM-1 β-lactamase therefore comprises a typical case of relatively weak, yet significant, trade-offs between the new and original function (Wang et al. 2002; Khersonsky et al. 2006).

We made use of three populations of TEM-1's genes that were drifted as previously described (Bershtein et al. 2006, 2008). Each population, after separate mutagenesis, underwent purifying selection by exposure to an appropriate concentration of ampicillin. For the "high" selection pressure (*Lib250*), a concentration of 250 μg/ml of ampicillin was chosen (the maximal concentration at which wild-type TEM-1 still confers 100% survival to freshly transformed cells is 500–1,000 μg/ml [Bershtein et al. 2006]). The "low" selection pressure was just above the lowest effective concentration of ampicillin (12.5 μg/ml; *Lib12.5*). No ampicillin was applied for the *Lib0* that thus drifted under no selection. The survived TEM-1 genes were subjected to the next round of mutagenesis and purifying selection. Altogether, we performed 18 successive rounds of mutagenesis and selection during which the drifting populations were never exposed to cefotaxime. The loss of

Table 1
The Frequency of Cefotaxime-Resistant Clones in the Drifting Populations of TEM-1

Library	Round of Mutagenesis and Selection on Ampicillin	Average Number of Mutations per Gene (n)	No. of Survived Colonies ($\times 10^{-5}$)	
			0.2 μg/ml Cefotaxime	0.5 μ g/ml Cefotaxime
Lib250 (≥1,000 μg/ml ampicillin)[a]	2	1.7 ± 1.2[b]	170 ± 43[c]	81 ± 6
	5	5.2 ± 2.2	386 ± 97	86 ± 7
	10	9.4 ± 3	423 ± 117	100 ± 7
	15	16 ± 5.3	207 ± 52	98 ± 8
	18	21.6 ± 3.7	121 ± 30	43 ± 3
Lib12.5 (≥100 μg/ml ampicillin)[a]	2	3.5 ± 1.6	220 ± 55	54 ± 15
	5	7.3 ± 3.1	225 ± 56	56 ± 16
	10	10.7 ± 3.5	131 ± 47	17 ± 4
	15	17.6 ± 4.9	65 ± 16	22 ± 6
	18	22.4 ± 6.1	26 ± 7	6 ± 2
Lib0	2	3.9 ± 1.6	108 ± 27	36 ± 3
	5	9.5 ± 3.7	38 ± 19	11 ± 1
	10	20.7 ± 2.8	1 ± 1	0
	15	29.8 ± 5.3	0	0
	18	38.2 ± 3.1	0	0

[a] Threshold of ampicillin resistance in IC measurements (the selection on 12.5 or 250 μg/ml of ampicillin was performed by directly plating transformed cells, in oppose to growing colonies in liquid medium prior to the IC measurements).
[b] Standard deviations from the average value were calculated on the basis of sequencing of up to 42 randomly picked variants per round from each library.
[c] Standard deviations were calculated on the basis of two, or three, independently performed experiments (see Materials and Methods).

diversity observed was ≤50% per round, and $\geq 10^6$ independent clones per library were maintained throughout.

Following the above-described drift, the three TEM-1 libraries (*Lib0*, *Lib12.5*, and *Lib250*) were retransformed, plated on agar plates carrying 0.2 or 0.5 μg/ml cefotaxime. The lower concentration (0.2 μg/ml) corresponded to the level of resistance conferred by the Gly238Ser TEM-1, whereas the higher level requires at least one additional mutation (Hall 2002; Wang et al. 2002). The cefotaxime surviving colonies were counted (table 1), and representative variants were chosen at random, sequenced, and their ampicillin and cefotaxime IC values determined (supplementary table 1, Supplementary Material online).

