Genomic Evolution of Vesicular Stomatitis Virus Strains with Differences in Adaptability[†]

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Virus strains with a history of repeated genetic bottlenecks frequently show a diminished ability to adapt compared to strains that do not have such a history. These differences in adaptability suggest differences in either the rate at which beneficial mutations are produced, the effects of beneficial mutations, or both. We tested these possibilities by subjecting four populations (two controls and two mutants with lower adaptabilities) to multiple replicas of a regimen of positive selection and then determining the fitnesses of the progeny through time and the changes in the consensus, full-length sequences of 56 genomes. We observed that at a given number of passages, the overall fitness gains observed for control populations were larger than fitness gains in mutant populations. However, these changes did not correlate with differences in the numbers of mutations accumulated in the two types of genomes. This result is consistent with beneficial mutations having a lower beneficial effect on mutant strains. Despite the overall fitness differences, some replicas of one mutant strain at passage 50 showed fitness increases similar to those observed for the wild type. We hypothesized that these evolved, high-fitness mutants may have a lower robustness than evolved, high-fitness controls. Robustness is the ability of a virus to avoid phenotypic changes in the face of mutation. We confirmed our hypothesis in mutation-accumulation experiments that showed a normalized fitness loss that was significantly larger in mutant bottlenecked populations than in control populations.

Evolutionary success depends on the survival of individuals and their progeny. Survival is the result of multiple environmental and genetic factors operating in a given population. Among such genetic factors are fitness, adaptability, and robustness (26, 50). Fitness can be defined as the overall ability of an organism to generate viable progeny, and it depends on the capacity of an organism to use available resources. In other words, fitness is a measurement of the degree of adaptation to the environment. Adaptability is the potential of a genome to acquire beneficial mutations and increase in fitness. Because bottlenecks and environmental changes may lead to a suboptimal adaptation of virus populations, adaptability is important for long-term survival, particularly in the presence of competitors that may be less fit initially but have the potential for larger increases in fitness under selection. Robustness is the ability of a genome to avoid phenotypic changes in the face of mutation; robustness increases when the neutral mutation rate increases, and it is particularly beneficial when this increase occurs at the expense of the deleterious mutation rate.

RNA viruses are excellent systems to carry out experimental tests of evolutionary theory and ecological principles (7, 12). Because of their high mutation rates, large population sizes,

and rapid replication, these microbes have the potential to evolve a millionfold faster than DNA-based organisms (7). In addition, their small genomic sizes allow determinations of multiple full-length genomic sequences, so phenotypic changes can be linked to genotypic changes. Vesicular stomatitis virus (VSV) is one of the most successful models used for these types of studies (23). VSV is the prototype of the family *Rhabdoviridae* among the *Mononegavirales* (viruses with nonsegmented, negative-stranded RNA genomes) (39). The particles are enveloped and contain a ribonucleocapsid composed of 11.2 kb of viral RNA in complex with nucleoprotein (N), phosphoprotein (P), and the large component of the RNA-dependent RNA polymerase (L). The membrane envelope is covered on the interior side with the matrix (M) protein and on the exterior side with glycoprotein (G). The natural replication cycle of VSV includes a step of insect infection, including sand flies, and a step of mammalian infection, usually pigs, horses, and cows (18, 37). The role of these mammals as natural reservoirs for VSV is unclear, except for feral pigs (37). Infected female insects can transmit the virus transovarially (46), and there is evidence suggesting that insect infection is the most important determinant of viral evolution (51). However, vertical transmission is inefficient, and the mammal is needed for virus survival (20).

Because RNA viruses have high mutation rates, they experience significant selection pressure to minimize their mutation load (11, 41, 45). Quasispecies theory (6, 11) predicts that at high mutation rates, selective pressure results in the selection of mutationally robust genomes (47, 49, 50). In a viral popu-

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FIG. 1. Passage history of control and mutant populations. All populations derive from wt VSV (Indiana serotype, Mudd-Summers strain). Black squares indicate large population passages, white squares indicate progenitor populations, and circles indicate plaque-to-plaque passages.

lation at a fitness peak, most mutations will be deleterious and result in progeny of lower fitness. An increase in robustness will result in more progeny as fit as the progenitor and fewer progeny with deleterious mutations, and thus, the population as a whole will be more fit and better able to survive. de la Torre and Holland provided the first evidence of a high-fitness VSV strain losing in competition against a presumably more robust but less fit competitor (5), and Burch and Chao later confirmed this result by using phage $\phi 6$ in their analysis (2). More recently, Sanjuán and coworkers reported that VSV strains replicating in the presence of mutagens increase their robustness (40). Martin et al., on the other hand, found no evidence of changes in robustness in lymphocytic choriomeningitis virus that replicated in the presence of mutagenic drugs (19). Phage $\phi 6$ strains evolved in regimens where selection is weakened by complementation seem to have a lower robustness than phages evolved under regimens without complementation (21).

