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Review

Pathways to extinction: beyond the error threshold

Susanna C. Manrubia^{1,*}, Esteban Domingo^{1,2,3} and Ester Lázaro¹

¹*Centro de Astrobiología, INTA-CSIC, Ctra. de Ajalvir km. 4, 28850 Torrejón de Ardoz, Madrid, Spain*

²*Centro de Biología Molecular ‘Severo Ochoa’, CSIC-UAM, Cantoblanco 28049, Madrid, Spain*

³*Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas, 08036 Barcelona, Spain*

Since the introduction of the quasispecies and the error catastrophe concepts for molecular evolution by Eigen and their subsequent application to viral populations, increased mutagenesis has become a common strategy to cause the extinction of viral infectivity. Nevertheless, the high complexity of virus populations has shown that viral extinction can occur through several other pathways apart from crossing an error threshold. Increases in the mutation rate enhance the appearance of defective forms and promote the selection of mechanisms that are able to counteract the accelerated appearance of mutations. Current models of viral evolution take into account more realistic scenarios that consider compensatory and lethal mutations, a highly redundant genotype-to-phenotype map, rough fitness landscapes relating phenotype and fitness, and where phenotype is described as a set of interdependent traits. Further, viral populations cannot be understood without specifying the characteristics of the environment where they evolve and adapt. Altogether, it turns out that the pathways through which viral quasispecies go extinct are multiple and diverse.

Keywords: quasispecies; viral extinction; mutagen; antiviral therapy; genotype–phenotype map; complex phenotype

1. INTRODUCTION

The idea of a critical mutation rate in replication fidelity above which a viral population would get extinct arises from quasispecies theory. That theory was devised in the context of a prebiotic world constituted by populations of replicating molecules which, due to the absence of correction mechanisms, would see their length limited by the frequent errors during template copying (Eigen 1971). The theory predicted that the maximal mutation rate compatible with faithful replication of the best adapted genotype would be inversely proportional to the sequence length. This defined an error threshold and a particular pathway to extinction, characterized by the transition from a population dominated by a master genotype of replicative superiority to a population constituted by an unstructured cloud of mutants of low replicative ability. Previously, Leslie Orgel had coined the term ‘error catastrophe’ to describe the effect of errors in amino acid incorporation during cellular protein synthesis, a process that may contribute to a collapse of regulatory networks in the process of ageing (Orgel 1963).

The concepts coined in the framework of prebiotic molecular quasispecies were quickly applied to other

fields describing the evolution of populations affected by high mutation rates, notably to viruses (Domingo *et al.* 1978). Virus quasispecies are defined as ensembles of viral genomes subjected to a dynamic process of genetic variation, competition and selection, so that the population is a highly complex and continuously changing mutant spectrum or mutant cloud. Because of the adverse effect that viral replication, in the presence of mutagenic agents, has on virus infectivity (Holland *et al.* 1990; Lee *et al.* 1997; Crotty *et al.* 2001), it has often been assumed that evolution has selected mutation rates close to the theoretical error threshold. Altogether, the idea of increased mutagenesis did not take long to emerge as a feasible strategy to suppress viral infectivity (Eigen 2002). However, the kinetics of infectivity loss upon treatment of virus populations with mutagens do not differ from those followed when the same populations are endowed with viral replication inhibitors. If the increased number of mutations in the presence of the mutagen is associated to the production of a larger amount of lower fitness variants causing values of the basic reproductive ratio R_0 below one, this could equally lead to the extinction of the population without involving the disorganization of the quasispecies through an error catastrophe transition (Bull *et al.* 2007).

Many other important dynamic mechanisms and relevant features of viral adaptation were not considered in the initial model of molecular quasispecies. The population size was kept constant (often infinite)

* Author for correspondence (scmanrubia@cab.inta-csic.es).

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and lethal mutations were absent, as well as any complex fitness landscape mapping sequence into replicative ability, the latter being the only phenotypic property considered. These notwithstanding, both the idea of extinction under a sufficiently large increase in the mutation rate and the pathway to extinction yielded by quasispecies theory have pictured a paradigmatic scenario of how viral extinction comes about. Nevertheless, the fact that viruses are much more complex than simple replicators, with fitness values arising from the optimization of several features affected in different ways by an increase in the error rate, implies that quasispecies theory does not suffice to explain lethal mutagenesis. Other particular characteristics of virus populations have to be taken into consideration.

