Supplementary informations – Variablity in the durability of CRISPR–Cas immunity

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1 Primers used for CRISPR PCR and sequencing

1.1 PCR Protocol

The PCR mix contained 5 μL of Multiplex Qiagen, 1 μL of each primer, 2 μL of sterile water and 1 μL of 1% bacteria. The PCR program involves 15 minutes at 94°C, 35 cycles of 30 seconds at 94°C, 90 seconds at 60°C (CRISPR1 and 2) or 56°C (CRISPR3 and 4), 1 minute at 72°C and ended by 10 minutes at 60°C.

1.2 Primers

Table S1: Primer sequences used for the PCR of *S. thermophilus* loci.

CRISPR	Forward Primer (5'-3')	Reverse primer (5'-3')
1	TGCTGAGACAACCTAGTCTCTC	GGATCCGGATCCGTTGAGGCCTTGTTC
2	GCCCCTACCATAGTGCTGAAAAATTAG	CCAAATCTTGTGCAGGATGGTCG
3	GGTGACAGTCACATCTTGTCTAAAACG	GCTGGATATTCGTATAACATGTC
4	CCTCATAGAGCTTTGAAAGATGCTAGAC	GTTCTTCTTGATGCTTGTCGAGGC

2 Characterization of BIMs

Table S2: BIMs spacer sequence and protospacer position in 2972 phage genome. BIMs are named with the following nomenclature: NC or ORF indicates if the BIM targets a non-coding or a coding sequence respectively. When appropriate, the number following the _ sign indicates the targeted *orf*. If multiple BIMs target the same *orf*, they are distinguished by capital letters.

Name	Spacer sequence (5'-3')	Protospacer position in the phage genome	*orf* targeted in the phage genome
NC	AGGAGGTGGACATATTGGGCTAAATCAACG	954 – 983	non-coding
ORF_2	GCTCTACGACTTCTTCCACGAGTTCCTGCC	1 199 – 1 228	2
ORF_5	CCATCTCGTTGTCCTTACGACGACCAGACT	3 223 – 3 252	5
ORF_9	AGATATTGATTATGGTGTTAAAGCAGACCA	7 020 – 7 049	9
ORF_17	AAGCAAGTTGATATATTTCTCTTTCTTTAT	10 270 – 10 299	17
ORF_19	TTATCTGATTTTTTCCCCTTGATTTCGGGG	16 226 – 16 255	19
ORF_20	TAAGGCAAACGAGACCGAGAGAGCTGCAGC	21 022 – 21 051	20
ORF_21	TTGACGATTGGGAACCGTGGAAGGAATTTG	23 067 – 23 096	21
ORF_24	AACACAGATGTTTTAGACCATGCGCAGAAG	24 326 – 24 355	24 + non-coding
ORF_27	TATTTGTACGTGAGTGGAAGTGCTTAGACT	25 544 – 25 473	27 + non-coding
ORF_33	TTTCATCGTCAATTTCCATGTTATAAATCT	27 003 – 27 032	33
ORF_37_A	TCGTTTTCAGTCATTGGTGGTTTGTCAGCG	29 988 – 30 017	37

