Following the very initial growth of biological RNA viral clones

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Due to their extremely high genetic diversity, which is a direct consequence of high mutation rates, RNA viruses are often described as molecular quasispecies. According to this theory, RNA virus populations cannot be understood in terms of individual viral clones, as they are clouds of interconnected mutants, but this prediction has not yet been demonstrated experimentally. The goal of this study was to determine the fitness of individual clones sampled from a given RNA virus population, a necessary previous step to test the above prediction. To do so, limiting dilutions of a vesicular stomatitis virus population were employed to isolate single viral clones and their initial growth dynamics were followed, corresponding to the release of the first few hundred viral particles. This technique is useful for estimating basic fitness parameters, such as intracellular growth rate, viral yield per cell, rate at which cells are infected and time spent in cell-to-cell transmission. A combination of these parameters allows estimation of the fitness of individual clones, which seems to be determined mainly by their ability to complete infection cycles more quickly. Interestingly, fitness was systematically higher for initial clones than for their derived populations. In addition to environmental changes, such as cellular defence mechanisms, these differences are attributable to high RNA virus mutation rates.

INTRODUCTION

RNA virus populations are extremely variable and their ability to adapt to changing environments is remarkable (Novella et al., 1999; Weaver et al., 1999; Turner & Elena, 2000). Due to their high mutation rates, in the range of $10^{-3} - 10^{-5}$ substitutions per nucleotide site and replication cycle (Wain-Hobson, 1996; Drake & Holland, 1999), RNA virus populations are often described as molecular quasispecies (Eigen et al., 1988). In this theory, the mutation–selection balance is expressed in terms of kinetic equations and the replication–mutation dynamics lead to a stationary mutant distribution around a master sequence, which displays the maximum replication rate but is in a low proportion in the population, due to the steady-state generation of closely related mutants. The extremely heterogeneous nature of RNA viruses, which is a direct consequence of their high mutation rates, shows that quasispecies may be a natural and relevant approach to understanding RNA virus evolution (Domingo et al., 1978, 1985), but the theory has been the object of some controversy (Eigen, 1996; Wain-Hobson, 1996; Smith et al., 1997; Jenkins et al., 2001; Domingo, 2002), its detractors pointing out that it still lacks support in natural systems (Holmes & Moya, 2002; Moya et al., 2004).

One of the key predictions of the quasispecies theory is that natural selection targets the population as a whole, rather than the individuals it comprises. This is an extremely important issue in understanding the evolution of RNA viruses, whose high mutation rates imply the constant production of lethal or deleterious mutations and, thus, a mutational load much higher than that for DNA-based organisms (Elena & Moya, 1999). Viral populations usually reach very large population sizes (Neumann et al., 1998), allowing natural selection to remove deleterious mutations efficiently and increasing the probability of adaptive changes appearing, thus leading to rapid fitness increases (Novella et al., 1995). On the contrary, when viral populations are reduced, the strength of selection is minimized and deleterious mutations can be fixed, thus leading to strong fitness decreases and even extinction, in a process called Muller’s ratchet (Muller, 1964; Chao, 1990; Duarte et al., 1992). However, high population sizes might be unable to guarantee virus survival and adaptation per se (Elena & Moya, 1999), so that additional explanations, such as mutational robustness, need to be considered (Wagner & Stadler, 1999; Wilke & Adami, 2003). The evolution of robustness against deleterious mutations may appear as a result of different mechanisms (reviewed by de Visser et al., 2003, e.g. group selection. The latter is expected under high mutation rates, because clouds of mutants are unavoidably generated around each individual genotype, such that the fitness of these closely related mutants may drive the evolutionary fate of individual genotypes. Despite
some indirect experimental evidence (Burch & Chao, 2000), this prediction is mainly supported by theoretical work (Schuster & Swetina, 1988; Wilke et al., 2001; de Visser et al., 2003).

