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Experimental Evolution of Gene Duplicates in a Bacterial Plasmid Model

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Abstract. The fate of gene duplicates subjected to diversifying selection was tested experimentally in a bacterial system. The wild-type TEM-1 *β-lactamase* gene confers resistance to ampicillin but not to cefotaxime. Point mutations confer cefotaxime resistance, but they compromise ampicillin resistance. Thus, selection for both drug resistances in a bacterium with two copies of *β-lactamase* should favor the divergence of one copy to improve cefotaxime resistance while maintaining the other copy to preserve ampicillin resistance. This selection was performed on a bacterium with identical sequences of *β-lactamase* on two separate, compatible plasmids. As expected, one plasmid evolved increased cefotaxime resistance when appropriately strong cefotaxime selection was applied. However, the cefotaxime-resistant plasmid maintained sufficient ampicillin resistance to tolerate the concentration of ampicillin used, and the other plasmid was lost. Hosts carrying both the cefotaxime-resistant and wild-type plasmids were then subjected to various higher concentrations of both drugs to find conditions that would ensure the maintenance of both plasmids. In a striking contradiction to our model, no such conditions were found. The fitness cost of carrying both plasmids increased dramatically as antibiotic levels were raised, and either the wild-type plasmid was lost or the cells did not grow. This study highlights the importance of the cost of duplicate genes and the quantitative nature of the tradeoff in the evolution of gene duplication through functional divergence.

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Introduction

Gene and genome duplication have been recognized as major sources of material for evolutionary innovations in eukaryotes (Ohno 1970, 1999). Recent analyses have revealed that even organisms with compact genomes have high levels of duplication. Roughly a fifth to a half of *Drosophila melanogaster*, *Caenorhabditis elegans*, and *Saccharomyces cerevisiae* functional genes are the result of duplication events (Gu et al. 2002; Rubin et al. 2000). These duplications allow for increases in complexity. In arthropods, tandem duplication of *Hox* genes led to increased segmental specialization (Lewis 1978). In vertebrates, genome duplication enabled specialized tissues to evolve in concert with tissue-specific gene isoforms (Kortschak et al. 2001; Ono-Koyanagi et al. 2000).

Despite the evolutionary importance of gene duplication, we know little about pathways involved in the retention and divergence of gene duplicates (e.g., Walsh 2003). Under one model, gene duplicates subfunctionalize (Lynch and Force 2000), with each duplicate performing part of the original function (e.g., differential tissue-specific expression). A second model—the one we investigated—relies on new, beneficial mutations driving adaptive divergence of duplicates (Hughes 1994). Under either model, divergence in function would lead to a selective

