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High mutation rates, bottlenecks, and robustness of RNA viral quasispecies

Susanna C. Manrubia[a], Cristina Escarmís[b], Esteban Domingo[a,b,c], Ester Lázaro[a,*]

[a]*Centro de Astrobiología (INTA-CSIC), Instituto Nacional de Técnica Aeroespacial, Ctra de Ajalvir km 4, 28850 Torrejón de Ardoz, Madrid, Spain*
[b]*Centro de Biología Molecular "Severo Ochoa" (CSIC-UAM), Universidad Autónoma de Madrid, Cantoblanco, 28049 Madrid, Spain*
[c]*Centro de Investigación en Sanidad Animal (CISA-INIA), Valdeolmos 28130, Madrid, Spain*

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Abstract

Population bottlenecks are stochastic events that strongly condition the structure and evolution of natural populations. Their effects are readily observable in highly heterogeneous populations, such as RNA viruses, since bottlenecks cause a fast accumulation of mutations. Considering that most mutations are deleterious, it was predicted that the frequent application of bottlenecks would yield a population unable to replicate. However, in vitro as well as in vivo systems evolving through bottlenecks present a remarkable resistance to extinction. This observation reveals the robustness of RNA viruses and points to the existence of internal mechanisms which must confer a high degree of adaptability to fast mutating populations. In this contribution, we review experimental observations regarding the survival of RNA viruses, both in laboratory experiments and in natural populations. By means of a simple theoretical model of evolution which incorporates strong reductions of the population size, we explore the relationship between the number of replication rounds that a single founder particle undergoes before the next bottleneck is applied, and the mutation rate in a particular environment. Our numerical results reveal that the mutation rate has evolved in a concerted way with the degree of optimization achieved by the population originated from the founder particle. We hypothesize that this mechanism generates a mutation–selection equilibrium in natural populations that maximizes adaptability while maintaining their structure.

Keywords: Error threshold; Population size; Replication rounds; Mathematical model

1. Introduction

Evolution of RNA viruses is governed by three main features. They present the highest mutation rates found in nature (Drake, 1993; Domingo and Holland, 1997), on the order of 10^{-3} to 10^{-5} errors per nucleotide and replication cycle. In addition to this, RNA viruses have very short replication times and generate very large populations, two characteristics that strongly accelerate evolution. The high error rate inherent to RNA virus replication is probably due to the absence of proofreading activities of RNA replicases and retrotranscriptases. Although most of the mutations produced upon replication have a negative effect on fitness, advantageous mutants reproduce faster than deleterious, such that a mutation–selection equilibrium arises. This balance between the continuous generation of new mutants and the action of positive and negative selective forces acting on complex ensembles of replicating units leads to a very dynamic, though highly organized population (Domingo et al., 1978, 2001) composed by a very large number of different, but related, genomes. The structure observed in RNA virus populations has been considered a real example of the molecular quasispecies proposed to explain the

Abbreviations: FMDV, foot-and-mouth disease virus; VSV, vesicular stomatitis virus; HIV, human immunodeficiency virus; W, fitness value; n, number of descendants of a viral genome in a single replication round; r, number of replication cycles; m, mutation rate; k, number of mutations per sequence in a single replication round; p, probability of deleterious mutations; q, probability of advantageous mutations.

* Corresponding author. Tel.: +34 915206457; fax: +34 915201074.
E-mail address: lazarolm@inta.es (E. Lázaro).

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evolution of populations of ideal molecules subjected to error-prone replication (Eigen and Schuster, 1979; Eigen and Biebricher, 1988).

In addition to internal mechanisms, such as mutation–selection in large populations in a given environment, the evolution of RNA viruses is also influenced by processes inherent to population dynamics (such as genetic drift) or by external disturbances (such as population bottlenecks). Those may strongly condition the mutant distribution of the new viral population (Domingo et al., 2001; Wilke, 2002; Moya et al., 2004). Genetic bottlenecks are stochastic events that cause strong reductions in the effective population size and take place when only a few individuals of a population found a new one. Therefore, bottlenecks reduce the genetic diversity of a population. When a few, or even a single genome, of a viral quasispecies is randomly chosen to generate a new population, there is a high probability that it carries a deleterious mutation relative to fitter genomes of the parental population. This mutation will be transmitted to all the members of the new population. Hence, bottlenecks accelerate the accumulation of mutations in the consensus sequence of the quasispecies (Fig. 1).

That small asexual populations should accumulate deleterious mutations in an irreversible way was theoretically predicted by Muller (Muller, 1964). Muller's ratchet hypothesis implies that the wild-type sequence can be lost by chance solely due to fluctuations in the population size or to genetic drift. The process is particularly effective when the mutation frequency is high, as in RNA viruses. If one further assumes that the least mutated genome is the fittest one, its elimination results in irreversible fitness losses and, eventually, in the extinction of the population. Since Muller's ratchet hypothesis was formulated, a number of theoretical studies have been carried out to analyze the effect of deleterious mutations during the transmission of pathogens through population bottlenecks (Wagner and Gabriel, 1990; Kondrashov, 1994; Gordo and Charlesworth, 2000).

