JOURNAL OF Evolutionary Biology

doi: 10.1111/j.1420-9101.2010.02107.x

Selection for thermostability can lead to the emergence of mutational robustness in an RNA virus

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Keywords:

bacteriophage Qβ;
mutagenesis;
plastogenetic congruence;
robustness;
thermostability.

Abstract

Mutational robustness has important evolutionary implications, yet the mechanisms leading to its emergence remain poorly understood. One possibility is selection acting on a correlated trait, as for instance thermostability (plastogenetic congruence). Here, we examine the correlation between mutational robustness and thermostability in experimental populations of the RNA bacteriophage Qβ. Thermostable viruses evolved after only six serial passages in the presence of heat shocks, and genome sequencing suggested that thermostability can be conferred by several alternative mutations. To test whether thermostable viruses have increased mutational robustness, we performed additional passages in the presence of nitrous acid. Whereas in control lines this treatment produced the expected reduction in growth rate caused by the accumulation of deleterious mutations, thermostable viruses showed no such reduction, indicating that they are more resistant to mutagenesis. Our results suggest that selection for thermostability can lead to the emergence of mutational robustness driven by plastogenetic congruence. As temperature is a widespread selective pressure in nature, the mechanism described here may be relevant to the evolution of mutational robustness.

Introduction

Robustness, defined as the phenotype resilience to perturbations, is central to many evolutionary processes (Wagner, 2005a; Borenstein & Ruppin, 2006). Much work has been devoted to understanding the origin, mechanisms and consequences of robustness, particularly in relation to mutational robustness (the ability to tolerate mutations) (Ancel & Fontana, 2000; de Visser *et al.*, 2003; Kitano, 2004; Wagner, 2005a; Borenstein & Ruppin, 2006; McBride *et al.*, 2008; Elena & Sanjuán, 2008; Szöllosi & Derenyi, 2009). For instance, mutational robustness is important for protein evolution, as it can potentially allow for the accumulation of genetic variation, which may become advantageous for survival over the long term and foster the appearance of novel functions (Wagner, 2005a; Bloom *et al.*, 2006; Ciliberti *et al.*, 2007; Wagner, 2008). Also, recent work with bacteriophages has shown that mutational robustness increases the ability to adapt to stressful conditions such as high temperatures (McBride *et al.*, 2008; Ogbunugafor *et al.*, 2009). However, robustness reduces the strength of selection and hence can also slowdown adaptation at least in the short term, as has been discussed theoretically (Lenski *et al.*, 2006; Kim, 2007) and tested using digital organisms (Elena & Sanjuán, 2008) and RNA viruses (Cuevas *et al.*, 2009). Recent work has confirmed that robustness can facilitate or impede adaptation depending on population size, mutation rate and the structure of the fitness landscape (Draghi *et al.*, 2010). Mutational robustness has also practical implications, because it may

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determine the susceptibility of viruses to therapeutic actions based on increasing their mutation rate (lethal mutagenesis) (Bull *et al.*, 2007; Manrubia *et al.*, 2010).

The evolutionary origin and maintenance of mutational robustness remains an unresolved issue (de Visser *et al.*, 2003; Wagner, 2005b, 2008). In principle, genotypes that tend to generate less deleterious mutations could be directly favoured by natural selection, as has been suggested for RNA secondary structure (Borenstein & Ruppin, 2006). However, mutations increasing robustness can only be beneficial in mutated genetic backgrounds and, therefore, mutational robustness may be directly favoured only if genomic mutation frequencies are high, as should be the case of systems with a high per-site mutation rate (Schuster & Swetina, 1988; Wilke *et al.*, 2001; Codoñer *et al.*, 2006) or large genomes (Szöllosi & Derenyi, 2009). Mutation accumulation caused by random genetic drift in small populations may also contribute to selecting mutational robustness (Krakauer & Plotkin, 2002), although low population sizes reduce the effectiveness of selection too (Wagner, 2005a; Elena *et al.*, 2007). Interestingly, even in RNA viruses, whose replication is highly error-prone (Drake & Holland, 1999), direct selection for robustness has only been observed after increasing the mutation rate artificially through chemical mutagenesis (Sanjuán *et al.*, 2007).