Recent work has shown how important it is to devise more realistic models for the evolution of populations of quasispecies. On the one hand, it has been shown that the ways to cause viral extinction through an increase in the mutation rate are multiple; on the other hand, simple models grounded on empirical observations improve our understanding of the non-trivial dynamics of viral populations (Manrubia & Lázaro 2006). The formal introduction of compensatory (Lázaro *et al.* 2003) and lethal mutations (Wagner & Krall 1993; Wilke 2005; Takeuchi & Hogeweg 2007), the consideration of different fitness landscapes (Saakian & Hu 2006) and our steadily improving understanding of how redundancy of genotypes (i.e. the large number of genotypes coding for the same phenotype; Grüner *et al.* 1996a; Bull *et al.* 2005; Takeuchi *et al.* 2005) conditions the composition of a population and its ability to recover optimal phenotypes (Cowperthwaite & Meyers 2007; Stich *et al.* 2007) are substantially changing the overall picture describing viral evolution. In our view, two main ingredients of realistic quasispecies models should be the description of populations in terms of phenotypes and the inclusion of more than one phenotypic trait subject to selection.

The genotype–phenotype map. Our ideas on the topology of the ensemble of genotypes yielding the same phenotype (often called the neutral network of genotypes) have been largely shaped by the investigations on the relationship between RNA sequences and their corresponding structures of minimal energy (Schuster 2006). This model yields very rough fitness landscapes and suggests that neutral networks (at least of abundant phenotypes) might span the whole genome space. Further, few mutations might suffice to turn one phenotype into a completely different one (Grüner *et al.* 1996b). The evolution of computational populations of RNA sequences with selection on their folded states reveals that the number of truly beneficial mutations or of those able to compensate the effect of deleterious ones—and thus able to generate a sequence with higher fitness—is non-negligible (Huynen *et al.* 1996; Stich *et al.* 2010).

In natural systems, the existence of many genotypes with the same phenotype has been demonstrated for complex phenotypes as well, including functional viruses (Lázaro *et al.* 2003; Koelle *et al.* 2006), viroids (Sanjuán *et al.* 2006) and ribozymes (Schultes &

Bartel 2000). The intrinsic degeneracy (or redundancy) between the space of genotypes and the space of phenotypes confers high robustness to quasispecies and immediately requires that beneficial and compensatory mutations be taken into account. Therefore, when many different genotypes can be equally fit, the definition of a master sequence becomes nonsensical. This scenario qualitatively changes the original quasispecies model, where the error threshold was associated with the loss of a precise genotype. Actually, selection acts on the phenotype (Kun *et al.* 2005), and the loss of the master sequence becomes irrelevant in evolution and adaptation if there are other genotypes with the same or similar phenotypic properties. In fact, there is no experimental assay where the extinction of a virus population through increased mutagenesis has been associated exclusively with the elimination of the master sequence. Also the survival of virus populations containing only suboptimal viable phenotypes speaks for the great plasticity and adaptive properties of viruses (Lázaro *et al.* 2003).

Complex phenotypes. The number of phenotypic traits relevant to some natural systems such as viruses is remarkable. To name just a few, the viral genome itself and its encoded proteins may affect particle stability and their environmental sensitivity, uncoating of the viral genome to initiate viral gene expression and genome replication, interaction with cellular structures and with cellular macromolecules, interactions with small molecules that may affect the rate of genome replication (through levels of nucleotide substrates, allosteric interactions with viral enzymes, etc.), or the assembly and exit from the cell. The effect of the increase of the mutation rate in a system as complex as a virus population is not easy to determine. The same mutation can have different or even opposed consequences on different fitness traits, causing pleiotropic effects or trade-offs between different functions. Also epistatic interactions among mutations can lead to unexpected effects when they are present together in the same or in different genes. Though there are indications of a relevant degree of neutrality in viruses, it is not possible to ascertain how many genomic nucleotides, if any, will behave as strictly neutral with regard to the complexity of the virus life cycle in its intracellular and extracellular phases.

Other relevant unsolved questions in natural populations are the accessibility of genomes from any given one and the shape of fitness landscapes. At the present moment, many theoretical assumptions seem to be a very narrow representation of the properties of real systems. Though it is not reasonable to try to include all known mechanisms in formal models (it is also useless, since one would get lost in the details), we should keep in mind that the predictions of a model are strictly dependent on the assumptions made. If one of such assumptions does not hold in a natural system, the predictions of the model cannot be trusted.

2. BENEFITS OF INCREASED MUTAGENESIS

As it could have been foreseen in light of the above, experimental implementation of increased mutagenesis has yielded unexpected results, different from

the straight predictions of simple theories (Eigen 2002). A remarkable effect of increases in the mutation rate is, in certain situations, an improvement in the adaptive ability of viral populations.