The empirical implementation of Muller's ratchet operation has often involved RNA viruses. A classical experiment to analyze the effect of an increased rate of mutations fixation is the plaque-to-plaque transfer of clonal populations of a virus (Fig. 2). Fitness losses caused by this experimental protocol have been reported for a number of viruses, including bacteriophage ϕ6 (Chao, 1990), vesicular stomatitis virus (VSV) (Duarte et al., 1992; Clarke et al., 1993; Novella and Ebendick-Corpus, 2004), foot-and-mouth disease virus (FMDV) (Escarmís et al., 1996, 2002; Lázaro et al., 2003), human immunodeficiency virus (HIV) (Yuste et al., 1999), and the bacteriophage MS2 (de la Peña et al., 2000). However, in most of these studies, the number of transfers performed has been rather low and only in the case of HIV and in few FMDV clones (Escarmís et al., 2002) real extinctions have been observed.

In nature, bottlenecks are very frequent during the transmission of many viruses and may affect the severity of disease outbreaks. However, although there are some documented extinctions of particular virus strains (Wittke et al., 2002), viruses do not get easily extinct despite the high frequency of bottlenecks in natural environments. Therefore, it is reasonable to assume that there must be mechanisms able to counterbalance the negative effect of bottlenecks. In the case of vertically transmitted viruses, bottlenecks may be particularly severe, since only a small amount of viruses are able to cross the barriers to infect the embryo. In addition, competition among genomes can only take place inside the infected host, so the action of selection is very reduced. Bottlenecks are also very frequent in horizontally transmitted viruses and during the inter-organ transmissions inside an infected organism. Under horizontal transfer, each virus can infect every susceptible individual of the host population, and competition can also happen at the inter-host level (Wilson et al., 1992; Chao et al., 2000). In HIV, new infections typically contain a smaller diversity of sequences than long-term infected individuals, indicating that, initially, the disease was likely transmitted through a low number of viral particles (Nowak et al., 1991; Pang et al., 1992). Bottlenecks also occur in many diseases transmitted via respiratory droplets, because most droplets only contain one or a few virions (Gerone et al., 1966). The inter- and intra-host competition that takes place in horizontally transmitted viruses should mitigate the effect of bottlenecks. This hypothesis has been suggested as an explanation to the observation that vertically transmitted pathogens evolve to lower virulence than do their horizontally transmitted relatives (Bergstrom et al., 1999).

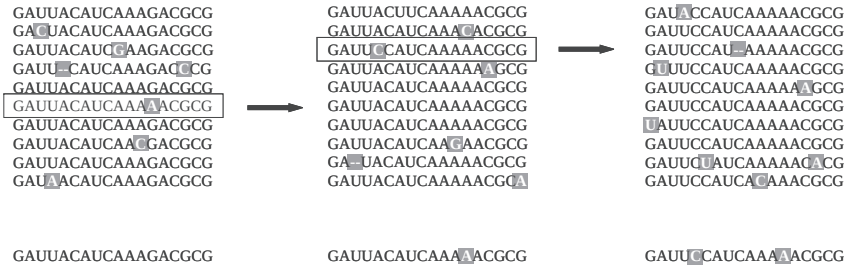

Fig. 1. Accumulation of mutations in the consensus sequence of a heterogeneous population when a single genome (in the box) is selected to found a new population. All the mutations that are present in this genome will be transmitted to the progeny and, as a consequence, they will be fixed in the consensus sequence (below) of the new population.

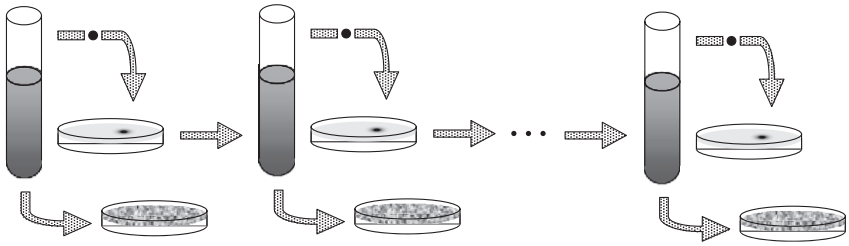

Fig. 2. Schematic representation of the process of plaque-to-plaque transfers of viral populations. At each transfer clonal populations are diluted and plated for isolation of individual plaques (representing the progeny of a single virus) and for the determination of the number of infective units.