Alternatively, mutational robustness might evolve in response to selection favouring an intrinsically correlated trait as, for instance, environmental robustness. The correlation between these two forms of robustness is termed 'plastogenetic congruence' (Ancel & Fontana, 2000). Temperature being an important environmental factor (Knies *et al.*, 2006), plastogenetic congruence could take the form of a positive correlation between thermodynamic stability and mutational robustness (Ancel & Fontana, 2000; Shu *et al.*, 2007; Szöllosi & Derenyi, 2009). Using predicted RNA structures, it has been shown that thermostability and mutational robustness can be simultaneously favoured, although the former can evolve without the latter (Meyers *et al.*, 2004). Plastogenetic congruence is also supported by theoretical and experimental work with proteins. For instance, Bloom *et al.* developed a model for predicting the effects of amino acid substitutions from thermodynamic parameters and concluded that increasing thermostability provides a free energy 'cushion' that allows the protein to better tolerate mutations, a result which was also experimentally validated (Bloom *et al.*, 2006). Similarly, extensive *in vitro* mutagenesis has shown that proteins isolated from thermophiles tend to be more robust than those from mesophiles (Besenmatter *et al.*, 2007), although these differences can critically depend on structural details and the particular substitutions involved (Woycechowsky *et al.*, 2008). Also, thermostability can have an associated fitness cost, because it can compromise performance at low temperatures (Vihinen, 1987; Závodszky *et al.*, 1998; Petsko, 2001; Knies *et al.*, 2006; Besenmatter *et al.*, 2007).

Plastogenetic congruence provides a potentially powerful mechanism for the evolution of mutational robustness, because selection favouring environmental robustness or thermostability should be stronger than direct selection for mutational robustness. RNA phages are a good system for testing this model as they allow us to observe rapid evolution under laboratory conditions while keeping a sense of evolution in a natural system. Here, we use the bacteriophage Qβ, a positive-stranded RNA virus belonging to the family *Leviviridae* with a 4.2 kb genome encoding a replicase subunit, a viral coat, a maturation protein and another protein resulting from read-through of the coat gene (Calendar, 2006). We show that, starting from plaque-purified viruses, serial passages alternated with periodic 52 °C heat shocks result in rapid evolution of thermostable variants. Subsequent evolution in the presence of a chemical mutagen suggests that lines selected for thermostability are more tolerant to mutation accumulation. We find that thermostability may appear through different mutational pathways and does not necessarily involve a fitness cost at 37 °C.

Material and methods

Bacteriophage and cells

We obtained bacteriophage Qβ from an infectious clone originally provided by Dr. René C. Olsthoorn (Leiden University) and adapted it to *Escherichia coli* C strain IJ1862 (obtained from Prof. James J. Bull, University of Texas) (Bull *et al.*, 2004) in our laboratory conditions by performing 90 serial passages (Domingo-Calap *et al.*, 2009). This adapted virus was used as reference in all growth assays. Evolution experiments were started from six viral plaques isolated from this stock to remove preexisting genetic variability (each plaque is assumed to come from a single infectious particle).

Selection of thermostable viruses

We carried out serial passages of six lines, each started from a single plaque of our reference virus. Exponentially growing IJ1862 cells (OD_{600} = 0.15) were inoculated with 10^5 plaque forming units (pfu) in 0.5 mL of LB medium and incubated at 650 rpm and 37 °C for 2 h in a Thermomixer 24-tube shaker (Eppendorf, Hamburg, Germany). This was the time required for the virus to reach a titre of approximately 10^9 pfu mL^{-1}. Cells were removed by centrifugation, and the supernatants were incubated at 52 °C for 10 min (heat shock), resulting in a titre loss of approximately two orders of magnitude as determined in preliminary assays. A new culture was infected, followed by another heat shock, until completing six such cycles. In parallel and starting from the same founder clones, we propagated six control lineages under

exactly the same conditions except that heat shocks were replaced by a 1/100 dilution.