To discern whether selection acts at the individual or group level, it is critical to determine fitness or, at least, fitness-related parameters of isolated individuals so that they can be compared with the fitness values measured in whole populations. The assessment of relative fitness for RNA virus populations is a well-established technique (Vandepol et al., 1986; Martínez et al., 1997; Escarmís et al., 1998). It has also been widely shown that the intrinsic growth rate is the most important parameter in determining fitness (Pienta & Groupé, 1967; Cromeans et al., 1989; Gong et al., 1996; Elena, 2001) because, according to their high replication rates, viruses are subject to r-selection, especially for viruses adapted to in vitro cell cultures (Bulmer, 1994), where faster replication is strongly favoured. The intrinsic growth rate can be easily measured by using classical one-step growth curves (Ellis & Delbrück, 1938). In contrast, fitness or growth-rate measures for individual viral particles are technically difficult to carry out. Although it is easy to isolate biological clones from populations and measure their fitness by standard assays, high mutation rates mean that these clones rapidly generate clouds of mutants as replication proceeds (Holland et al., 1991). For this reason, we propose a new procedure that monitors the initial replication dynamics of individual clones to obtain the closest approximation to their real fitness values. In this scenario, the classical exponential model does not describe viral growth satisfactorily. We have therefore developed a new model that allows a more accurate estimation of growth rates, besides fitness-related traits, such as cellular viral yield, intracellular replication rate, rate at which cells are infected and time spent in cell-to-cell transmission. The relations between these parameters, as well as between individual and population fitness estimates, are discussed.

**METHODS**

**Vesicular stomatitis virus (VSV) populations.** We started the experiment with a wild-type (WT) clone (Whelan et al., 1995) of VSV belonging to the Mudd–Summer strain of the Indiana serotype, which was adapted to hamster BHK21 cells for 35 passages (~150 generations) by carrying out massive passages at a low m.o.i. (0-01) (Novella et al., 1995). Adapted populations were kept at ~80°C in 0·1 ml aliquots.

**Cell lines and culture conditions.** BHK21 cells were grown as monolayers under Dulbecco’s modified Eagle’s minimum essential medium (DMEM) containing 10% heat-inactivated fetal calf serum. Cells were grown in 25 cm² plastic flasks for the preliminary adaptation experiments, in 96-well plates for clonal growth-curve determinations, in 24-well plates for standard growth curves and in 100 cm² plates for routine maintenance. Cells were incubated at 37°C, 95% relative humidity and in a 5% CO₂ atmosphere.

**Clonal growth curves.** Biological clones (Holland et al., 1991) were isolated from the adapted population by using a limiting-dilution process. To do this, ~10⁶ BHK₂₁ cells were trypsinized and suspended in 10 ml culture medium. This cell suspension was mixed with ~50 infectious units (IU) and 96-well plates were then seeded with 100 μl aliquots of this mix. Each well therefore contained, on average, 10⁶ cells and less than a single IU. This initial infection was done with suspended cells to favour cell–virus contact. In 96-well plates, superficial tension is too high to allow efficient infection of a monolayer culture. It is also important to note that each of these units can contain various aggregated viral particles, analogous to natural conditions. Disaggregating them (for example, by using a sonication bath) would lack biological sense. Cell suspensions became a monolayer in ~2–3 h. No virus production occurred prior to 3·5 h post-infection (p.i.). Sampling was done by extracting all of the supernatant and replacing it with fresh medium at 4, 4·5, 5·5, 6, 6·5, 7, 7·5, 8, 9, 10 and 11 h p.i. After the last time point, viral growth was allowed to proceed until cytopathic effect was evident (typically, at 30 h p.i.) and cell suspensions were then stored at ~80°C. Approximately two to three of the wells remained uninfected after this time. Viral titres obtained at each time point were enumerated by plaque assays using confluent BHK₂₁ cell monolayers under DMEM solidified with 0·4% agarose. It is necessary to note that the growth dynamics of a single particle can only be measured once, so that statistical replication is impossible.