When VSV strains are subjected to plaque-to-plaque genetic bottlenecks, there is a decline in fitness (8, 28), presumably because of the stochastic fixation of deleterious mutations. The bottlenecked strains also show consistent defects in their ability to increase fitness as much as (or as rapidly as) their nonbottlenecked counterparts (24, 34). Here, we report further studies of two such mutants with low adaptability. We hypothesized that these mutants differ from the controls with higher adaptability in their distribution of mutational effects. We subjected multiple replicas of two control populations and two mutant populations to a regimen of positive selection (25) and confirmed that the extent of fitness gains was larger in control populations than in mutant populations. Analysis of the mutant distribution in these viruses showed that one of the mutants had decreased robustness compared to that of controls. A comparison of the numbers of mutations accumulated in controls and mutant populations showed that the numbers are similar for both, while fitness increases are larger for control strains than for mutant strains. This result indicates that the average beneficial effect of mutations in control populations is

FIG. 2. Mutation-accumulation regimens in evolved MARM U (Jack and Steve) and evolved MRb (Bonnie, Clyde, and Marco). Evolved MARM U and MRb are depicted as slashed squares. Black squares indicate large population passages, white squares indicate progenitor populations, and circles indicate plaque-to-plaque passages.

larger than that of mutations in mutant populations. We carried out further tests that showed that in cases where mutant progeny was capable of increasing in fitness as much as controls, the progeny had a lower robustness than control progeny. The combination of the lower average effect of beneficial mutations and lower genomic robustness explains why bottlenecked mutants cannot outcompete the nonbottlenecked wild type (wt) despite initial neutral fitness.

MATERIALS AND METHODS

Cells and viruses. The host cells used were baby hamster kidney cells (BHK-21), from John Holland's laboratory (University of California at San Diego), grown to a density of about 10^5 cells/cm². We used minimal essential medium (MEM) supplemented with 7% heat-inactivated bovine calf serum and 0.05% proteose peptone 3 (PP3) (Difco) for cell growth; for plaque assays, we used the same medium without PP3. For virus passages and competitions, we used MEM supplemented with 7% fetal bovine serum. Douglas Lyles (Wake Forest University) provided I1 hybridoma cells (17). All the VSV populations derive from strain Mudd-Summers (Indiana serotype). The wt is the standard reference virus and the progenitor of all other populations, and its fitness was arbitrarily set at 1 (9, 15). MARM U is a clone of the wt selected in the presence of an I1 monoclonal antibody (MAb) and amplified twice in BHK-21 cells at a low multiplicity of infection (MOI) (0.1 PFU/cell). MARM U differs from the wt in a single nucleotide substitution that confers resistance to the I1 MAb (15) and has the same fitness as that of the wt (1.00 ± 0.05) (24). (Our definition of fitness is explained in "Fitness determinations" below.) MRb and MRr are the result of MARM U subjected to 20 plaque-to-plaque passages and one amplification passage at an MOI of 0.1 (24). The fitness of MRb is also the same as that of the wt (1.01 ± 0.05), but MRr has a low-fitness phenotype (0.43 ± 0.08) (26). The history of MARM U, MRb, and MRr is depicted in Fig. 1. Bonnie and Clyde are the progeny of MARM U after 50 large-population passages at an MOI of 0.1, and they have fitnesses of 8.0 ± 1.1 and 8.7 ± 1.4 , respectively (Fig. 2). Jack, Marco, and Steve are the progeny of MRb after 50 large-population passages at an MOI of 0.1, and their fitness values are 7.2 ± 1.1 , 6.8 ± 0.8 , and 9.7 ± 0.4 , respectively (Fig. 2). MARM N is a low-fitness strain generated after 20 plaque-to-plaque passages and one large-population passage in BHK-21 cells (25) (Fig. 1).

Virus passages. We carried out eight replicas of large-population passages for the wt and MARM U and 12 replicas of large-population passages for MRb and MRr. We used 2×10^5 PFU for each infection (MOI of 0.1 PFU/cell). At 20 to 24 h postinfection (p.i.) the production of viral progeny was on the order of 5×10^{10} PFU; we diluted this progeny as needed to perform the next passage under the same conditions. We determined the fitnesses of the wt, MARM U, and MRb

after 25 and 50 large-population passages. We also determined the fitness of MRr after 10 and 25 large-population passages.