Mutation accumulation using nitrous acid

Viruses in supernatants were exposed to nitrous acid as described in previous work (Domingo-Calap *et al.*, 2009). Briefly, 4 μL of virus-containing LB were mixed with 50 μL of a freshly prepared 4 : 1 (volume ratio) mixture of acetate buffer 0.3 M pH 4.3 and sodium nitrite 5 M. The reaction was quenched by adding 200 μL of acetate buffer 1 M pH 7.0 containing 100 μg mL^{-1} of BSA. The time of exposure to nitrous acid was approximately 4 min but was adjusted at each passage such that the final titre was 10^6 pfu mL^{-1}, which was the minimal titre required to initiate the next passage. In total, we completed 18 mutation accumulation passages in duplicate for each of the six thermostable and six control lines.

Growth rate assays

Infections in liquid culture were performed as detailed above with an incubation time of 2 h 10 min. Preliminary assays showed that the reference virus grows exponentially during this time range. We calculated growth rates as the change in natural log titre per hour. In all cases, the reference and evolved viruses were assayed in the same experimental block and we carried out three replicate assays.

Sequencing

RT-PCR products were obtained directly from viral supernatants, column-purified (Zymoresearch, Orange, CA, USA) and sequenced by the Sanger method using the Big-Dye Terminator v3.0 Cycle Sequencing Ready Reaction kit (Applied Biosystems, Carlsbad, CA, USA) in an ABI Prism 373 machine (Applied Biosystems).

Analysis of variance

We tested for differences in growth rates before and after mutagenesis using an ANOVA with the following factors: founder clone (six-class random factor), presence or absence of mutagenesis (two-class fixed factor) and mutagenesis line (two-class random factor nested within clone). The fitness cost of thermostability was tested using the growth rate values of nonmutagenized viruses and a two-way ANOVA with thermostability as a fixed factor and the founder clone as a random factor.

RNA and protein folding

We used the RNAFold program implemented in Vienna RNA Package (Hofacker, 2003) (http://www.tbi.univie.ac.at/~ivo/RNA) to predict the secondary structure minimum free energy of the six thermostable and the reference virus genomes. To obtain the predicted free energy of protein structures, we used MUPro (Cheng *et al.*, 2005; 2006) (http://www.ics.uci.edu/~baldig/mutation.html), FoldX (Guerois *et al.*, 2002; Schymkowitz *et al.*, 2005a,b) (foldx.crg.es) and Eris (Yin *et al.*, 2007a,b) (troll.med.unc.edu/eris/login.php) programs. The crystal structures of the coat and replicase proteins are available as pdb files at http://www.pdb.org (1QBE.pdb for the coat protein and 3MMP.pdb for the replicase). However, the pdb file of the replicase was too large to be handed by the above program servers. Hence, for all proteins except the coat we could only use MUpro, which does not require pdb inputs.

Results

We performed six infection passages alternated with heat shocks of 10 min at 52 °C for each of six Qβ replicate lines, and we did the same for six control lines in the absence of heat shocks. To quantify thermostability, we carried out heat degradation curves for the control and selected lines simultaneously (Fig. 1). The average rate of titre loss estimated by ordinary linear regression was -0.082 ± 0.007 $\log_{10}$ units min^{-1} for the selected lines and -0.428 ± 0.016 $\log_{10}$ units min^{-1} for the controls (Mann–Whitney test, $N = 12$, $P = 0.002$). Hence, thermostability evolved rapidly in response to heat shocks.