Our group has made a detailed study of the evolution of several clones of FMDV subjected to a very large number of plaque-to-plaque transfers (Escarmís et al., 2002). Analysis of the changes in fitness shows that the evolution of the viral titer (the parameter used in our assays as a measure of fitness) as a function of the passage number follows a biphasic dynamics (Escarmís et al., 2002; Lázaro et al., 2003). There is a first phase in which the number of individuals generated at each transfer decreases and, after a variable number of passages, the decreasing trend disappears and a stationary state of infectivity values is reached. In the stationary phase, the number of individuals generated at each transfer showed large fluctuations around a constant mean value (Escarmís et al., 2002; Lázaro et al., 2003; Manrubia et al., 2003). In contrast to this, the number of mutations fixed in the consensus sequence grew linearly in the stationary state as well, despite the stabilization of the average fitness (Escarmís et al., 2002). These results are unexpected in the light of the classical Muller's ratchet hypothesis. To identify the main mechanisms at play in the system, we developed a theoretical model of evolution through bottlenecks (Lázaro et al., 2002; Manrubia et al., 2003). The essential features to reproduce the behavior observed in the experiments are (i) the occurrence with low probability of advantageous mutations, a mechanism not considered in most previous theoretical models (Kondrashov, 1994; Gordo and Charlesworth, 2000), and (ii) the presence of an extinction threshold below which individuals are removed. This results in an equilibrium between the elimination of least fit individuals and the selection for the next transfer of a virus able to replicate, an ability that is maintained thanks to the appearance of compensatory mutations.

The results obtained in the simulations suggest that, even during the development of a lytic plaque, an heterogeneous population is generated and a fraction of the mutants should have advantageous mutations. In the plaques generated at the stationary state, many mutants are probably eliminated, increasing in this way the probability of selecting for the next transfer a virus with compensatory mutations. The main biological consequence of this behavior is, as observed, a remarkable resistance of bottlenecked viruses to extinction. These results also contrast with experimental studies showing that small increases in the mutation rate through the use of mutagens can lead viral populations to extinction (Sierra et al., 2000; Pariente et al., 2001; Crotty et al., 2001; Grande-Pérez et al., 2002; Severson et al., 2003), a fact expected attending to theoretical studies on molecular evolution (Eigen and Biebricher, 1988; Eigen, 2002). Those studies predicted the existence of an upper limit for the error rate of replication (inversely proportional to the genome length), beyond which the genetic information of a population is lost.

In this paper, we use the previous numerical model modified in order to include a microscopic description of mutations. Our goal is to quantify the relevance of two additional parameters: the mutation rate and the number of replication rounds that a virus undergoes before it is transmitted to a new host. While the mutation rate is a character subject to selection (Earl and Deem, 2004) and likely determines the ability of RNA viruses to overcome frequent environmental changes, the number of replication rounds is directly related to the degree of optimization that a pathogenic population can attain when replicating inside the host. We discuss how these two parameters relate to each other and eventually decide the ability of viruses to reach a stable mutation–selection equilibrium under frequent bottlenecks followed by optimization periods.

2. Materials and methods

The essential features of the model of viral evolution through bottlenecks are described in Lázaro et al. (2002). Viral infection starts with a single particle with fitness value W, a real number in the range $[1, \infty]$. At every replication round, this particle produces a number n of descendants, which can replicate at the next round. The process is iterated up to r replication cycles. The number n of descendants of each individual virus is randomly drawn from a Poisson distribution of average W. This implies that the replication rate is directly proportional to fitness. The average number of mutations per replication event is denoted by m, and the actual number of mutations, k, follows a Poisson distribution of average m. Each individual mutation can have a deleterious, a positive, or a neutral effect on fitness, with probabilities p, q, and $1-p-q$, respectively (the values used in all the simulations we have carried out correspond to p=0.5 and q=0.1). We assume that there are no epistatic interactions among mutations and that the total change in

fitness, ΔW, is given by the sum of the individual effects of the mutations occurring in the same sequence.

$$\Delta W = \sum_{i=1}^{k} (\delta W)_i, \quad (1)$$

where the sum runs over the actual number of individual mutations k, and the absolute value of each of them $|\delta W|$ is obtained from an exponential probability distribution of average one:

$$P(|\delta W|) = \exp\{-\delta W\}. \quad (2)$$

The sign of the change is negative with probability p and positive with probability q. Particles with fitness less than one are eliminated from the system. This defines an extinction threshold for viruses unable to replicate as a consequence of the deleterious effect of mutations. Finally, there are a maximum number of particles, N_m, per lytic plaque.

In this way, the founder particle generates a heterogeneous population of daughter sequences. The variance of this distribution (the degree of diversity of the population) depends on the mutation rate and on the number of generations elapsed between consecutive bottleneck events. Bottlenecks are implemented by randomly choosing a particle from the heterogeneous population generated and using it as a seed for a new infection.