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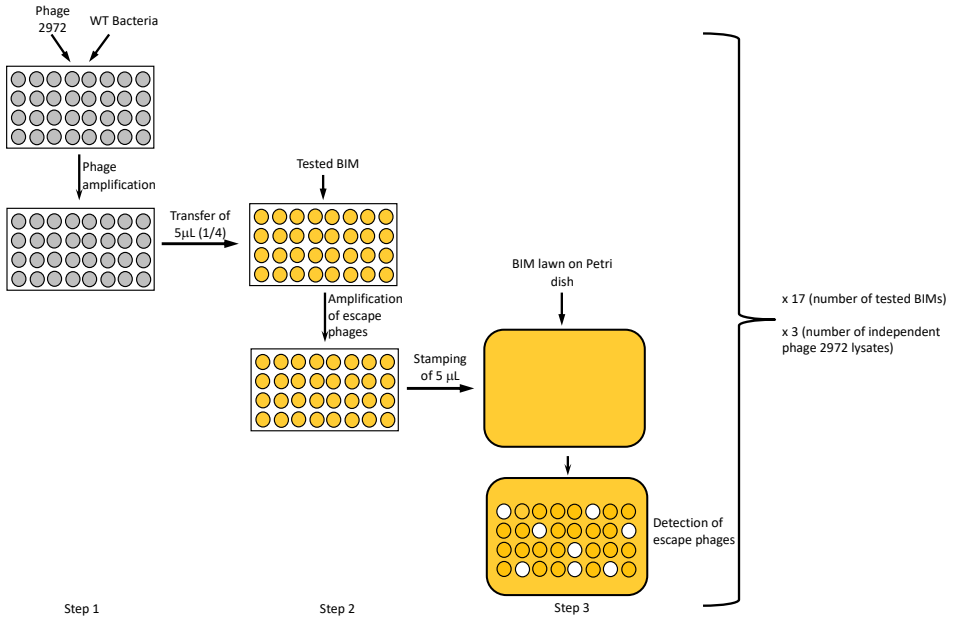
Name	Spacer sequence (5'-3')	Protospacer position in the phage genome	*orf* targeted in the phage genome
ORF_37_B	AGAAGCACCTCTTGCGTTGATAAAAGTATT	30 369 – 30 398	37
ORF_38_A	ATATTCATATTCCCTGCTCATGTTTGATAG	31 055 – 31 084	38
ORF_38_B	CTTTATACTCGTTAAGAATGGCATCTACGA	31 132 – 31 161	38
ORF_38_C	CACATATCGACGTATCGTGATTATCCCATT	31 709 – 31 737	38
ORF_44	AGCCTAGATAGCGAAGTTGATCGTATCTAT	34 587 – 34 616	44

3 Graphic overview of Luria-Delbrück protocol

The Luria-Delbrück protocol is composed of three steps. In the first step, phage 2972 is amplified on the WT bacterium (in gray) in 96 well plates (96 replicates). In a second step, 25% of the lysate of each replicate is used to inoculate a bacteria population with a single resistance (BIM, in orange). If the replicate does not contain any mutant, the phage cannot grow, but if the replicate contains even a tiny amount of escape mutants, these mutants can amplify on the resistant bacteria. This amplification allows their detection in a third step by spot assay on a lawn of resistant bacteria (BIM, in orange). The number N_E of replicates that do contain an escape mutant (in white) is recorded and yields $P_E = N_E/96$. The entire protocol has been used for 17 BIM and has been replicated three times per BIM.



4 Impact of centrifugation on phage titre in lysate

Table S3: **Impact of centrifugation on phage titre. Centrifugation does not modify phage titre.**

Titre before centrifugation (PFU/mL)	Titre after centrifugation (PFU/mL)
8.2×10^8	5.6×10^8
1.0×10^9	7.8×10^8
1.2×10^9	6.6×10^8
9.2×10^8	9.2×10^8

W=14.5, p-value=0.081 : No statistical differences in phage titer before and after centrifugation.

5 Primers used for escape phages sequencing

Table S4: **Primers used for sequencing escape phages.**
Phages are named with the following nomenclature: 2972 indicates that it is a phage derived from 2972 phage; NC or ORF indicates whether the protospacer is part of a non-coding or a coding sequence respectively. When appropriate, the number following the _ sign indicates the *orf* in which the protospacer is located. If a given *orf* contains several protospacers, they are distinguished by a capital letter.