**Standard population growth-curve assays.** We used ~5 × 10⁵ viral particles to infect 24-well plates containing ~10³ cells and allowed the population to grow for 7 h. Preliminary assays showed that exponential growth occurred within this interval. In fact, ~1/100 of the maximum viral titre was reached and, hence, viral growth was still far from saturation. All assays were done in triplicate.

**Growth-curve assays in the presence of pre-infected media.** Approximately 10⁴ cells were infected (at a m.o.i. of 0-01) with WT virus and incubated until ~10⁷ IU ml⁻¹ were produced. The supernatant was harvested without freezing and mAb I1, which efficiently inactivates WT viruses (Vandepol et al., 1986), was added 1:1 (v:v). Fresh cells were incubated with this mix for 3 h. Then, clonal and standard growth assays were done by using this pre-infected culture supernatant. A mAb-resistant strain, MARM C (Holland et al., 1991), was used to perform these growth assays. Plating viruses in the presence and absence of I1 antibody confirmed that the WT was inhibited efficiently. As mock experiments, MARM C growth curves with non-pre-infected media, supplemented with antibody I1 1:1 (v:v), were carried out. The growth rate of the MARM C genotype in the presence of antibody was close to the growth rate of the WT in antibody-free medium.

**Growth models.** Viral growth always takes place in discontinuous events of intracellular replication, followed by periods of inactivity outside the cell. When large cellular populations are considered, viral growth can be regarded as an approximately continuous process that can be summarized by an exponential model (model I) as follows:

\[ N_t = N_0 e^{rt} \quad (\text{equation 1}) \]

where \( N_0 \) is the initial population size and \( r \) is the intrinsic growth rate. However, in the case of clonal growth curves, the number of infected cells is much lower (typically one) and it is thus necessary to take into account the discontinuous nature of viral growth (model II). We propose to use a logistic model to describe the intracellular viral dynamics. Initially, assuming that a single cell has been infected:

\[ N_t = N_{0ij} = \frac{K}{1 + e^{c_{ij} - \rho t}} \quad (\text{equation 2}) \]

where \( K \) is the cellular viral yield (carrying capacity), \( \rho \) is the
intracellular growth rate and $\theta_0$ is a constant that sets the initial conditions. This initial infection will be completed when $K$ viruses are released. However, each of these $K$ particles will initiate a second infection cycle that will be delayed a time, $\tau$, relative to the first cycle. Thus, the amount of viruses produced during these two cycles can be expressed as:

$$N_i = N_{(0)t} + N_{(t)l} = \frac{K}{1 + e^{-\rho t}} + \frac{K(K-1)}{1 + e^{-\rho t - \theta t}}$$

where $K(K-1)$ can be approximated by $K^2$ as $K \gg 1$.

We call $t_{(1)} = N_{(t)l}^{-1}(1) = \theta_0 - \log(K-1)$ and we set $N_{(t)l}(t_{(t)} + \tau) = K$, so that $\theta_0 = \tau$. Also, $N_{(t)l}(t_{(t)} + \tau t) = Kn^* for any $n$. Hence, at any time $t$,

$$N_i = \sum_{n=1}^{\infty} \frac{K^n}{1 + e^{-\rho t - \theta t}}$$ (equation 3).

Model II can be refined by taking into account the fact that RNA viruses have to diffuse in the time between cell infections (model III). If the random variable ‘time spent by a viral particle in wandering from cell to cell’ follows any distribution $D$, equation (2): $N_{(0)t} = N_{(0)t}$ remains valid and for the initial cycle and subsequent cycles:

$$N_i = N_{(0)t} \sum_{n=1}^{\infty} (1 + Kn)D_{(0)t}$$ (equation 4)

where $D_{(0)t}$ is a probability function. Here, we assume that $D$ is a logistic distribution:

$$D_{(0)t} = \frac{1}{1 + e^{-\delta_0 t}}$$

Parameter $\delta_0$ can be solved as:

$$\delta_0 = \log(K^n - 1) - t_{(t)}d$$

by setting the condition $KnD_{(0)}(t_{(t)} + \tau t) = 1$.