To analyze mutant distributions in MRr, MRb, and MARM U, we diluted a stock from each population and infected multiple T-25 flasks so that no more than five plaques were present in each flask. Infection mixtures were incubated for 48 h to ensure that small plaques would be visible and our sampling would not be biased. After incubation we picked all well-isolated plaques from each flask, resuspended the virus sample in MEM, and froze the sample at -80°C until use. Fitness was measured in direct competition against the wt as described below. We picked and analyzed at least 200 plaques for each population.

We carried out mutation accumulation experiments with five populations: two populations derived from MARM U after 50 large-population passages (Bonnie and Clyde) and three populations derived from MRb after 50 large-population passages (Marco, Jack, and Steve). From each population we diluted stocks and infected BHK-21 monolayers. We added agarose (0.2 to 0.3%) to the MEM overlay so that well-isolated plaques could be identified after 20 to 24 h of incubation at 37°C . We picked a plaque randomly from each flask and transferred the progeny virus to a vial with MEM. We then diluted the sample and infected fresh cell monolayers to generate another set of well-isolated plaques, and we again randomly picked one. We repeated this process for 20 passages, and we used samples of the passage 20 progeny to determine the fitness of the bottlenecked populations. We carried out six replicas of bottleneck passages for each progenitor (Fig. 2).

Fitness determinations. To determine fitness we competed each evolved population against a reference virus and monitored changes in the ratio of the two competitors as previously described (9, 15). In summary, we mixed test virus with genetically marked reference virus, and we determined the ratio of the two competitors in the mixture (R0) by triplicate plaque assay in the presence and absence of the I1 MAb. We used the mixture to infect BHK-21 monolayers at an MOI of 0.1 PFU/cell, and after 20 to 24 h, we harvested the progeny and determined the ratio after competition (R1) in the same manner. For clonal analysis of MARM U, MRr, and MRb we used the wt as the reference, and we carried out three consecutive competition passages. We then calculated the slope (m) of the linear regression of the log-transformed MARM-to-wt ratios against passage number. Fitness (w) can then be obtained as follows: $w = \exp(m)$. For fitness determinations of evolved populations, we used the wt as the reference for evolved MARM U, MRr, MRb, and MARM U for the evolved wt. For some of the evolved populations (particularly progeny of MRb, MARM U, and the wt), we could not use several consecutive competition passages because the very fit mutant outcompetes the wt after 1 or 2 passages. In such cases we carried out 3 to 6 independent competitions and then determined fitness as the fraction R1/R0 for each competition and calculated the fitness value as the average of all the determinations.

Data analysis. We carried out all statistical analyses with R, version 2.8.1 (35), with one exception. We used SAS software, version 9.1 (SAS Institute Inc., Cary, NC), to fit fitness data of bottlenecked strains to a linear mixed model. We used the standard R function density to calculate kernel density estimates (42) of fitness distributions, t -test to perform t tests (44), and $ks.test$ to perform Kolmogorov-Smirnov tests (44). For the linear mixed model, we used the SAS function proc mixed and used the Satterthwaite method to estimate the significance of the fixed effects (44).

Sequencing and sequence analysis. We determined the full-length sequence of a total of 56 genomes, including all replicas of the evolved wt and MARM U after 25 and 50 passages as well as the sequences of all replicas of evolved MRr at passage 25 and evolved MRb at passage 50. Our choice of time point was based on previous results: we had observed frequently that there are no changes in the consensus sequences of several strains after 10 and even 20 passages despite significant fitness increases (26). We interpreted this observation to be the result of an increase in the frequency of beneficial variation that did not reach sufficiently high levels to become apparent in the sequencing chromatograms. We then expected that the additional 5 passages would allow a sufficient increase in the frequency of beneficial mutants to identify substitutions in most of the evolved populations derived from the wt, MARM U, and MRr. We had previously observed that multiple replicas of MRb/wt competitions continued after passage 25, but only a few went beyond passage 50, so we chose passage 50 to compare the mutations accumulated in the evolved wt and MARM U controls with evolved MRb. All the methods for sequence determinations were described elsewhere previously (31). Briefly, we extracted RNA from virions using a QiaAMP viral RNA amplification kit, carried out reverse transcription using Superscript III polymerase, and amplified 10 overlapping fragments encompassing the genome. To amplify the termini we used rapid amplification of cDNA ends (RACE). We used these amplified cDNAs as templates in sequencing reactions to generate a level of redundancy of 2- to 5-fold from clean readings.