The Frequency of Deleterious and Advantageous Mutations

We developed a simple model that accounts for the observed adaptation dynamics in the three drifting libraries (table 1) and derives the frequency of mutations with the potential to provide cefotaxime resistance (f_+) and of deleterious mutations (f_-). In the simplest terms, the frequency of cefotaxime-resistant variants (P_{ctx}) in a drifting polymorphic population is

$$P_{ctx} = 1 - e^{-f_+ \cdot n}, \quad (1)$$

where f_+ is the fraction of mutations endowing cefotaxime resistance within the entire ensemble of randomly accumulating mutations and n is the average number of mutations per gene. Because $f_+ << 1$ (adaptive mutations are very rare), and in our experiment $n < 40$, then $f_+ \cdot n << 1$ and:

$$1 - e^{-f_+ \cdot n} \approx f_+ \cdot n = P_{ctx}. \quad (2)$$

Equation 2 assumes that the higher is the frequency of mutations accumulated per gene (n), the higher is P_{ctx} (the frequency of library variants exhibiting cefotaxime resistance). However, because a randomly drifting population also accumulates deleterious mutations that lead to nonfunctionalization (primarily due to destabilizing mutations that reduce protein levels), the fraction of the cefotaxime-resistant genes would be accordingly reduced

$$P_{ctx} = f_{ctx} \cdot n \cdot (\text{fraction of viable variants in the population}). \quad (3)$$

The fraction of "viable" genes in a randomly drifting population correlates with the average fitness values of the drifting TEM-1 populations ($W(n)$) as follows (Bershtein et al. 2006):

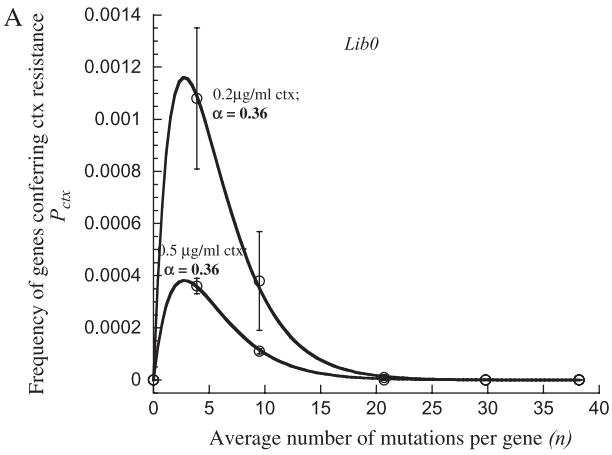
$$W(n) = (1 - f_-)^n = e^{-\alpha n}, \quad (4)$$

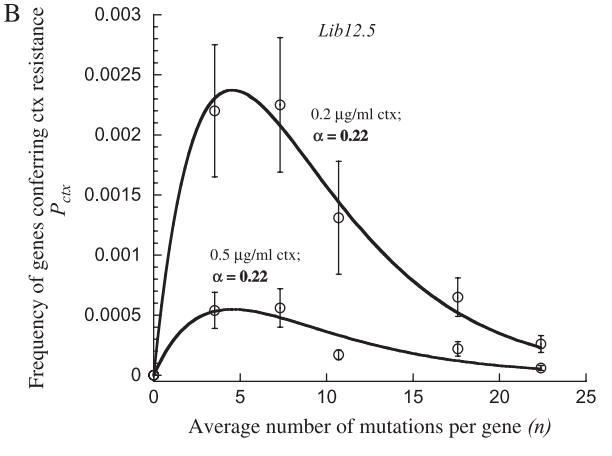
where f_- is the fraction of the deleterious mutations (out of the entire ensemble of randomly accumulating mutations) and $\alpha = -\ln(1 - f_-)$. Note that for $\alpha \leq 0.5$, $f_- \approx \alpha$.

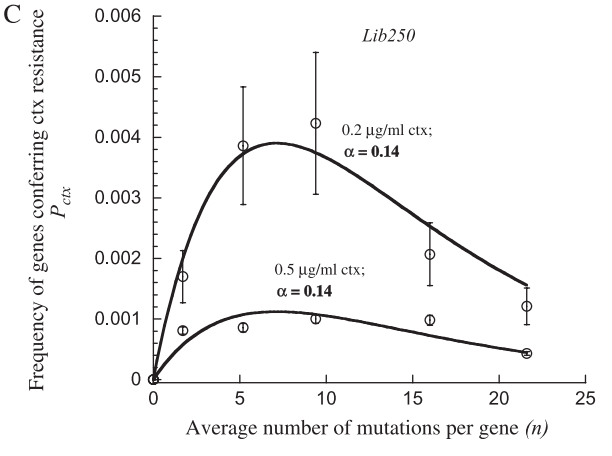
Hence, the model predicts that the frequency of genes conferring cefotaxime resistance (P_{ctx}) corresponds to the average number of mutations per gene in the following manner:

