Adaptation to Fluctuating Temperatures in an RNA Virus Is Driven by the Most Stringent Selective Pressure

Maria Arribas1, Kirina Kubota2, Laura Cabanillas1, Ester Lazaro1*

1 Centro de Astrobiologia (INTA-CSIC), 28850 Torrejon de Ardoz, Madrid, Spain, 2 Facultad de Ciencias, Universidad Autonoma de Madrid, 28049 Cantoblanco, Madrid, Spain

Abstract

The frequency of change in the selective pressures is one of the main factors driving evolution. It is generally accepted that constant environments select specialist organisms whereas changing environments favour generalists. The particular outcome achieved in either case also depends on the relative strength of the selective pressures and on the fitness costs of mutations across environments. RNA viruses are characterized by their high genetic diversity, which provides fast adaptation to environmental changes and helps them evade most antiviral treatments. Therefore, the study of the adaptive possibilities of RNA viruses is highly relevant for both basic and applied research. In this study we have evolved an RNA virus, the bacteriophage Qβ, under three different temperatures that either were kept constant or alternated periodically. The populations obtained were analyzed at the phenotypic and the genotypic level to characterize the evolutionary process followed by the virus in each case and the amount of convergent genetic changes attained. Finally, we also investigated the influence of the pre-existent genetic diversity on adaptation to high temperature. The main conclusions that arise from our results are: i) under periodically changing temperature conditions, evolution of bacteriophage Qβ is driven by the most stringent selective pressure, ii) there is a high degree of evolutionary convergence between replicated populations and also among populations evolved at different temperatures, iii) there are mutations specific of a particular condition, and iv) adaptation to high temperatures in populations differing in their pre-existent genetic diversity takes place through the selection of a common set of mutations.

Introduction

The frequency of change in the selective pressures together with the fitness effects of mutations across environments are major determinants of the evolutionary pathway followed by populations. When selective pressures remain constant for a long time, specialist organisms, which perform well in a particular environment and poorly elsewhere, are favoured [1–7]. In contrast to this, when the selective pressures change frequently, fitness costs of mutations across environments can limit adaptation [6], [8–10], promoting the selection of specialist organisms that can cope with a variety of conditions [8], [11–15]. Generalists frequently select genotypes containing mutations of beneficial effects under all the selective pressures that are to be encountered. Another possibility is to maintain polymorphisms advantageous in some environments but deleterious under alternative conditions. The existence of different targets of selection through time together with the high frequency of fitness trade-offs has led to the proposition that generalism could have an associated fitness cost [7], [11], [14], [16–18].

Many of the studies concerning the selection of generalism and specialization have been carried out through experimental evolution, which requires systems that evolve quickly and that allow to establish accurate correspondences between phenotypes and genotypes. Since RNA viruses are able to adapt in very short times to environmental changes [19], and also possess small genomes, they have been used oftenly [20–33]. The high adaptive capacity of RNA viruses lies on their elevated error rates that promote the generation of populations composed by a mutant spectrum with a very wide phenotypic and genetic heterogeneity [19], [34]. In these populations, mutants dominant under previous selective pressures can be kept as minority variants of the mutant spectrum and be rapidly selected when the virus is exposed again to the same condition. This reservoir of variants provides a molecular memory [35–37] which could be very useful during adaptation to fluctuating selective pressures. Typically, studies carried out with RNA viruses compare the results obtained when a selective pressure, commonly the virus host or some abiotic factor, either remains constant or alternates between different values. In most cases a pattern of specialization under constant conditions was found, which was occasionally accompanied by a cost in alternative environments [21], [22], [25], [26], [28], [30], [31]. Populations that alternated different environments in some cases presented a generalism-associated fitness cost [14], although in others they showed equal or superior improvement in each environment than the lineages evolved constantly in the same conditions [22], [24], [27–29], [32], [33].
In this study we have propagated a heterogeneous population of an RNA virus, the bacteriophage Qb, at its optimal growth temperature (37°C) and at two new temperatures, one above the optimal value (43°C) and another one below (30°C). To compare the virus populations in constant versus deterministically fluctuating environments, we carried out evolution experiments in which the temperature was kept invariable throughout the process, together with other ones in which the temperature alternated periodically among the three values assayed, using two patterns of change. The analysis of the evolved populations allowed us to determine the evolutionary pathway followed by the virus under the different propagation conditions. We also analyzed the genomic changes candidate to be responsible for adaptation and the degree of evolutionary convergence between independent replicated experiments as well as among populations evolving under different conditions, including changing environments. Finally, we investigated whether the genetic changes that allow adaptation to a particular condition (in this case, high temperature) were the same or not in populations differing in their initial genetic diversity.

Materials and Methods

Viruses and bacteria: Standard procedures for infection

Bacteriophage Qb was routinely propagated by infecting log-phase cultures of Escherichia coli, strain Hfr (Hayes) in NB medium (8 g/l Nutrient Broth from Merck and 5 g/l NaCl). The virus, which was provided by Prof. C. K. Biebricher, was adapted to replicate in liquid cultures in our laboratory by infecting 1 liter of an exponential phase E. coli culture at a multiplicity of infection (moi) of 1 plaque forming unit (pfu) per bacterium. The culture was incubated at 37°C with aeration during 90 min and subsequently an aliquot was used to infect another fresh exponential culture of E. coli at the same moi. This process was repeated for a total of 5 serial transfers [38]. The lysate at the fifth transfer was clarified by incubating with a few drops of chloroform for 15 min at 37°C and differential sedimentation [38]. The virus population obtained in this way was called Qb₀, which was used as ancestor population for most of the evolution experiments described in this paper. We chose to use a heterogeneous population instead of a clone to better reproduce the action of natural selection in natural virus populations, which normally show high diversity.