FIG. 3. Fitnesses of evolved strains. Symbols show fitness measurements for individual replicates, and horizontal bars indicate the mean fitness over all replicates. (A) wt, MARM U, and MRb at passages 25 and 50. (B) MARM N and MRr at passages 10 and 25.

Readings of insufficient quality were discarded, and we generated new and better templates, cDNA, amplified DNA, or readings as needed. The primers used for amplification and readings were described previously by Rodriguez et al. (38), with some modifications to match our wt sequence, and they are available upon request. We used MacVector to assemble the readings of each genome and to align and compare genomes of different origins. The high-quality sequencing results allowed a visual inspection of the chromatograms to identify polymorphic positions that the program may miss. Based on the relative frequencies of wt and mutant peaks in the chromatograms, we scored the presence of mutations as follows: 1 indicates a mutant peak that is consistently present in all readings but at a very low frequency, 2 indicates a mutant peak that is at a substantial frequency but lower than that of the wt, 3 indicates a mutant peak that is at a frequency equal to that of the wt, 4 indicates that the wt is still present but that the mutant peak is at a higher frequency, and 5 indicates that the mutant peak has replaced the wt.

For all mutations, we identified whether they were located in an open reading frame (ORF) or not. For mutations within ORFs, we determined whether the mutations were synonymous or nonsynonymous and, if the latter, which codon and amino acid changes they caused. For each genome, we counted the total number of synonymous and nonsynonymous mutations and calculated the fraction of nonsynonymous changes per nonsynonymous site (dN) and the fraction of synonymous changes per synonymous site (dS) using the counting method described previously by Nei and Gojobori (22). To determine systematic changes in codon bias, we divided all codons into two groups. We considered codons with a relative synonymous codon usage (RSCU) (43) of greater than 1 as preferred and codons with an RSCU of <1 as nonpreferred. We then defined mutations from a major codon to another major codon coding for the same amino acid or from a minor codon to another minor codon coding for the same amino acid as conservative synonymous mutations and defined all other synonymous mutations as nonconservative. We then counted the number of conservative and nonconservative synonymous mutations for each genome. We did a similar analysis for nonsynonymous mutations. We considered nonsynonymous mutations as conservative if the Grantham distance (14) between the original and mutated amino acids was smaller than 100 and as nonconservative otherwise.

Clonal analysis. Genomic variation in each population was assessed by clonal analysis of the M ORF. RNA was obtained and reverse transcribed as described above, and 3 μl of cDNA template was used to amplify a fragment encompassing nucleotides (nt) 2250 to 2939. The reaction was done with forward primer 5'-TCT AAG TGT TAT CCC AAT CC-3' (nt 2223 to 2242 of the viral sequence), reverse primer 5'-CGG GGA TTG TTC AGA AGC-3' (nt 2956 to 2973 of the viral sequence), and high-fidelity *Pfx* polymerase. Amplified cDNA was purified by using Qiagen PCR cleanup kits and cloned into pCR-BLUNT (Invitrogen). *Escherichia coli* TOP-10 cells were transformed according to the manufacturer's recommendations. At least 50 clones were sequenced using plasmid forward and reverse primers.

RESULTS

Differential fitness increases during regimens of positive selection. Large-population passages of strain MARM U and the wt led to significant increases in fitness after 25 passages and further increases after 50 passages (Fig. 3A). As expected,

both strains improved similarly, in agreement with their identical relative fitnesses and with the similar potentials of either one to outcompete the other in long-term competitions (24). All replicas of passages from each population gained fitness, with average values of 3.98 ± 0.52 for MARM U and 4.20 ± 0.36 for the wt at passage 25 and 8.97 ± 0.54 for MARM U and 7.47 ± 0.53 for the wt at passage 50 (Fig. 3A).

MRb populations also showed fitness increases in all replicas at passage 25 and passage 50, from a fitness of 1.0 to average values of 2.69 ± 0.17 and 5.49 ± 0.56 , respectively (Fig. 3A). However, even at passage 25, the overall gain was not as much as the gain observed for the wt or MARM U, and this difference remained at passage 50 (all $P < 0.05$ by two-tailed t tests). This result is consistent with the behavior of MRb in long-term competitions against the wt, where in multiple replicas, we consistently found a period of stable coexistence of variable length followed by a frequency decrease and the eventual extinction of MRb (24). It is important that MRb has a fitness of 1.0, as measured in competitions against the wt for 6 to 8 passages. Thus, an initially lower fitness cannot explain the lower fitness gains of MRb. The results here show that MRb has the potential to generate beneficial variation but that this potential is lower than the potentials of the wt and MARM U; in other words, the wt and MARM U have a higher adaptability than MRb.