$$P_{ctx}(n) = f_+ \cdot n \cdot e^{-\alpha n}. \quad (5)$$

A fit of the frequencies of the cefotaxime-resistant variants in *Lib0* to equation 5 with both f_+ and α values floating yielded values of $\alpha = 0.35$ and 0.37 for 0.2 and 0.5 μg/ml cefotaxime, respectively. As expected (see Model Validity), the α values for both cefotaxime levels were essentially identical, and the data fitted equally well ($R > 0.99$) with an average α value of 0.36 (fig. 1*A*). For *Lib12.5*, the







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cefotaxime (μg/ml)	*f*+ (*Lib0*) **α = 0.36**	*f*+ (*Lib12.5*) **α = 0.22**	*f*+ (*Lib250*) **α = 0.14**
0.2	$1.1 \cdot 10^{-3}$	$1.4 \cdot 10^{-3}$	$1.5 \cdot 10^{-3}$
0.5	$3.9 \cdot 10^{-4}$	$3.3 \cdot 10^{-4}$	$4.3 \cdot 10^{-4}$

FIG. 1.—The observed frequency of cefotaxime-resistant clones in the drifting TEM-1 populations. Plotted, for the various libraries and generations, are the measured frequencies (P_{ctx}) versus the average number of mutations per gene (table 1). Note the different scales for both the *y* axis and the *x* axis. Error bars represent deviations of two–three independently performed experiments. The line corresponds to a fit to equation 5 that yielded the fraction of the cefotaxime resistance conferring mutations (f_+) and deleterious mutations (α). (*A*) Fitting the frequencies in *Lib0* at 0.2 and 0.5 μg/ml cefotaxime with floating f_+ and extracted α values were 0.21 and 0.23, and a subsequent fit made use of an average α value of 0.22 (fig. 1*B*). For *Lib250*, α values were 0.15 and 0.13, and α = 0.14 was applied for the subsequent fit (fig. 1*C*). The derived α values correspond to the fraction of deleterious mutations (f_-) under the various selection regimes and f_+ to the fraction of cefotaxime resistance mutations (fig. 1).

Model Validity

The above model accounts for the observed adaptation dynamics throughout the length ("generations") of the experiment, despite its simplicity, and inevitable error range in the measurements of P_{ctx} (to rigorously estimate these errors, repetitive measurements were obtained by two–three independent transformations and cefotaxime selections). Several observations support the validity of the proposed model.

First, the extracted α values are expected to be identical for both levels of cefotaxime, as is largely the case. This stems from the fact that the fraction of deleterious mutations is a function of the drift only (i.e., the level of purifying selection for ampicillin resistance) and because this drift occurred independently of the eventual exposure to cefotaxime. For the same reason, the α values obtained from the cefotaxime adaptation model should correlate with the α values obtained from the entirely independent ampicillin fitness measurements of the same drifting libraries (Bershtein et al. 2006). Indeed, the α value obtained here from the fit of *Lib0* (0.36) is comparable to the previously reported fitness decline for ampicillin at high levels (0.28 at 2,000 and 0.37 at 2,500 μg/ml ampicillin) (Bershtein et al. 2006). At these high ampicillin levels, the negative epistatic effects were negligible, and the fitness level was reliably predicted by equation 4 (Bershtein et al. 2006). Further, the measured fraction of deleterious mutations under no selection ($f_- \approx \alpha = 0.36$ for *Lib0*) is very similar to the experimentally determined fraction of the deleterious mutations (~33%) in various proteins subjected to random mutagenesis (Camps et al. 2007). The α value obtained from the ampicillin measurements of *Lib12.5* is 0.18 at 2,500 μg/ml ampicillin (supplementary fig. 1, Supplementary Material online) and is comparable to the α value of 0.22 obtained from the cefotaxime adaptation model. Because the selection threshold of the *Lib250* was very close to the maximal ampicillin degrading capacity of wild-type TEM-1, the resulting fitness decline was small. The data are rather noisy, and α values cannot be reliably extracted. Nevertheless, the maximal concentration under which all variants in the drifting *Lib250* populations grew was 1,000 μg/ml (Bershtein et al. 2006), and the fraction of deleterious mutations measured independently in *Lib250* at 2,000 μg/ml ampicillin (~0.05) was, as expected, much smaller than for *Lib12.5* and *Lib250* and in agreement with the smaller value derived from the cefotaxime fit (0.14).