3. Results

In this paper, we study the role played by two parameters: the number of replication cycles r that take place during the development of the lytic plaque, and the mutation rate, m. The parameter r presumably influences the degree of optimization of the cohort generated by the founder particle before the next cycle of infection starts. The larger the r value, the closer is the new population to the mutation–selection equilibrium of quasispecies. The mutation rate, m, determines the genetic variability produced at each infection cycle. A too large value of m may cause an excess of deleterious mutations that selection cannot compensate, whereas a too low value of m may be insufficient to originate enough genetic diversity, necessary for the appearance of rare advantageous mutants. As the time allowed for the production and selection of fitter genomes depends on the number of replication cycles, we hypothesize that the optimal value of m is related to the value of r. Therefore, transmission cycles of different viruses might be associated with values of m that are dependent on the average number of replication cycles of the virus in a particular host. This value may vary in the course of a natural infection depending on many factors, including the type of virus and the moment at which the progeny viruses are transmitted to a new host. In the plaque-to-plaque transfer experiments, r usually takes a fixed value that may be altered if the plaques are allowed to develop for different times (Escarmís et al., 2002). However, there is a lower limit in that value to obtain plaques of observable size and an upper limit that is usually constrained by the viability of the cells and by the time that they are susceptible to infection. The other parameter under study, m, experiences less variations and, as pointed above, has been likely optimized for each particular virus to ensure its survival according to the transmission cycle established in nature. It is very difficult to do experiments in which the values of m and r are altered simultaneously to demonstrate if a concrete m value is associated with a particular transmission regime. In this sense, theoretical models and numerical simulations motivated by the real system can be very useful.

3.1. Optimization of quasispecies between bottlenecks

In all the simulations described across this paper, we used the model described in the Materials and methods. The results obtained do not qualitatively differ from those obtained with a simpler version of the model (Lázaro et al., 2002; Manrubia et al., 2003) pointing out that the model is robust and likely valid to qualitatively study the effect of other parameters involved in viral evolution through bottlenecks.

First, we studied the dynamics of the viral yield through successive bottlenecks once the system has attained the stationary state of fitness (Fig. 3). We have tested several values of m and r, other variables being fixed. As most mutations are deleterious, an increase in the mutation rate results in lower yields on the average (Fig. 3C) and in alterations in the pattern of fluctuations between consecutive passages (Fig. 3A and B). When r=7 (Fig. 3A) and the mutation rate is the lowest assayed (m=0.1), the system reaches the maximum number of particles permitted almost in all passages and there are few fluctuations of the viral titer, despite the frequent bottlenecks. This result is indicating that, when m is low and r is high enough, the system is optimized to obtain the highest viral yields permitted. The almost complete absence of fluctuations indicates that an optimal mutation–selection equilibrium is reached, and most of the plaques generated come from high-fitness genomes. As a consequence, the system presents a relatively low diversity. However, this simultaneously implies low adaptability, since when r decreases (Fig. 3B), and m is maintained at the value of 0.1, there is almost no possibility of generating advantageous genomes in few replication cycles. When m increases (m=1 and m=4 for r=7), the fluctuations become more frequent and have larger amplitude, and the fraction of passages at which the maximum yield is reached decreases. Since p takes values higher than q, the negative effect of most mutations translates into a decrease of the average viral yield but, at the same time, the greater diversity originated at each passage causes larger fitness fluctuations allowing the