We previously showed that in a single replica of large-population passages, MRr was unable to recover fitness in 6 passages (24). This observation was in contrast with the results obtained with other virus strains that typically gain fitness significantly under comparable protocols (25, 29). Low-fitness strains tend to gain fitness faster than strains with an initial fitness of 1 during the first 5 to 10 passages (25), and thus, a comparison of the fitness increases between low-fitness MRr and the wt or MARM U may be misleading. For that reason we compared the fitness gains of 12 additional replicas of MRr with the fitness gains of strain MARM N (25), which has an initial fitness of 0.43 ± 0.05 , similar to that of MRr. For this report we carried out 12 additional replicates of large-population passages for MRr, and we tested the progenies at passage 10 and passage 25. MARM N reached a fitness of 1 in 6 to 7 passages (25), and other low-fitness strains increased fitness 2- to 6-fold in 6 passages or fewer (24). The fitness of evolved MRr had overall increases at passage 10 to 0.70 ± 0.05 and at passage 25 to 1.76 ± 0.14 (Fig. 3B), but the evolved fitness was substantially lower than that of MARM N at the same passage ($P < 0.005$ in both cases by two-tailed t tests).

Differences in the types of mutations present in different populations. The differences in fitness gains between mutants and controls could be explained if the overall mutation rate of the former was lower than the overall mutation rate of the latter. However, the sequences of polymerases (P and L proteins) and the associated N protein from MRr and MRb are identical to those of the polymerases of the controls, so assuming that the structural M and G proteins have no effect, we can rule out this explanation. Note that the sequences represent a consensus, and it is possible that some populations will contain a minority fraction of mutants, and even though these consensus sequences were obtained from high-quality readings and that the frequency of such mutations has to be very low, such minority mutants may be playing a role. Next, we hypothesized

FIG. 4. Kernel density estimates of the fitness distributions of individual clones isolated from strains MRr, MARM U, and MRb. Fitness values of clones were normalized to the fitness values of the originating strains (the complete data set of all fitness determinations is presented in Table S1 in the supplemental material). Clones of MARM U and MRb have comparable fitness distributions with means close to the originating strains' fitness values. Clones of MRr tend to have lower normalized fitness values than clones of both MARM U and MRb.

that there could be differences in the effects of mutations generated in each genetic background. To test this idea, we carried out a clonal analysis of MARM U, MRb, and MRr. We isolated at least 200 clones (plaques) from each population, measured the fitness of each clone, and normalized this fitness value by the average fitness of the original population (see Table S1 in the supplemental material). We found that MARM U and MRb had similar distributions ($P = 0.47$ by Kolmogorov-Smirnov test), with averages close to the average fitness of the respective population (Fig. 4). This result is consistent with the ability of MRb to coexist for extended periods of time (up to 60 passages) in competition against the wt. In contrast, the distribution of MRr clones was strikingly different (Fig. 4) ($P < 10^{-10}$ for MRr compared separately to both MRb and MARM U by Kolmogorov-Smirnov test). Nearly all the MRr clones had fitnesses that were significantly lower than the population average, and none of the clones had a fitness that was higher than the population average. We concluded from these observations that MRr has a higher deleterious and (possibly) lower beneficial mutation rate than either MARM U or MRb. In other words, MRr is less robust than the other two strains.

We next performed a sequence analysis of molecular clones to look at the mutation frequency and distribution of mutations found in the wt, MARM U, MRr, and MRb populations. We chose the M ORF because in past studies, and also here, we found that there are both conserved and highly variable regions. We found a relatively small number of mutated sequences among these clones. Fewer than 10% of all sequences had one mutation, and only a single sequence out of 206 sequences had two mutations (see Table S2 in the supplemental material). The overall mutant frequency was in the order of 10^{-4} substitutions/nucleotide, and there was no obvious difference among strains in number and type of mutations among clones (Tables S2 and S3), but we identified mutations in conserved areas (nt 2320 to 2540).