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an average α value of 0.36. (*B*) The frequencies in *Lib12.5* at 0.2 and 0.5 μg/ml fitted with an average α value of 0.22. (*C*) The frequencies in *Lib250* fitted with an average α value of 0.14. (*D*) Summary of f_+ and average α values obtained from the fit to equation 5.

Second, the obtained f_{ctx} values for the low cefotaxime concentration (0.2 μg/ml) are within the expected range. These were found to be similar in all TEM-1 populations regardless the intensity of the purifying selection operating on the library ($f_{ctx} = (1.1–1.5) \times 10^{-3}$; fig. 1*D*). Resistance to 0.2 μg/ml cefotaxime can be obtained by a single Ser238Gly substitution seen in all clinical isolates (Jacoby and Bush 2008) and laboratory evolution experiments (Orencia et al. 2001; Hall 2004). Indeed, this mutation appeared in 93% of the sequenced cefotaxime-resistant variants (supplementary table 1, Supplementary Material online). The Ser238Gly mutation corresponds to a single nucleotide substitution (G to A) that comprises the dominant substitution (95%) at TEM-1's G sites under the applied random mutagenesis protocol (Bershtein et al. 2006). Given a 840-nt length gene, 25.8% G content, and an overall frequency of mutations at G sites of 8.06% (Bershtein et al. 2006), the expected f_{ctx} is: $1/840 \times 0.95 \times 0.0806/0.258 \approx 0.4 \times 10^{-3}$ and is indeed close to the value of f_{ctx} predicted by the model.

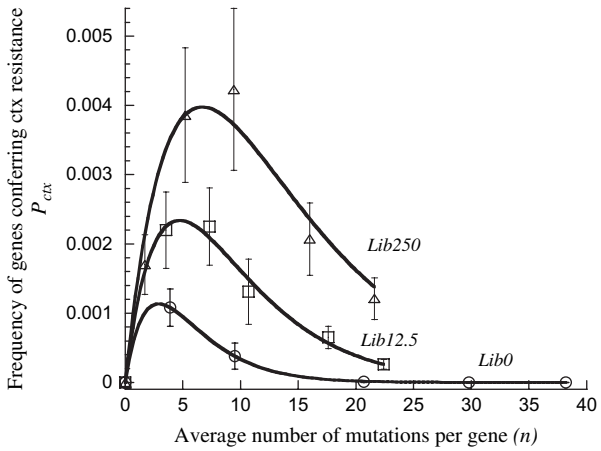

FIG. 2.—Direct comparison of the measured frequencies of the cefotaxime-resistant clones in TEM-1 populations drifting under various strengths of purifying selection. The frequencies of cefotaxime-positive clones obtained for each library (*Lib250*, triangles; *Lib12.5*, squares; and *Lib0*, circles) at the low cefotaxime level (0.2 μg/ml) were fitted to equation 5 using with the parameters provided in figure 1. Error bars represent standard deviations of two–three independently performed experiments.

Role of Global Suppressors

As in most experimental evolution studies, the conditions applied in this study deviate from the natural ones. For example, the high mutation rate (two mutations per gene per generation) that was necessary to compress millions of years of natural drifts into a laboratory timescale. One consequence of the high mutational load was an enrichment in global suppressor mutations that increased TEM-1's thermostability and thereby facilitated the accumulation of more mutations (Bershtein et al. 2008). The most frequently enriched global suppressor Met182Thr is also found in cefotaxime-resistant TEM-1 (Jacoby and Bush 2008; Huang and Palzkill 1997; Wang et al. 2002). It compensates the loss of stability associated with the Gly238Ser mutation, thereby increasing the levels of functional protein and the levels of both cefotaxime and ampicillin resistances (Orencia et al. 2001; Wang et al. 2002). Along side with other global suppressors such as Arg120Gly and His153Arg, this mutation was found in cefotaxime-resistant variants isolated in our experiment and, in particular, in those selected at the high cefotaxime level (0.5 μg/ml; supplementary table 1, Supplementary Material online). Another important mutation is Glu104Lys. This mutation yields increased level of cefotaxime resistance and is frequently occurs alongside Gly238Ser (Orencia et al. 2001; Wang et al. 2002). This mutation is probably also a weak global suppressor because it was shown to slightly increase the TEM-1's thermostability (Raquet et al. 1995). Unsurprisingly, Glu104Lys mutation appears in 22% of the cefotaxime-resistant variants (and almost always paired with Gly238Ser) isolated from the drifting TEM-1 populations (supplementary table 1, Supplementary Material online).