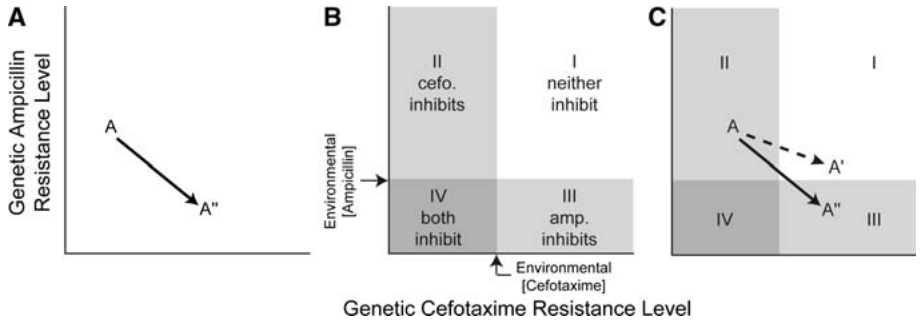

Fig. 1. **A** Tradeoff in drug resistance. Each point (A and A′) represents the joint level of resistance to each antibiotic separately. If A and A′ are different mutants of the same gene, they depict a tradeoff in drug resistance, because increases in resistance to one antibiotic causes a decrease in resistance to the other. **B** Four zones in an environment containing fixed levels of the two antibiotics. Shaded areas represent concentrations of the two antibiotics in the environment where a specific plasmid would (I) not be inhibited by either antibiotic, (II, III) be inhibited by one antibiotic, or (IV) be inhibited by both antibiotics. When antibiotic concentration levels include zones II and III, two copies of the gene may be necessary for host growth. For example, if the first copy is inhibited by cefotaxime at a level depicted by zone II, and a second copy of the gene is inhibited by ampicillin at a level depicted by zone III, both copies are required to permit growth with that combination of antibiotic concentrations. **C** Conditional maintenance of a gene duplication. Starting at point A, if the drug resistance tradeoff follows the trajectory to A′, then a single copy confers resistance to both antibiotics in this environment. If the tradeoff boundary extends instead to A″, then no single copy will permit growth, and the cell must maintain two copies (A and A″) to persist in this environment.

advantage for retaining both genes because each gene cannot perform the functions of the other copy. Although the divergence in function may occur before or after the duplicate's sojourn to fixation, this study focuses on divergence following fixation.

We developed an experimental model system to test factors involved in maintenance and divergence of duplicates. Our model utilized the antibiotic resistance properties of the gene TEM-1 *β-lactamase*, hereafter referred to as *β-lactamase*. This gene confers resistance to ampicillin by catalyzing the hydrolysis of the antibiotic to inactive products (Palzkill et al. 1994). Native *β-lactamase* confers resistance to high levels of ampicillin (30 mg/ml) and to very low levels of the cephalosporin-type antibiotic, cefotaxime (0.05 μg/ml). Mutations confer increased resistance to cephalosporin antibiotics but decrease resistance to ampicillin, creating a tradeoff (Long-McGie et al. 2000; Palzkill et al. 1994). This tradeoff prevents a single copy of the gene from achieving the high level of resistance to both antibiotics that could be achieved by two, diverged copies.

The conceptual foundation for our design follows the fitness set methodology introduced by Levins (1968) for two environments (Fig. 1), with some simple modifications. A specific cell or plasmid type has an intrinsic level of resistance to each antibiotic, which we may represent as the maximal concentration at which the cell (containing the plasmid) is able to grow. The joint maximal tolerated concentrations of two antibiotics may thus be plotted as a point in this space for each gene copy. Whether a tradeoff actually leads to the maintenance of two diverged copies depends on the shape of the tradeoff as well as the selection imposed by the environment (Fig. 1C). Here we explore the landscape of the model by varying antibiotic concentrations to estimate the fate of fixed duplicates subjected to diversifying selection.

Methods

Media and Culture Conditions

All cells were grown at 37°C in LB broth (10 g NaCl, 10 g Bacto-tryptone, 5 g Bacto-yeast extract per liter water) or on LB plates (agar at 15 g/L). For growth of standard strains, ampicillin was used at 0.1 mg/ml, cefotaxime at 0.05 μg/ml, tetracycline at 12.5 μg/ml, and kanamycin at 25 μg/ml. Higher concentrations of ampicillin and cefotaxime were used in some experiments, as described.

Genetics

β-Lactamase genes were introduced into cells on the compatible plasmids pACYC177 and pBR322. Both plasmids are moderate copy number (~10/cell), and the sequence of the *β-lactamase* gene is identical on both. The start and stop positions for *β-lactamase* on pBR322 are 4048 and 3296, respectively (GenBank accession number J01749). The start and stop positions for *β-lactamase* on pACYC177 are 3696 and 612, respectively (GenBank accession number X06402). A cell with both plasmids was considered equivalent to a cell with duplicate copies of *β-lactamase*, because the two plasmids are maintained independently, hence a cell can stably maintain an evolved copy on one plasmid and a wild-type copy on the other. Each plasmid carries a separate, unique antibiotic resistance: tetracycline (*Tet*) resistance on pBR322 and kanamycin (*Kn*) resistance on pACYC177. These additional antibiotic resistances provided easy detection of each plasmid in the cell. After transformation, individual colonies isolated on these double antibiotics were grown in LB with ampicillin (1 mg/ml) to log-phase, frozen in 20% glycerol, and used as starting stocks for all experiments.