Infections in liquid medium were always carried out using fresh exponential phase E. coli cultures (with an optical density at 550 nm between 0.6 and 0.8) that were infected with the virus at the moi indicated in each experiment. After 2 h of incubation at 37°C with aeration, the virus supernatants were collected as described above and the virus titres were estimated by plaque assay. The amount of phage suspensions containing 10⁶ pfu (cultures performed at 30°C and 37°C) or 10⁴ pfu (cultures carried out at 43°C) was used to infect a fresh E. coli culture. The procedure was repeated for a total of 15 transfers under constant conditions to obtain the virus populations Qb₃₀, Qb₄₃, and Qb₃₀₄₃ (Fig. 1). The approximate number of replication rounds experienced by the virus during the 15 transfers were 235 (Qb₄₃), 200 (Qb₃₀), and 120 (Qb₃₀₄₃). Adaptation to deterministically fluctuating temperatures took place by alternating 37°C, 43°C, and 30°C in this order, with the temperature change occurring either every transfer (populations Qb₃₀₄₃) or every five transfers (populations Qb₃₀₄₃) (Fig. 1). The culture conditions for each temperature were the same as described for experiments carried out at constant temperatures. In all cases replicate lineages were distinguished with numbers 1 and 2.

Experiments designed to study adaptation in populations differing in their pre-existent genetic diversity (Fig. 2) were carried out using as ancestor population a clonal population of bacteriophage Qb (cQb). The clonal population was obtained upon expression of the plasmid pBR77Qb which contains a cDNA of the wild type virus cloned in the plasmid pBR322 [39]. Briefly, we transformed E. coli DH5α with the plasmid pBR77Qb, and used the supernatant from an overnight culture of a transformed colony to infect a lawn of E. coli Hfr in semisolid agar. A lytic plaque was isolated, and the viruses contained on it were extracted and adapted to replicate at 37°C or 43°C as described above (Fig. 2).

Fitness determinations

Growth rate values at 37°C, 30°C, or 43°C were used as a surrogate of fitness at each temperature. All the determinations were carried out by using liquid cultures that contained 10⁷ bacteria which were inoculated with 10⁶ pfu of the virus population indicated in a final volume of 1 ml. After two hours at 37°C with aeration, the virus supernatants were collected as described above and titrated to estimate the virus yield. Preliminary assays showed that bacteriophage Qb grew exponentially under these conditions. Each determination was carried out in triplicate, and fitness was expressed as the log₂ [virus₀/virusₙ], where virus₀ was the initial input of virus in each experiment and virusₙ was the number of pfu obtained at the end of the experiment.

RNA extraction, cDNA synthesis, PCR amplification and nucleotide sequencing

Virus RNA was prepared following standard procedures [38], [40]. RNAs were amplified by RT-PCR using Avian Myeloblastosis Virus RT (Promega) and Expand High Fidelity DNA polymerase (Roche). The cDNAs were purified with a Qiagen purification kit and subjected to cycle sequencing with Big Dye Chemistry (Applied Biosystems; Perkin Elmer). The following pairs of oligonucleotide primers were used for RT-PCR: 5’GGAATCTTTCCAGACGGCATTCC3’ and 5’AAACGGTTAACCGCTTCTTCCGACACC3’ to amplify from nucleotide position 15 to 1497; 5’TCGAATCCCAGGGTGGGATACATCC3’ and 5’CAAAATCGGCCGATGACAGCAAC3’ to amplify from nucleotide position 1407 to 2817; and 5’TGTCACATAAGGTTTGGACT3’ with 5’GATCCCTCCCTTCTTCTACTGT3’ to amplify from nucleotide position 2254 to 4195. Sequences were aligned with the consensus sequence of the wild type phage with Clustal W. Mutations relative to the consensus sequence were identified using the program BioEdit. Nucleotides were numbered according
to the sequence of the cDNA of bacteriophage Qβ cloned in the plasmid pBR322 [39].

Statistics
The statistical significance of the fitness differences between the ancestor and the evolved populations was evaluated through the Student’s t-test. A nested ANOVA, including the temperature of evolution as main factor and the triplicate measures of fitness as random variables, was performed for each temperature at which fitness was determined. A two-way ANOVA was carried out to compare fluctuating populations with those evolved under each constant temperature condition. The factors considered were the temperature of evolution and the temperature of the fitness assay. Each replicate population was characterized by three fitness values, each one corresponding to the average of three determinations carried out at each temperature. Calculations were carried out using Mathematica 5 (Wolfram Research).