We have discussed how increases of the error rate through the use of mutagenic agents have negative consequences for RNA viruses. These observations agree with the statement that most mutations have deleterious effects on fitness, and consequently their accumulation can lead to the extinction of the virus population if adaptive mechanisms, able to counteract the negative effect of the mutagen, do not emerge. Nonetheless, the amount of beneficial mutations arising in a population depends on several factors, including the capacity of adaptation to the selective pressures at play and the variability of the environment (Sniegowski *et al.* 2000; de Visser & Rozen 2005; Denamur & Matic 2006). Since any change in the environmental parameters displaces optimized populations towards lower fitness regions, where the amount of beneficial mutations is higher, these populations could benefit from an increase in the error rate. In bacteria, there are many reported examples of the selection of hypermutator strains under conditions of experimental stress, a clear example of the fact that in nature, mutation rates can be modified to optimize the balance between the negative effects of mutations and the need to adapt (Mao *et al.* 1997; Matic *et al.* 1997; Sniegowski *et al.* 1997). Also, recent results obtained with a DNA virus replicating in the presence of a mutagen show that fitness can increase at genomic error rates by two or three orders of magnitude above the natural value (Springman *et al.* 2010). Despite the high error rates of RNA viruses, there are also some examples of the selection of strains with increased mutation rates when the environmental conditions vary drastically, as happens with changes in the host where the virus replicates (Schultz *et al.* 1991; Suárez *et al.* 1992), or during treatment with antiviral agents (Mansky & Bernard 2000). In this sense, a striking example is the selection of a mutant of foot-and-mouth disease virus (FMDV), which has a modestly lower fidelity polymerase that provides increased resistance to treatment with ribavirin (Sierra *et al.* 2007; Arias *et al.* 2008). These findings raise the question of whether there could be situations where an increase in the error rate by artificial means could have adaptive benefits for RNA viruses. Obviously, for it to have such a beneficial effect, this increase should not be as high as to impede the fixation of the possible beneficial mutations generated (Orr 2000), a fact that is influenced by the particular recombination rate of each virus. The relationships among mutation rate in populations that have undergone long-term selection, amount of beneficial mutations and mean fitness have been studied in simulations of replicating RNA molecules (Stich *et al.* 2010). This study reveals that whereas the amount of beneficial mutations at equilibrium is higher in populations optimized at high mutation rates, these mutations do not increase population fitness, because of the difficulties of their fixation at high error rate. Despite the advantages that could be provided by an increase in

the mutation rate in adapting populations, a study of Lee and co-workers (Lee *et al.* 1997) concluded that there are negative effects in the adaptability of vesicular stomatitis virus upon treatment with chemical mutagens. However, it would be desirable to carry out further research to investigate the behaviour of other mutagenized RNA viruses in response to a wider range of selective pressures.

RNA viruses can also be displaced from the adaptive optimum by evolving through serial population bottlenecks. In the case of certain viruses suffering from a large enough amount of deleterious mutations, the increased fixation of mutations caused by this process might induce fitness losses large enough for the population to be extinguished. However, in a very complete study carried by Escarmís and collaborators subjecting several FMDV clones to a large number of plaque-to-plaque transfers, a noticeable resistance of the virus to extinction through this process of accumulation of mutations was found (Lázaro *et al.* 2002, 2003; Escarmís *et al.* 2006), in agreement with previous results obtained in the framework of the classical theory of drift load (Poon & Otto 2000). At the first transfers, the virus loses fitness as expected. However, once a low fitness value is reached, there are no additional net fitness losses, and the virus titre fluctuates around a constant mean value. This situation, which has been interpreted as the result of an increase in the amount of compensatory mutations in low fitness populations, again demonstrates the contingent nature of mutations and their relative contextual value, and raises doubts on the adverse effect of mutagens in viruses of low fitness. Actually, a mutagenic treatment during the development of lytic plaques increases the replicative ability of the individual components of virus clones generated from low fitness viruses (Cases-González *et al.* 2008). Moreover, individual virus clones, when isolated from mutagenized populations that were doomed to extinction, could replicate under 5-azacytidine (AZC) mutagenesis during a number of plaque-to-plaque transfers. The increase in the fraction of beneficial mutations in less adapted genotypes is a basic property of Fisher's model observed as well in other systems (Poon & Otto 2000; Orr 2005; Martin & Lenormand 2006). The analysis of the viruses composing the mutant spectrum generated when replication takes place in the presence of AZC reveals that, even in pre-extinction populations, there can be an improvement of the replicative ability of individual viruses (figure 1). However, these 'improved' viruses cannot be maintained, possibly because of the generation of additional deleterious mutations and the coexistence with non-viable, highly mutated genomes that can interfere with their replication. If the mutagen is removed before the virus infection is cleared, these high-fitness mutants could be selected, in this way hampering the treatment of the infection. These results have important implications on lethal mutagenesis as they document two important facts: (i) there are circumstances in which mutagenesis can improve the replicative ability of some viruses, and (ii) the elimination through bottleneck events of the defective and highly mutated genomes generated at high error rate

