SUPPLEMENTARY MATERIALS.

Supplementary text: Analysis of deep sequencing data.

Randomized PAM

For the randomized PAM experiment 3,429,406 reads were obtained for crR6 and 3,253,998 for R6. It is expected that only half of them will correspond to the PAM-target while the other half will sequence the other end of the PCR product. 1,623,008 of the crR6 reads and 1,537,131 of the R6 reads carried an error-free target sequence. The occurrence of each possible PAM among these reads is shown in supplementary file. To estimate the functionality of a PAM, its relative proportion in the crR6 sample over the R6 sample was computed and is denoted r_{ijklm} where I,j,k,l,m are one of the 4 possible bases. The following statistical model was constructed:

$\log(r_{ijklm}) = \mu + b2_i + b3_j + b4_k + b2b3_{i,j} + b3b4_{j,k} + \varepsilon_{ijklm}$,

where ε is the residual error, b2 is the effect of the 2nd base of the PAM, b3 of the third, b4 of the fourth, b2b3 is the interaction between the second and third bases, b3b4 between the third and fourth bases. An analysis of variance was performed:

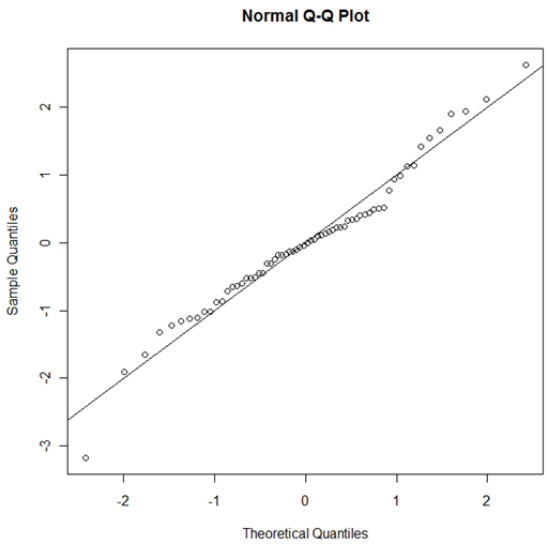
Anova table

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
b3	3	151.693	50.564	601.8450	< 2.2e-16	***
b2	3	90.521	30.174	359.1454	< 2.2e-16	***
b4	3	1.881	0.627	7.4623	6.070e-05	***
b3:b2	9	228.940	25.438	302.7738	< 2.2e-16	***
b3:b4	9	3.010	0.334	3.9809	5.227e-05	***
Residuals	996	83.680	0.084			

When added to this model, b1 or b5 do not appear to be significant and other interactions than the ones included can also be discarded. The model choice was made through successive comparisons of more or less complete models using the anova method in R. Tukey's honest significance test was used to determine if pairwise differences between effects are significant.

NGGNN patterns are significantly different from all other patterns and carry the strongest effect (see table below).

In order to show that positions 1, 4 or 5 do not affect the NGGNN pattern we looked at theses sequences only. Their effect appears to be normally distributed (see QQ plot below), and model comparisons using the anova method in R shows that the null model is the best one, i.e. there is no significant role of b1, b4 and b5.



QQ plot for the NGGNN sequences

Model comparison using the anova method in R for the NGGNN sequences

```
Model 1: ratio.log ~ 1
Model 2: ratio.log ~ b1 + b4 + b5
  Res.Df    RSS Df Sum of Sq      F Pr(>F)
1     63 14.579
2     54 11.295  9    3.2836 1.7443 0.1013
```

Partial interference of NAGNN and NNGGN patterns

NAGNN patterns are significantly different from all other patterns but carry a much smaller effect than NGGNN (see Tukey's honest significance test below).

Finally, NTGGN and NCGGN patterns are similar and show significantly more CRISPR interference than NTGHN and NCGHN patterns (where H is A,T or C), as shown by a bonferroni adjusted pairwise student-test.

Pairwise comparisons of the effect of b4 on NYGNN sequences using t tests with pooled SD

```
Data: b4

  A       C       G
C 1.00    -       -
G 9.2e-05 2.4e-06 -
T 0.31    1.00    1.2e-08
```

Taken together, these results allow concluding that NNGGN patterns in general produce either a complete interference in the case of NGGGN, or a partial interference in the case of NAGGN, NTGGN or NCGGN.