Results
Evolution of bacteriophage Qβ at constant temperatures
A heterogeneous population of bacteriophage Qβ (Qβ0), which had previously been adapted to replicate at 37°C [see Materials and Methods] was propagated during 15 serial transfers at 3 different temperatures (30°C, 37°C, and 43°C). All experiments
were carried out in duplicate and the populations obtained at the last transfer were denoted \( Q_{B0} \), \( Q_{B37} \), and \( Q_{B43} \) respectively (Fig. 1). Fitness values were determined at the three temperatures assayed for both the ancestor population and the evolved populations. Population \( Q_{B0} \) had the highest fitness value at 37°C (20.50±0.19), which agrees with the optimal growth temperature of the bacterial host, an intermediate value at 30°C (17.92±1.26), and the lowest value at 43°C (9.50±0.38). Therefore, replication at 43°C was the selective pressure which produced a strongest decrease in the virus growth rate. All evolved populations experienced small, although significant, fitness gains at 37°C relative to the ancestor population \( (P<0.05, \text{Student}' t \text{ test; Fig. 3, upper panel}) \). There were no significant differences among populations that could be attributable to the temperature of evolution \( (\text{nested ANOVA 30°C: } F_{2,3} = 2.0, P>0.05) \). The fact that populations propagated at 30°C and 43°C also gained fitness at 37°C probably indicates that population \( Q_{B0} \) was not well adapted to the propagation conditions used in the adaptation experiment described in Fig. 1, and further optimization was still possible, independently of the temperature at which the virus was propagated. The generalized fitness gains at 37°C also imply that evolution at 30°C and 43°C does not have strong fitness costs on the replication of the virus at 37°C. In contrast to these results, fitness values determined at 30°C did not increase relative to the ancestor population in any of the evolved populations (Fig. 3, medium panel), and fitness at 43°C only increased significantly \( (P<0.05, \text{Student}' t \text{ test}) \) in the populations propagated at this temperature (Fig. 3, lower panel). Fitness determinations at both 30°C and 43°C were significantly affected by the temperature at which populations had evolved \( (\text{nested ANOVA 30°C: } F_{2,3} = 25.5, P<0.025; \text{nested ANOVA 43°C: } F_{2,3} = 10.8, P<0.05) \). The two populations evolved at 43°C had significantly lower fitness at 30°C than population \( Q_{B0} \) \( (P<0.05, \text{Student}' t \text{ test}) \), suggesting the existence of a fitness trade-off between adaptation to 43°C and replication at 30°C. Although evolution at 43°C was carried out by using larger culture volumes (10 ml) than evolution at 30°C or 37°C (1 ml), the fitness gains attained at 43°C (observed in an assay performed in 1 ml volume) indicate that the culture volume is not confounding the interpretation of the results.

**Evolution of bacteriophage Qβ at deterministically fluctuating temperatures**

Population \( Q_{B0} \) was also evolved at deterministically fluctuating temperatures that alternated 37°C, 43°C, and 30°C in that order, with the temperature change occurring either every transfer \( (\text{populations } Q_{B37}) \) or every five transfers \( (\text{populations } Q_{B43}) \) (Fig. 1). Determination of the fitness values of populations obtained at transfer number 15 allowed us to determine whether the fluctuating conditions assayed are compatible with adaptation. The results obtained (Fig. 4) show that both \( Q_{B37} \) and \( Q_{B43} \) resembled populations \( Q_{B13} \). They presented small though significant fitness gains at 37°C (with the only exception of \( Q_{B43,20} \), fitness losses at 30°C (although they were significant only in one of the replicated populations), and significant fitness gains at 43°C. In consonance with these observations, a two-way ANOVA showed that there was no significant interaction between the temperature of evolution and the temperature of the fitness assay when populations \( Q_{B37} \) and \( Q_{B43} \) were compared to populations \( Q_{B13} \) \( (F_{1,3} = 1.1, P = 0.417) \), whereas both factors interacted significantly when the same populations were compared to \( Q_{B30} \) \( (F_{1,3} = 6.0, P = 0.013) \) or \( Q_{B43,20} \) \( (F_{1,3} = 7.1, P = 0.007) \). It is remarkable that the fitness trade-off shown at 30°C by the populations that evolved at 43°C (Fig. 3) did not preclude adaptation to 43°C when the virus alternated these temperatures during its propagation. Evolution of bacteriophage Qβ at fluctuating temperatures shows a clear example in which the most stringent selective pressure drives adaptation. In general, fitness gains at 43°C were higher for populations evolved constantly at this temperature than for those that evolved under fluctuating temperature conditions. This could have been expected, since the former were adapting to 43°C for a larger number of transfers.

![Figure 3. Fitness values of bacteriophage Qβ populations evolved at constant temperatures.](https://www.plosone.org/content/images/evolution.png)
Figure 4. Fitness values of bacteriophage Qβ populations evolved at fluctuating temperatures. Fitness values at 37°C (upper panel), 30°C (medium panel), and 43°C (lower panel) of the two replicas of populations QβF1, and QβF5. Each bar represents the average of three parallel determinations carried out as described in Materials and Methods. The error bars represent the standard deviation. The fitness value obtained for the ancestor population Qβ0 at each temperature is indicated by a discontinuous line. Thus, bars above this line represent fitness increases relative to the ancestor and bars below the line correspond to fitness decreases. Asterisks mean that the average fitness for a given population is significantly higher (black ones) or lower (red ones) than the fitness value of population Qβ0 ($P<0.05$, Student’ t test).

doi:10.1371/journal.pone.0100940.g004
Table 1. Genomic substitutions present in the consensus sequence of the populations obtained upon evolution of Qb0 at constant and deterministically fluctuating temperatures.