The presence of these global suppressors accounts for unexpectedly high frequency of cefotaxime variants with higher resistance level (0.5 μg/ml). The frequency of double mutants (f_+) is expected to be roughly the square of f_+ for a single mutation ($\sim 10^{-3} \times 10^{-3} = 10^{-6}$). The $>$ 100-fold higher than expected f_+ values calculated at 0.5 μg/ml cefotaxime ($\sim 4 \times 10^{-4}$; fig. 1*D*) is therefore explained by the fact that several different mutations act in concert with Gly238Ser to increase cefotaxime resistance (Glu104Lys and different global suppressors). These mutations accumulated as neutral or were even positively enriched under selection to maintain the original penicillinase activity. Although the same effects, and the very same mutations, play a role in natural evolution, they are undoubtedly overrepresented under our experimental conditions. A more valid comparison of the adaptation dynamics is therefore provided for the low cefotaxime level (0.2 μg/ml) where f_+ correlated primarily with a single mutation (Gly238Ser mutation) and exhibited the expected value of $\sim 10^{-3}$ (fig. 2).

Trade-Offs Constraints

The combined results (fig. 2) show that the frequency of genes conferring cefotaxime resistance in the drifting populations is remarkably sensitive to the fraction of accumulating deleterious mutations. The gradual changes in α values (0.36, 0.22, and 0.14, fig. 1) led to dramatic differences in the dynamics of cefotaxime adaptation. The ability of the unselected *Lib0* to generate cefotaxime-resistant clones was not only significantly lower (4-fold at the peak) but was also exhausted by the 10th generation (fig. 2). Thus, unless deleterious mutations are efficiently purged under selection for ampicillin, nonfunctionalization is a most likely outcome.

Given the dominant effect of purifying selection, the question arises whether an even higher selection pressure could further increase the potential for adaptation. The answer in this case is no. In the TEM-1 model case, the trade-offs between the original and new function constrain the maximal levels of purifying selection. Gly238Ser—the mutation that primes cefotaxime resistance—decreases the

levels of ampicillin resistance of TEM-1 (Wang et al. 2002) to the selection threshold of *Lib250* (250 μg/ml of ampicillin with direct plating after transformation, which corresponds to ~1,000 μg/ml in IC measurements following growth in liquid culture). Indeed, all the representative cefotaxime-resistant clones analyzed in detail show an IC for ampicillin that is ≥1,000 μg/ml. Of these, the ones capable of resisting wild-type–like ampicillin levels (~3,000 μg/ml) appeared mostly under selection at high cefotaxime concentration (0.5 μg/ml) and carry at least one global suppressor (supplementary table 1, Supplementary Material online). Thus, purifying selection to maintain the wild-type levels of ampicillin resistance would lead to the purging of many, if not most, of the variants with adaptive potential. For example, plating the fifth generation of *Lib250* on 500 μg/ml, or 1,000 μg/ml of ampicillin, resulted in a decrease of 50% and 70%, respectively, in the frequency of cefotaxime-resistant variants relative to their frequency after purifying selection at 250 μg/ml of ampicillin.