The strain of *E. coli* cells, W3110, has slight native antibiotic resistance to ampicillin and cefotaxime (1 μg/ml and 5 ng/ml, respectively), orders of magnitude lower than the concentrations used for selection. Cells can evolve some minimal amount of resistance to antibiotics by changing lipopolysaccharides on the

Table 1. Experimental design and resulting evolution

Design			Outcome				
			Plasmid[c]				
Experiment[a]	[Ampicillin], mg/ml	[Cefo-taxime], [b] μg/ml[b]	A	B	A + B	Final cefo-taxime resist.	*β-lactamase* mutations
L1	0.1	—	1	—	—	1×	—
L2	—	0.05	1	—	—	1×	—
L3	0.1	0.05↑	—	4	—	100×	G238S + S268G + E104K[d]

[a] L, liquid culture.
[b] ↑ denotes increased concentration of cefotaxime over time during experiment.
[c] Number of replicates with each plasmid present in final assay (A = pACYC177; B = pBR322).
[d] For example, notation G238S designates a change in amino acid 238 from glycine (G) to serine (S).

outer membrane (Livermore and Pitt 1986). Therefore, we periodically transformed plasmids into naïve cells to eliminate host evolution as a factor for resistance to antibiotics.

Liquid Culture Selection

Four experiments were conducted in liquid (L1–L3; Table 1). Approximately 10^6 suspended cells were added to tubes containing 2 ml of LB with the appropriate antibiotics (see Table 1). Cells were grown with aeration for 8 h (to log-phase). Every 8 h, approximately 10^6 cells were transferred to new tubes with the same antibiotic treatments. Aliquots of cultures at log-phase were frozen with 20% glycerol.

Selection of Drug Resistance

In experiment L3, parallel cultures were grown in two concentrations of cefotaxime: one known to permit growth and one 5- to 10-fold higher. If the culture at the higher concentration grew (implying evolution of increased resistance), it was used to continue the line. Periodically, plasmid DNA was isolated (Wizard *Plus* SV Minipreps DNA Purification System, A1330; Promega, Inc.) and transformed into fresh W3110 cells to check evolution of host resistance; passages were continued using these transformed naïve cells. Increases in resistance were assayed by growing cells in concentrations of cefotaxime that were 2- and 10-fold above the known resistance level. Plasmid genes were sequenced at each stage of detectable increase in resistance to cefotaxime that was not due to host evolution.

Sequence Analysis

PCR products from individual colonies or whole plasmid preps were used to sequence *β-lactamase*, the ORI (origin of replication initiation) and ROP (replication of plasmid; only present in pBR322). Sequence was obtained for both the forward and the reverse reactions. Sequence files were manipulated with SeqMan and aligned in MegAlign (DNAStar, Inc., 1993–2000). Amino acid position notations are taken from Ambler et al. (1991).

Measurement of Copy Number

We grew cells containing wild-type and/or evolved plasmids in liquid culture with ampicillin and cefotaxime to log-phase. We calculated the number of cells per microliter after the culture reached log-phase by plating on ampicillin. We then extracted plasmid DNA (Gene Elute Plasmid Miniprep Kit, PLN350; Sigma-Aldrich, Inc.) and measured the amount of DNA recovered per cell using a UV spectrophotometer (Biophotometer, Eppendorf).

Competitive Fitness Assays

In preparation for assaying differences in fitness, cell stocks with each of six plasmid combinations were prepared:

(i) pACYC177^{wt},
(ii) pBR322^{wt},
(iii) pBR322$^{3\Delta}$,
(iv) both wild-type plasmids,
(v) pACYC177^{wt} with pBR322$^{1\Delta}$, and
(vi) pACYC177^{wt} with pBR322$^{3\Delta}$.

Superscript *wt* represents the wild-type sequence; 1Δ the *β-lactamase* gene with mutation G238S; and 3Δ the gene with mutations E104K, G238S, and S268G. Each was grown as an overnight culture with ampicillin plus the other appropriate antibiotic(s) to ensure that each plasmid type was maintained. The overnight cultures were then transferred to new media with 0.2 mg/ml ampicillin and grown separately for an additional 3 h to equilibrate the cultures to the same media.

