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Impact of increased mutagenesis on adaptation to high temperature in bacteriophage Qβ

María Arribas [a,1], Laura Cabanillas [a,1,2], Kirina Kubota [a], Ester Lázaro [a,b,*]

[a] Centro de Astrobiología (INTA-CSIC), Torrejón de Ardoz, Madrid, Spain
[b] Grupo Interdisciplinar de Sistemas Complejos (GISC), Madrid, Spain

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ABSTRACT

RNA viruses replicate with very high error rates, which makes them more sensitive to additional increases in this parameter. This fact has inspired an antiviral strategy named lethal mutagenesis, which is based on the artificial increase of the error rate above a threshold incompatible with virus infectivity. A relevant issue concerning lethal mutagenesis is whether incomplete treatments might enhance the adaptive possibilities of viruses. We have addressed this question by subjecting an RNA virus, the bacteriophage Qβ, to different transmission regimes in the presence or the absence of sublethal concentrations of the mutagenic nucleoside analogue 5-azacytidine (AZC). Populations obtained were subsequently exposed to a non-optimal temperature and analyzed to determine their consensus sequences. Our results show that previously mutagenized populations rapidly fixed a specific set of mutations upon propagation at the new temperature, suggesting that the expansion of the mutant spectrum caused by AZC has an influence on later evolutionary behavior.

1. Introduction

The high error rates of RNA viruses (Drake and Holland., 1999; Sanjuán et al., 2010), together with their short replication times and large population sizes, give rise to a huge genetic diversity and a great capacity to adapt to most environmental changes (Domingo et al., 2012). The structure of RNA virus populations is often described as that of a quasispecies, which can be defined as an ensemble of variants interconnected through mutations and shaped by the action of natural selection (Andino and Domingo, 2015; Domingo et al., 2006; 2012; Lauring and Andino, 2010). According to quasispecies theory, the genetic information contained in a replicative system can only be maintained below a value of the error rate which is known as error threshold (Eigen and Schuster, 1979; Biebricher and Eigen, 2005). This hypothesis suggests that, if RNA viruses behaved as theoretical quasispecies, slight increases in their error rates would make them cross the error threshold, which would be incompatible with the proper expression of their genetic information and, thus, with their performance as infective agents. According to this, the idea of extinguishing virus infectivity through the artificial increase of the error rate arose as a promising new antiviral therapy named lethal mutagenesis (Eigen, 2002; Loeb et al., 1999; Loeb and Mullins, 2000; Perales et al., 2011).

Lethal mutagenesis has been successful in many *in vitro* studies (Agudo et al., 2009; Crotty et al., 2001; Dapp et al., 2009; Grande-Pérez et al., 2002; Pauly and Lauring, 2015; Severson et al., 2003) and has also been assayed in some studies carried out *in vivo* (Arias et al., 2014; Dietz et al., 2013; Hicks et al., 2013; Mullins et al., 2011). Although the loss of genetic information could not be demonstrated in any of these works, virus extinctions were explained in terms of the negative effects that most mutations have on fitness (Bull et al., 2007), and on the change of the type of interactions within the mutant spectrum (Grande-Pérez et al., 2005b). Despite these promising results, there are still some questions that need to be investigated in order to guarantee the safety of lethal mutagenesis based therapies. An important issue is whether the expansion of the mutant spectrum caused by incomplete mutagenic treatments could represent adaptive advantages for the virus. The success of lethal mutagenesis depends on the distance of the virus error rate to the error threshold, which in turns depends on several factors such as population robustness (Graci et al., 2012; Sanjuán et al., 2007), frequency of population bottlenecks (Paff et al., 2014), or probability of co-infection (Froissart et al., 2004; Novella et al., 2004). All this means that the response of a particular virus to increased mutagenesis is not easy to predict. There are prior studies showing the increased

* Corresponding author at: Centro de Astrobiología (INTA-CSIC), Torrejón de Ardoz, Madrid, Spain.
E-mail address: lazarole@cab.inta-csic.es (E. Lázaro).
[1] These authors contributed equally to this work.
[2] Present address: Department of Microbiology and Immunology, University of Rochester, Rochester, New York, USA.

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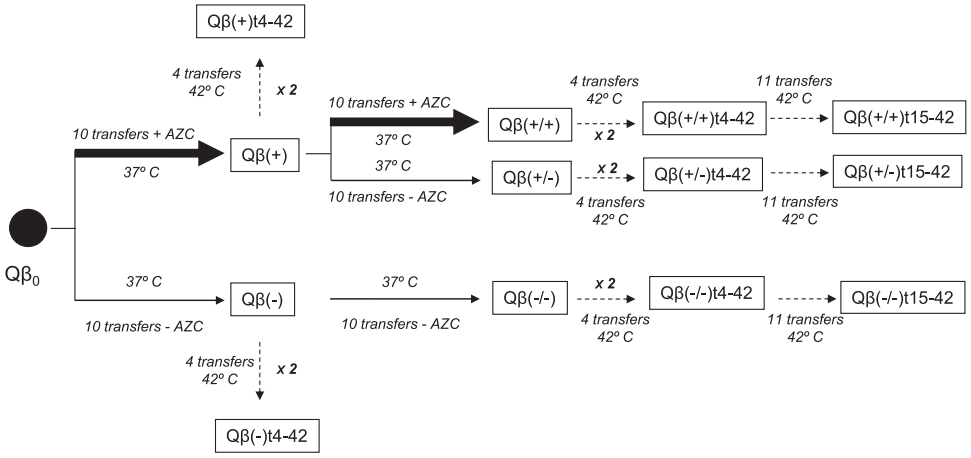