To study the genetic basis of thermostability, we sequenced the genomes of the six thermostable lines (Fig. 2). Parallel evolution was evident at genome positions 190, 1173, 1776, 1971 and 2350, suggesting that these changes might be important for thermostability. We also sequenced these specific positions for the six control lines and found that substitutions U190G and

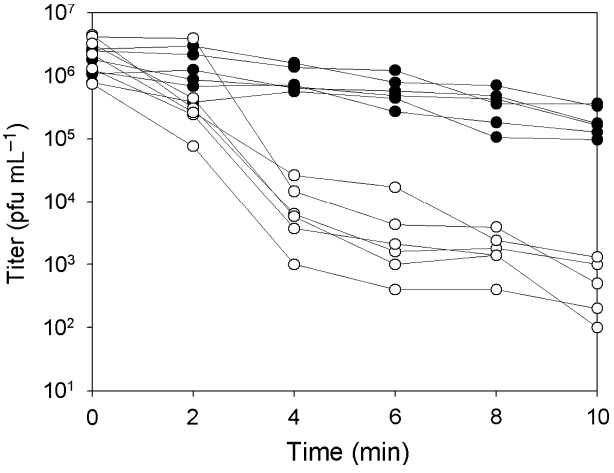

Fig. 1 Heat degradation curve of bacteriophage Qβ-free virions at 52 °C. Black circles correspond to viruses previously passaged six times in the presence of heat shocks (52 °C, 10 min) and white circles to control lines.

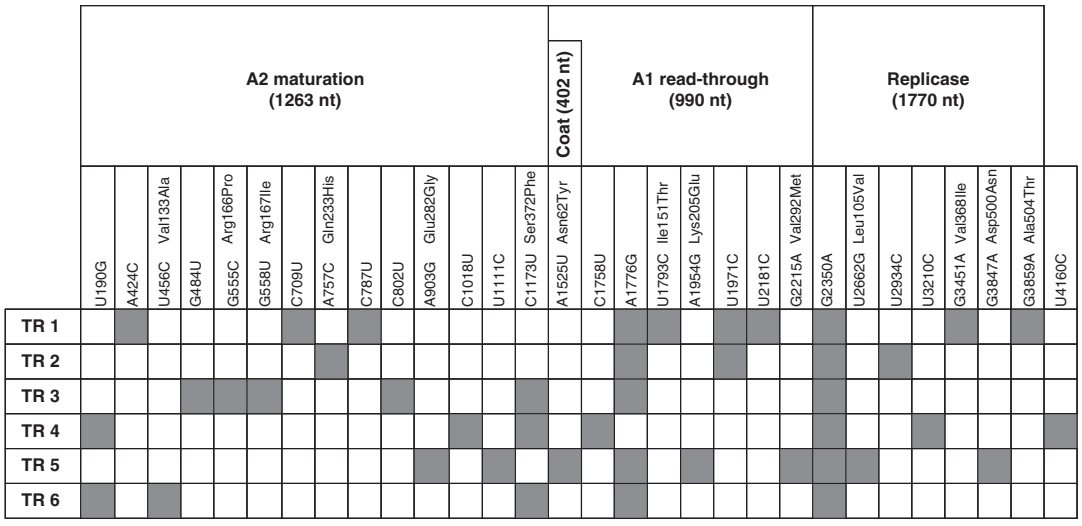

Fig. 2 Nucleotide substitutions observed in the consensus sequence of the six thermostable lines of bacteriophage Qβ. The type and genome position of each substitution is shown. For nonsynonymous substitutions, the amino acid position and change are indicated. The last position shown corresponds to a noncoding region.

C1173U were absent, further suggesting their association with thermostability. In contrast, substitutions A1776G, U1971C and G2350A were found in two or more control lines, indicating that they were probably generally advantageous but not relevant to thermostability. Finally, there were also substitutions occurring only in one line. The latter might confer thermostability, be generally advantageous, or selectively neutral.