Tukey multiple comparisons of means

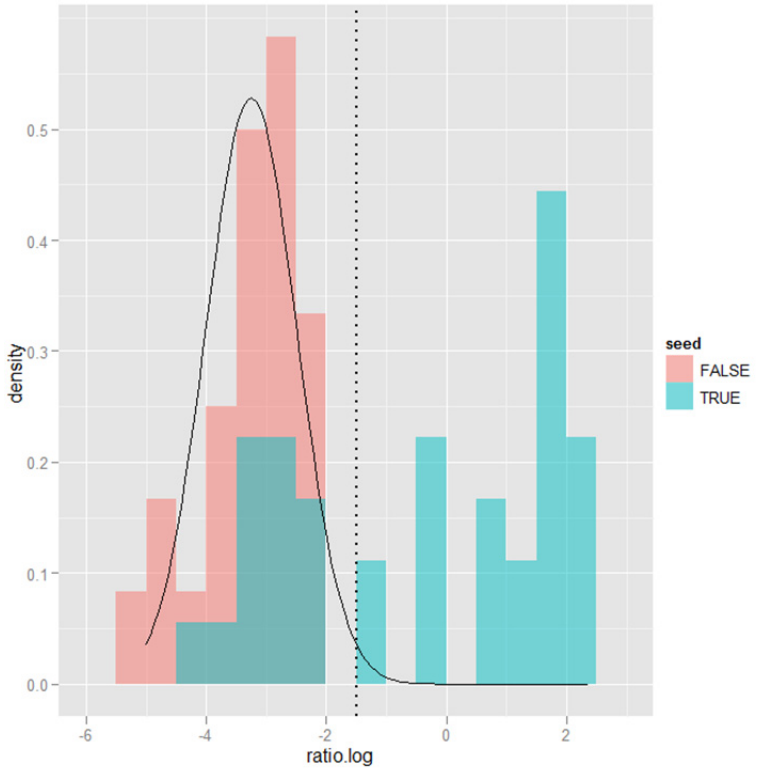
95% family-wise confidence level

```
$b2:b3

          diff      lwr       upr       p adj
G:G-A:A -2.76475 -2.94075 -2.58875 <1E -07
G:G-C:A -2.79911 -2.97511 -2.62311 <1E -07
G:G-T:A  -2.7809  -2.9569  -2.6049 <1E -07
G:G-A:C -2.81643 -2.99244 -2.64043 <1E -07
G:G-C:C -2.77903 -2.95504 -2.60303 <1E -07
G:G-G:C -2.64867 -2.82468 -2.47267 <1E -07
G:G-T:C -2.79718 -2.97319 -2.62118 <1E -07
G:G-A:G -2.67068 -2.84668 -2.49468 <1E -07
G:G-C:G -2.73525 -2.91125 -2.55925 <1E -07
G:G-T:G  -2.7976 -2.62159  -2.9736 <1E -07
G:G-A:T -2.76727 -2.59127 -2.94328 <1E -07
G:G-C:T -2.84114 -2.66513 -3.01714 <1E -07
G:G-G:T -2.76409 -2.58809 -2.94009 <1E -07
G:G-T:T -2.76781 -2.59181 -2.94381 <1E -07
G:G-G:A -2.13964 -2.31565 -1.96364 <1E -07
G:A-A:A -0.62511 -0.80111  -0.4491 <1E -07
```

```
G:A-C:A -0.65947 -0.83547 -0.48346 <1E -07
G:A-T:A -0.64126 -0.46525 -0.81726 <1E -07
G:A-A:C -0.67679 -0.50078 -0.85279 <1E -07
G:A-C:C -0.63939 -0.46339 -0.81539 <1E -07
G:A-G:C -0.50903 -0.33303 -0.68503 <1E -07
G:A-T:C -0.65754 -0.48154 -0.83354 <1E -07
G:A-A:G -0.53104 -0.35503 -0.70704 <1E -07
G:A-C:G -0.59561  -0.4196 -0.77161 <1E -07
G:A-T:G -0.65795 -0.48195 -0.83396 <1E -07
G:A-A:T -0.62763 -0.45163 -0.80363 <1E -07
G:A-C:T -0.70149 -0.52549  -0.8775 <1E -07
G:A-G:T -0.62445 -0.44844 -0.80045 <1E -07
G:A-T:T -0.62817 -0.45216 -0.80417 <1E -07


$b3:b4
         diff      lwr       upr       p adj
G:G-G:A -0.33532 -0.51133 -0.15932   <1E-07
G:G-G:C -0.18118 -0.35719 -0.00518 0.036087
G:G-G:T -0.31626 -0.14026 -0.49226   <1E-07
```