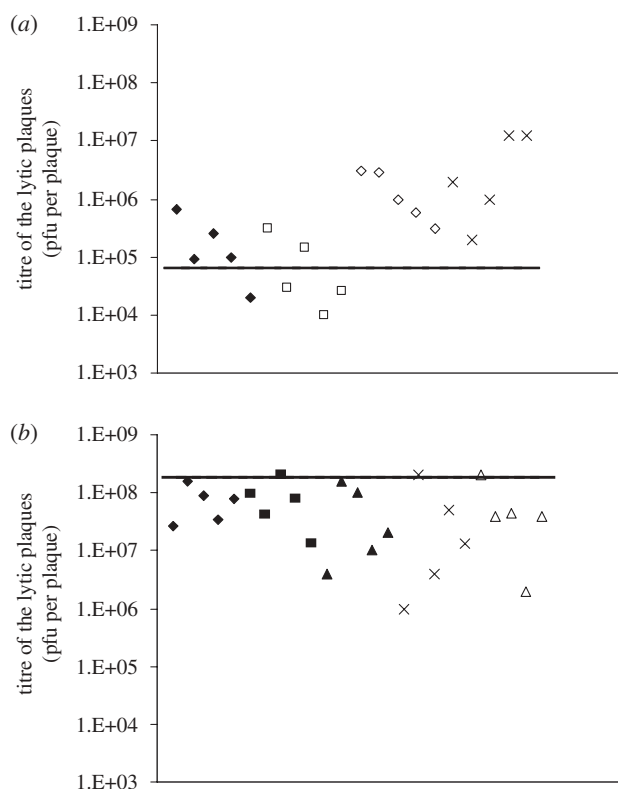


Figure 1. Increased mutagenesis can improve the replicative ability of some viruses. Titres of the lytic plaques originated from individual viruses isolated from the supernatant of clonal virus populations of bacteriophage Q β replicating in liquid medium in the presence of different concentrations of 5-azacytidine (AZC). Observe that whereas low-fitness viruses (virus clone C3pt40 obtained upon 40 plaque-to-plaque transfers in *a*) can be optimized in the presence of the mutagen, high-fitness viruses (virus clone C12 isolated from an optimized population of bacteriophage Q β in *b*) always produce lower fitness descendants. In both cases, the experiment was carried out by infecting 300 μ l of exponential phase bacteria (*Escherichia coli* Hfr, Hayes) with 4000 plaque forming units (pfu) of the corresponding virus in the presence of indicated AZC concentration in a final volume of 1 ml. After 2 h of incubation at 37°C, the supernatants were extracted, and plated to obtain well-isolated lytic plaques that were titrated following standard procedures (Cases-González *et al.* 2008). In both panels, the solid line represents the titre of the virus clone exposed to the mutagenic conditions. The symbols represent the titres of the lytic plaques isolated from the supernatants obtained for each AZC concentration. Supernatants obtained when virus clone C3pt40 replicated in the presence of 80 and 120 μ g ml⁻¹ of AZC represent pre-extinction populations. (*a*) Filled diamonds, AZC (0 μ g ml⁻¹); open squares, AZC (40 μ g ml⁻¹); open diamonds, AZC (80 μ g ml⁻¹); crosses, AZC (120 μ g ml⁻¹); solid line, C3pt40. (*b*) Filled diamonds, AZC (0 μ g ml⁻¹); filled squares, AZC (40 μ g ml⁻¹); filled triangles, AZC (80 μ g ml⁻¹); crosses, AZC (120 μ g ml⁻¹); open triangles, AZC (160 μ g ml⁻¹); solid line, C12.

can favour the sustained replication of viruses in the presence of mutagenic agents.

Another common treatment to fight viral infections is the use of non-mutagenic viral inhibitors (Pariente *et al.* 2003). This procedure cannot be applied for long periods, since inhibitor-resistant mutants appear with relative ease and are readily selected, thus

suppressing any effect of the inhibitor. For this reason, it is usual to apply combination therapies, where two or more inhibitors of viral replication are dispensed together, a strategy whose benefits have been supported experimentally and by theoretical studies (Bonhoeffer *et al.* 1997; Domingo 2003). Combination therapy is standard practice to treat some virus infections, such as HIV, for which highly active anti-retroviral treatment is frequently applied. However, the simultaneous administration of an inhibitor and a mutagenic agent might bring about unwanted behaviour, since two opposing mechanisms are acting. On the one hand, the inhibition of replication reduces the production of viral progeny in significant amounts, and thus diminishes the absolute diversity in the population. But, on the other hand, and apart from generating a larger amount of lethal or defective mutants, the mutagen works towards increasing diversity, thus favouring the appearance of resistant forms. It has been shown that, under fairly general conditions that depend on intrinsic properties of the virus (J. Iranzo *et al.* 2010, unpublished results), it is advisable to use sequential therapies, where first the inhibitor and later the mutagen are applied, instead of combining them in a single treatment (Perales *et al.* 2009*a,b*).

