Loss of dispensable genes is not adaptive in yeast

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A substantial share of genes identified in yeast can be deleted without visible phenotypic effects. Current debate concentrates on the possible roles of seemingly dispensable genes. The costs of maintaining unnecessary functions has attracted little attention. The hypothesis of antagonistic pleiotropy postulates that adaptations to different constituents of the environment are likely to interfere with each other, and therefore loss of unnecessary functions is potentially advantageous. We tested an entire collection of nonessential yeast gene deletions in a benign and nutritionally rich environment in which the number of dispensable genes was particularly high. We applied a series of competition experiments that could detect differences in relative fitness of ≈0.005. No beneficial deletions were found, except perhaps for the deletion of about a dozen genes that slightly improved competitive ability; however, a functional explanation of the fitness advantage is lacking. The paucity of beneficial gene deletions is striking because genetic adaptations to laboratory conditions are regularly observed in yeast. However, it accords with the finding that the gene contents of four species of *Saccharomyces* are nearly identical, despite up to 20 million years of independent evolution and extensive DNA sequence divergence. Such extreme conservation of functions would be improbable if there were periods of selection promoting the loss of temporarily dispensable genes. The evident cohesion of the yeast genomes may be their evolved feature or an intrinsic property of complex genetic systems.

antagonistic pleiotropy | fitness | gene deletion | genome | *Saccharomyces cerevisiae*

According to the hypothesis of antagonistic pleiotropy, it is unlikely that evolution in a heterogeneous environment will lead to a single superior type, providing that genes that increase fitness under some conditions are likely to decrease it under different circumstances (1–4). The necessity to trade off resources between conflicting functions is believed to be a crucial factor not only in ecological specialization but also in the evolution of life-history traits (5–7). In microorganisms, evidence of antagonistic fitness effects comes from experimental evolution of bacteria in structurally and nutritionally novel environments (8, 9). Direct links between adaptation to laboratory environments and loss of unused or less needed genes have been found in *Escherichia coli* (10–12). Extensive gene loss and advanced ecological specialization is most characteristic for bacterial endosymbionts and parasites. However, antagonistic pleiotropy is typically not invoked to explain either the loss of functions or loss of DNA in these organisms. The decay of functions is probably brought about by relaxation of selection because life within a host leads to redundancy of numerous metabolic pathways and intensification of genetic drift (13, 14). The deletion of unnecessary genetic material is most likely the result of mutational bias favoring loss over expansion of DNA (15, 16). Results of laboratory experiments and inferences derived from comparisons of bacterial genomes are disparate but not necessarily contradictory. It is possible that the loss of genes is occasionally adaptive, perhaps soon after environmental change, but most reductive evolution is caused by nonselective loss of genetic material. The important role of mutational decay has been recognized in more recent concepts of ecological specialization (17, 18).

The budding yeast is a free living microbe containing genes that, in general, are not mutationally degenerated. Nevertheless, most do not have an effect on viability (19). Numerous nonessential genes have also been found in other organisms (20–22). These genes may actually back up essential functions, fine-tune their regulation, or code for functions that remain unrecognizable under laboratory conditions (23). Although all these explanations may be true, the fate of a gene in a given environment is determined not by its potential function but by its current selective value. Consequently, the question arises whether genes that become functionally dispensable may also become deleterious to fitness. In the budding yeast, it is possible to search systematically for the fitness effects caused by inactivation of single genes after a collection of gene deletion strains has been generated (24). The fitness of deletion strains is estimated by competing all strains in a common culture and recording the changes in their frequency by using gene-chip technology (25). This high-throughput technique has proved valuable in searches for relatively large fitness effects. Small effects, of about one to a few percent change of fitness, have been difficult to identify even in well replicated experiments (26). The existence of slight differences in fitness is routinely verified in competition experiments that involve only a few strains and extend over many generations of growth (27). In an experiment of this type, a sample of 34 strains with a single gene disrupted by a transposon was tested in a nutritionally rich laboratory environment. Among 18 strains whose competitive ability was different from that of the wild-type strain by no more than 1%, there were 9 that had a statistically significant decrease in fitness and 2 with statistically significant increases (28). Extrapolated to the whole genome, this result suggests that as many as hundreds of gene disruptions may be associated with not only negative but also positive fitness effects of so low value that they could have been overlooked both under standard laboratory propagation and in massive competition experiments. The prediction that gene inactivation can often be adaptive is especially intriguing and directly related to the problem of the prevalence of antagonistic pleiotropy.