| Substitution | Protein¹ | Amino acid change² | Repetitions³ |
|--------------|----------|--------------------|-------------|
| U920A        | A2       | Phe280Tyr          | 1           |
| A1088G       | A2       | Asp342Gly          | 3           |
| U1198C       | A2       |                   | 1           |
| U1763C       | A1       |                   | 1           |
| G1773A       | A1       | Gly143Arg          | 2           |
| C1806U       | A1       | Pro154Ser          | 4           |
| G1817A       | A1       |                   | 3           |
| A1823G       | A1       |                   | 1           |
| A1930G       | A1       | Gln195Arg          | 4           |
| U2006A       | A1       | Ser220Arg          | 1           |
| A2187C       | A1       | Ser281Arg          | 7           |
| U2297C       | A1       |                   | 1           |
| U2776C       | Rep      | Val141Al           | 3           |
| U3402C       | Rep      | Ser350Pro          | 6           |
| U3731C       | Rep      |                   | 2           |
| G3945A       | Rep      | Gly531Ser          | 1           |
| C4031U       | Rep      |                   | 1           |

¹Protein encoded by the gene where the nucleotide substitution indicated is located.
²Amino acid replacement produced by the nucleotide change. In the cases where no amino acid change is indicated, the substitution is synonymous.
³Number of populations where a given substitution appears either fixed or as a polymorphism.

doil:10.1371/journal.pone.0100940.t001

Analysis of consensus sequences

To identify the genomic substitutions potentially responsible for the observed fitness changes we determined the consensus sequences of all evolved populations and compared them to that of population Qb0. To make a more thorough analysis, the mutations, either fixed or polymorphic, detected in each population were visually inspected in the chromatograms of the remaining populations to evaluate their presence as polymorphisms not recognized by the sequence analysis programs. In total we detected 42 mutations, 35 of which were present as polymorphisms (Tables 1 and 2). There were 17 different substitutions, 8 of which were present in single populations whereas the rest were repeated in at least two populations (Table 1). The total amount of repeated mutations was 84%. The percentage of coincident changes between replicated populations was higher for populations evolved under constant conditions (67% for Qb30, 60% for Qb30, and 43% for Qb30) than for those evolved under fluctuating conditions (29% for Qb31 and 11% for Qb35). When populations evolved under different conditions were compared, the percentages of coincident changes were 60% for Qb37 with Qb30, 11% for Qb37 with Qb31, and 9% for Qb30 with Qb31. In good agreement with the results obtained in the fitness assays, fluctuating populations showed a higher amount of coincident changes with populations evolved at 43°C (56% Qb31 and 33% Qb35) than with populations evolved at 37°C (25% Qb31 and 9% Qb35) or at 30°C (20% Qb31 and 8% Qb35). A high amount of mutations (76%) were non synonymous. The ratio dn/dS for the whole set of mutations was 1.1, which increased to 1.7 when the mutations represented in several populations were the only considered. The distribution of the substitutions along the Qb genome (Table 2) shows a significant accumulation (P<0.025, chi squared test, 3 d.f.) in the region of the A1 protein that does not overlap with the coat protein (Fig. 5).

The analysis of the consensus sequences of the populations evolved under constant conditions shows that substitutions C1806U and A1930G were selected at 37°C and 30°C, and substitution A2187C was selected at the three temperatures (Table 2). There were also several substitutions that only appeared in the populations evolved at 30°C (U3731C and G3945A), or at 43°C (A1088G, G1773A, G1817A, U2297C, U2776C, and U3402C) (Table 2). In contrast to this, the populations evolved at 37°C did not show any specific mutations. From all these substitutions, the changes G1773A, C1806U, G1817A, A1930G, A2187C, and G3945A had previously been detected in our laboratory during the propagation of bacteriophage Qb, suggesting that they are beneficial mutations of general effect under the culture conditions used in our lab [40], [41]. Therefore, they are good candidates to be responsible for the small fitness gains observed at 37°C in populations evolved at 37°C, 30°C, and 43°C. Substitutions exclusive to a particular temperature, and not detected during the evolution of the virus under unrelated selective pressures, probably represent adaptive mutations for that particular condition. In this way, substitution U3731C, only detected in populations Qb30, is a candidate to promote adaptation to 30°C. Similarly, substitutions A1088G, U2297C, U2776C, and U3402, could contribute to the fitness gains observed at 43°C.

The comparison of the substitutions found in the consensus sequences of the populations evolved under constant and fluctuating temperatures allowed us to determine whether the similarities shown at the phenotypic level by Qb31 and Qb35 with Qb30, are also displayed at the genetic level. None of the populations Qb31 and Qb35 showed the substitutions exclusive to the population evolved at 30°C. In contrast to this, all substitutions but one (U2297C) of those exclusive to the populations evolved at 43°C were present at least in one of the populations evolved under fluctuating conditions (Table 2),
Table 2. Genomic location of the nucleotide positions mutated in the consensus sequences of the populations obtained upon evolution of Qβ at constant and deterministically fluctuating temperatures.

| Population | Replicase | Coat | A1 (Non-overlapping region with the coat protein) |
|------------|-----------|------|--------------------------------------------------|
| Qβ0        |           |      |                                                  |
| Qβ37(1)    |           |      |                                                  |
| Qβ37(2)    |           |      |                                                  |
| Qβ30(1)    |           |      |                                                  |
| Qβ30(2)    |           |      |                                                  |
| Qβ43(1)    |           |      |                                                  |
| Qβ43(2)    |           |      |                                                  |
| QβF1(1)    |           |      |                                                  |
| QβF1(2)    |           |      |                                                  |
| QβF5(1)    |           |      |                                                  |
| QβF5(2)    |           |      |                                                  |

The asterisks indicate the presence of a mutation at the position indicated. All mutations were polymorphic at transfer number 15, except those shown in brackets which were fixed.