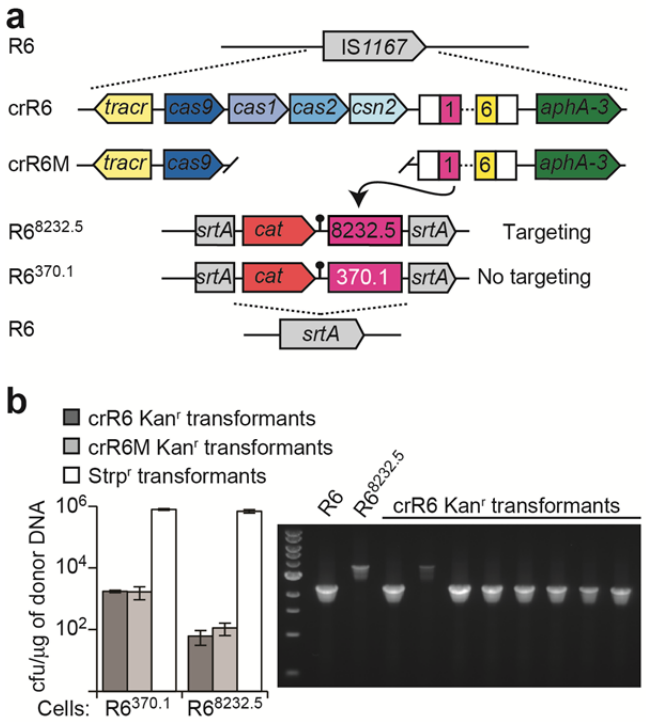
Randomized target

For the randomized target experiment 540,726 reads were obtained for crR6 and 753,570 for R6. As before, only half of the reads are expected to sequence the interesting end of the PCR product. After filtering for reads that carry a target that is error-free or with a single point mutation, 217,656 and 353,141 reads remained for crR6 and R6 respectively. The relative proportion of each mutant in the crR6 sample over the R6 sample was computed (figure 2C). All mutations outside of the seed sequence (13-20 bases away from the PAM) show full interference. Those sequences were used as a reference to determine if other mutations inside the seed sequence can be said to significantly disrupt interference. A normal distribution was fitted to theses sequences using the fitdistr function of the MASS R package. The 0.99 quantile of the fitted distribution is shown as a dotted line in Fig. 2C.