Phage	Left Primer	Right Primer
2972_NC	TAGCGGAATTTTCACGGTCT	CCTGTAGCGGCATTTAGCTC
2972_ORF_2	CTTGCTTAGCCGTTGGGTAG	GGCTCATTTGTGGGTTGTCT
2972_ORF_5	CGGATAGGATTGCCAGCTAA	GTCATCGGTAGCACAGAGCA
2972_ORF_9	AAAACGACCGTCAACAGCTT	GTAGATGCAGCCTTGCGAAT
2972_ORF_17	AGAGCGCTAGACATGCCATT	AGAGGCGACCGAGTAAGTGA
2972_ORF_19	TCAGAGCCTTGCACAACATC	GCGGCACTTTCTTGTATGGT
2972_ORF_20	AGAGATGGAAGCCAAAGCAA	AAGATCCCGTTCTCGATGTG
2972_ORF_21	ATGGAAAGCCTAGCGTTGAA	TGTGGCTAGCTCCTTCGTTT
2972_ORF_24	TCGGATTGCTACCGAAAATC	CAATCTGCTCCACTGCGTTA
2972_ORF_27	AATACCGTGCCAAGTCTGGT	GGGATCCCATTTTCTCATTACT
2972_ORF_33	AATGTCTGCCTCAAGCGACT	GTGTGCGGAGTGCAACTAAA
2972_ORF_37_A	CTTGCATGTTCCCAATTCCT	ACCGATATCCCACTTCCAGA
2972_ORF_37_B	AAGGAATTGGGAACATGCAA	ACTCGGCTAGGGCGTTATTT
2972_ORF_38_A	TCCCATCCGTTTATGGTAGG	ACCCTCGAAAATGGGAAAGT
2972_ORF_38_B	ACCCTCGAAAATGGGAAAGT	TCCCATCCGTTTATGGTAGG
2972_ORF_38_C	TTGCCATTATCGAAGGGAAG	CGAGTGGAAACGACATCTGA
2972_ORF_44	TCGCAAGGAAATCCAAGAGT	CGTTTAACACTTTCCTTTTCAAGA

6 Sequences of escape phages protospacer

Table S5: Sequences of escape phages protospacer and PAM. Mutations are highlighted in grey and their PAM is framed. WT and lower case letters indicate if the sequence corresponds to the WT phage 2972 or an escape mutant.

Phage escape mutants	Protospacer + PAM
2972_NC_WT	AGGAGGTGGACATATTGGGCTAAATCAACGAC AGAA
2972_NC_a	AGGAGGTGGACATATTGGGCTAAATCGACGAC AGAA
2972_NC_c	AGGAGGTGGACATATTGGGCTAAATCAACGAC AGAG
2972_NC_d	AGGAGGTGGACATATTGGGCTAAACCAACGAC AGAA
2972_NC_e	AGGAGGTGGACATATTGGGCTAAATCACCGAC AGAA
2972_ORF_2_WT	GCTCTACGACTTCTTCCACGAGTTCCTGCCTC AGAA
2972_ORF_2_a	GCTCTACGACTTCTTCCACGAGTTCCTGCCTC ATAA
2972_ORF_2_b	GCTCTACGACTTCTTCCACGAGTTCCTGCCTC AAAA
2972_ORF_2_c	GCTCTACGACTTCTTCCACGAGTTCCTTCCTC AGAA
2972_ORF_5_WT	CCATCTCGTTGTCCTTACGACGACCAGACTTG AGAA
2972_ORF_5_a	CCATCTCGTTGTCCTTACGACGACCATACTTG AGAA
2972_ORF_9_WT	AGATATTGATTATGGTGTTAAAGCAGACCATA AGAA
2972_ORF_9_a	AGATATTGATTATGGTGTTAAAGCAGAGCATA AGAA
2972_ORF_9_b	AGATATTGATTATGGTGTTAAAGCAGAAAATA AGAA
2972_ORF_17_WT	AAGCAAGTTGATATATTTCTCTTTCTTTATTA AGAA
2972_ORF_17_a	AAGCAAGTTGATATATTTCTCTTTCTTTATTA AGAG
2972_ORF_17_b	AAGCAAGTTGATATATTTCTCTTTCTTTGTTA AGAA
2972_ORF_17_d	AAGCAAGTTGATATATTTCTCTTTCTTTATTA ATAA
2972_ORF_19_WT	TTATCTGATTTTTTCCCCTTGATTTCGGGGAT AGAA
2972_ORF_19_a	TTATCTGATTTTTTCCCCTTGATTTCGCGGAT AGAA
2972_ORF_19_b	TTATCTGATTTTTTCCCCTTGATTTCTTGGAT AGAA
2972_ORF_20_WT	TAAGGCAAACGAGACCGAGAGAGCTGCAGCCG AGAA
2972_ORF_20_b	TAAGGCAAACGAGACCGAGAGAGCTGCAGCCG AGAC
2972_ORF_21_WT	TTGACGATTGGGAACCGTGGAAGGAATTTGCA AGAA
2972_ORF_21_a	TTGACGATTGGGAACCGTGGAAGGAATTTGCA AGAC
2972_ORF_21_c	TTGACGATTGGGAACCGTGGAAGGAATTTGCA AGTA
2972_ORF_21_d	TTGACGATTGGGAACCGTGGAAGGAATTTGCA AAAA