The sign of the effect caused by increases in the mutation rate is critically dependent on the state of the population (Stich *et al.* submitted). At equilibrium, it will mainly increase the amount of deleterious mutations and the fitness of the population will on average decrease. But in populations out of mutation-selection equilibrium, a larger mutation rate might be beneficial by increasing the variability of the population. In this way, faster adaptation (Cases-González *et al.* 2008) or enhanced generation of resistant forms (Perales *et al.* 2009*b*) becomes possible.

3. SELECTION OF RESISTANCE MECHANISMS TO MUTAGENIC AGENTS

One of the main problems for the treatments of the diseases caused by RNA viruses is the emergence of inhibitor-resistant viruses (Domingo 2003). The increase of the error rate caused by mutagenic agents is not an exception, as it constitutes a change in the environmental conditions at which viruses try to adapt. In this case, however, the generation and stability of the resistant mutants is also influenced by the selective pressure created by the mutagen. Whereas high error rates can favour the generation of mutants able to replicate in the presence of the mutagen, the increase in the error rate also can result in the missing of these mutants because of the generation of additional mutations that reduce the fitness of the resistant mutants. Therefore, the success of selection to counteract the negative effects of the increase in the error rate is a non-trivial issue (E. Lázaro & M. Arribas 2010, unpublished results).

Up to now, several strategies that permit RNA viruses to replicate in the presence of mutagenic agents have been identified. Two of them involve the replicative machinery of the virus and consist of the selection of mutant polymerases of increased fidelity

(Wainberg *et al.* 1996; Pfeiffer & Kirkegaard 2003; Vignuzzi *et al.* 2006) or an enhanced ability to discriminate between the correct nucleotide and the mutagenic analogue (Sierra *et al.* 2007). The evolution of mutational robustness by the selection of genotypes in which mutations have less negative impact is another possibility that must be taken into consideration (Sanjuán *et al.* 2007), although the selection of this strategy possibly involves much longer times than those taking place during mutagen exposure (Martín *et al.* 2008). Recent studies suggest that multiple mechanisms that attenuate the adverse effect of continuous mutagenesis can be selected during replication of viruses in a highly mutagenic environment (R. Agudo *et al.* 2010, unpublished results). These mechanisms have in common the occurrence of specific amino acid substitutions in viral proteins that result in higher frequencies of viable progeny genomes. It is unlikely that an increase in resistance to a mutagenic agent can occur by mechanisms other than specific amino acid substitutions in viral proteins, and it has been documented that virus robustness is unlikely to increase in the course of a mutagenic treatment (Martín *et al.* 2008).

Mutations that decrease the sensitivity of a virus to a mutagenic agent can prevent extinction by high doses of the same mutagen. However, extinction can be achieved by treatment with alternative mutagenic agents that produce a different distribution of mutants within the quasispecies (Perales *et al.* 2009a). The resistant mechanisms need not be the same for different mutagens, although this is still a highly unexplored subject. In addition to the use of alternative mutagens, an adequate choice of inhibitor and mutagen doses, used either sequentially or in combination (depending on a number of parameters that may characterize a given infection—population size, virus diversity, etc.), may also contribute to efficient virus extinction, despite the inherent capacity of viruses to find resistance pathways. A careful examination of the parameters that mediate the irreversible fitness decrease associated with virus extinction may result in improved protocols of administration of one or more mutagenic agents in sub-toxic doses, along with classical inhibitors of viral replication. By applying theoretical concepts, new prospects for antiviral designs are currently being opened. It should also be said that there might be no need to aim at virus extinction solely as a result of treatment. A sufficient reduction of viral load as a result of treatment may provide the host immune system with an opportunity to clear the virus from the infected organism.

4. THE ROLE OF DEFECTIVE FORMS

One of the side effects of increased mutagenesis is an intensified production of defectors, i.e. genomes by themselves unable of completing the full reproductive viral cycle but capable of surviving within a complex population. In some situations, defectors play a capital role in the extinction of the quasispecies.