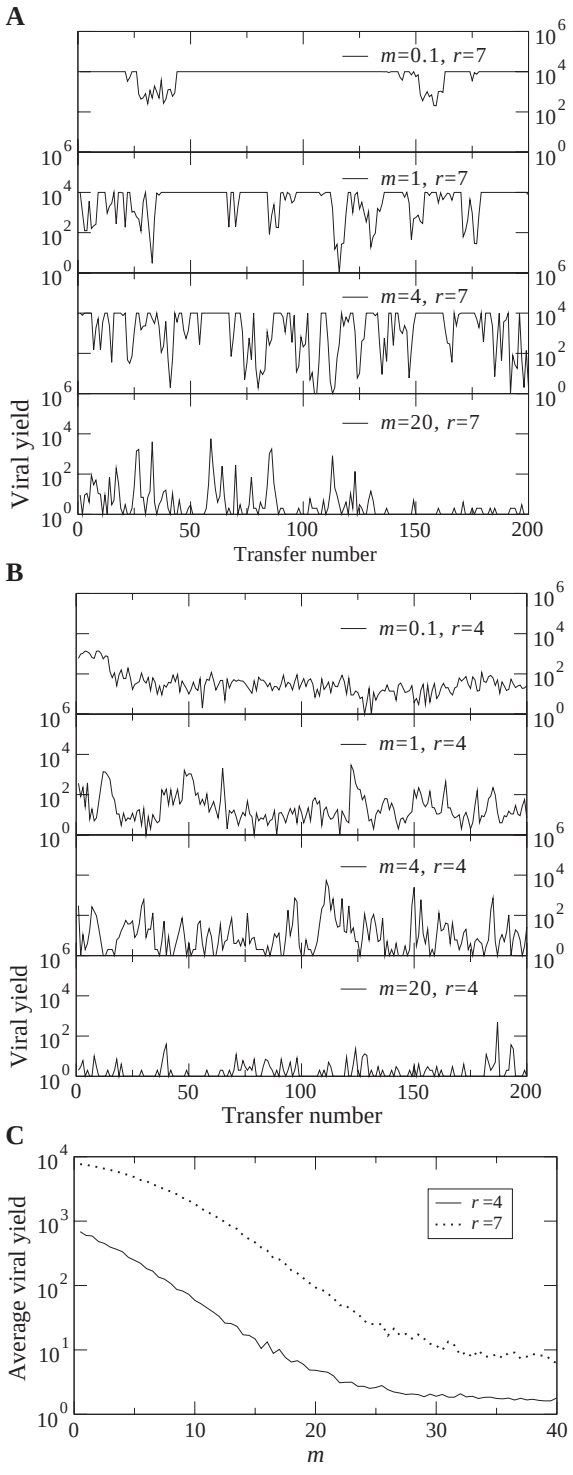

Fig. 3. Dynamics of the viral yield as a function of the mutation rate. (A) r=7 and four different values of m, as shown in the legend. (B) Same, for r=4. Each series corresponds to a single realization of the process. (C) Average viral yield as a function of the mutation rate for the two cases shown in panels (A) and (B). The results have been calculated averaging over 20,000 transfers.

system to recover from low fitness values in a short time. In these conditions, a different mutation–selection equilibrium is attained. However, if the value of m is increased too much (m=20 in the simulations assayed), the large number of mutations produced limits the adaptability of the system as well as the average yield. These results are clearer for small r (Fig. 3B). Then, for m=0.1, the system remains attached to low titers for a large number of passages without reaching the highest number of particles permitted. The number of replication cycles clearly affects the characteristics of the stationary state of fitness. The results of Fig. 3C show that the increase in the value of r leads to higher average yields for all the values of m assayed. From a different viewpoint, this means that the population is better optimized at each passage the larger is the number of replication cycles, since a larger r permits the selection of fitter variants. It is important to discuss the fact that, when m reaches a certain value (m=30, for this combination of parameters, see Fig. 3C), the average yield does not decrease significantly. This is due to the specific implementation of the computer model, where degradation of particles has not been explicitly considered, and where it is not necessary to have a minimum number of viral particles produced for the plaque to be visible. Note that even if the seed particle fails to replicate, it can be used to "start" the new plaque, since it can always be localized. This is not the case in the experimental setting, so extrapolation of our quantitative numerical results to real systems is not possible in this case.

For low values of m, the amplitude of the fluctuations increases as the mutation rate grows, and so does the difference between yields at consecutive passages (Fig. 4). For r=4, for instance, there is an optimum value of m ($m \approx 2$) at which this difference attains its largest value. We equate this maximum with maximal adaptability, since the recovery from low yield states is the fastest possible. The consequence is clear: given m, there is an optimal combination of parameters (p, q, and r) for which the probability of survival of the quasispecies is the highest, despite the frequent occurrence of bottlenecks. At that value of m, there is a

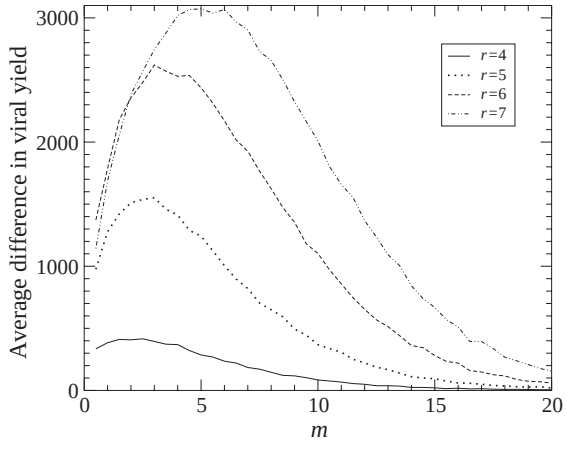

Fig. 4. Average difference between viral yield at consecutive transfers as a function of the mutation rate for several values of the number of replication cycles that take place during the development of a lytic plaque. The results have been calculated averaging over 20,000 transfers of the process with the parameters of Fig. 3A and B.

balance between the yield of the infection and the ability to generate the necessary diversity to produce optimized particles for the next transfer. Once more, large values of m cause too many deleterious mutations, prevent to attain high yields, and cause a mutation–selection equilibrium less stable than the one obtained for lower values of m. In some of the plaque-to-plaque transfer experiments, we also explored the effect of changing the value of r (Escarmís et al., 2002) by allowing the same viral clones to develop for 48 h instead of the standard time of 24 h. The result obtained, in good agreement with our simulations, was a lower fitness loss for longer times of plaque development.

In Fig. 4, we also observe that the larger the number of replication cycles during the development of the lytic plaques, the higher is the mutation rate corresponding to maximal adaptability. This means that when the population generated from the founder virus grows during a large number of generations, it can stand a higher mutation rate. In this case, selection has time to eliminate the least fit genomes and to fix fitter variants. However, if the number of generations is low, the action of selection is limited, and least fit genomes may remain in the population, reducing adaptability. One has to be cautious with the extrapolation of these results to the in vivo transmission of real viruses. In the latter case, m may be modulated by other factors, such as the type and state of cells where the virus is replicating or a number of extracellular components that are not present in the experiments carried out in the laboratory. Therefore, the direct effect of the number of generations on the survival of the quasispecies may be partly hidden by the presence of these additional factors.