doi:10.1371/journal.pone.0100940.t002

When all the genetic diversity is generated de novo, it determines whether the same mutations could also be selected during adaptation, we sequenced the two replicated populations cQβ37 that were pre-adapted at 37°C (Table 2). Upon propagation of cQβ at 37°C, both replicated populations cQβ37(1) and cQβ37(2) fixed substitutions A2187C, U2776C, and U3402C, which were not selected during the propagation of bacteriophage Qβ at 43°C (Table 2). Although this experiment shows the selection of a common set of mutations when populations differing in their pre-existent genetic diversity evolve at 43°C, we cannot exclude the possibility that those substitutions were generated during the propagation of cQβ at 37°C, and, thus, were already present, at undetectable frequency by the sequencing methods employed, in the mutant spectrum of population cQβ37(1). To determine whether the same mutations could also be selected when all the genetic diversity is generated de novo during adaptation, we sequenced the two replicated populations cQβ43(1) and cQβ43(2) generated from cQβ directly evolved to 43°C (Fig. 2). We detected the same set of polymorphic substitutions (A1088G, U2776C, and U3402C) in both of them, confirming that substitutions A1088G, U2776C, and U3402C represent an adaptive pathway to high temperature that is accessible from different initial conditions. Substitution U3784C, which was not selected during the adaptation of Qβ at 43°C, could also be involved in adaptation to 43°C or be a substitution that increases the growth rate by a mechanism independent of temperature.

Discussion

Living organisms have to cope with frequent environmental changes that limit their survival if they are not able to adapt to the new conditions. When the selective pressures remain constant for a long time, populations usually evolve to perform optimally under those conditions, even if that entails a decrease of fitness in alternative environments [1–7]. On the contrary, when the selective pressures change frequently, populations do not have...
the time necessary to achieve the best adaptive solution for each condition [42]. The situation gets further complicated when mutations selected in a particular environment have deleterious effects in a different environment encountered by the population. The study of the evolutionary pathways followed by populations adapting to constant versus fluctuating environments is particularly interesting in the case of RNA viruses whose great capacity to adapt to new selective pressures hamper most antiviral treatments [43–45]. We have addressed this question by exposing an RNA virus, the bacteriophage Qβ to three constant environments that differed in the temperature (37 °C, 43 °C, and 30 °C), and to two deterministically fluctuating environments in which the temperature alternated among 37 °C, 43 °C, and 30 °C with two patterns of change.

Propagation of bacteriophage Qβ at different constant temperatures does not result in a common pattern of adaptation

We found that propagation of bacteriophage Qβ at any of the temperatures assayed increased the performance of the virus at 37 °C, indicating that either the fitness effects of some of the mutations selected in lineages exposed to different treatments correlate across different temperatures or selection is acting on viral traits unrelated to this character. Since in all cases the virus was propagated according to a similar transmission regime, fitness increases at 37 °C can arise as a consequence of adaptation to the culture conditions used. The coincidence of several mutations in populations evolved under different temperature conditions indicates that the common behaviour observed at 37 °C has a genetic basis. The fact that several of those coincident mutations had also been detected in previous experiments in which bacteriophage Qβ was propagated in our lab under unrelated selective pressures also agrees with a general beneficial fitness effect on the adaptation of the virus to the culture conditions used [40], [41]. Fitness gains at a temperature different from that at which the virus was propagated have also been reported for vesicular stomatitis virus (VSV) adapted to 29 °C and 37 °C [33]. Another study that compared the adaptation to 44 °C in the related viruses PhiX174 and G4 showed the existence of a fitness cost at 37 °C in PhiX174 but not in G4 [46]. In our study, the general fitness gains observed at 37 °C had no counterpart at 30 °C or 43 °C. In fact none of our populations increased fitness at 30 °C, and only populations Qβ3 improved their performance at 43 °C. This result suggests that the inhibition of virus growth that takes place at 30 °C and 43 °C masks the beneficial effect of the mutations that provide selective advantages unrelated to temperature. The observed difficulties for adaptation to 30 °C agree with the hypothesis that argues that the rate-depressing effects of low temperature on biochemical reactions are difficult to overcome by genetic changes [47].

Populations adapted to 43 °C performed worse at 30 °C than the ancestor population, suggesting that some of the mutations responsible for adaptation to high temperature are deleterious when this parameter decreases below the optimal value. A possible explanation for this trade-off is that adaptation to high temperature selects for mutations that render more thermodynamically stable proteins, whose performance worsens when the temperature is lowered [48–51]. The main argument for the trade-off between function and stability lies in the belief that catalytic proteins must not be too stable to allow for function-related flexibility [52], a hypothesis supported by the fact that the stability of real proteins is not too high. Also there could be a trade-off between growth rate and survival, particularly at high temperatures, a possibility that deserves to be further analyzed.

Bacteriophage Qβ populations evolved under deterministically fluctuating temperatures perform similarly to populations propagated under the most restrictive condition

The fitness trade-off between adaptation to 43 °C and virus performance at 30 °C could pose a conflict when the virus has to thrive in environments in which the two temperatures alternate. However, populations evolved under fluctuating conditions showed striking similarities with populations evolved at 43 °C. This result agrees with the selection of an evolutionary pathway that favours adaptation to the most restrictive condition, in this case 43 °C, even if that means that the virus decreases its performance under other less stringent selective pressure (30 °C). This pathway is probably favoured because the fitness cost at 30 °C is lower than the growth inhibition that takes place at 43 °C in non-adapted viruses. It is striking that we observe the same pattern of fitness across environments in the populations Qβ3. This result supports the hypothesis that the fitness gains responsible for adaptation to high temperature are deleterious temperature on biochemical reactions are difficult to overcome by genetic changes [47].