Here, a series of competitive experiments revealed that adaptive deletions are very rare or actually nonexistent. In the first experiment, we used a complete collection of yeast nonessential gene deletions and looked for those that resulted in an enhancement of fitness in a rich and benign laboratory environment. In the second, we showed that, in most cases, the observed fitness gains were caused not by the deletions but, most probably, by spontaneous beneficial mutations. Finally, using newly prepared and strictly isogenic constructs, we determined that only about a dozen of the yeast gene deletions were slightly advantageous. Those few identified cases of increased competitive ability could not be interpreted functionally, and therefore their adaptive status is unclear.

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Abbreviation: YPD, yeast extract, peptone, glucose.

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Materials and Methods

Strains and Media. The yeast strain BY4743 derived from S288C served as the genetic background in the generation of yeast deletion strains (29). A mixture of homozygous diploid deletions BY4743 *MAT***a**/α (95401.H1Pool, Invitrogen) was used in an initial selection of the most fit strains. Collections of individual haploid deletions, BY4741 *MAT***a** and BY4742 *MAT*α (YSC1063, YSC1064, Open Biosystems, Huntsville, AL), were used in confirmatory experiments. The deletion strains contain a genetic marker of resistance to geneticine conferred by the *kan*MX4 cassette inserted into a deleted ORF. To obtain differently marked competitors, we introduced nourseothricine resistance to the wild types BY4741, BY4742, and BY4743 by inserting *nat*MX4 into the *HO* locus. Previous experiments established that these markers are neutral when compared with unmarked wild-type strains (30, 31). All experiments were carried out at 30°C on YPD (yeast extract, peptone, glucose) agar medium, or in liquid YPD microcultures with shaking at 1,000 rpm. The microcultures were kept in standard titration plates with 96 wells.

Selection of Strains with Enhanced Fitness. A frozen sample of mixed diploid deletions was thawed and then immediately diluted and dispensed as 150-μl aliquots into four titration plates. The microcultures were transferred daily with a 96-pin replicator. A single pin transferred $\approx$1.5 μl of overnight culture to fresh YPD. During these initial transfers, which may be considered a preselection experiment, deletion strains competed only with each other, and the outcome of the competition was not monitored.

In the main selection experiment, 75 μl of overnight cultures of the preselected mixtures of deletions was mixed with 75-μl aliquots of an overnight culture of the wild-type BY4743 *ho*::*nat*MX4 strain. The resulting microcultures were transferred as described above between fresh aliquots of YPD without antibiotics. Every 7 days, early microcultures (i.e., immediately after transfer to fresh medium) were printed with the replicator onto two selective agar media, with geneticine or nourseothricine added. The resulting surface cultures were photographed after 48 h of incubation. In a pilot experiment, we prepared five mixed cultures of the geneticine and nourseothricine resistant strains in the following ratios: 50:50, 70:30, 90:10, 95:5, and 99:1. Photographs of prints obtained from these cultures served as standards for visual classification of the relative abundance of the competing *nat* and *can* populations into the five ratios. To estimate selection coefficients, log-normal values of the ratios were regressed over number of generations, assuming $\log_2 100 = 6.64$ generations per day. There were eight time points of regression in competitions with no winner. In competitions in which a geneticine-resistant strain won, there were usually six to seven time points because only the first 99:1 score was included into the analyses.

A single geneticine-resistant colony was isolated from every microculture in which a definite decline in the density of the *nat* strain was observed. The barcode of the deletion cassette was amplified and sequenced to identify a winner deletion strain (24).