3.2. Fitness distributions of the founder genomes and probability of extinction through bottlenecks

The analysis of the average fitness distributions of the founder particles for several values of m and two values of r is shown in Fig. 5. These distributions represent averages of the dynamical processes shown in Fig. 3A and B. For r=4, the highest probability corresponds to founder particles with low fitness values, irrespectively of the value of m considered. When the number of replication cycles is low, there is little chance for the occurrence of advantageous mutations able to improve the fitness of the seed particle. In contrast, a large enough number of replication cycles (r=7) shifts the maximum probability towards higher fitness values. Once more, this points out to the efficient optimization of the viral population within the lytic plaque during its development. Indeed, even for a remarkably high mutation rate (e.g., m=20 and r=7) fitter mutants appear and constitute a measurable fraction of the total number of generated particles, such that they are often selected to found the new plaque. There is a positive feedback between the development in the plaque and the random selection at each bottleneck event such that the average fitness of the process notably increases.

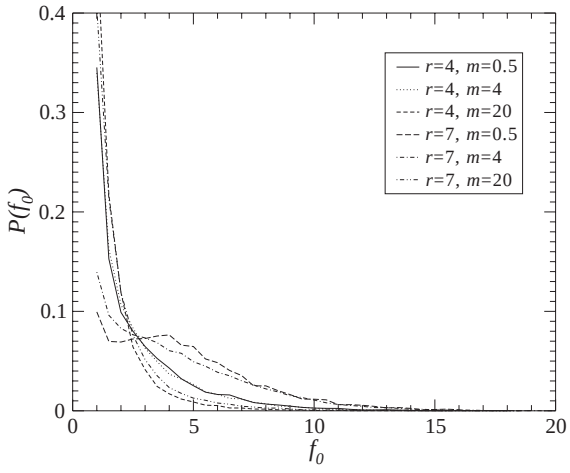

Fig. 5. Distribution of the fitness values of the founder particle for two values of the number of replication rounds during the development of the lytic plaques and three values of the mutation rate, as shown in the legend. Each distribution corresponds to a single realization averaged over 20,000 transfers.

Although we have observed a remarkable resistance of bottlenecked viruses to extinction, it might happen that the seed particle does not replicate for r replication cycles (in the computer simulation) or for the time fixed in the experimental protocol (24 or 48 h). This situation can be understood as a real extinction of infectivity in both, the experimental system (Escarmís et al., 2002; Yuste et al., 1999) and in the numerical simulations. The probability of extinction averaged over a large number of passages is represented in Fig. 6. There is a fast increase in the probability of extinction when m grows. In contrast, the probability of extinction tends slowly to the asymptotic value one, which is probably attained only in the limit $m \to \infty$. However, it is important to point out that for high enough values of m, we obtain extinctions (as defined above) in most passages. In agreement with previous results, we see that, the higher the number of replication cycles, the lower is the probability of extinction.

The typical deviation of the average viral yield, which quantifies the presence of fluctuations in the virus titer when the system is at the stationary state, has been also calculated as a function of the error rate (Fig. 6). We can observe (r=7) that there is an initial growth in the fluctuations when the error rate increases. This value reaches a maximum value and then decreases. This reflects the fact that, for low m, the system systematically reaches the highest yield permitted, while for m large enough it stays close to the extinction threshold. For intermediate values of m, the yield oscillates broadly between those two limit states, and this explains why the typical deviation has a maximum. However, this fact can also be related to the appearance of a higher fraction of mutants (both with higher and lower fitness) for intermediate m. The presence of a maximum in the typical deviation agrees with the observed optimum value of m that confers maximal ability to recover from low fitness values (Fig. 4).

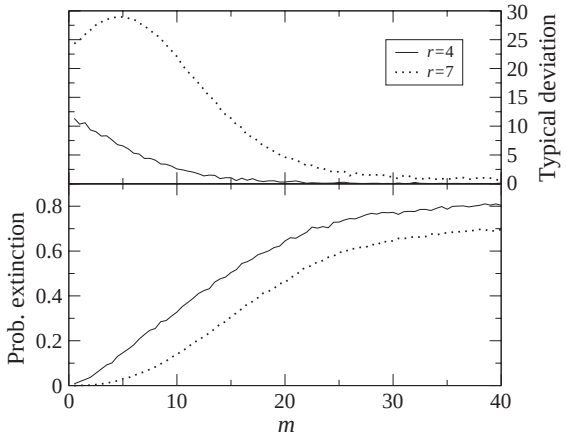

Fig. 6. Standard deviation from the average yield (shown in Fig. 3C) and probability that the seed particle fails to replicate in r replication cycles as a function of the mutation rate.