Mutants with low adaptability accumulate at least as many mutations as controls during adaptation. We interpreted the differences in adaptability in MRb and MRr compared to MARM U and the wt in terms of the rate and effect of beneficial mutations. The potential to generate beneficial variation depends on two variables, the rate at which beneficial mutations are produced and the magnitude of the beneficial effect of these mutations. A strain with reduced adaptability may have a reduced beneficial mutation rate, beneficial mutations of reduced magnitude, or both. To distinguish between these possibilities, we sequenced the full-length genomes of evolved control strains (MARM U and wt progeny) and the evolved strains with low adaptability (MRr and MRb progeny). By definition, the consensus sequence represents the average (or most abundant) nucleotide at each site, and therefore, based on our scoring of mutant nucleotides (see Materials and Methods), we considered all mutations in groups 3, 4, and 5 in our analysis. Minor polymorphisms, which are identified by a score of 1 or 2, may represent beneficial mutations on their way to fixation, but they may also be transient, and in most cases, we cannot distinguish between the two possibilities. A complete list of substitutions in all 56 genomes is presented in Table S2 in the supplemental material. We did find some replicas without changes in the consensus, but all populations had at least minor polymorphisms (see Fig. S1 and S2 in the supplemental material). None of the mutations identified in these evolved genomes corresponded to the mutations identified in the clonal analysis of stock populations (Table S2 and Fig. S3).

We found that MRr at passage 25 had accumulated 2.75 nucleotide substitutions of groups 3, 4, and 5 on average, compared to 1.12 substitutions for wt replicates and 1.88 substitutions for MARM U replicates (Table 1). The mean number of substitutions for MRr was significantly larger than that for the wt ($P = 0.007$ by t test) but similar to the mean number of substitutions in MARM U ($P = 0.216$ by t test). If we considered only completely fixed mutations (group 5 mutations), MRr had an average of 1.50, which is significantly greater than the numbers of substitutions for both the wt (average, 0.13; $P = 0.004$) and MARM U (average, 0.25; $P = 0.013$). We were surprised to find more mutations in MRr than in the wt and MARM U. None of the strains analyzed are at a mutation-selection balance; instead, they are all under positive selection, and their fitness is increasing exponentially. Thus, it is unlikely that the strains with low adaptability are generating beneficial mutations that are not increasing in frequency, and this result shows that MRr does not generate fewer mutations than controls.

We found no significant differences in the numbers of mutations accumulated in MRb replicates compared to either the wt or MARM U at passage 50 (all $P > 0.05$ by t tests). Considering mutation groups 3, 4, and 5, we found an average of 5.17 substitutions for MRb, compared to 5.38 for the wt and 5.75 for MARM U (Table 2).

We found mutations in all regions of the VSV genome and could not identify clear differences in the locations of mutations for the wt, MARM U, MRb, and MRr. Thus, our results indicate that the numbers and the distributions of mutations generated in each strain are similar, and the differences in adaptability are the result of differences in the selective values of mutations.

TABLE 1. Numbers of mutations in MRr compared to the wt and MARM U at passage 25

Parameter	wt at cutoff of:					Mean value $\pm$ SEM for group ^d					MRr at cutoff of:				
	1					MARM U at cutoff of:					1				
	1	3	5	1	3	1	3	5	1	3	1	3	5	1	3
No. of substitutions ^a	4.38 $\pm$ 0.89	1.12 $\pm$ 0.35*	0.12 $\pm$ 0.12*	5.38 $\pm$ 0.62	1.88 $\pm$ 0.40	5.38 $\pm$ 0.62	1.88 $\pm$ 0.40	0.25 $\pm$ 0.25*	4.67 $\pm$ 0.47	2.75 $\pm$ 0.39	4.67 $\pm$ 0.47	2.75 $\pm$ 0.39	1.50 $\pm$ 0.38	4.67 $\pm$ 0.47	2.75 $\pm$ 0.39
dS ^c	0.00016 $\pm$ 0.00008	0.00000 $\pm$ 0.00000*	0.00000 $\pm$ 0.00000	0.00062 $\pm$ 0.00011*	0.00016 $\pm$ 0.00011	0.00062 $\pm$ 0.00011*	0.00016 $\pm$ 0.00011	0.00005 $\pm$ 0.00005	0.00024 $\pm$ 0.00010	0.00014 $\pm$ 0.00006	0.00024 $\pm$ 0.00010	0.00014 $\pm$ 0.00006	0.00004 $\pm$ 0.00003	0.00024 $\pm$ 0.00010	0.00014 $\pm$ 0.00006
dN ^d	0.00038 $\pm$ 0.00009	0.00009 $\pm$ 0.00005*	0.00002 $\pm$ 0.00002*	0.00040 $\pm$ 0.00005	0.00015 $\pm$ 0.00003*	0.00040 $\pm$ 0.00005	0.00015 $\pm$ 0.00003*	0.00002 $\pm$ 0.00002*	0.00047 $\pm$ 0.00004	0.00027 $\pm$ 0.00004	0.00047 $\pm$ 0.00004	0.00027 $\pm$ 0.00004	0.00017 $\pm$ 0.00004	0.00047 $\pm$ 0.00004	0.00027 $\pm$ 0.00004
dN - dS	0.00022 $\pm$ 0.00007	0.00009 $\pm$ 0.00005	0.00002 $\pm$ 0.00002*	-0.00023 $\pm$ 0.00012*	-0.00001 $\pm$ 0.00011	-0.00023 $\pm$ 0.00012*	-0.00001 $\pm$ 0.00011	-0.00004 $\pm$ 0.00004*	0.00023 $\pm$ 0.00010	0.00013 $\pm$ 0.00008	0.00023 $\pm$ 0.00010	0.00013 $\pm$ 0.00008	0.00014 $\pm$ 0.00005	0.00023 $\pm$ 0.00010	0.00013 $\pm$ 0.00008