Slowly replicating variants, in isolation, are as viable as variants replicating faster, as long as they maintain their infective capacity. Further, because of the

possibility of complementation among viral types occurring in persistent infections or in lytic infections at high multiplicity of infection (MOI), the selection pressures are reduced (Froissart *et al.* 2004; Novella *et al.* 2004), and populations can sustain a degree of diversity higher than those where each viral particle needs to code correctly for all the products required for survival. High diversity, a quantity that depends also on the environment (and not only on the intrinsic mutation rate), has implications on the pathogenesis (Vignuzzi *et al.* 2006) and adaptability properties of viral populations (Montville *et al.* 2005; Perales *et al.* 2009b). In highly diverse populations permitting complementation among types, a large fraction of defective, parasitic types can be sustained. Parasites are unable to produce themselves the correct products to ensure their viability, but are often able to use *in trans* the products correctly encoded by other types. The extreme version of complementation is represented by fragmented genomes encapsidated in different particles, a situation where co-infection becomes a must for viability (García-Arriaza *et al.* 2004; Manrubia *et al.* 2006; S. Ojosnegros *et al.* 2010, unpublished results).

Mild increases in the mutation rate augment the amount of defective genomes in viral populations. When populations are finite, fluctuations in the relative number of defective versus wild-type genomes are able to produce the disappearance of the wild-type, thus leading to the extinction of the whole population. This extinction mechanism, termed lethal defection (Grande-Pérez *et al.* 2005), is significantly different from those formerly described. Extinction through lethal defection was first observed in persistent infections of lymphocytic choriomeningitis virus (LCMV) treated with the mutagen 5-fluorouracil (5-FU). About 90 h post-infection, the ability of the virus to produce infective particles disappeared, though its replicative capacity, understood as the capacity of an RNA virus to produce RNA progeny, was not affected (Grande-Pérez *et al.* 2005). The interpretation of the experiment was as follows. In persistent infections, competition among all different viral genomes inside the cell effectively selects for efficient replicators. However, since the infection of new susceptible cells is not a requirement at that stage, genomic sequences coding for proteins only implied in the infection step behave as neutral characters. As a result, they might accumulate mutations that may eventually result in the loss of the infective ability. When the replication time inside the cell is long enough (a characteristic that depends on the environment), defectors can get fixed in the population and cause a halt of infection propagation.

Actually, stochastic extinction owing to defectors might supervene at any value of the mutation rate. At natural mutation rates, however, the typical time for this pathway to extinction to occur is long enough that it is not observed. However, as soon as the equilibrium between the fraction of defectors and the fraction of wild-type forms is disturbed through increases in the mutation rate, the extinction of the population in short times owing to lethal defection becomes increasingly likely (Iranzo & Manrubia 2009). In the next section we will briefly describe a

formal model that captures the dynamics implied in lethal defection. To this end, it is mandatory to include two phenotypic traits, replicative ability and infective capacity, subjected to different selection pressures. The defective class will be represented by those genomes able to replicate but unable to infect susceptible cells on their own.

5. MULTI-TRAIT PHENOTYPES

Several experimental observations highlight an obvious characteristic of natural systems largely forgotten in theories of evolution and extinction: phenotype is a multi-trait feature. The experiment carried out with persistent infections of LCMV described in the previous section is an example of the relevance of complex phenotypes in quasispecies dynamics. Different traits vary or adapt under the action of different selection pressures, and simultaneous optimization of several traits is usually not possible. Phenotypic traits cannot be truly independent: the sole consideration of the small number of genes in relation to the number of phenotypic traits suffices. Very clear evidence has accumulated that one cellular or viral protein can exert multiple (and some times disparate) biological functions. This is dramatically so for viral proteins, notably those that are produced as a result of processing of a polyprotein precursor.

Growth rate and yield (Novak *et al.* 2006), robustness and evolvability (Lenski *et al.* 2006), neutrality and adaptability (Aguirre *et al.* 2009) or virulence and replicative ability in competition assays (Herrera *et al.* 2007) are traits that have been simultaneously considered. Their relationship has been a matter of discussion, and their interdependence is certainly a non-trivial issue. Actually, the degree of optimization of either trait critically depends on the selective pressures acting in the environment where the population evolves, as indicated above. Let us deepen our discussion on this relevant point with some examples. Lytic infections necessarily imply the entry of the virus into new susceptible cells: when efficient infectivity is required, virulence is positively selected. Persistent infections do not require the continued infection of fresh cells, so the selective pressure to maintain high virulence is released. In some cases, however, the dynamics of virus replication and cell duplication is such that a coevolution of both biological entities takes place in the course of a persistent infection. This was first documented for reovirus (Ahmed *et al.* 1981), and then extended to other systems including FMDV (de la Torre *et al.* 1988).