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Phage escape mutants	Protospacer + PAM
2972_ORF_24_WT	AACACAGATGTTTTAGACCATGCGCAGAAGGGAGAA
2972_ORF_24_c	AACACAGATGTTTTAGACCATGCGCAGA-GGGAGAA
2972_ORF_27_WT	TATTTGTACGTGAGTGGAAGTGCTTAGACTTTAGAA
2972_ORF_27_a	TATTTGTACGTGAGTGGAAGTGCTTAGACTTTAAAA
2972_ORF_27_d	TATTTGTACGTGAGTGGAAGTGCTTAGTCTTTAGAA
2972_ORF_33_WT	TTTCATCGTCAATTTCCATGTTATAAATCTCTAGAA
2972_ORF_33_a	TTTCATCGTCAATTTCCATGTTATAAATCTCTAAAA
2972_ORF_33_b	TTTCATCGTCAATTTCCATGTTATAAATCTCTTGAA
2972_ORF_33_c	TTTCATCGTCAATTTCCATGTTATAAATTTCTAAAA
2972_ORF_37_A_WT	TCGTTTTCAGTCATTGGTGGTTTGTCAGCGAAAGAA
2972_ORF_37_A_a	TCGTTTTCAGTCATTGGTGGTTTGTCAGCGAAAGAG
2972_ORF_37_B_WT	AGAAGCACCTCTTGCGTTGATAAAAGTATTGCAGAA
2972_ORF_37_B_a	AGAAGCACCTCTTGCGTTGATAAAAGTTTTGCAGAA
2972_ORF_37_B_b	AGAAGCACCTCTTGCGTTGATAAAAGCATTGCAGAA
2972_ORF_37_B_c	AGAAGCACCTCTTGCGTTGATAAAAGTATTGCAAAA
2972_ORF_37_B_d	AGAAGCACCTCTTGCGTTGATAAAATTATTGCAGAA
2972_ORF_38_A_WT	ATATTCATATTCCCTGCTCATGTTTGATAGCAAGAA
2972_ORF_38_A_a	ATATTCATATTCCCTGCTCATGTTTGAAAGCAAGAA
2972_ORF_38_A_b	ATATTCATATTCCCTGCTCATGTTTGTTAGCAAGAA
2972_ORF_38_A_e	ATATTCATATTCCCTGCTCATGTTCGATAGCAAGAA
2972_ORF_38_B_WT	CTTTATACTCGTTAAGAATGGCATCTACGACAAGAA
2972_ORF_38_B_a	CTTTATACTCGTTAAGAATGGCATCTTCGACAAGAA
2972_ORF_38_B_c	CTTTATACTCGTTAAGAATGGCATCTACGACAATAA
2972_ORF_38_C_WT	ACATATCGACGTATCGTGATTATCCCATTCAAGAA
2972_ORF_38_C_a	ACATATCGACGTATCGTGATTATACAATTCAAGAA
2972_ORF_38_C_b	ACATATCGACGTATCGTGATTATCCCATTCATGAA
2972_ORF_38_C_c	ACATATCGACGTATCGTGATTATCCCCTTCAAGAA
2972_ORF_38_C_e	ACATATCGACGTATCGTGATTATGCCATTCAAGAA
2972_ORF_44_WT	AGCCTAGATAGCGAAGTTGATCGTATCTATTTAGAA
2972_ORF_44_b	AGCCTAGATAGCGAAGTTGATCGTATCTGTTTAGAA