Parameter $\tau$ is now defined as the time that a viral particle spends inside the cell. This definition stays valid for model II.

**Model fitting.** Parameter estimation was done by fitting the log-experimental data ($\log(N_x)$) to equations (3) and (4) by using the sequential quadratic programming method implemented in the SPSS package, version 11.5. As we are interested in the very initial growth, we only included a small number of infection cycles ($n=5$) in equations (3) and (4). Initial estimations for $\rho$ and $K$ were done graphically: $\rho$ is the initial slope of $\log(N_x)$ and $K$ is the $N_x$ value corresponding to the first observed transient plateau. The initial estimation of $\theta_0$ was calculated as $\log(KN_{(t)l} - 1)$. Initial values for $\tau$ and $d$ were fixed at $\tau = 3$ and $d = 1$. Fitting to equation (1) was done by ordinary linear regression.

**Estimation of clonal fitness.** Whenever a population grows following model I and, in general, if density-dependent phenomena are not acting, fitness ($W$) equals $e^r$ (Crow & Kimura, 1970). For models II and III, estimators $\rho$, $K$ and $\tau$ can be used in equations (3) and (4) to predict the population size reached by a given clone at any time $t$. The slope of a linear regression of the log-predicted values against time would give the closest approximation to the overall growth rate, $r$. However, a simple expression for $r$ can be obtained analytically: let us define $m$ as the smallest integer that fulfills $m > ut$. At any time $t$, all of the elements of equation (3) for which $n > m$ can be considered as null, as they refer to infection cycles that have not yet begun. With respect to the rest of the elements, when growth at a scale greater than the cell is considered, equation (3) can be approximated by:

$$N_i = \sum_{n=1}^{m-1} K^n + \frac{K^m}{1 + e^{-\rho(m - ut)}}$$

After a time $\tau$,

$$N_{t+\tau} = \sum_{n=1}^{m} K^n + \frac{K^{m+1}}{1 + e^{-\rho(m - ut)}} = K(1 + \sum_{n=1}^{m-1} K^n) + K^m \frac{1}{1 + e^{-\rho(m - ut)}}$$

$N_{t+\tau} = K(N_{(t)l} + 1) \approx KN_i$

and, thus, the predicted population size can be approximated, with a precision $1/K$, by the exponential $N_i = N_{(0)t} e^{rt}$, where

$$r = \frac{\log K}{\tau}$$ (equation 5).

This simple expression for the overall growth rate determines the fitness of single biological clones for model II. For model III, an analogous rationale can be used: let’s find the time necessary to increase population size $K$ times (i.e. to complete an infection cycle). With a precision $1/K$, this is equivalent to finding the time $\Delta t$ that fulfills the equation $D_{(0)+\tau} = D_{(0)t}$. After some algebra, and again assuming that $K(N_t + 1) \approx KN_i$, the solution is $\Delta t = logK/d + \tau$ and, therefore:

$$r = \frac{\log K}{\tau + logK/d}$$ (equation 6).

Notice that equations (5) and (6) hold, regardless of the intracellular growth model chosen (here, logistic), provided that growth proceeds until an upper boundary ($K$) is reached.