Our results differ from those obtained by Alto et al. during the adaptation of VSV to deterministically changing temperatures (29 °C alternating with 37 °C), where a generalist population with increased performance at each temperature was selected [33]. These results could have been facilitated by the absence in VSV of fitness trade-offs across the two temperatures assayed. Studies carried out with viruses challenged to alternate between different hosts sometimes also show equal or superior fitness gains on each host compared to viruses constantly evolved in the same host [22], [24], [27–29], [32], [33]. These fitness gains in the generalist...
lineages can even occur when trade-offs across different hosts exist [22].

The consensus sequences of the evolved bacteriophage Qβ populations show coincident mutations at different temperatures together with mutations exclusive to a particular condition

Most of the mutations that we found in the consensus sequences of the evolved bacteriophage Qβ populations were polymorphisms. A possible explanation is that not enough generations were allowed for mutations to reach fixation. An alternative interpretation could be that different beneficial mutations occurred in different lineages and competed with each other, such that interference would delay their fixation [53–55]. Antagonistic epistatic interactions among different beneficial mutations could also contribute to prolong the time that mutations remain as polymorphisms, especially if some of them cannot coexist in the same genome. This kind of interactions has been described in different viruses [56–58], and was shown to be responsible for the prolonged co-existence of two beneficial mutations during the adaptation of bacteriophage Qβ to a mutagen [41]. Investigating this question would require an analysis of the distribution of mutations in the individual components of the mutant spectra of the evolved Qβ populations. Propagation of the virus for a larger number of transfers would also help to determine whether a defined consensus sequence can be obtained.

The analysis of the consensus sequences of the evolved populations show a high number of coincident mutations. This fact, together with the high value of the ratio dn/ds calculated for the repeated mutations, is indicative of the action of natural selection. In the case of RNA, selection may also act at the level of synonymous mutations, whose fitness effect might appear through modifications of the RNA secondary and tertiary structures or by affecting genomic domains required for interaction with substrates. The high degree of convergent evolution here uncovered is similar to that found in other studies carried out with viruses adapting to a constant selective pressure [59–61].

In addition to the mutations repeated in populations propagated under different conditions, other mutations only appeared in the sequence of the virus exposed to a particular temperature. Some of these mutations also coincided in replicated populations, and thus are likely responsible for adaptation to a given temperature. Specifically, substitution U3731C could promote adaptation to 30°C, although this effect was not reflected in fitness increases at 30°C, which could mean that it acts on a trait with no influence on the growth rate. The other substitution exclusive to 30°C but only present in one of the replicated populations was G3945A, which was previously demonstrated to have a general beneficial fitness effect in experiments carried out in both our lab [41] and in others [62]. In the same way, substitutions A1088G, G1817A, U2776C, and U3402C, exclusive to populations evolved at 43°C, could be responsible for the fitness gains observed at this temperature. There were no mutations exclusive to 37°C, which agrees with the fact that the ancestor population Qb0 had previously been propagated at 37°C and, therefore, the supply of beneficial mutations specific to this temperature could be exhausted. To establish unambiguous correspondences between particular mutations and their fitness effect, it would be desirable to build single site-directed mutants and determine their fitness at the temperature of interest, either in competition with the wild-type virus or through the estimation of the growth rate.

Populations evolved under fluctuating conditions, which were phenotypically similar to the constant ones evolved at 43°C, also resembled those populations at the genetic level. They presented some of the common mutations selected at different constant temperatures, did not have any of the mutations exclusive of 30°C, and showed most of the mutations exclusive of the populations evolved at 43°C, confirming their implication in the adaptation of bacteriophage Qβ to high temperature.

Evolution at 43°C of bacteriophage Qβ populations differing in their pre-existent genetic diversity leads to the selection of a common set of mutations

The high degree of parallel evolution shown by the populations analyzed in this work could be due to the fact that the ancestor population was heterogeneous, and, thus, it could contain in its mutant spectrum some of the mutations that consistently appeared during evolution in different lineages. To investigate this possibility we compared the mutations selected during adaptation to 43°C in populations that differed in their pre-existent genetic diversity. We observed that a clonal population and a heterogeneous population, obtained upon transmission at 37°C of the clonal population, coincided in the selection of the polymorphic substitutions A1088G, U2776C, and U3402C, which had previously been identified during the adaptation of population Qβ0 to 43°C. The result indicates that there are few pathways for adaptation of bacteriophage Qβ to high temperature. The population adapted directly to 43°C showed the additional selection of U3784C, which could also provide selective advantages. Pre-adaptation of the clonal population of bacteriophage Qβ to 37°C led to the fixation of a single substitution, A2187C, in two replicate experiments. Therefore, at least the most repeated substitution across all evolutionary lineages analyzed in this work could also be generated de novo during propagation of cQβ at 37°C. We did not detect any of the other substitutions to which we had attributed general beneficial effects, meaning that they were probably present in the mutant spectrum of population Qβ0 and reached high frequency upon its propagation.