Fig. 1. Evolutionary lines of bacteriophage Qβ. The scheme shows the transmission regimes experienced by a biological clone of phage Qβ (virus $Q\beta_0$, see Section 2) to give rise to the different evolutionary lines used in this work (shown in boxes). Thick lines represent transfers in the presence of AZC (40 μg/ml), whereas thin lines refer to transfers in the absence of AZC. Continuous lines are used for transfers at 37 °C and discontinuous for transfers at 42 °C. Transfers were carried out as described in Section 2. In the cases indicated (× 2), populations were propagated in duplicate. Populations evolved at 37 °C were named Qβ, followed by a parenthesis that includes the signs + (to indicate 10 transfers in the presence of AZC), - (10 transfers in the absence of AZC), or combinations of both. Populations exposed to 42 °C, were named with the same nomenclature of their precursor populations, followed by t4–42 or t15–42 to indicate the number of transfers experienced at 42 °C.

appearance of novel phenotypes after single episodes of mutagenesis (Bull, 2008; Vignuzzi et al., 2006). However, the effect of prolonged mutagenesis on virus adaptability has been less investigated. A study carried out with the DNA phage T7 has recently shown that a population surviving high mutagenesis exhibited enhanced adaptation in some environments and little negative consequences in others (Paff et al., 2014). Thus, for a better implementation of lethal mutagenesis therapies, it would be convenient to know whether these results also extend to RNA viruses.

In order to investigate the consequences of mutagenic treatments under conditions of environmental change it is necessary to consider that the effect of mutations on fitness is not constant but depends on several factors, among which the most relevant are the genomic context where new mutations appear (Martin et al., 2007; Phillips, 2008; Sanjuán et al., 2004) and the degree of adaptation of the population (Elena and Lenski, 2003; Fisher, 1930; Rokyta et al., 2005; Silander et al., 2007; Stich et al., 2010). Well-optimized populations usually have replicated during a large number of generations in a constant environment, meaning that the pool of beneficial mutations is almost exhausted and, thus, they are prone to experience a larger amount of deleterious mutations. On the contrary, environmental perturbations displace populations towards lower fitness regions, where the number and the effects of beneficial mutations increase, making it possible to obtain some advantage from higher error rates. The genomic context also has a great impact on adaptation of mutagenized viruses, due to the fact that the increased number of mutations previously accumulated may influence the selective value of the new mutations that arise after an environmental change.

In the current study we have explored the consequences of sublethal prolonged increases in the error rate on the subsequent adaptation of an RNA virus, the bacteriophage Qβ, to a new selective pressure that consisted in a change in the replication temperature, from the optimal value of 37 °C to 42 °C. The error rate was increased through the exposure to a cytidine analogue, 5-azacytidine or AZC, which is mutagenic for Qβ (Arribas et al., 2011; Cabanillas et al., 2014; Cases-González et al., 2008). The main finding was that propagation at 42 °C of previously mutagenized Qβ populations led to the fixation of a set of mutations that were not present in the absence of mutagenic treatment, showing that sustained replication at increased error rate below the extinction threshold can strongly influence virus future evolution.

2. Materials and methods

2.1. Viruses and bacteria. Standard procedures for infection

Virus infections were carried out in *Escherichia coli*, strain Hfr (Hayes) in NB medium (8 g/l Nutrient Broth from Merck and 5 g/l NaCl). The plasmid pBRT7Qβ, which contains the cDNA of bacteriophage Qβ cloned in the plasmid pBR322 (Barrera et al.,1993) was used to transform *E. coli* DH5-α. The supernatant of an overnight culture obtained from a transformed colony was used to isolate a biological clone (virus $Q\beta_0$), which was the founder of all Qβ populations analyzed in this work. Sequencing of the virus $Q\beta_0$ revealed the presence of the genomic substitution U1193C.

Infections in liquid medium were always carried out using fresh log-phase *E. coli* cultures with an OD_{550} between 0.6 and 0.8, which were infected at the multiplicity of infection (moi) indicated in each experiment. After 2 h of incubation either at 37 °C (optimal temperature) or 42 °C (selective temperature) with good aeration (250 rpm), cultures were treated with 1/20 vol of chloroform for 15 min at 37 °C with shaking (300 rpm). Virus supernatants were harvested upon centrifugation at 13,000 × *g*. Virus titers were determined by plaque assay and expressed as the number of plaque forming units (pfu) per ml of the phage suspension.

Virus populations were used to obtain biological clones that corresponded to lytic plaques obtained in semisolid agar. They were isolated by punching and removing the top and the bottom agar around well-separated lytic plaques. The agar containing each plaque was transferred into a tube with 1 ml of phage buffer (1 g/l gelatine, 0.05 M Tris–HCl, pH 7.5, and 0.01 M $MgCl_2$) and 50 μl of chloroform, and incubated for 1 h at 28 °C with shaking (300 rpm). After centrifugation at 13,000 × *g* for 15 min to clarify the supernatant, the latter was stored over 25 μl of chloroform.

2.2. Propagation of bacteriophage Qβ under mutagenic and non-mutagenic conditions

The virus $Q\beta_0$ (see above) was the precursor of all the evolutionary lines established in this work (Fig. 1). Infections were carried out as described above using an moi of 0.01 pfu/cell in a volume of 10 ml (containing ~10^9 bacteria) either in the absence or in the presence of 40 μg/ml of AZC. After 2 h of incubation at 37 °C, viral supernatants were collected, and approximately 10^7 pfu of each phage suspension were used to infect a fresh *E. coli*