Cells of different types were mixed in concentrations to facilitate easy detection of changes in frequencies and grown for 8 h. Cells were plated on ampicillin at the beginning and end (8 h) of the competition, whence these colonies were replica-plated on appropriate antibiotics to detect plasmid type and possible resistance to cefotaxime. Per-generation fitness (w) of one plasmid type relative to another was determined by $w^{16} = [P_1(1 - P_0)]/[P_0(1 - P_1)]$, where, P_0 is the initial frequency, and P_1 is the frequency at 8 h; generation time was taken as 30 min, hence 16 generations during the competition. Slower generation times (i.e., >30 min) would make our estimates of fitness conservative because fewer generations would have led to the observed differences.

Results

Static Antibiotic Levels

Experiments L1 and L2 (one trial each) were conducted as controls with antibiotics maintained at starting levels. Both L1 and L2 lost pBR322 and the *β-lactamase* gene and the ORI sequence of pACYC177 lacked detectable changes (Table 1). A direct competition assay between cells containing three

Table 2. Three-way competitive fitness assays

Competitors[a]	[Ampicillin], mg/ml	[Cefotaxime], μg/ml	Plasmid type with higher fitness	n	Fitness/gen.	
					Avg.	SE
A^{wt} vs. B^{wt} vs. (A^{wt} + B^{wt})	0.1	0.05	$pACYC177^{wt}$	3	1.071[b]	0.0455
A^{wt} vs. $B^{3\Delta}$ vs. (A^{wt} + $B^{3\Delta}$)	1	5	$pBR322^{3\Delta}$	4	1.172[cd]	0.0359
A^{wt} vs. $B^{3\Delta}$ vs. (A^{wt} + $B^{3\Delta}$)	5	5	$pBR322^{3\Delta}$	3	1.331[c]	0.1299
A^{wt} vs. $B^{3\Delta}$ vs. (A^{wt} + $B^{3\Delta}$)	15	1	$pBR322^{3\Delta}$	3	1.355[e]	0.0466
A^{wt} vs. $B^{3\Delta}$ vs. (A^{wt} + $B^{3\Delta}$)	10	1	$pBR322^{3\Delta}$	3	1.029[ef]	0.0178
A^{wt} vs. $B^{3\Delta}$ vs. (A^{wt} + $B^{3\Delta}$)	10	2.5	$pBR322^{3\Delta}$	3	1.203[f]	0.0465

[a] A^{wt} , $pACYC177^{wt}$; B^{wt}, $pBR322^{wt}$; $B^{3\Delta}$, $pBR322^{3\Delta}$. (A + B) indicates cultures with two plasmids types in each cell.
[b] z-test: $z = 1.573$, df = 2, $p = 0.058$.
[c] Unpaired t-test: $t = 1.36$, df = 5, $p = 0.116$.
[d] Two trials were removed from analyses due to inconsistent results. Three additional trials supported results from the first trial. Total number of replicates used in analyses = 4.
[e] Paired t-test: $t = 7.60$, df = 2, $p = 0.017$.
[f] Paired t-test: $t = 4.61$, df = 2, $p = 0.022$.

combinations of a single or both wild-type plasmids (only pACYC177, only pBR322, and cells with both pACYC177 and pBR322) yielded a higher fitness of pACYC177, consistent with these results (Table 2).

Increasing Cefotaxime Levels

In all four replicates of L3, cefotaxime resistance evolved from changes in the *β-lactamase* gene of pBR322. Cefotaxime concentration was increased as resistance increased, and even higher levels of resistance evolved. In all replicates, three nonsynonymous substitutions accumulated in the same order; there were no synonymous substitutions (Table 1, Fig. 2). pACYC177 was lost sometime during passages between the second and the third mutations on pBR322. No changes were observed in ORI or ROP on pBR322.