Elimination of Spontaneous Mutations. Haploid strains of both mating types with the same deletions as the winning diploid strains were obtained from a new supplier. Diploid homozygous deletion strains were produced by mating the haploids. For each winning deletion, three pairwise competitions were initiated by mixing 15 μl of overnight cultures of the *MAT***a** haploid, *MAT*α haploid and the diploid deletion strains with 135 μl of overnight cultures of wild-type competitors of the same ploidy and mating type. The relative abundance of both competitors was estimated by diluting the cultures serially and spreading on Petri dishes. Scores obtained on YPD plates supplied with geneticine and on plain YPD plates were used to calculate the ratio of deletions to wild types. Selection coefficients were obtained by regressing log-normal values of these ratios over time. In most cases, sampling was done every 7 days, and this resulted in five time points in the analysis.

Verification of Adaptive Deletions. Those ORFs that were positively tested in the preceding experiment were deleted *de novo* in the wild-type BY4741 and BY4742. The deletion cassettes together with $\approx$150 bp of upstream and downstream sequence were amplified from the collection of haploid deletions. Diploid homozygous deletions were obtained by mating the haploids. Competition experiments were initiated in a similar way to that described in the preceding experiment, except that the initial ratio of the tested deletion strain and the unmarked strain was 5:95. The competition lasted for 4 weeks, or 180 generations. The relative abundance of the competitors was assayed by printing samples of the cultures onto agar in the same way as in the "selection of the most fit clones" experiment. The prints were made onto YPD with geneticine and plain YPD at six time points. The outcome of a single competition was determined by assigning the result into one of two classes: the *kan* competitor continued to be as rare as at the beginning, or, its density increased systematically and reached at least the 30:70 ratio. The were no ambiguities in the results; i.e., all cases could be assigned to either of the classes.

Results

Selection of Strains with Enhanced Fitness. Selection of adaptive deletions can be done in several ways, including individual competition of every deletion with a neutral reference strain or assessment of all deletions in one common culture. Here, we have chosen an intermediate option. A mixture of equally abundant 4,653 viable homozygous diploid deletions was diluted and partitioned into 384 populations of 50 cells on average. As a result, a single deletion strain was present in $(384 \times 50)/4653 = 4.13$ populations, on average. The initial populations reached sizes of 3×10^7 cells after the first cycle of growth and were then serially propagated by transferring 1/100 of an overnight culture to fresh medium. Each of the 50 deletions competing in one population was represented by $(1/50) \times (3 \times 10^7) \times (1/100) = 6{,}000$ cells at the time of first transfer (the first population bottleneck). With this arrangement, the number of cultures was manageable, the effects of selection were readily visible, genetic drift was minimal, and the competitive interference between adaptive deletions was small. A caveat is that some deletions may not have been included during the establishment of initial populations, although the probability of being omitted was not high, $(1\text{–}50/4{,}653)^{384} = 0.016$.

The populations containing deletions were transferred for 4 weeks or 180 generations to enhance the proportion of the most fit deletions. The populations were then mixed 50:50 with a standard competitor, i.e., a differently marked wild-type diploid. The ratio of deletions to the wild-type strain was monitored for up to 54 days or 360 generations. The initial ratio of the deletions and the reference strain remained unchanged in 251 cultures. In the remaining 133 cultures, the ratio of deletions steadily increased up to at least 90:10. There were no cultures in which the frequency of the reference strain visibly exceeded the expected 0.5.

The ratios of competitors were used to calculate relative fitness for all 384 populations of deletions. Their distribution is shown in Fig. 1. A single clone from each winning population was isolated, and the deleted ORF was identified. There were 54 deletions that won in only one population and 20 deletions that won in several populations. Distribution of the repeatedly win-