To get a deeper insight into the mechanisms explaining the resistance of viruses to extinction despite the accumulation of mutations, we carried out a study of the dependence of the ΔW values (see Materials and methods) on m (Fig. 7). Since the probability of deleterious mutations is higher than the probability of advantageous ones, one could naively expect that, for high enough m, the total change of fitness per sequence should present always negative values. This however depends on how individual mutations add up in each sequence. With the prescription used, that is, absence of epistasis and simple sum of individual effects, there is always a finite fraction of positive changes. To check this point, we have calculated the distribution of values ΔW as defined above for different values of m. In the limit of large m, and given that ΔW is the sum of identically distributed and independent random variables, the distribution $F(\Delta W)$ converges to a Gaussian function. Fig. 7A represents the main results of this analysis. As m increases, the average of the distribution $F(\Delta W)$ decreases proportionally to m:

$$\langle \Delta W(m) \rangle \approx m(q-p) \tag{3}$$

The distribution broadens at a comparable rate, as the typical deviation shows:

$$\langle \Delta W(m) \Delta W(m) \rangle - \langle \Delta W(m) \rangle^2 \approx m(p-q)(2+p-q) \tag{4}$$

Since the ratio between the average and the typical deviation is a constant independent of m, there is a persistent tail entering into the region of positive changes, meaning that, though small, there is a probability of selecting for the next bottleneck a daughter sequence with fitness higher than its parent. The presence of these advantageous mutants will be conditioned by the population size: at m large enough such that the probability of positive changes falls below the inverse of the population size, the quasispecies cannot be sustained.

We have also calculated the fraction of mutants with positive, neutral, and negative changes in fitness for increasing m. The numerical results are summarized in Fig. 7B. If there is an initial fraction of neutral mutations, then an increase in m can imply a higher fraction of advantageous mutants with respect to those obtained with lower m. This observation confers an additional robustness to our previous results indicating the existence of an optimum value for m. In the numerical simulations, a sequence can accumulate an arbitrarily large number of mutations. Since there is no memory of past mutations, only of their effects on fitness, we observe that, on the average, every sequence accumulates m mutations per replication cycle and transfer. In the experimental system of plaque-to-plaque transfer, we do not observe a saturation of the accumulation of mutations after more than 130 transfers. Possibly, not all combinations of mutations are permitted, but as it happens in our simulations, there are viable mutants, able to replicate and infect, despite

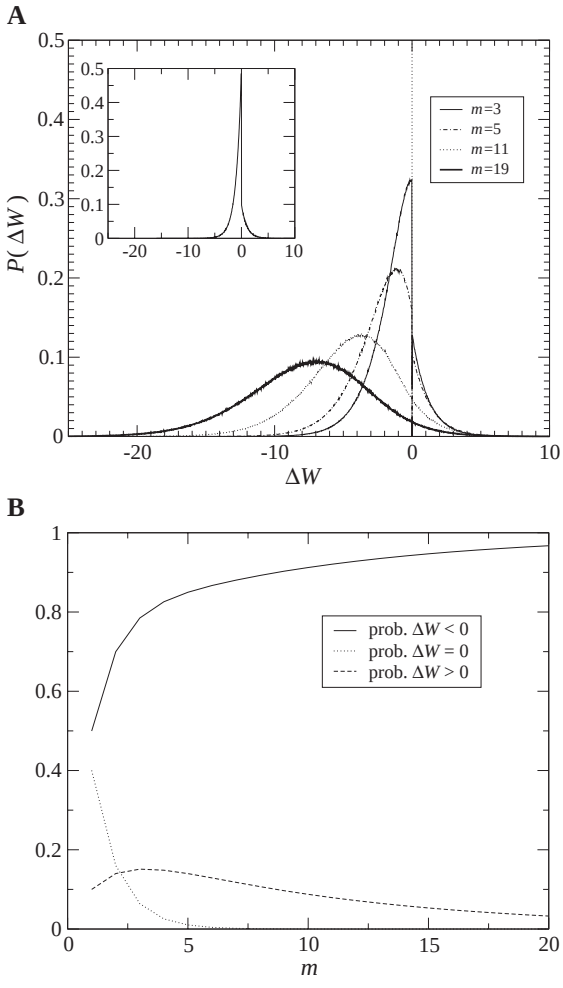

Fig. 7. Changes in the fitness of the progeny sequences generated from the founder one as a function of the mutation rate. (A) Main plot—distribution of changes in fitness for increasing values of the number of mutations per replication. Insert—distribution for m=1 with the corresponding weights for p=0.5 and q=0.1. (B) Fraction of positive, negative, and neutral changes in fitness as a function of the mutation rate.

having accumulated a large number of mutations (Escarmís et al., 2002). Bottlenecks could select those mutants and eventually condition the survival of the viral population. If those viable genomes would be accompanied by a large cloud of deleterious mutants, as it happens in the absence of bottlenecks when mutagenesis increases, they would be likely suppressed and the probability of extinction would increase.

4. Discussion

4.1. Resistance to extinction of bottlenecked viruses

In our previous studies (Escarmís et al., 1996, 2002), we observed a remarkable resistance to extinction of several bottlenecked FMDV clones. In our understanding, the continuous generation of mutants within a lytic plaque and the selection for the next transfer of a genome able to replicate gives rise to a state of dynamical equilibrium. During the development of a lytic plaque many unviable genomes appear and, therefore, there are many extinctions in the system. However, if a particular combination of mutations renders a genome able to replicate and to form a plaque, there are good chances that this plaque is selected for the next transfer. This results in an enhanced effect of positive selection between passages that stabilizes the system, despite the continuous generation of deleterious mutants.