The first pBR322 substitution (residue 238) resulted in an eightfold increase in resistance to cefotaxime (Fig. 2). In one replicate, the change was from glycine (GGT) to serine (AGT), but in the other three replicates, the change was to alanine (GCT). This amino acid substitution resulted in no apparent effect on resistance to ampicillin. At this step, all replicates contained both plasmids and there were no sequence changes in pACYC177. A single direct competition assay between cells carrying $pBR322^{wt}$ (wild-type) and $pACYC177^{wt}$ versus cells carrying $pBR322^{1\Delta}$ and $pACYC177^{wt}$ showed that the mutation in pBR322 had a selective coefficient of 0.16 at starting antibiotic concentrations (0.1 mg/ml ampicillin, 0.05 μg/ml cefotaxime). This selective advantage would cause cells harboring $pBR322^{1\Delta}$ to increase in frequency, even at low antibiotic concentrations.

The second mutation (S268G), in conjunction with the first mutation, yielded a combined 50-fold increase in resistance to cefotaxime and a concomitant 33% decrease in the maximal concentration of ampicillin tolerated (Fig. 2). Sequences of pBR322 *β-lactamase* were obtained from plasmid preps of the whole culture following this increase in resistance and all four replicates were fixed (i.e., the sequence electropherograms for forward and reverse sequencing reactions had single peaks at all bases) for the S268G substitution. Additionally, the three replicates that previously had a G238A were now fixed for serine at that position.

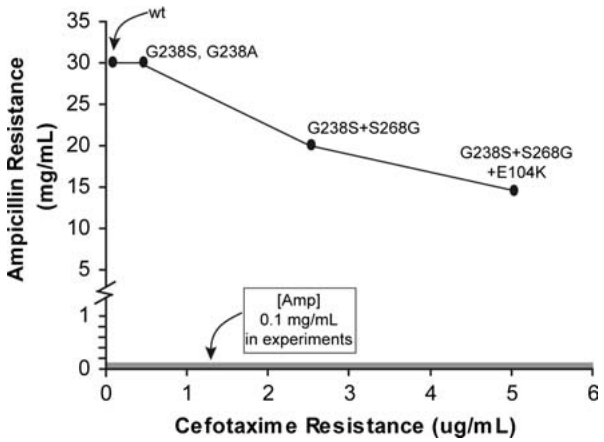

Fig. 2. Resistance to ampicillin decreased progressively as *β-lactamase* accumulated mutations. Amino acid substitutions are noted along the line. Cultures were grown separately with each antibiotic to determine maximal levels that permit growth. The thin gray bar designates the concentration of ampicillin (0.1 mg/ml), which was held constant during liquid culture experiment L3. The level of ampicillin was too low to inhibit the mutated copy of *β-lactamase*; therefore, there was no tradeoff between accumulating mutations that increased resistance to cefotaxime and reduced resistance to ampicillin (i.e., not in area III from Fig. 1).

A third substitution (E104K) in combination with G238S (the first substitution) led to a 100-fold increase in resistance to cefotaxime but a 50% decrease in ampicillin resistance compared to wild-type (Fig. 2). However, based on sequences of whole cultures, all four lines were polymorphic for this third mutation. Two lines reverted to the wild-type amino

Table 3. Estimation of copy number

Plasmids in cell cultures	Concentration of culture (cells/μl)	Concentration of plasmid DNA (ng/μl)	Plasmid DNA/cell (ng/cell)	Standardized DNA quantity[a]
pACYC177^{wt}	11.4	30.2	2.65	0.77
pBR322^{wt}	8.9	30.6	3.44	1.00
pBR322$^{3\Delta}$	11.4	19.0	1.67	0.48
pACYC177^{wt} + pBR322^{wt}	10.7	23.0	2.15	0.63
pACYC177^{wt} + pBR322$^{3\Delta}$	11.3	48.8	4.31	1.25

[a] DNA concentration in cells harboring pBR322^{wt} was used to standardize all other quantities.