**RESULTS**

**Describing the growth of isolated viral clones**

We followed the initial growth dynamics of 24 biological clones isolated from a WT VSV population to obtain the closest approximation to their real growth parameters. When we plotted the $\log$-population sizes against time, we observed an initial phase of linear growth, followed by a second linear phase with a reduced slope. Both phases were sometimes separated by a short, transient plateau. This pattern was repetitive (Fig. 1), although the time frame in which it occurred was highly variable (the initial burst ranging from 4 to 7 h.p.i., with mean of 4.909 ± 0.202). Experimental data were fitted to three alternative growth models. Model I, which is the classical exponential, fitted relatively poorly to the data. The percentage of explained variance ranged between 78.0 and 95.9% for each of the 24 datasets. According to the exponential model, viral clones sampled from the WT population had a mean intrinsic growth rate of 1.753 ± 0.121 h$^{-1}$. According to model II, viruses grow in successive infection cycles, each one described by an intracellular logistic dynamic model. For each of the 24 curves, the percentage of variance
explained by this model ranged from 95.0 to 99.9%. Taking all 24 samples together, model I explained 89.1% of the total variance associated with the growth of clones sampled from the WT population, whereas this percentage grew to 98.6% for model II. This improvement was significant according to a partial $F$-test ($F_{48,56} = 7.985; P < 0.0001$). Hence, model II provided a more accurate description of the dynamics of single clones isolated from a WT population. A simple biological interpretation of this can be given by assuming that, at the beginning of the infection, viral particles released into the medium reflected exponential growth occurring inside the first infected cell (characterized by parameter $r$). Later on, the maximum viral yield per cell ($K$) was reached because the synthesis of viral products exhausted cellular resources. Then, particles released by this cell initiated a new infection cycle with a time delay $\tau$ with respect to the first cycle. The overall growth rate $r$ of each clone according to this model was calculated by linear regression of the log-predicted values against time, as well as by using equation (5), giving a mean overall growth rate of $1.957 \pm 0.094$ and $1.930 \pm 0.074$ h$^{-1}$, respectively. Model III takes into account the fact that, upon transmission, viruses have to diffuse in the medium before they can infect a new cellular host. Fig. 2 shows various growth patterns that can be predicted by using model III, compared to models I and II. It is easy to realize that the former reflects more truly the patterns shown in Fig. 1. In fact, for individual biological clones, the percentage of variance explained

**Fig. 1.** Growth patterns followed by 24 clones isolated from a WT population. For clarity, growth curves have been separated arbitrarily into six panels.
by this latter model ranged from 99.0 to 100%. Overall, this model fitted the data excellently, because it explained >99.9% of the total observed variance and it significantly improved on model II (partial $F_{24,32} = 2.621; P = 0.005$). Therefore, we will use this model hereafter to make estimations of fitness-related parameters.

**Fitness components**

The mean intracellular growth rate was $6.069 \pm 0.244$ h$^{-1}$, a value that is much above the overall replication rate (see below), indicating that the delay imposed by transmission is strong. The estimation of $K$, which is expected to reflect cellular carrying capacity, was $K = 166 \pm 15$ IU. The third fitness-related trait is defined as the mean time spent by a viral particle on a single cell infection. Its estimation is $t = 1.060 \pm 0.070$ h. Finally, the rate at which viruses diffuse in the medium is $d = 0.693 \pm 0.080$ h$^{-1}$. Therefore, the mean time spent by a viral particle in ‘wandering’ between cells is $1/d = 1.443$ h. Parameter $d$ is determined by environmental properties, such as medium temperature, viscosity or cellular density. Hence, although it is included in the overall growth rate, it can not be regarded as a viral fitness component. The mean overall growth rate $r$ (log-fitness) obtained by using a linear regression of the log-predicted values (equation 4) against time is $r = 0.629 \pm 0.040$ h$^{-1}$, which is quite similar to the estimation obtained with equation (6), $r = 0.604 \pm 0.045$ h$^{-1}$.