Our numerical results support the great ability of the system to recover from the negative effect of bottlenecks when the number of replication cycles is high enough, even for large mutation rates. We can translate these results to the framework of the experimental results obtained with different viruses subjected to plaque-to-plaque transfers. Systematic extinctions of bottlenecked viruses have only been observed in the case of HIV (Yuste et al., 1999). Viral titers obtained from HIV plaques are lower than those obtained with other viruses such as VSV or FMDV. Therefore, we suggest that the number of replication rounds and the possibility of competition among genomes forming HIV plaques could be very restricted, giving rise to lower optimization of the quasispecies within each plaque.

During transmission of viruses in nature, there is also an intra-host interval of optimization of the population generated starting from the particles that initiated the infection. In the plaque-to-plaque transfer system, this period is equivalent to the plaque development time. In the case of horizontally transmitted viruses, viral particles are released at different moments during the progression of the infection. This means that an inter-host competition among viruses selects the more virulent forms, which have greater chances of infecting a new host. The analogous property in the plaque-to-plaque transfer experiments is the capability of fitter genomes to generate lytic plaques and thus to be selected for the next transfer.

4.2. Error catastrophe and bottlenecks

In experiments with viruses experiencing a high replication error rate due to the action of mutagens, but in the absence of bottlenecks (Sierra et al., 2000; Pariente et al., 2001; Crotty et al., 2001; Grande-Pérez et al., 2002; Severson et al., 2003), real extinctions of infectivity are observed. A possible interpretation, in agreement with early molecular evolution theories (Eigen and Biebricher, 1988; Eigen, 2002), is that viral replication indeed operates very close to the error threshold. When this limit is crossed, the population enters into an "error catastrophe," and the genetic information is lost. Near this threshold, however, RNA viruses can maintain a population structure organized in quasispecies, where the variability is maximal and where the fittest viruses can be rapidly selected. When this limit is crossed, the population disorganizes and forms an ensemble of random sequences unable to transmit the genetic information. This leads eventually to extinction. In real systems, the presence of bottlenecks can positively select and isolate particles that still keep the ability to infect cells. Given that the frequency of reversions is extremely low, with a high probability, these particles are endowed with a combination of mutations, some of them compensatory and some of them deleterious, which synergically allow the recovery of the viral infectivity. In this direction, recent studies of viral evolution through bottlenecks have identified sequences carrying certain combinations of mutations never seen in natural isolates of the virus (Escarmís et al., 2002; Yuste et al., 2000; Novella and Ebendick-Corpus, 2004). Thanks to the bottleneck, these minority particles are separated from the unstructured group of mutants and allowed to generate a new population without the interfering effect of a highly complex quasispecies. This provides an additional evidence to claim that mutation rates cannot be separated from the transmission mode used by viruses in nature. The optimum mutation rate for a certain virus should have evolved according to the frequency of transmission through bottlenecks and to the degree of optimization intra- and inter-host that takes place before the next bottleneck occurs. Error rates can also be modulated by many other parameters that are not considered in this paper.

4.3. Emergence of drug-resistant mutants and bottlenecks

The results of our numerical simulations might be interesting in the context of the appearance of drug-resistant mutants, a problem that causes difficulty in the eradication of diseases caused by many RNA viruses. The mutation rates of RNA viruses allow the stable coexistence of small subpopulations of mutants that are resistant to the action of antivirals with a wide spectrum of other phenotypic variants. In the absence of the inhibitor, and considering that harmful mutations are more probable than beneficial ones, the resistant subpopulation has lower fitness than most viruses in the initial, optimized popula-

tion. However, in the presence of the inhibitor their relative fitness is higher, this small number of individuals is selected and, in practice, the population undergoes a bottleneck. As replication proceeds in the presence of the inhibitor, the generation of new mutations (probably compensatory mutations) permits fitness recovery. At this point, the treatment starts to fail. Too low mutation rates probably prevents the existence of the genetic diversity required to maintain a mutant spectrum containing drug resistant variants (see results for m=0.1 in Fig. 3A and B). In contrast to this, too high mutation rates strongly reduce the fitness of the resistant subpopulation, since they increase the mutational load (Fig. 3A and B, m=20). The relationships between the efficacy of the treatment and the mutation rate of the pathogen have been studied by Gerrish and García-Lerma (2003). They postulate that the efficacy of antivirals can be increased if they are administered simultaneously with mutagens, a fact that has been experimentally demonstrated (Pariente et al., 2001). However, mutagens can also enhance the appearance of compensatory mutations able to improve fitness in the resistant subpopulation. Therefore, detailed simulations of the process of real infections combined with experiments could be very useful to gain further insight into the mechanisms that increase the efficacy of antiviral treatments in diseases such as AIDS, where the problem of drug-resistant mutants is a major, on-going problem.

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