To test the putative association between thermostability and mutational robustness, we further evolved each of the above 12 lines by exposing them to nitrous acid after each passage (in the absence of heat shocks). Two replicate sublines were established for each line in this second phase, thus making 24 mutation-accumulation lines in total. Nitrous acid causes oxidative deamination of adenine and cytosine in single stranded RNA and thus introduces mutations (mainly transitions) throughout the genome. However, it also produces virion degradation by damaging the RNA or the capsid. Hence, we first checked that thermostable and control viruses showed no differences in their rates of nitrous acid-induced degradation (Fig. 3). We also verified that initial differences in thermostability remained significant after mutation-accumulation passages 10 and 18 (not shown). Finally, we performed growth rate assays for thermostable and control lines before and after mutation accumulation (passage 18). On average, the change in growth rate after mutagenesis was −0.124 ± 0.070 for control lines and −0.026 ± 0.073 for thermostable lines (Fig. 4). Using the change in growth rate for the 24 lines, differences between control and thermostable lines were marginally significant (Mann–Whitney test: $N = 72$, $P = 0.076$; $P = 0.038$ for a one-tail test). An ANOVA accounting for random differences between lines indicated that the growth rate of thermostable lines did not change significantly before and after mutagenesis ($F_{1,5} = 0.468$, $P = 0.524$), whereas a significant reduction took place in control lines (ANOVA, $F_{1,5} = 7.684$, $P = 0.039$).

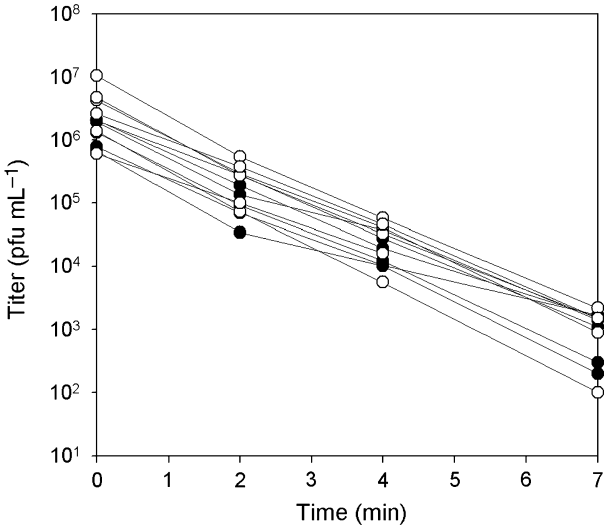

Fig. 3 Nitrous acid degradation curve of bacteriophage Qβ-free virions. Black circles correspond to thermostable viruses and white circles to control lines. The rate of titre loss was similar for both groups (−0.460 ± 0.0177 and −0.479 ± 0.019 $\log_{10}$ min^{-1}, respectively; $P = 0.552$).

The above data suggest that direct selection for thermostability may lead to the evolution of increased mutational robustness. However, whether mutational robustness can be maintained once the selective pressure

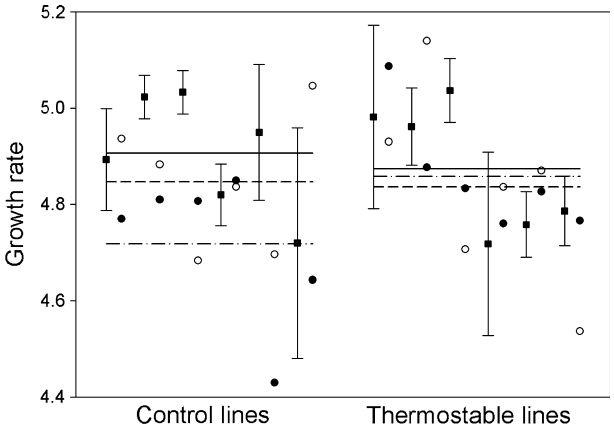

Fig. 4 Changes in growth rate following mutation accumulation in control and thermostable lines. Black squares represent each of the six control/thermostable lines before mutation accumulation. Solid lines indicate the average growth rate of these starting viruses. White circles and dashed lines indicate the individual and mean growth rates for the first replicate of mutagenesis, whereas black circles and dashed-dotted lines correspond to the second replicate.

favouring thermostability disappears depends on the fitness cost associated to thermostability/robustness. The fact that differences in thermostability were maintained for 18 passages in the absence of heat shocks suggests that this cost was low. Further, growth assays of thermostable and control viruses indicated that there were no significant differences in growth rate between the two groups (ANOVA: $F_{1,5} = 1.845$, $P = 0.232$).