These estimations are well below those obtained with models I and II, which do not take diffusion into account. Notice that diffusion imposes an upper boundary to the overall growth rate, $r_{\text{up}} = d$. The estimation of $r$ is quite close to $d$, therefore suggesting that growth rates are highly optimized. As shown in equation (6), the overall growth rate is determined by two terms: $logK$ and the time spent to complete an infection cycle, $(\Delta t = logK/d + t)$. The first fitness component, $logK$, measures the ability of the virus to exploit cellular resources. By using parameter $\tau$, we can define a second fitness component, $1/\tau$, called the cellular infection rate. Finally, remember that the diffusion rate ($1/d$) is not regarded as a fitness component here. As shown in Fig. 3, $1/\tau$ and $logK$ were negatively correlated (Pearson correlation coefficient: $R = -0.524$, 23 d.f., $P = 0.009$), thereby indicating a trade-off between the ability to propagate and the ability to exploit host resources. Given that both fitness traits cannot be maximized simultaneously, it is important to discern what is more efficient for viruses at the cellular level: maximizing $1/\tau$ to the detriment of $K$, maximizing $K$ to the detriment of $1/\tau$ or finding intermediate solutions. The answer depends on the real contribution of each of these two parameters to the overall

Fig. 2. Graphical representations of the growth patterns predicted by models I, II and III: model I (a), model II (b) and some examples of the different hypothetical situations that can be predicted by model III (c–f).
growth rate. Values of \( \log K \) are in the range 4.24–5.97, whereas values of \( 1/\tau \) are in the range 0.48–2.11 for the 24 clones analysed. Fig. 4 illustrates the influence of these two components on the overall growth rate for these parameter ranges. It can be appreciated that \( r \) is much more sensitive to \( 1/\tau \) than to \( \log K \). Indeed, \( 1/\tau \) showed a strong positive correlation with observed \( r \) values (\( R=0.900 \), 23 d.f., \( P<0.0001 \)) but, in contrast, this correlation did not exist for \( \log K \) (\( R=-0.194 \), 23 d.f., \( P=0.364 \)). Therefore, the optimal solution for these viruses should be to maximize their cell-turnover rates to the detriment of viral productivity, simply because the former parameter has a deeper influence on fitness than the latter.

The time spent to complete an infection cycle, calculated as \( \Delta t = \log K/d + \tau \), was approximately 8 h. By excluding passive diffusion, which represented as much as 87% of this time, the duration of an infection cycle depends on two processes: the rate at which viruses are produced in the cell (\( \rho \)) and the time of transmission between cells (\( T \)). Interestingly, \( T \) can be estimated by using equation (3). If the virus did not spend any time in being transmitted, then the intracellular growth rate \( \rho \) would equal the total growth rate \( r \) but, actually, \( \rho \gtrsim \log K/\tau \). Hence, the delay imposed by transmission can be estimated as:

\[
T = \tau - \frac{\log K}{\rho}.
\]

Its mean value was \( T=0.229\pm0.058 \) h. This means that viruses spend approximately one-fifth of their active time in transmission processes, such as cell adhesion and viral entry, whereas the rest of the time is employed in replication and formation of viral particles.