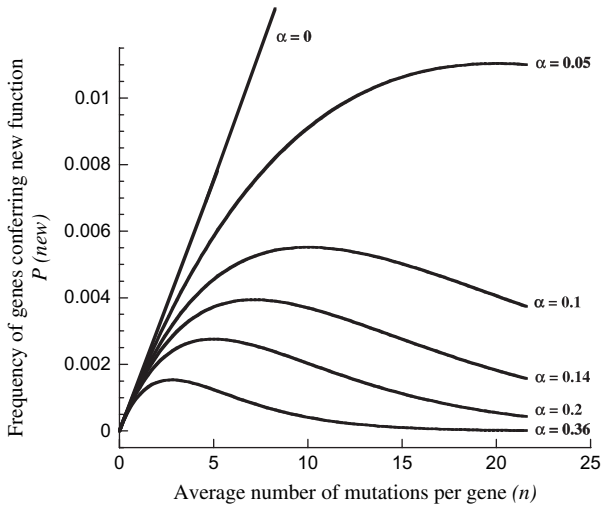

FIG. 3.—The predicted frequency of variants conferring new function (P_{new}) as the function of the fraction of deleterious mutations (α). The proposed model (eq. 5) was applied to predict the frequency of variants (or alleles) conferring a new function at a range of α values (0.36–0) and under a constant frequency of potentially adaptive mutations ($f_+ = 10^{-3}$).

Discussion

Our experiment revealed the adaptation dynamics of TEM-1 drifting under different levels of purifying selection. A simple model was developed that accounts for these dynamics (eq. 5; fig. 2) and provides the desired parameters 1) the frequency of potentially adaptive mutations (f_+); 2) the frequency of deleterious mutations (f_- or α); and 3) the trade-off—that is, the dependency of f_+ on the level of purifying selection. Although these results concern one enzyme and one adaptation route, they can be extended to many other cases. The extrapolated value for f_+ is $\sim 10^{-3}$ and is probably typical for a single mutation in a gene of ~1-kbp length. The frequency of deleterious mutations in the absence of any purifying selection (0.36) is also typical for proteins of TEM-1's size (Camps et al. 2007). Based on these results, and the model, the adaptation dynamics can be simulated for different α values (fig. 3)—namely, in selection regimes that vary from no selection whereby all mutations can accumulate ($\alpha = 0.36$), to the maximal level of purifying selection whereby no deleterious mutation accumulate ($\alpha = 0$).

This case study reveals the inherent difficulty in Ohno's route of divergence. At the highest α value, the frequency of alleles carrying the new function (P_{new}) is low ($<2 \times 10^{-3}$) and is very rapidly exhausted as the drift progresses. The purging of deleterious mutations dramatically increases the likelihood of emergence of a new function. Thus, if we assume no negative trade-offs, deleterious mutations possess nothing but a threat for the original as well as the new protein function. In this case, purifying selection at the maximal level brings the frequency of the deleterious mutations in the drifting populations to zero ($\alpha = 0$), and adaptation occurs at the fastest rate possible (fig. 3). This is the case in gene sharing scenarios when different functions are performed by the very same coding region (Piatigorsky 2007). However, a scenario that is purely gene sharing is inapplicable to TEM-1's adaptation and to numerous other cases because of negative trade-offs—the key adaptive mutation (Gly238Ser) is partially deleterious to the original function and is therefore purged under highly stringent purifying selection.

The strength of the trade-off therefore dictates an optimum level of "relaxation" in the purifying selection pressure and an interplay between f_+ and α. The proposed model can predict the adaptation dynamics provided that the level of trade-off is known. In TEM-1's case, the value of f_+ remains constant under a wide range of purifying selection stringencies (*Lib0* to *Lib250*), and the adaptation dynamics are dictated by differences in α values in the range of 0.36–0.14 (fig. 1). However, at higher selection pressures, when α becomes lower than 0.14, the Gly238Ser mutation is purged, namely f_+ goes down, and so does the rate of adaptation. The simulation provided in figure 3 therefore holds only as long as f_+ does not trade-off with α. In cases of strong trade-offs, f_+ will have a strong dependency on α such that, at low α values, f_+ will be effectively zero, and a new function can diverge only when the selection to retain the existing function is significantly, or even totally, relieved ($\alpha = 0.36$ in TEM-1's case and probably for other proteins [Camps et al. 2007]).

That adaptation proceeds at the fastest rate when the pressure of purifying selection is relaxed but not entirely relieved, favors a revision of Ohno's model, as well as of the gene sharing models, and lends support to the alternative scenarios detailed below.