^a Cutoff indicates the minimum frequency of the mutation group considered (e.g., a cutoff of 3 corresponds to all mutations scored as 3, 4, and 5). * indicates a significant difference ($P < 0.05$) in comparison to MRr.
^b The number of substitutions gives the genome-wide count of nucleotide differences.
^c dS gives the number of synonymous substitutions per synonymous site.
^d dN gives the number of nonsynonymous mutations per nonsynonymous site.

FIG. 5. Changes in the consensus sequences of the wt, MARM U, MRr, and MRb during adaptation to BHK-21 cells, including mutations scored as 3, 4, and 5. The top shows the nucleotide length. Each virus gene (N, P, M, G, and L) is depicted in gray, and intergenic regions are shown in white. (A) Mutations in the wt, MARM U, and MRr at passage 25. (B) Mutations in the wt, MARM U, and MRb at passage 50. In each case we present substitutions compared to their respective ancestors. The original wt sequence is available in the GenBank database (accession number EU849003).

ficial mutant in a genome that lacks the first beneficial mutation. In the case of these VSV populations, we demonstrated that the first wave of fixation comes from mutant populations that are present as preexisting variation, and the corresponding initial period of fitness improvement corresponds to the increase in the frequency of these mutants (10).

Contribution of differences in robustness to long-term survival. Interestingly, some of the replicas of evolved MRb populations at passage 50 had increased their fitness to levels similar to those found in evolved wt and MARM U controls (Fig. 3). This result suggested that MRb would have the potential to adapt as well as controls and should be capable of outcompeting the wt occasionally, but we never found such a result (24). However, we did observe some replicas of long-term competitions in which MRb maintained its initial frequency for over 50 passages, confirming that, in some cases, MRb was capable of gaining as much fitness as the wt during this period of time. Nevertheless, the result of these competitions was invariably MRb extinction. We hypothesized that even when capable of sufficient fitness increases, evolved MRb strains may have a lower robustness than controls. Thus, once the two competing populations are close to the top of a fitness peak, a lower deleterious mutation rate would give a selective advantage to the wt.

We tested this hypothesis in mutation-accumulation experiments using two strains of evolved, high-fitness MARM U (Bonnie and Clyde) and three strains of evolved, high-fitness MRb (Marco, Jack, and Steve). For each strain we carried out six replicates of 20 plaque-to-plaque passages, and we determined the fitness of the progeny (Fig. 2). We assessed whether strains derived from MRb experienced a significantly more severe fitness loss than strains derived from MARM U by fitting the normalized and log-transformed fitness data to a mixed linear model. We normalized the fitnesses of the bottlenecked strains by the fitnesses of their high-fitness ancestors (Bonnie, Clyde, Marco, Jack, and Steve). In the linear model, we treated the fitness effects of the progenitors MRb and

MARM U as fixed and treated the effects of all evolved strains (Bonnie, Clyde, Marco, Jack, and Steve as well as bottlenecked progeny) as random. We found that the effect of MRb was significant ($P = 0.018$) and amounted to a reduction of 0.7172 in normalized log fitness. In other words, bottlenecked strains originally derived from MRb had an approximately 50% lower normalized fitness than bottlenecked strains originally derived from MARM U (Fig. 6). The overall results indicated a more severe fitness loss in MRb-derived populations than in MARM U-derived populations (Fig. 6). This behavior was particularly evident in Marco, with a fitness loss of over 80%, compared to a 40 to 50% loss in the control strains.