**Individual and population fitness**

Each isolated clone was allowed to replicate until approximately \( 10^7 \) IU were obtained. Then, standard growth curves were performed for each clone-derived population. Also, equation (4) was used to predict the population sizes under model III. This allows comparison of the growth rates of each clone and its derived population. Observed titres were systematically lower than their expectation by using equation (4) (paired \( t \)-test, \( t_{23}=10.315 \), \( P<0.0001 \)). This result can be attributed to several factors. Firstly, it is possible that the expected values under model III were biased toward higher overall growth rates. Although this cannot be absolutely discarded, it does not seem likely to be the case, considering that models I and II provide estimations of \( r \) higher than that of model III and that model I is commonly used to describe the growth of viral populations. Secondly, these results could be a consequence of high mutation rates. This being the case, mutations would occur in a significant proportion of the replication events, most of them being deleterious. The initial viral particle would then degenerate into a cloud of mutants, the latter being under a mutation–selection balance. In contrast, beneficial mutations are expected to be scarce, especially for viruses previously adapted to their cellular environment, as was the case here. We observed no correlation between the overall growth rate estimated for the initial viral clone and its derived population (\( R=0.181 \), 23 d.f., one-tailed \( P=0.099 \)), indicating that the fitness measure of a single biological clone was not sufficient to predict the fitness of its derived population. This can be a consequence of measure error, but is also in accordance with the fact that
mutation is a random process, in which the expected fitness effect of these mutations might be high enough to make fitness unpredictable. The superiority of the initial viral clone over its derived population, as defined by Eigen et al. (1988), estimated in terms of the overall growth rate as \( \sigma = \frac{r_{r_{+}}}{r_{r_{-}}} \), would be \( \sigma = 0.161 \pm 0.017 \). Thirdly, our results can also be attributed to environmental changes. During the initial moments of infection, viruses would propagate without any impediment. However, the physicochemical properties of the medium can change as a consequence of cell lysis and, more importantly, infection could activate a cellular secretion pathway leading to the release of antiviral cytokines. To explore this possibility, we performed both clonal and population (standard) growth-rate assays in the presence and absence of a previously infected culture supernatant. This supernatant was obtained from a standard massive infection by using the WT virus. Prior to growth-rate assays, cells were incubated with this supernatant for 3 h. The results (Fig. 5) show that, in the presence of pre-infected culture supernatant, viral growth is reduced significantly (two-way ANOVA, \( F_{1,35} = 9.735, P = 0.0036 \)). However, even when the physicochemical factor is taken into account, differences between clonal and population growth rates remain highly significant (two-way ANOVA, \( F_{1,35} = 21.622, P = 0.0004 \)). Therefore, both mutation and putative cytokine secretion seem to be responsible for the observed superiority of the initial clone over its derived population.

![Fig. 5. Observed titres for clonal and population growth curves in the absence and presence of pre-treatment with medium obtained from a previous infection with WT virus. In both treatments, a 1:1 proportion (v:v) of mAb I1 was added to the medium. The viral genotype employed in these curves is a mutant MARM C resistant to antibody I1, which completely inhibits the replication of the WT genotype. Error bars represent 95% confidence intervals.](http://vir.sgmjournals.org)

**DISCUSSION**

The isolation of RNA virus biological clones from their original population is a well-established technique (Holland et al., 1991). However, due to the typically high mutation rates of RNA viruses (Drake & Holland, 1999), these clones rapidly become a cloud of mutants, making it difficult to measure the fitness of a single virion. To minimize this problem, we followed the initial dynamics of populations derived from isolated biological clones. Our observations are compatible with a model in which viral growth takes place through discrete episodes. This model assumes that exponential growth occurs within infected cells. The validity of this assumption depends on the mechanism by which viruses replicate. If the progeny of the initial particle are able to become a template for further replication, exponential intracellular growth will occur. However, if the replication conforms to a ‘stamping machine’ (Chao et al., 2002), the parental virus being the only template used for intracellular production of progeny, then growth will be linear. Our data are highly compatible with the former model, although a more precise follow-up of this initial phase would be required to distinguish between these two alternatives. Regardless of the intracellular dynamics, a maximum viral yield per cell (\( K \)) will be reached when cellular resources are exhausted, while a new infection cycle begins. It is important to notice that the expressions that we derived for the overall growth rate of single clones remain valid, regardless of the intracellular growth model assumed.