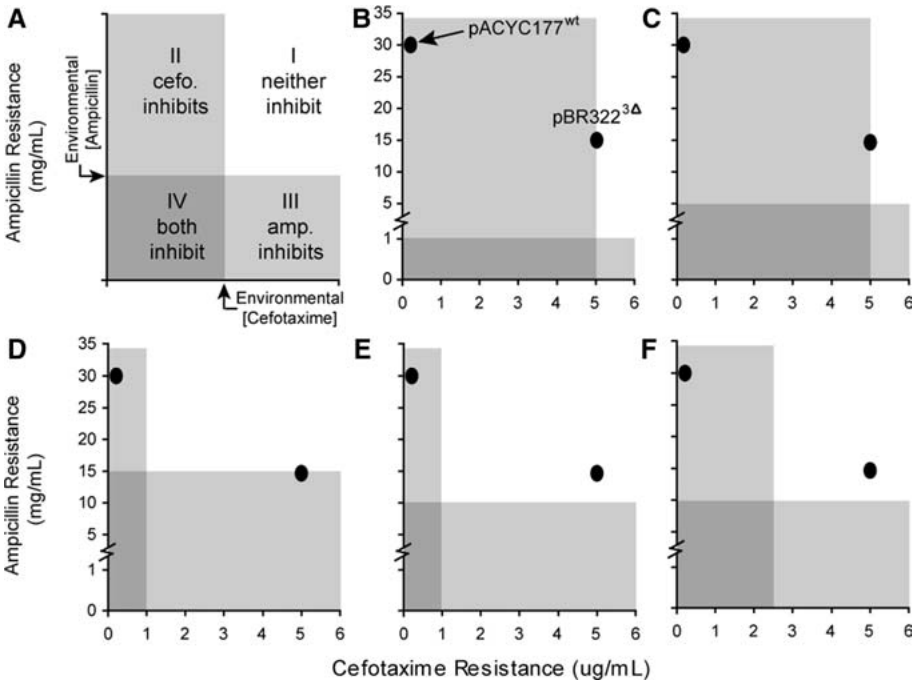

Fig. 3. **A** Zones of inhibition are depicted similarly to those in Fig. 1. In the example, A, plasmids in the clear area (zone I in A) are not inhibited by either antibiotic and plasmids in zones II and III are inhibited by only one antibiotic at the particular concentration depicted. **B–E** Antibiotic concentrations for the final five competitive fitness assays in Table 2. Points in the figures represent the joint level of resistance to each antibiotic for each plasmid type. (Assays of antibiotic resistance were performed separately for each antibiotic.) We predicted that, as antibiotic concentrations increased, the cost of maintaining both plasmids would decrease. pACYC177^{wt} (wild-type) would be required to inactivate ampicillin and pBR322$^{3\Delta}$ (with mutations E104K, G238S, and S268G) would be required to inactivate cefotaxime. Cells containing pBR322$^{3\Delta}$ alone had a higher fitness than cells with both pACYC177^{wt} and pBR322$^{3\Delta}$ in all assays. Attempts to grow cells at higher concentrations of antibiotics (e.g., 20 mg/ml ampicillin with 1 μg/ml cefotaxime) failed and are not illustrated.

acid at position 268 (the second mutation) and the other two lines were polymorphic for that mutation. The second substitution was seemingly unnecessary for the 100-fold increase in resistance. All three of the mutations observed in this study were observed in previous work and known to increase resistance to cefotaxime (Gniadkowski et al. 1998, Long-McGie et al. 2000; Petrosino et al. 1999; Stapleton et al. 1999).