Discussion

Plastogenetic congruence has been previously studied by comparing proteins or RNAs from extant species with different levels of thermostability (Bloom *et al.*, 2006; Besenmatter *et al.*, 2007) and the evolution of robustness has been inferred from the comparison between natural and artificial molecules (Ancel & Fontana, 2000; Fontana, 2002; Meyers *et al.*, 2004; Borenstein & Ruppin, 2006; Szöllosi & Derenyi, 2009). However, few studies have tested experimentally the ability of plastogenetic congruence to promote the evolution of mutational robustness. Here, we have used bacteriophage Qβ as a model system to determine whether direct selection for thermostability can result in the emergence of mutationally robust viruses. Consistent with previous work with the DNA bacteriophage G4 (Holder & Bull, 2001), we observed rapid evolution of thermostability. Further, our thermostable viruses turned out to be also more tolerant to chemical mutagenesis, suggesting that there is an intrinsic association between these two forms of robustness. Altogether, the rapid appearance of thermostable genotypes, the variety of potential mutations conferring this phenotype, and the emergence of mutational robustness suggest that plastogenetic congruence is a plausible mechanism for the evolution of mutational robustness in our model system.

The link between thermostability and mutational robustness has also been explored using the double stranded RNA bacteriophage Ø6. Genotypes with increased ability to tolerate mutations were shown to be more evolvable than less robust genotypes, as measured by their ability to adapt to 45 °C heat shocks (McBride *et al.*, 2008). Interestingly, the robust viruses were found to be more stable at intermediate temperatures (39–44 °C), suggesting that pastogenetic congruence paved the path for the evolution of thermostability in robust viruses (Ogbunugafor *et al.*, 2009). However, the association between mutational and environmental robustness was not found to be a general one, because mutationally robust viruses did not have increased ability to tolerate UVC-induced virion damage. Therefore, work with bacteriophage Ø6 is generally consistent with our findings and supports the notion that direct selection for thermostability (but not necessarily for other kinds of environmental robustness) may lead to the emergence of mutational robustness.

In our control (nonthermostable) lines, the average growth rate loss after 18 mutagenic passages was only modest (2.5% on average), yet statistically significant despite we only had six founder populations with two replicate lines for each. The most likely explanation for this small change in fitness is the use of nitrous acid as a mutagen. On one hand, as virions are nonspecifically and passively mutagenized by nitrous acid, the evolution of genotypes resistant to mutagenesis (something that we wanted to avoid here) seems more difficult than for other mutagens such as nucleoside analogues which are incorporated into the active site of the viral polymerase and for which several forms of resistance have been described (Pfeiffer & Kirkegaard, 2003; Sierra *et al.*, 2007). Also, as nitrous acid is applied extracellularly, the physiology of the host cell was not modified. On the other hand, nitrous acid mutagenesis is discontinuous and thus not very efficient, because it allows selection to purge deleterious mutations during the virus growth phase. Further, nitrous acid rapidly degrades the virions and as a result, the amount of mutagenesis that can be applied is smaller than for other chemicals as, for instance, hydroxylamine. Use of a weak mutation-accumulation system and the relatively small number of replicate lines limited our statistical power and as a consequence, our results are not fully conclusive. However mild mutagenesis may better reflect the effect of mutagens in nature.