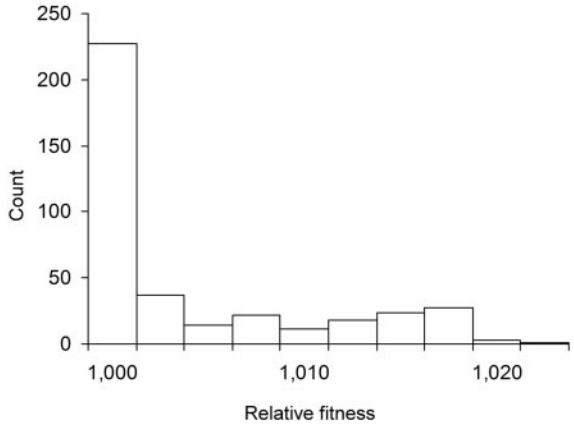

Fig. 1. Selection for advantageous gene deletions. A total of 384 cultures each containing 50 deletion strains were competed against a reference strain (isogenic except for the studied deletions). The graph shows the frequency distribution of the relative fitness, *w*, calculated for the deletion strains. In most cultures, the deletion strains did not outcompete the reference strain (the modal bar). A total of 133 competitions were classified as won by the deletion strains (see *Materials and Methods* and *Results* for details). These cultures were all located in the right tail of the presented distribution.

ning deletions did not indicate cross-contamination between the wells of microplates.

Elimination of Spontaneous Mutations. The 74 identified deletions may have outcompeted reference strains either due to the beneficial effects of adaptive deletions or spontaneous mutations. New deletions were obtained for the 74 winners on three genetic backgrounds, that is, two haploids of opposite mating type and a diploid. These strains were competed in pairs with the respective wild-type strains (see *Materials and Methods* for details). The competitions were at least duplicated. No signs of fitness enhancement in the haploids or the diploid were found for a substantial number of the tested deletions. This finding suggested that the formerly winning deletions were in fact hitchhiking with spontaneous beneficial mutations. The second class of deletions were those that in the new tests were associated with enhanced fitness in only one haploid strain and sometimes also in the diploid one, indicating a mating type-dependent adaptive deletion or a spontaneous mutation that appeared during the construction of the deletions, i.e., before they were mated to yield homozygous diploids. Finally, there were deletions for which both the two haploids and the diploid were more fit than their wild-type competitors. These were true adaptive deletions or spontaneous mutations that occurred during the construction of one haploid and were passed on to the other if the latter was obtained by switching of mating types and not independently. The last two classes comprised 24 deletions and were subject to further analyses aiming at final discrimination between the spontaneous beneficial mutations and the adaptive deletions.

Verification of Adaptive Deletions. We constructed the 24 tentatively adaptive deletions *de novo* and independently in the two haploid backgrounds. Homozygous diploid deletions were obtained by mating the haploids. The subsequent pairwise competitions with respective wild-type strains were devised to unequivocally assess whether a deletion was adaptive or not. To this end, we replicated 10-fold each competing pair and scored clear wins of the deletion strain (see *Materials and Methods*). In almost all cases, $24 \times 3 = 72$, there were either 10 positive or 10 negative scores. There were six exceptions when the score of wins was not 10 but nevertheless highly biased to this end. (Note that the expected number of wins was as low as 0.5; see *Materials and Methods* for details.) Partition of wins between deletions was also clear. Either both the two haploids and the diploid won (12 deletions) or none of the three strains won (the remaining 12 deletions). Consequently, we unambiguously confirmed that only 12 deletions were associated with reproducibly adaptive effects. The reference strain never won, indicating that spontaneous mutations were not frequent or strong enough to overshadow the impact of deletions on fitness.

Table 1. Selectively advantageous gene deletions

ORF/gene	No. of wins	Relative fitness
YIL001W	2	1.012
YIL006W	2	1.005
YIL041W	3	1.013
YIL077C	5	1.010
YIL087C	6	1.011
YJL215C	5	1.007
YJL150W	1	1.009
YLR104W	4	1.008
YLR207W/*HRD3*	3	1.012
YGR035C	6	1.011
YHL014C/*YLF2*	1	1.010
YNL027W/*CRZ1*	3	1.015

Table 1 lists the 12 confirmed adaptive deletions. The table also shows how many times a particular deletion was found among the winning populations of the first selection experiment. Recall that most of the 74 winning deletions identified in the first experiment were found only once. In case of the 12 confirmed adaptive deletions, as many as 10 were found in more than one population, and an average score was 3.4. The expected average, based on the partitioning of 4,653 strains among 384 populations, was 4.13. The difference is small and implies that very few adaptive deletions remained unrecognized in the first experiment. If the number of adaptive deletions was high and only a small sample of them was found, a particular deletion would be encountered usually only once. The congruence between the expected and observed data again indicates that our assays, including the first selection experiment, permitted the repeatable identification of small fitness differences and were not biased by spontaneous beneficial mutations.