Molecular mechanism of action of the mutations fixed during adaptation

Most mutations selected in lineages evolved at different temperatures were placed in the A1 protein and corresponded to the replacement of different amino acids by arginine residues. The A1 protein incorporates in 3–10 copies per virion and is essential for producing infectious virus particles, although its precise function remains unknown. It has recently been suggested that some domains of the A1 protein can be involved in protein interactions that would be necessary to reach the virus fivefold and threefold symmetry axes [63]. Therefore, our results could mean that structural constraints on the virus capsid limit replication under the transmission regime used, and selection of the A1 mutants helps to overcome those constraints. Some of the lineages evolved at 43°C showed the selection of substitution G1817A, also placed in the A1 protein. Although this is a synonymous substitution, we cannot discard that it has some beneficial effect either in stabilizing the viral RNA or in altering somehow its interaction with other molecules.

Adaptation to 43°C was associated to the selection of substitution A1088G (Asp342Gly) in the A2 protein, which is involved in the recognition of the conjugative pilus as receptor in F+ bacteria, the escort of the genomic RNA into the host cytoplasm, and the lysis of the bacteria to release the virus progeny. High temperatures might impair any of these processes, which would be restored, at least in part, in the mutant. It is interesting that during the evolution of bacteriophage phi6 in a
novel temperature environment where heat shock imposed extreme virus mortality, a single amino acid substitution in the viral lysis protein was also selected [51].

The replicase substitutions selected at 43°C, U2776C, U3402C, and U3578C rendered the non-synonymous changes Val141Ala, Ser147Pro, and Ile147Thr respectively. Ser320 is placed in the palm domain of the QB replicase, whereas Val141 is placed in the fingers domain, and Ile477 is in thumb domain [64], [65]. The three residues are not conserved among members of the virus family Leviviridae, which suggests that they do not form part of the active center of the QB replicase. Moreover, the related viruses MX1 and M11 contain proline instead of serine at position 350, the viruses NL95, GA, MS2, and FR contain alanine instead of valine at position 141, and threonine instead of isoleucine is found in the viruses NL95, SP, F1, and MX1 [64], [65]. The most probable explanation for the beneficial effects of these substitutions at high temperatures is that they increase the thermal stability of the replicase. The finding that some QB-related viruses possess in their wild type form the same amino acids as the high temperature QB adapted mutants suggests that what we have selected through experimental evolution in the lab was already selected in nature. Since we have shown that high temperature is the dominant force driving evolution in bacteriophage QB when it is exposed to fluctuations in this condition, the selection of thermostable replicases must not be an unusual fact in viruses transitory temperature increases. The determination of whether those viruses have higher tolerance to high temperatures would be very useful to check the validity of our interpretation.

Acknowledgments

We are very grateful to S Manrubia for useful discussions and critical reading of the manuscript.

Author Contributions

Conceived and designed the experiments: EL. Performed the experiments: MA KK LC. Analyzed the data: EL MA KK LC. Contributed to the writing of the manuscript: EL.

References

1. Levin R (1968) Evolution in changing environments. Princeton Univ. Press, Princeton, NJ.
2. Lynch M, Gabriel W (1987) Environmental tolerance. Am Nat 122:745–764.
3. Futuyma DJ, Moreno G (1983) The evolution of ecological specialization. Annu Rev Ecol Syst 19:207–233.
4. Via S (1990) Ecological genetics and host adaptation in herbivorous insects: the experimental study of evolution in natural and agricultural systems. Annu Rev Entomol 35:421–446.
5. Stearns SC (1992) The evolution of life histories, Oxford Univ Press, Oxford UK.
6. Hereford J (2009) A quantitative survey of local adaptation and fitness trade-offs. Am Nat 173:579–580.
7. Legros M, Koella JC (2010) Experimental evolution of specialization by a macrosporidian parasite. BMC Ecol Biol 10:159.
8. Gandon S (2004) Evolution of multilocus fitness. Evolution 58:455–469.
9. Kawecki TJ, Ebert D (2004) Conceptual issues in local evolution. Ecol Lett 7:1223–1241.
10. Magathhes S, Blanchet E, Egas M, Olivier I (2009) Are adaptation costs necessary to build up a local adaptation pattern? BMC Evol Biol 9:182.
11. Poulin R (1998) Large-scale patterns of host use by parasites of freshwater fishes. Ecol Lett 1:118–120.
12. Krause BR, Poulin R, Sherbon GL, Mouillot D, Khokhlova IS (2004) Ectoparasitic "jacks-of-all-trades": relationship between abundance and host specificity in fleas (Siphonaptera) parasitic on small mammals. Am Nat 164:506–516.
13. Palaima A (2007) The fitness of generalization: present limitations and future possible solutions. Bio J Linn Soc Lond 90:583–590.
14. Coffey LL, Vignuzzi M (2011) Host alternation of chikungunya virus increases fitness while restricting population diversity and adaptability to novel selective pressures. J Virol 85:1025–1035.
15. Deardoff ER, Fitzpatrick KA, Jerzak GV, Shi PY, Kramer LD, et al. (2011) West Nile virus experimental evolution in vivo and the trade-off hypothesis. PLoS Pathog 7(11):e1002333.
16. Bedhomme S, Lafforgue G, Elena SF (2012) Multihost experimental evolution of a plant RNA virus reveals local adaptation and host-specific mutations. Mol Biol Evol 29:1481–1492.
17. Alto BW, Wasik BR, Morales NM, Turner PE (2013) Stochastic temperatures impede virus adaptation. Evolution 67:969–979.
18. Louring AS, Andino R (2010) Quasispecies theory and the behavior of RNA viruses. PLoS Pathog 6(7):e1000195.
19. Ruiz-Jarabo CM, Arias A, Baranski E, Escarmís C, Domingo E (2000) Memory in viral quasispecies. J Virol 74:3543–3547.
20. Domingo E, Ruiz-Jarabo CM, Sierra S, Arias A, Pariete N, et al. (2002) Emergence and selection of RNA virus variants: memory and extinction. Virus Res 82:39–44.
21. Briones C, Domingo E (2008) Minority report: hidden memory genomes in HIV-1 quasispecies and possible clinical implications. AIDS Rev 10:93–109.
22. Cases-González C, Arribas M, Domingo E, Lázaro E (2000) Beneficial effects of population bottlenecks in an RNA virus evolving at increased error rate. J Mol Biol 304:1120–1129.
23. Barraza I, Schappi D, Sogo JM, Weber H (1993) Different mechanisms of recognition of bacteriophage QB plus and minus strand RNAs by QB replicase. J Mol Biol 232:512–521.
24. Arribas M, Cabanillas I, Lázaro E (2011) Identification of mutations conferring 5-azacytidine resistance in bacteriophage QB. Virology 417:343–352.
25. Domingo E, Ruiz-Jarabo CM, Arias A, Baranski E, Escarmís C, Domingo G, et al. (2000) Viral quasispecies and the problem of vaccine-escape and drug-resistant mutants. Prog Drug Res 48:99–128.
26. Domingo E, Menéndez-Arias I, Quiñones-Mateu ME, Holguín A, Gutiérrez-Rivas M, et al. (1997) Viral quasispecies and the problem of vaccine-escape and drug-resistant mutants. Prog Drug Res 48:99–128.
27. Ruíz-Jarabo CM, Lázaro E, Vignuzzi M (2012) Biodrugs implications of viral mutation and evolution. Future Virology 7:391–402.
47. Knies JL, Kingsolver JG, Burch CL (2009) Hotter is better and broader: thermal sensitivity of fitness in a population of bacteriophages. Am Nat 173:419–430.