Divergence prior to Duplication

This model, which in a way comprises an extension of gene sharing, is gathering growing levels of support (Hughes 2002; Aharoni et al. 2005; Kondrashov 2005). Most recently, it has been formalized under the term innovation–amplification–divergence (Bergthorsson et al. 2007). In many (but certainly not all) cases, the emergence of a new protein function can proceed via "generalist"

intermediates, namely by acquisition of a new function without severely compromising the original one, for example, when a weak, promiscuous function such as the ability of TEM-1 to degrade cefotaxime at very low efficiency develops with little loss of the existing function (Aharoni et al. 2005; Bergthorsson et al. 2007). Duplication may initially enable an increase in protein levels that compensates for the low enzymatic efficiency (see below) and the eventual development of much higher level of the new function. The latter is likely to be accompanied by significant, or even complete, loss of the original function and thus lead to a new "specialist" (Khersonsky et al. 2006).

This model is also supported by the notion of neutral networks. Numerous theoretical, computational, and experimental works indicate that the potential for adaptation can develop under "neutrality" ([Bornberg-Bauer and Chan 1999; Ancel and Fontana 2000; Amitai et al. 2007; Nei 2007] and references therein)—namely, under selection to maintain the original function and structure. Many mutations of adaptive potential, such as Glu104Lys, and global suppressors, such as Met182Thr, are completely neutral or even advantageous under neutrality. Indeed, the evidently wrong assumption that neutral mutations cannot possess an adaptive potential is the main factor behind the absurdly low probabilities for neofunctionalization seen in Behe's simulations (Behe and Snoke 2004).

When divergence is capitalizing on a minor, or promiscuous activity in an existing protein, immediate selective advantage can be provided by increasing protein doses (Patrick et al. 2007). Duplication is known to occur under such selection pressures and under this scenario, both duplicates will be maintained under selection to maintain both the primary function and the minor one (Bergthorsson et al. 2007). Mutations that increase the latter, and the trade-offs between these two activities, will eventually direct one copy to neofunctionalization.

Subfunctionalization

Duplication may well be a neutral, coincidental event, as in Ohno's model. However, once duplication has occurred, both copies are likely to be maintained under purifying selection. Earlier versions of this model include the Dykhuizen–Hartl hypothesis by which seemingly neutral mutations can fix in either the original or the copy owing to the relief in selection pressure afforded by gene duplication (Dykhuizen and Hartl 1980; Zhang 2003). This may occur under the subfunctionalization (duplication–degeneration–complementation [DDC]) model (Force et al. 1999). Following duplication, the two copies may acquire complementary loss-of-function mutations such that both genes are now required to maintain the function of a single ancestral gene (Lynch and Force 2000). Although this model was devised for complex gene functions and regulatory elements, it can readily be extended to simple enzymatic functions. Indeed, a study of yeast genome duplications indicated that the outcome of duplication is a relaxation in selection pressure afforded by a redundant gene pair and the increased accumulation of activity-reducing mutations (ARMs) (Scannell and Wolfe 2008). The appearance of these mutations enforces both copies to be maintained viable as in the DDC model. Because, the ARMs are mutations that decrease enzyme dose and activity, they are readily compensated by the activity of the second gene copy. As demonstrated here, mutations with adaptive potential, such as Gly238Ser, clearly belong to the category of ARMs. Thus, duplication, followed by the fixation of both copies under purifying selection, provides the key to rapid divergence owing to the fact that duplication allows, and is sustained, by ARMs.

Concluding Remarks

In our view, Ohno's model and the gene sharing model comprise the edges, or extreme points, within a wide spectrum of scenarios. Although the extreme scenarios no doubt occur, they are not necessarily the most common, or most feasible, scenarios. In some cases, a new function is exerted with no sequence changes (Piatigorsky 2007). In other cases, strong trade-offs between the existing and the new function may not enable the divergence of the new function under selection for the existing one (Vick et al. 2005), and even a single mutation can lead to an abrupt switch of enzymatic specificity (Varadarajan et al. 2005; Watts et al. 2006). However, in many cases, where weak trade-offs dominate, relaxation of selection, rather than total relief, prompts adaptation. This trend is seen not only in this experiment but also in the clinic where long treatments under low doses promote the emergence of antibiotic resistance (Siegel 2008). Given the model and parameters derived from the TEM-1 experiment, and while assuming that these reflect other cases, the intermediate scenarios described above can be simulated under conditions that resemble natural evolution, as previously preformed for Ohno's model, and thus reveal how the rate of acquisition of new gene functions is facilitated beyond what is generally assumed.

Supplementary Material

Supplementary figure 1 and table 1 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

Acknowledgments

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