Quantitative Test of the Tradeoff Model

For the triple-mutant, pBR322$^{3\Delta}$, the decrease in ampicillin resistance still enabled growth at 15 mg/ml (down from 30 mg/ml), whereas the selection experiments used the much lower concentration (0.1 mg/ml). Therefore, this tradeoff was never manifested as a cost to pBR322$^{3\Delta}$; the experiment evolved to ampicillin and cefotaxime resistance that would be represented in region I in Fig. 1B. To directly test the tradeoff model in this system, we subjected existing plasmids to higher concentrations of antibiotics. The clear prediction is that high levels of ampicillin will push the system into zones II and III in Fig.1, where one plasmid confers resistance to ampicillin (wild-type pACYC177 in zone II) and the other confers resistance to cefotaxime (evolved pBR322 in zone III), therefore forcing the maintenance of both plasmids in the same cell. We based these expected resistance levels on combined resistance to both antibiotics of individual plasmids. In contrast to this

straightforward prediction, either the pACYC177^{wt} plasmid was lost or the cells did not grow depending on the antibiotic concentration. This failure suggests that there is at most a narrow range of antibiotic concentrations favoring retention of both plasmids. In further contradiction to these simple expectations, competition assays of the double-plasmid cell relative to the single pBR322$^{3\Delta}$ showed that the double-plasmid fitness actually declined as higher levels of antibiotics were applied (Table 2); the model predicts that the double-plasmid fitness should increase. Figure 3 depicts the combinations of antibiotics used in competition experiments and the corresponding zones of inhibition as illustrated in Fig. 1.

Discussion

In a model popularized by Ohno (1970) for the evolution of gene duplicates, two copies of a gene in the original environment are redundant; one copy is free to evolve new function. The design of our experiment fits this scenario, with some modification. In our system, the second "copy" of the gene was redundant until drug concentrations were elevated, effectively changing the environment. In principle, one copy should have diverged in response to our changing environment, with an unaltered copy being retained to maintain the original function of ampicillin resistance. The high cost of maintaining two plasmids resulted in the loss of one plasmid in both plate and liquid culture experiments. In some cases, one copy of *β-lactamase* did evolve novel function; however, this evolved copy was still able to serve the ancestral function (inactivating ampicillin), which resulted in the loss of the wild-type plasmid (Fig. 2).

The model in Fig. 1 depicts a tradeoff between evolution of novel function and maintenance of the ancestral function of a gene. Whether duplicates will be maintained depends on the environment (gray zones in Fig. 1) and the tradeoff. In the case of liquid culture experiment L3, the level of ampicillin used was so low that the tradeoff would not have been expected to prevent loss of one copy. The evolved copy of *β-lactamase* fell into the "no inhibition" zone in Fig. 1C and alone was able to combat the effects of both antibiotics, even at elevated levels of cefotaxime. If the evolved plasmid would have been in zone III (A″ in Fig. 1C), both copies should have been maintained, under the model. Of course, when the divergence involves multiple steps along the tradeoff function, a delicate balance is required among the cost of the duplicate, the tradeoff shape, and the environmental selection to foster maintenance of both copies throughout the entire process of divergence.

After it became clear that the conditions of the selection experiments never approached the point that both plasmids should be maintained, competition assays were designed with sufficient levels of double antibiotics to expect the superiority of cells carrying both plasmids (Table 2, Fig. 3). Contrary to the model, we found that the double-plasmid state was not maintained, and even more striking, the fitness of double-plasmid hosts declined with increasing doses of antibiotics. Thus, the cost of carrying both plasmids was a variable that offset the advantage of carrying both.

These competition experiments, rather than being merely equivocal, provide a direct and incontrovertible refutation of our simple "fitness set" model. With the present data, we can only speculate on the causes of this failure, but two strong possibilities come to mind. (1) In view of the fact that fitness of the double-plasmid combination actually declines with increasing concentrations of the two drugs, it appears that the cost of plasmid carriage increases as the environment becomes more extreme. Possibly the second drug resistance genes (tetracycline on pBR322 and kanamycin on pACYC177) carried on the plasmids are important in this cost. For example, early chemostat experiments showed that tetracycline resistance was costly in chemostats (Dykhuizen and Hartl 1983), and as this resistance involves membrane transport, it should be considered that the high concentrations of *β*-lactam antibiotics may contribute to an increased sensitivity to foreign membrane proteins. (2) Ampicillin and cefotaxime may interfere with each other. The two copies of *β-lactamase* are similar enough that each will bind both antibiotics. Our model assumes that the wild-type protein hydrolyzes the ampicillin and the evolved protein hydrolyzes the cefotaxime, but in reality, both drugs are binding both antibiotics. Thus the cefotaxime may be reducing the efficacy of the wild-type gene to process ampicillin, or the ampicillin may be reducing the efficacy of the evolved gene to process cefotaxime. It has been demonstrated that cefotaxime acts as a competitive inhibitor of purified TEM-1 *β-lactamase* when it is present simultaneously with a *β*-lactam antibiotic that is a good substrate for the enzyme (Vakulenko et al. 1999). This possibility, in fact, identifies an additional factor in the functional divergence of gene duplicates that is not captured by the fitness set model, but may accrue far more generally than to our design.