7 Measure of phages fitness: determination of phage proportion during the competition experiment

The qPCR mix was composed of 3 μL of 2X Master Mix, 0.3 μL of primers at 10 μM, 1.7 μl of water and 1 μl of phages solution at 10^5 or 10^6 PFU/mL. To specifically target the referee phage (ie the phage with a 37-bp deletion), we used primers 5'-TAGACCATGCGCAGAAGGGA–3' and 5'-CCACGATTTCAACGATACGC-3'. To amplify all phages, we used 5'-GAAAATCAGCAGCAAATGGC-3' and 5'-TGACCA-CATCTTCTAAGCCGT-3'. The qPCR program was as follows: an initial denaturation at 95°C for 10 minutes, 45 amplification cycles of 15 seconds at 95°C, 20 seconds at 58°C, 25 seconds at 72°C. To obtain melting curves, temperature reached 95°C for 5 seconds, 60°C for a minute and rose to 97°C at a rate of 0.11°C per second. The DNA was cooled down at 40°C for 30 seconds. Calibration curve was obtained by applying this protocol to known-phage ten-times dilutions from 10^7 PFU/mL to 10^3 PFU/mL. To attribute an absolute number of phages to each qPCR point, these dilutions were titrated simultaneously (see above).

In our collection, the Reference phage is targeted by the BIM that target *orf24* and carries a 37 bp deletion in its protospacer.