The relative fitness of the 12 adaptive mutations is listed in Table 1. These are mean values of estimates obtained in the first selection experiment and in the ''elimination of spontaneous mutations'' experiment. The estimates were pooled because both experiments gave similar results; the average values of the 12 coefficients were 1.011 and 1.009, respectively, and the difference was insignificant ($t = 1.199$, df = 22, $P = 0.24$). The similarity of results is noteworthy because the protocols of the two fitness assays were different (see *Materials and Methods*). Both assays proved sensitive enough to detect differences in fitness of $\approx$0.005 (0.5%). There were no obvious characteristics shared by the 12 adaptive deletions. The cellular roles of the deleted ORFs are not known in most cases.

Discussion

A phenotype of increased fitness is a self-selecting trait under competitive conditions. Our experiments were founded on this simple assumption, but they failed to identify comprehensible examples of selectively advantageous gene deletions. The applied assays were sufficiently sensitive because we originally found >100 winning deletion strains whose fitness advantage was often as small as 0.5%. Subsequent experiments showed that most of these strains were spurious winners that

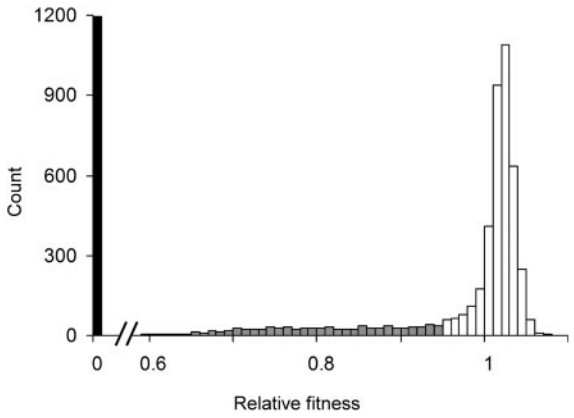

Fig. 2. The distribution of fitness effects caused by gene deletions. The filled and empty bars indicate lethal and neutral effects, respectively. The dotted bars indicate an arbitrarily delimited interval of deleterious effects. The distribution was obtained by making use of data published elsewhere (35). The relative fitness was calculated as a number of cell doublings completed by a deletion strain in relation to an average number of doublings in a culture containing all viable deletions. For this reason, the modal value of fitness is higher than one. For more information, consult www-deletion.stanford.edu/YDPM.

were driven by spontaneous beneficial mutations. Deletion may have resulted in adaptation in only a limited number of strains, i.e., about a dozen. The identification of few, slight, and functionally noninterpretable beneficial effects does not imply that gene deletions can be advantageous on any meaningful scale, but it verifies the sensitivity and repeatability of the applied experimental procedures. We propose that not antagonistic pleiotropy but the negligible cost of maintaining unnecessary functions is an important feature of the yeast genetic system.

Beneficial vs. Harmful Deletions. One purpose of the Yeast Deletion Project is to catalog phenotypic effects of strains in which single genes are absent (32–34). A complete set of selection coefficients for nutritionally rich YPD at 30°C can be found (35). We present these data in Fig. 2. There are 1,194 lethal and 724 or 959 deleterious deletions depending on whether the criterion of possible non-neutrality is set at a 10% or 5% departure from the modal value of fitness. Accepting the latter limit, we find that lethal and harmful deletions sum to 2,153 and outnumber the tentative 12 beneficial deletions by about 200-fold. Moreover, the average selection coefficient of the beneficial deletions is only ≈0.01 whereas, for the deleterious and lethals, it equals 0.19 and 1.0, respectively. Thus, the data obtained with the yeast gene deletions support the traditional view that the genetic variation in fitness is generated almost exclusively by deleterious mutations (36, 37).