For chromosomal genes, duplicates with neutral effects on fitness will be lost at a rate of $1 - (1/Ne)$ due to drift; any fitness cost will increase this rate (Walsh 2003). In experiments with no plasmid evolution, pBR322 was always lost. Competitive fitness assays established that the two plasmids, our proxy for duplicate genes, had differential fitness effects on cell growth. Cells containing pACYC177^{wt} were slightly more fit/generation than those containing pBR322^{wt}

in the presence of ampicillin or in ampicillin and low cefotaxime (Table 2). This asymmetry explains why pBR322^{wt} was lost if there was no evolution of *β-lactamase*. The exact mechanism for the difference in cost is unknown but could be due to a difference in replication machinery between the two plasmids or to the other drug resistance genes on each plasmid, as noted above for tetracycline resistance. High costs of the duplicate constitute the most severe limitation of this two plasmid system as a general model of gene duplication; this limitation could possibly be overcome by using lysogenic phages carrying *β-lactamase* or by using other means of chromosomal insertions of the gene.

A mechanism by which the system might evolve higher levels of resistance to cefotaxime (without mutations in *β-lactamase*) is to increase enzyme production, as with higher levels of mRNA expression or stability. As one such mechanism, substitutions in regulatory regions of β-lactamase could elevate production of or stability of mRNA at the level of the plasmid. Alternatively, increased plasmid copy number would produce more transcript. We tested whether copy number increased in several ways. First, no changes in the sequence of the copy control elements (promoter region, ORI, and ROP) were observed in any experiment. Second, we estimated the amount of plasmid DNA per cell. We found that wild-type and evolved plasmids, singly and in combination, had similar amounts of plasmid DNA per cell (Table 3).

In liquid culture experiment L3 some populations were polymorphic for the final two amino acid substitutions. This polymorphism could have been transient, as the new mutation increased in frequency. Conversely, it could also be stable because of density-dependent, frequency-dependent selection: because *β-lactamase* is diffusible, cells lacking the enzyme are somewhat protected by cells that produce this enzyme. In one scenario, a mutation that increases resistance to cefotaxime occurs and is at a low frequency in the population. As the concentration of cefotaxime is increased, the mutated copy that confers greater resistance to cefotaxime increases in frequency in the population. The evolved *β-lactamase* enzyme leaks out of cells, so copies that do not produce the mutated enzyme may be protected from antibiotics and remain in the culture at a low frequency. This explanation makes sense only if the mutations increase the cost of plasmid maintenance.

This study has illustrated the feasibility and difficulties underlying one of the main models for the evolution of novel function through gene duplication and divergence. The mere existence of a tradeoff between old and new functions is not sufficient to lead to retention of a gene duplicate. When a duplicate starts down the pathway toward acquisition of novel function, the environment must be such that the copy evolving novel function is no longer able to serve the original function adequately. This would then provide some benefit to maintaining both copies of the gene. Yet for duplicate genes to be maintained during the period of redundant function, costs must be low. Duplicates may survive under a very narrow range of environmental conditions if there is a cost to maintaining duplicates. In this model system, the outcome of each experiment was driven by the high cost of maintaining both copies. We have shown the difficulty of finding experimental conditions under which duplicates are maintained when duplicates are costly. There is no empirical evidence of the costs of gene duplicates in cases where the duplicate is incorporated into the genome.

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