8 Durability of CRISPR resistances

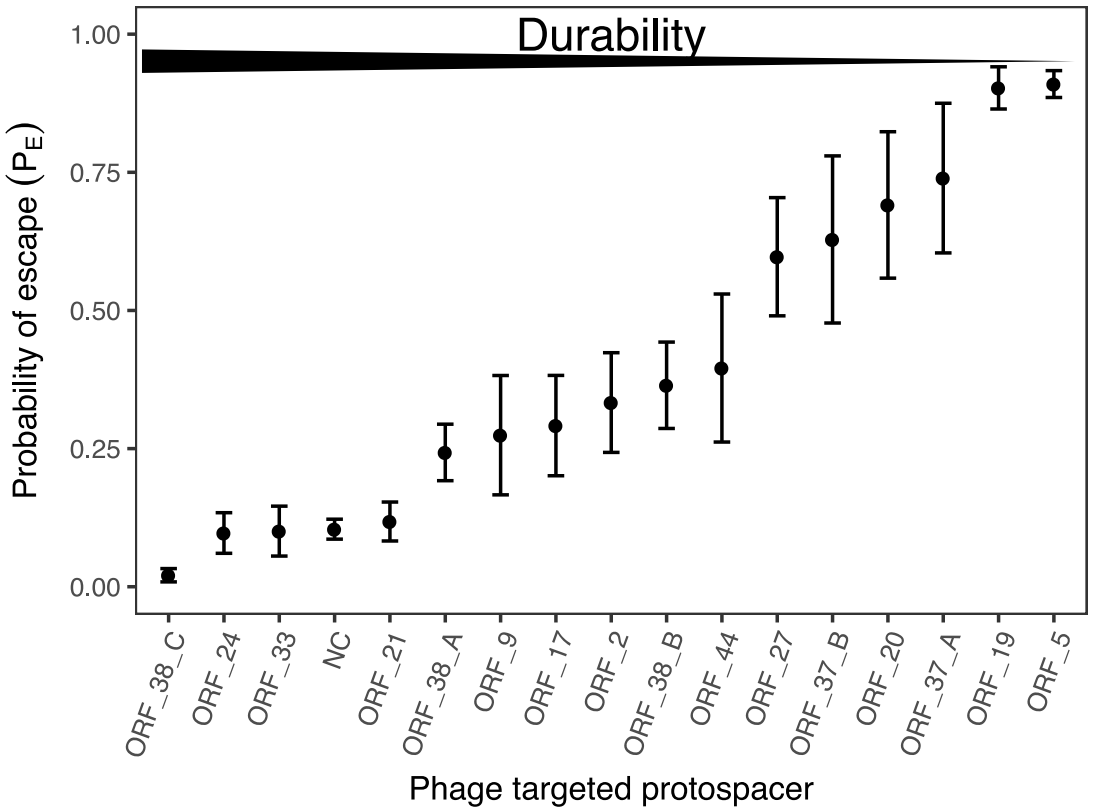

Figure S1: **Variability in the durability of CRISPR–Cas immunity.** The probability of escape P_E was measured for each BIM using fluctuation tests. Mutation rates of each protospacer can be found in supplementary information 9.

9 Mutation rate of CRISPR-targeted sequences

Table S6: Mutation rate and 95% Confidence Intervals of phage protospacers. Mutation rates were measured using fluctuation tests (see Materials and Methods).

Name	Mutation rate	95% Confidence Interval
NC	4.9×10^{-8}	$[3.1 \times 10^{-8}, 6.7 \times 10^{-8}]$
ORF_2	1.9×10^{-7}	$[6.8 \times 10^{-8}, 3.1 \times 10^{-7}]$
ORF_5	1.1×10^{-6}	$[8.6 \times 10^{-7}, 1.3 \times 10^{-6}]$
ORF_9	1.5×10^{-7}	$[2.7 \times 10^{-8}, 2.8 \times 10^{-7}]$
ORF_17	1.6×10^{-7}	$[4.5 \times 10^{-8}, 2.8 \times 10^{-7}]$
ORF_19	1.2×10^{-6}	$[6.2 \times 10^{-7}, 1.7 \times 10^{-6}]$
ORF_20	6×10^{-7}	$[2.5 \times 10^{-7}, 9.5 \times 10^{-7}]$
ORF_21	5.7×10^{-8}	$[2.1 \times 10^{-8}, 9.2 \times 10^{-8}]$
ORF_24	4.6×10^{-8}	$[1.0 \times 10^{-8}, 8.2 \times 10^{-8}]$
ORF_27	4.3×10^{-7}	$[2.2 \times 10^{-7}, 6.5 \times 10^{-7}]$
ORF_33	4.8×10^{-8}	$[4.6 \times 10^{-9}, 9.2 \times 10^{-8}]$
ORF_37_A	7.1×10^{-7}	$[2.9 \times 10^{-7}, 1.1 \times 10^{-6}]$
ORF_37_B	5.1×10^{-7}	$[1.6 \times 10^{-7}, 8.6 \times 10^{-7}]$
ORF_38_A	1.3×10^{-7}	$[6.5 \times 10^{-8}, 1.9 \times 10^{-7}]$
ORF_38_B	2.1×10^{-7}	$[9.3 \times 10^{-8}, 1.3 \times 10^{-7}]$
ORF_38_C	9.4×10^{-9}	$[-1.2 \times 10^{-9}, 2.0 \times 10^{-8}]$
ORF_44	2.5×10^{-7}	$[5.2 \times 10^{-8}, 4.4 \times 10^{-7}]$

10 Mutation profile of escape phages

Table S7: Profile of substitutions carried by phage escape mutants. 27 substitutions are transversions and 16 are transitions.

Type of substitution	Substitution	Number of occurences
Purine Transition	A → G	6
	G → A	6
Pyrimidine Transition	C → T	1
	T → C	3
Transversion	A → C	4
	C → A	4
	A → T	7
	T → A	1
	T → G	0
	G → T	8
	G → C	1
	C → G	2

11 Escape phage relative fitness

Table S8: Relative fitness of escape phages. Deleterious mutations are highlighted in dark grey and neutral mutation in medium grey.