FIG. 6. Relative fitnesses of replicates after 20 passages of plaque-to-plaque transfers. Symbols indicate fitness values for individual replicates, and horizontal bars indicate the mean fitness over all replicates. Bottlenecked strains originally derived from MRb have approximately 50% lower fitness than bottlenecked strains originally derived from MARM U.

DISCUSSION

Putting all our results together, we can explain the inability of MRb to outcompete the wt in long-term experiments by a combination of decreased overall adaptability and decreased robustness in populations that undergo sufficient fitness increases. We can also suggest how the quasispecies nature of RNA viruses provides (e.g., selection of high robustness) a mechanism that explains the results described previously by de la Torre and Holland (5), who observed a wt virus outcompeting a more fit strain when the frequency of the latter fell below 0.1%. Considering this frequency and the total population size during competition (about 2×10^5 PFU), we can interpret this behavior as a combination of a lower robustness in the high-fitness mutant and bottleneck effects when its total number of particles in the competition was too small to include in the sample progeny with enough fitness to compete against the initially less fit variant.

Here we have explored the mechanism leading to a loss of robustness. One possibility that we tested was that different strains have differences in their overall fidelity. A lack of non-synonymous mutations in L, P, and N, together with the results of clonal analysis, indicates that mutation rates are similar for all the strains. A second potential mechanism was that even though overall mutation rates were similar, there may be differences in the beneficial mutation rate. This seems unlikely, because under strong positive selection, the mutants accumulated as many beneficial mutations as the control strains. The last option is that the beneficial effect of mutations accumulated in MRr and MRb is lower than the beneficial effect of mutations accumulated in the wt and MARM U. In the case of evolved strains (Bonnie, Clyde, Marco, Steve, and Eric), another possible explanation for the differences in adaptability could be changes in the mutation rates (e.g., polymerase fidelity) in evolved MRb. If any of these mutants had a more error-prone polymerase, the overall mutation rate would increase along with the deleterious mutation rate. Such changes in polymerase fidelity are possible and can result from increased mutational pressure, as shown previously for picornaviruses replicating in the presence of the mutagen ribavirin (1, 32, 48). In the case of MRb-derived populations, we cannot rule out differences in fidelity as a factor contributing to the more substantial fitness loss because Jack, Marco, and Steve all have at least one nonsynonymous mutation in the L component of the polymerase that may lead to changes in copying accuracy.

Therefore, at least for MARM U, MRb, and MRr, the difference between mutants and controls is the value that specific mutations have in each parental background; in other words, the molecular basis leading to loss of adaptability and robustness is epistasis. The minimal differences in the consensus sequences of control and mutant populations suggest that epistatic interactions are important and widespread throughout the VSV genome.

It is interesting that, at least in theory, changes in robustness should affect both beneficial and deleterious mutation rates, and one might expect tradeoffs between adaptability and robustness leading to adaptability loss when robustness increases and *vice versa*. Thus, sometimes, selection may promote decreased robustness to favor higher adaptability, for instance, in

sequences that code for antigenic sites under strong immune selection (33). Surprisingly, our results showed that this may not always be the case, because MRr lost both robustness and adaptability, and all the changes in the neutral mutation rate resulted in deleterious mutations.

Quasispecies theory predicts the selection of high robustness. To our knowledge there are no studies analyzing the evolution of adaptability and robustness in natural virus populations, and these are not trivial experiments, but there is some work using experimental evolution that sheds some light on this subject. If selection promotes high robustness, we would predict that when selection is relaxed, robustness will decrease. MRr and MRb were the result of repeated plaque-to-plaque passages, where selection is highly minimized and can affect only mutants that are extremely deleterious or lethal. Therefore, our results are consistent with quasispecies dynamics. It is important that both the wt and MARM U were the result of viral adaptation to cell culture in the absence of mutagens, suggesting that typical mutation rates in RNA viruses are high enough to result in the selection of high robustness, and further increases by the use of mutagens are not required. Nevertheless, there is evidence that the addition of mutagens also results in increased robustness (40). Burch and Chao (2) also reported a loss of robustness of phage $\phi 6$ after plaque-to-plaque passages. A second method to relax selection is the use of high MOIs during passage. Under these conditions there is frequent coinfection, which leads to complementation and the maintenance of undesirable mutations (30). As expected, phage populations generated at a high MOI show a significant loss of robustness (21). Thus, our results and the results from other groups clearly show a correlation between selection and robustness.

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