Genetic Adaptability of Yeast. The used strain of the budding yeast, S288C, is not among those fastest growing in the laboratory, so improvements in its performance in such an environment are conceivable. In fact, adaptive mutations were demonstrated for nearly any strain and experimental arrangement tested. Fitness gains of 5–20% were found in a study involving haploid and diploid populations maintained in glucose-limited chemostats (38). Studies applying serial transfer cultures also suggest that fitness gains as high as 20% can occur in large evolving populations (39, 40). Rapid adaptation, reaching 40% after 100 generations, was described for yeast cultures propagated in chemostats at 37°C (41). Other experiments also detected fitness gains, although on a smaller scale (42, 43). Thus, the existence of mutations with relatively strong beneficial effects seems well documented (44). Another clear result is that evolved strains exhibited extensive chromosomal rearrangements (45, 46). The identified mutations included changes in the level of expression or in the number of gene copies; complete gene losses were not observed (47).

One study is particularly relevant to the present article (48). A yeast culture was subject to random mutagenesis that resulted in a few copies of the Ty1 element per genome. Only two Ty-mediated mutations were slightly adaptive, and only one mutation was associated with the loss of a gene (*FAR3*) rather than gene deregulation. The study had some caveats, such as possible interference between multiple mutations within a clone. Moreover, transposon-mediated mutagenesis was likely not only to abolish gene function, but also to change the gene product, alter gene expression, or trigger chromosomal rearrangements. In our study, however, both the experimental material and experimental design were chosen deliberately to avoid such uncertainties.

Stability of *Saccharomyces* Gene Content. The entire genomes of four *Saccharomyces sensu stricto* species have been compared (49). These species have evolved independently for up to 20 million years. Divergence of nucleotide sequences was considerable because, in the four species, nucleotides were not identical in ≈58% and 30% of corresponding positions in intergenic and genic regions, respectively. But the gene content did not diverge. When pairs of species were compared, ≈99% of their genes were the same and not degenerated. In another study, chromosomal fragments of *S. cerevisiae* and *Saccharomyces bayanus* containing in total 1,810 genes have been compared. Among a few dozen of detected gene losses, all were attributable to segmental duplication and differential decay of one copy of a duplicated gene whereas all single-copy genes remained functional in both species (50). There has also been a study comparing the DNA sequence of the laboratory strain S288C and a strain isolated from an AIDS patient, YJM789 (51). It is uncertain whether YJM789 has adapted to its unusual environment; the history of "domestication' of S288C is also unclear (52). Nevertheless, it is noteworthy that none of these strains exhibited signs of rapid loss of genetic material, as seen when closely related isolates of bacteria were compared (53).

The stability of the yeast gene content implies positive selection for maintenance of virtually all genes for remarkably long time periods. On the other hand, many genes are likely to be temporarily dispensable. The results of our study suggest that periods of dispensability are not associated with an enhanced risk of loss. It may be a result of evolution toward low costs of maintenance of conditionally dispensable genes. This hypothesis would explain why beneficial effects of gene deletions are so rare and weak, whereas harmful gene deletions and beneficial mutations of other kinds are not. Such an interpretation of the results here described seems conceptually attractive but would be difficult to test empirically.

Recent theoretical work indicates that the ability of organisms to buffer genetic variation can be an inevitable feature of complex genetic systems that requires no explanation in terms of evolution (54–56). The ubiquity of yeast genes whose deletion causes no visible phenotypic defects has been attributed to gene duplications, redundancy of metabolic pathways, or flexibility of transcription programs (57–59). A metabolic model predicts that only 37% of yeast genes will have non-zero flux in nutritionally rich medium, which explains why deletion of many genes is selectively neutral (60). It must be noted, however, that the proportion of expressed proteins was actually found to be about twice as high (61, 62). Generally, it would be difficult to specify which of these mechanisms is responsible for the paucity of adaptive gene deletions.

There is a very special case of gene loss in yeasts. All seven genes of the *GAL* pathway were lost three times independently in lineages leading to *Eremothecium gossypii*, *Candida glabrata*, and *Saccharomyces kudriavzevii* (63). These species represent different lifestyles; the first two are pathogenic. It is puzzling that ecological diversification was associated with a rare and uniform loss of genes. It is thinkable that the loss of the *GAL* pathway was not among the changes that were most relevant to the experienced ecological niche shifts but among those most tolerable for the gene network. If so, the evolutionary fate of single genes would be to a large extent dependent on properties of genetic systems.

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