Functional constraints can impose a trade-off between protein stability and performance (Meiering *et al.*, 1992; Shoichet *et al.*, 1995; Beadle & Shoichet, 2002). Previous work has shown that experimental populations of bacteriophage G4 adapted to growth at 44 °C (but not at 41.5 °C) paid a fitness cost below 35 °C (Knies *et al.*,

2006). In contrast, we have found that bacteriophage Qβ can strongly and rapidly increase its tolerance to 52 °C shocks without significantly compromising growth at 37 °C and that this ability is not lost upon serial passages in the absence of heat shocks. In the G4 study, selection was continuous because infected cultures were incubated at high temperatures whereas, here, we only applied periodic heat shocks to free virions. Therefore, our experimental design selected for virion thermostability but not for the ability to complete the entire infection cycle at high temperatures. This might explain why we did not observe strong fitness trade-offs.

Evolutionary parallelisms or convergences at the molecular level are abundant in bacterial (Betancourt, 2009), animal (Cuevas *et al.*, 2002; Remold *et al.*, 2008), and plant (Rico *et al.*, 2006; Agudelo-Romero *et al.*, 2008) RNA viruses, as well as ssDNA viruses (Bull *et al.*, 1997; Cunningham *et al.*, 1997; Wichman *et al.*, 1999; Crill *et al.*, 2000; Wichman *et al.*, 2000). Sequencing of thermostable and control lines confirmed this pattern here, although only two of the five evolutionary parallelisms detected here (U190G and C1173U) were likely relevant to the evolution of thermostability and U190G was a synonymous substitution. Still, this mutation might confer thermostability by allowing the viral RNA to stabilize its secondary structure. We found no polymorphisms at any of these two genome sites among sequences downloaded from the GenBank, although this might not be significant given the low number of sequences available. Both mutations were located in the maturation protein, which is necessary for absorption to the pilus F (Paranchych, 1975) and lysis (Karnik & Billeter, 1983). Although the majority of mutations observed (parallel or not) in the thermostable lines were located in this gene, we did not find statistical evidence for gene clustering, in agreement with previous work with bacteriophage G4 (Knies *et al.*, 2006).

We have not addressed experimentally the molecular mechanisms by which the observed mutations increased the virion thermostability. In principle, this could occur through changes in the minimum free energy of the RNA secondary structure or the protein folding. The RNA secondary structure of most of the viral genome has been experimentally determined (Skripkin *et al.*, 1990; Beekwilder *et al.*, 1996) and, according to this model, substitutions G484U and U1971C could establish new base pairs and hence increase stability. However, there were also seven mutations that disrupted previous base pairs, whereas four occurred in loops. We also obtained the predicted minimum free energy for the secondary structure of each sequenced genome using RNAFOLD (Hofacker, 2003), but no significant differences between the thermostable and reference viruses were observed. Concerning the protein structure, we used the MUPRO program (Cheng *et al.*, 2005; 2006) for predicting the effects of each single amino acid substitution on protein stability. In the case of the coat protein, the available crystal structure was used to obtain the prediction and two additional programs were used (FOLDX and ERIS). However, we obtained no conclusive results.

We have shown that congruent evolution of environmental and mutational robustness is possible in laboratory populations of an RNA bacteriophage. This represents a step forward in our understanding of the evolution of mutational robustness and contributes to generalize previous *in silico* and *in vitro* work with RNA and proteins. However, it remains unknown whether similar results will be obtained with more complex biological systems. A better understanding of the genetics and biochemistry of plastogenetic congruence will help to elucidate this question.

Acknowledgments

We thank Jim Bull and René C. Olsthoorn for supplying the viruses, cells and molecular clones, and Concha Hueso and Loles Catalán for technical assistance. This work was financially supported by grant BFU2008-03978/BMC from the Spanish MICIIN to R.S., Ramón y Cajal research program from the Spanish MICIIN to R.S., and a fellowship from the Generalitat Valenciana to P.D.-C.

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Received 8 June 2010; revised 15 July 2010; accepted 10 August 2010