The overall growth rate for individual viral clones can be assimilated to fitness, as no density-dependent processes are expected at these low population sizes. In classical growth curves (Ellis & Delbrück, 1938), two biologically relevant parameters are estimated: the intrinsic growth rate, which is equivalent to what we have called here the overall growth rate, and the total viral yield, which can be easily converted to a viral yield per cell if we divide it by the total number of infected cells. Equation (6) shows that the so-called intrinsic growth rate is indeed dependent on the viral yield per cell. Therefore, we suggest that the infection rate (\( 1/\tau \)) should be used instead of the overall growth rate to perform studies on \( r \)- and \( K \)-selection. According to the properties of their life cycles, RNA viruses are preferably under \( r \)-selection (Pianka, 1970), especially in the case of laboratory populations (Bulmer, 1994). Our results confirm these previous studies, because \( 1/\tau \) becomes the main determinant of the overall viral dynamics, rather than \( logK \). In fact, when \( K \)-regimes are simulated by retarding experimental times of transmission, the appearance of slow replicators (reduced \( r \)) rather than highly efficient host exploiters (higher \( K \)) is favoured (Sevilla et al., 1998; Borderia & Elena, 2002). Additionally, in these studies, fitness correlated with the intrinsic growth rate, but not with viral yield, suggesting that \( K \) is a parameter with limited evolutionary relevance in the context of serially transferred populations that are typically used for in vitro evolution experiments.
Our growth model allows us to assess the time spent in cell-to-cell transmission. To date, in vitro studies on transmission rates have been limited by the fact that transmission could only be regarded as a factor fixed by the experimenter. By using this approach, Elena (2001) and Cooper et al. (2002) showed, for VSV and nuclear polyhedrosis virus, respectively, that the earlier the transmission was carried out, the higher the virulence. Directly measuring transmission rate as a free parameter would shed more light on this issue. Such experiments can provide a tractable way to explore the trade-off between transmissibility and virulence (Ebert & Bull, 2003).

Growth parameters exhibited by individual biological clones are determined by their different genetic properties, but also by the physiological conditions that are imposed by the infected host cell. Adenoviral infection triggers defence mechanisms via secretion of chemokines (Muruve et al., 1999). This pathway is activated directly in kidney mouse cells by the viral capsid and is not dependent on the presence of immune cells (Borgland et al., 2000). Analogously, the diffusion of cytokines into baby hamster kidney cell-culture medium could slow down VSV infection. Our data suggest that, although the effect of cellular physiological conditions is significant, it does not account entirely for the observed reduction in viral growth ability. These results can therefore be also attributed to RNA virus intrinsic high mutation rates. As predicted by quasispecies theory (Eigen et al., 1988), RNA virus populations are composed of a master sequence, which is at low or even undetectable frequency in the population (Domingo et al., 1978), and a cloud of surrounding mutants. Our results show that, in the short term, clones grow systematically faster than their derived populations. When individual clones are sampled randomly and used to seed a new population, a new master sequence will be fixed. As pointed out by Schuster & Swetina (1988), this new population will initially consist exclusively of the new master and, hence, it will display its maximum growth rate. In the early stages of virus replication, the master will generate its own cloud of random mutants and fitness will decline. Finally, the population will reach a mutation–selection balance, in which mean fitness will be lower than the fitness of the master. This does not counter the observation that beneficial or compensatory mutations can appear (Novella et al., 1995), but rather says that these mutations will occur over a much larger timescale, simply because deleterious changes are much more frequent than beneficial ones (Miralles et al., 1999), especially for populations that are well-adapted to their environment. The lack of correlation between the fitness of an individual viral clone and the fitness of its derived population can be attributed to measure errors, but also to intrinsically high mutation rates, suggesting that the progeny of a given clone will carry a myriad of mutations that will affect fitness in an unpredictable (although probably deleterious) way. By using bacteriophage P6, Burch & Chao (2000) have already shown that the evolution of an isolated clone is determined by its mutational neighbourhood. As a consequence, if this clone has greater fitness than its mutational neighbourhood, it will evolve toward fitness losses, although random drift can also yield a similar outcome (Holmes & Moya, 2002; Moya et al., 2004). By using digital organisms, Wilke et al. (2001) showed that, at high mutation rates, selection does not necessarily favour the genotype showing the maximum replication rate. Rather, genotypes with reduced replication ability, located in flat regions of the fitness landscape, can be selected because of their mutational robustness, indicating that selection can act on clouds of mutants rather than on individuals. However, similar results confirming or rejecting this prediction have not been obtained experimentally (or observed in nature) for any biological entity. The method described here enables characterization of the distribution of individual fitness values that compose a viral population. Therefore, this approach can become a very useful tool in clarifying whether selection acts on individual genotypes or favours clouds of mutants with the highest average fitness.

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