48. Wang X, Minasov G, Shoichet BK (2002) Evolution of an antibiotic resistance enzyme constrained by stability and activity trade-offs. J Mol Biol 20:85–95.

49. Tokarzki N, Stricher P, Serrano L, Tawfik DS (2008) How protein stability and new functions trade off. PLoS Comput Biol 4(2):e1000002.

50. Lee KH, Miller CR, Nagel AC, Wichman HA, Joyce P, et al. (2011) First-step mutations for adaptation at elevated temperature increase capsid stability in a virus. PLoS One 6(9):e25640.

51. Dessau M, Goldhill D, McBride R, Turner PE, Modis Y (2012) Selective pressure causes an RNA virus to trade reproductive fitness for increased structural and thermal stability of a viral enzyme. PLoS Genet 8(1):e1002102.

52. DePristo MA, Weinreich DM, Hartl DL (2005) Missense meanderings in sequence space: a biophysical view of protein evolution. Nat Rev Genet 6:678–687.

53. Gerrish PJ, Lenski RE (1998) The fate of competing beneficial mutations in an asexual population. Genetica 102–103:127–144.

54. Sniegowski PD, Gerrish PJ (2010) Beneficial mutations and the dynamics of adaptation in asexual populations. Philos Trans R Soc Lond B Biol Sci 365:1255–1263.

55. Lang GI, Botstein D, Desai MM (2011) Genetic variation and the fate of beneficial mutations in asexual populations. Genetics 188:647–661.

56. Weinreich DM, Watson RA, Chao L (2005) Perspective: Sign epistasis and genetic constraint on evolutionary trajectories. Evolution 2005, 59:1165–1174.

57. Elena SF, Solé RV, Sardanyés J (2010) Simple genomes, complex interactions: epistasis in RNA virus. Chaos 20(2):026106.

58. Rokyta DR, Joyce P, Caudle SB, Miller C, Beisel CJ, et al. (2011) Epistasis between beneficial mutations and the phenotype-to-fitness Map for a ssDNA virus. PLoS Genet 7(6):e1002075.

59. Bull JJ, Badgett MR, Wichman HA, Huschenbeck JP, Hills DM, et al. (1997) Exceptional convergent evolution in a virus. Genetics 147:1497–1507.

60. Wichman HA, Badgett MR, Scott LA, Boulianne CM, Bull JJ (1999) Different trajectories of parallel evolution during viral adaptation. Science 283:422–424.

61. Rollback JP, Huschenbeck JP (2009) Parallel genetic evolution within and between bacteriophage species of varying degrees of divergence. Genetics 181:225–234.

62. García-Villada L, Drake JW (2013) Experimental selection reveals a trade-off between fecundity and lifespan in the coliphage Qβ. Open Biol 3(6):130043.

63. Rummicks J, Tars K (2011) Crystal structure of the read-through domain from bacteriophage Qβ A1 protein. Protein Sci 20:1709–1712.

64. Kidmose RT, Vasiliev NN, Chetverin AB, Andersen GR, Knudsen CR (2010) Structure of the Qbeta replicase, an RNA-dependent RNA polymerase consisting of viral and host proteins. Proc Natl Acad Sci USA 107:10884–10889.

65. Takeshita D, Tomita K (2010) Assembly of Qbeta viral RNA polymerase with host translational elongation factors EF-Tu and -Ts. Proc Natl Acad Sci USA. 107:15735–15738.