One can reasonably assume that competitive fitness is always under selection in persistent infections, because of the relatively high diversity that is presumably maintained inside the cell, and the subsequent competition among variants to replicate faster. The selective pressure to competitive fitness acts however differently in lytic infections. The degree of competitive fitness of the population will depend on the MOI of the virus and probably on the infection proceeding on a (three-dimensional) liquid medium, where diffusion is highly efficient or on (two-dimensional) plaques with limited mobility (Aguirre & Manrubia 2008). At high

MOI, fast replication confers an advantage and is thus positively selected. At low MOI, however, and particularly during plaque development, populations are clonal, and there might be limited competition for replication.

A clear illustration of the dependence between survival and environment at different error rates when two traits are under selection is summarized in figure 2. Consider a population of replicators formed by four different types. Fast replicators have an average of R offspring per replication cycle, and slow replicators have r , with $R > r$. Either type can be infective or non-infective. Non-infective forms are considered defective in the sense that the presence of infective forms is a requirement for the propagation of the infection and thus for the survival of the population as a whole. The arrows indicate the transition rates from one type to another (details can be found in a previous publication, Iranzo & Manrubia 2009). The essential point to be emphasized here is the following. At a relatively low mutation rate (upper panel on the right), there is a background of defective forms during a persistent infection (red curve). The latter are continuously generated by the infective type but are unable to invade the population. When the mutation rate is multiplied by a factor of three (in this case), fluctuations in the amount of each type increase significantly (mid panel on the right): in a short time, the infective population will go extinct. The dynamics changes when the typical time until cell lysis decreases, forcing the infection of new cells if the population has to survive. The lower panel on the right represents the same population as before, with a high mutation rate, but frequently cleaned up from defectors, thanks to the action of regular bottlenecks. The mutation rate required to cause the extinction in a population infecting two different hosts that differ in the characteristic times between cellular infection cycles might be dramatically different, as this example illustrates, and is independent of the intrinsic features of the viral population.

6. THE CRITICAL MUTATION RATE: A RELATIVE CONCEPT

The mutation rate at which a population goes extinct does not take a unique and fixed value. The above considerations indicate that it can depend on the environment, on the population size and on other parameters such as the diffusion of the virus in the medium in which it propagates. Spatial constraints, that are rarely taken into account (Altmeyer & McCaskill 2001; Aguirre & Manrubia 2008) can condition the degree of competition among genomes, the capacity of the virus to infect new cells and the degree of preservation of the resulting mutants, which have higher chances of surviving as diffusion decreases (Cases-González *et al.* 2008). The critical mutation rate has a genetic component (determined by the actual mutation rate, the type of mutants produced and the interactions established among them) and a demographic component, ascribed to reductions in the population size caused by the deleterious effects that most mutations have on fitness. Connection