Phage escape mutants	Relative Fitness
2972_NC_a	0.688
2972_NC_c	-2.135
2972_NC_d	-2.072
2972_NC_e	-2.590
2972_ORF_2_a	-3.086
2972_ORF_2_b	-1.573
2972_ORF_2_c	-0.754
2972_ORF_5_a	-5.005
2972_ORF_9_a	-1.134
2972_ORF_9_b	-1.056
2972_ORF_17_a	-1.462
2972_ORF_17_b	-3.013
2972_ORF_17_d	-3.864
2972_ORF_19_a	-2.424
2972_ORF_19_b	-4.333
2972_ORF_20_b	-2.959
2972_ORF_21_a	-4.134
2972_ORF_21_c	-4.431
2972_ORF_21_d	-2.401
2972_ORF_24_c	-3.074
2972_ORF_27_a	-0.093
2972_ORF_27_d	-0.890
2972_ORF_33_a	-2.861
2972_ORF_33_b	-0.426
2972_ORF_33_c	-0.657
2972_ORF_37_A_a	-0.840
2972_ORF_37_B_a	-5.290
2972_ORF_37_B_b	0.458
2972_ORF_37_B_c	-2.365

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Phage escape mutants	Relative Fitness
2972_ORF_37_B_d	-1.940
2972_ORF_38_A_a	-4.355
2972_ORF_38_A_b	-1.311
2972_ORF_38_A_e	-1.652
2972_ORF_38_B_a	0.415
2972_ORF_38_B_c	-4.282
2972_ORF_38_C_a	-3.166
2972_ORF_38_C_b	-6.212
2972_ORF_38_C_c	-2.674
2972_ORF_38_C_e	-0.215
2972_ORF_44_b	0.150

12 Impact of synonymous mutations on the fitness of phage escape mutants

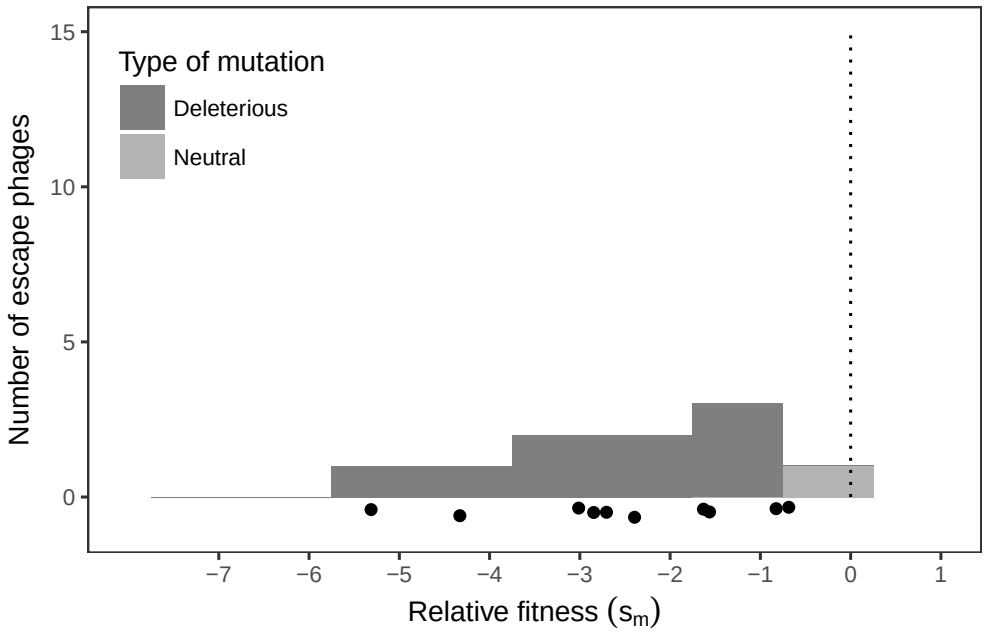

Figure S2: **Distribution of fitness effects of synonymous escape mutations in the phage.**

Relative fitness was measured through competition experiments with a collection of 10 escape phages with a synonymous mutation on their seed or PAM sequences. Phages that carry a neutral and deleterious mutations are represented in medium and dark grey respectively. Black dots show the relative fitness of each escape phage. The dotted segment represents the fitness of WT phage 2972. Fitness value of each escape phage is also provided in the supplementary informations 11.