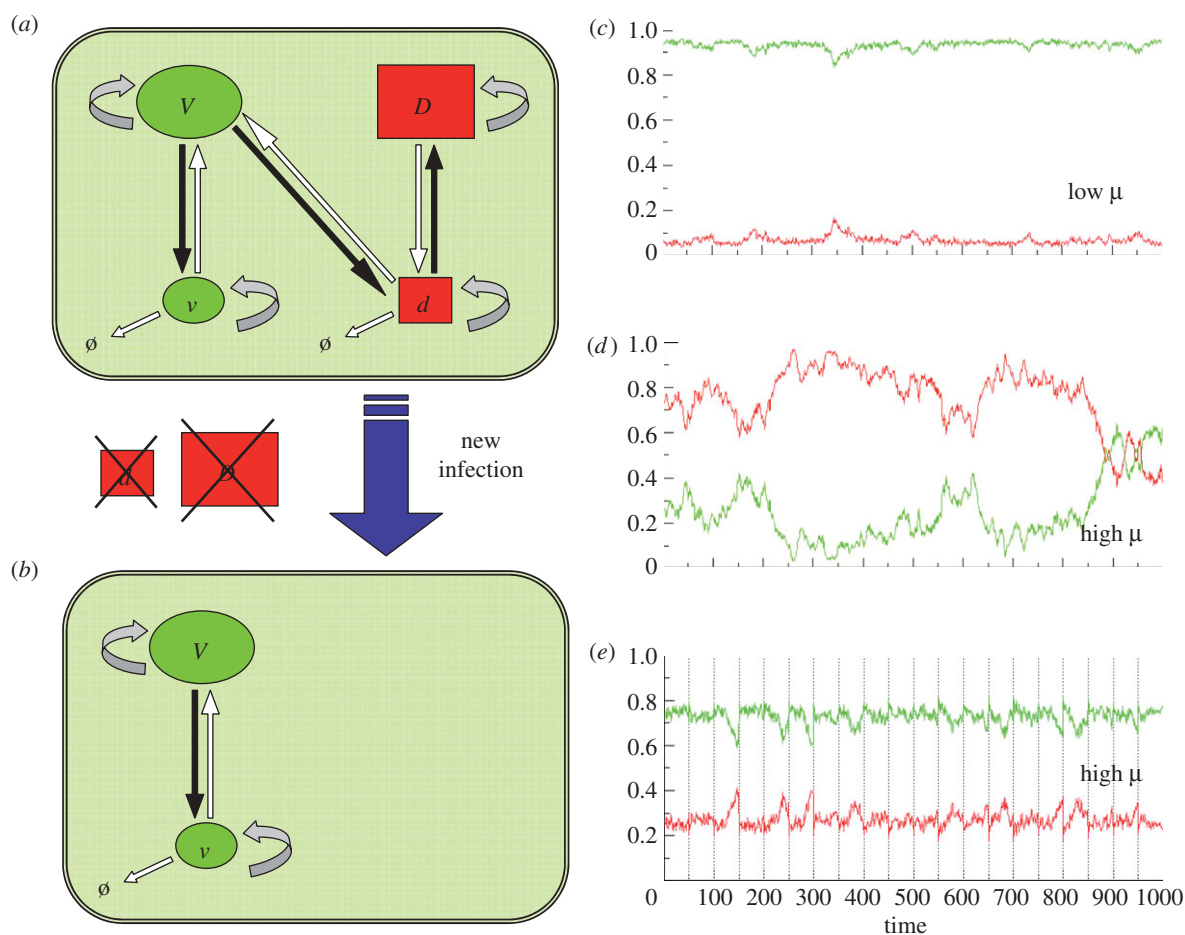


Figure 2. Survival or extinction depends on the environment. Dynamics of a population formed by two different types of replicators: infective and non-infective, both with two possible replicative ability levels. (a) Schematic representation of how the four different types generate each other. (b) Initial condition in a newly infected cell, free of defective genomes. As time elapses, defective forms will be regenerated by mutation from the viable forms. Panels on the right illustrate the dynamics of the total number of genomes of each type (wild-type and defective). (c) Inside a cell, at low mutation rates, the number of defectors is low. (d) In the same situation, population fluctuations increase (and lead more easily to extinction of the wild-type) when the mutation rate increases. (e) When bottlenecks are frequent, non-infective forms are regularly eliminated and the population survives.

between both components is what would determine the extinction of the population.

The production and maintenance of defectors and the selection of different traits in different environments may offer an alternative pathway to extinction, as has been suggested (Grande-Pérez *et al.* 2005) and theoretically demonstrated (Iranzo & Manrubia 2009). The key for a phenotypic trait to become useless requires (i) an environment where that trait is either neutral or not subject to strong selection and (ii) a finite number of individuals in the quasispecies, since extinction through the action of defectors is a purely stochastic phenomenon. The second condition is easily fulfilled because of the compartmentalization of infections in individual cells. Previous analyses of the effect of finite populations in quasispecies dynamics just considered how it affected the value of the critical mutation rate (Campos & Fontanari 1999; Altmeyer & McCaskill 2001). However, the intrinsic multiplicative nature of population dynamics causes large fluctuations in the composition of quasispecies that may result in the disappearance of any viable type in a finite time. This newly identified collective behaviour of quasispecies has a strong

corollary: de-structuring of the quasispecies through the elimination of positive selection on a basic trait can lead to the disappearance of a second trait that is under selection but depends on the first one, and thus to the extinction of the population as a whole. The mechanism can be extended to any number of traits, and may hold at any value of the mutation rate. We believe that the key to use that pathway as an efficient extinction mechanism relies in the identification of environments different from the one where the quasispecies has been optimized and where one basic trait would behave as neutral. In such a situation it could accumulate mutations and create a subpopulation of defectors able to induce the extinction of the whole when confronted again with the first environment (Grande-Pérez *et al.* 2005; Herrera *et al.* 2007).

Other systems provide evidence for the strong dependence of the critical mutation rate on particular phenotypic traits. Observations of critical mutation rates in populations of RNA where selection acts on the secondary structure reveal changes by a factor of 2 when different secondary structures act as target of selection (M. Stich & S. C. Manrubia 2010, unpublished results). It seems that phenotypes that can be

obtained from a larger number of genotypes are not only more abundant (Cowperthwaite *et al.* 2008), but substantially more robust to mutation.

The loss of viral infectivity can be achieved through different pathways. Increases in the mutation rate have demonstrated their ability to cause it; however, de-structuring of the quasispecies through loss of the master sequence, mutational meltdown, sequential dilution, stochastic extinction through lethal defection or cascades of extinction owing to temporary neutral characters are completely different pathways, described by different dynamics, population structures and formal dynamic transitions. The near future might bring new mechanisms that exploit those same properties that confer on viruses their high ability to diversify and adapt to effect host clearance.

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