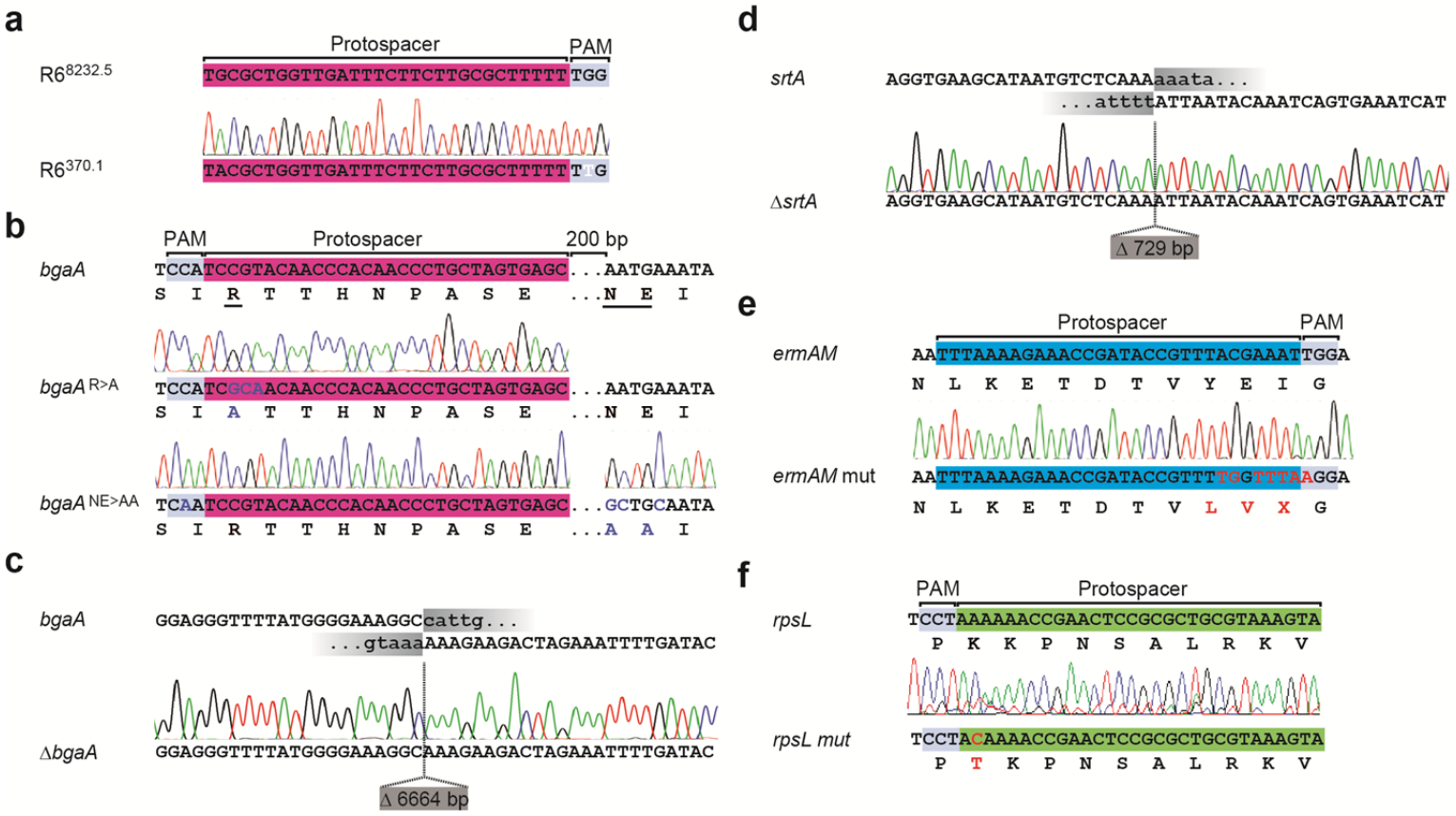


Histogram of the data density with fitted normal distribution (black line) and .99 quantile (dotted line)

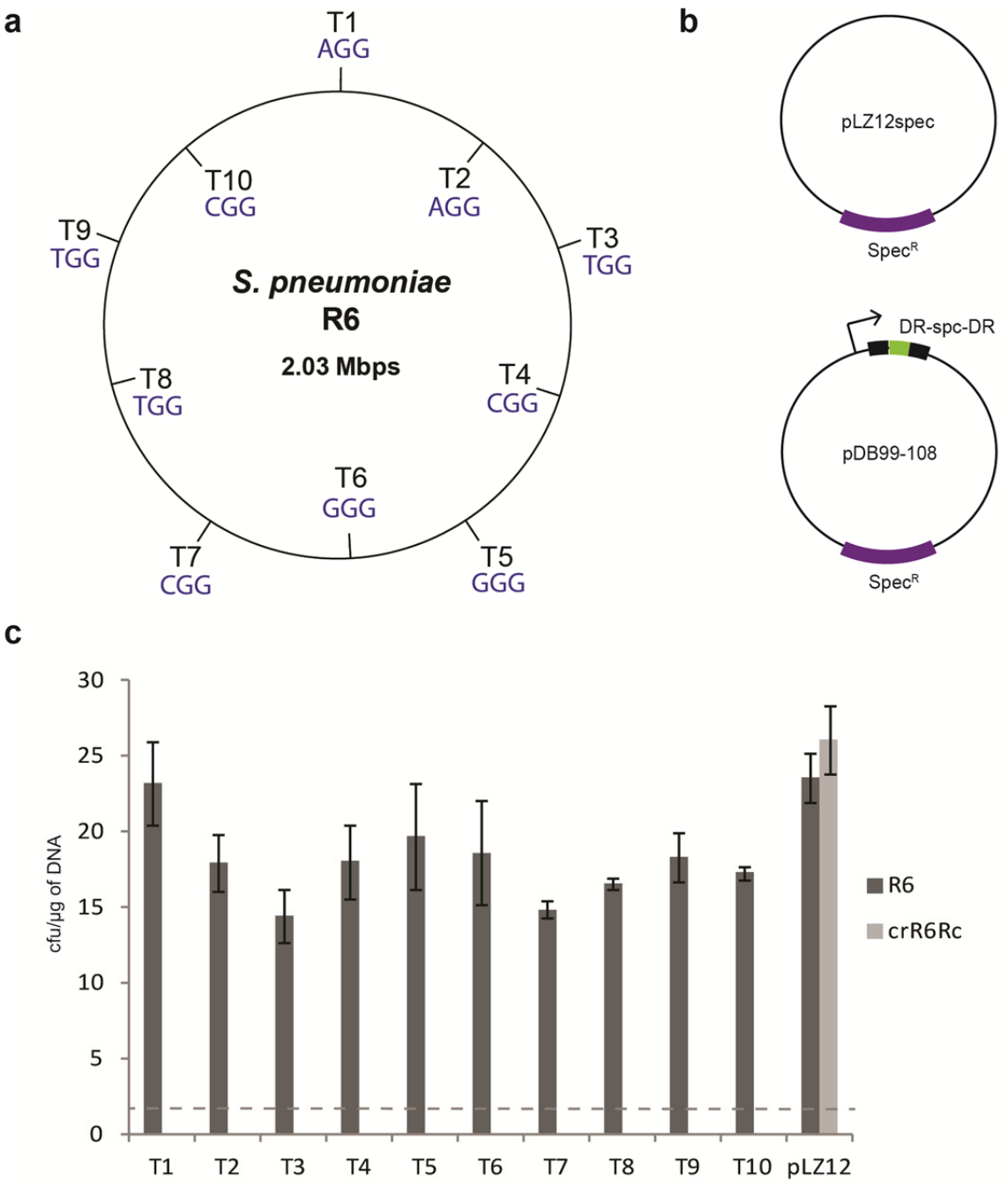
Supplementary figures.



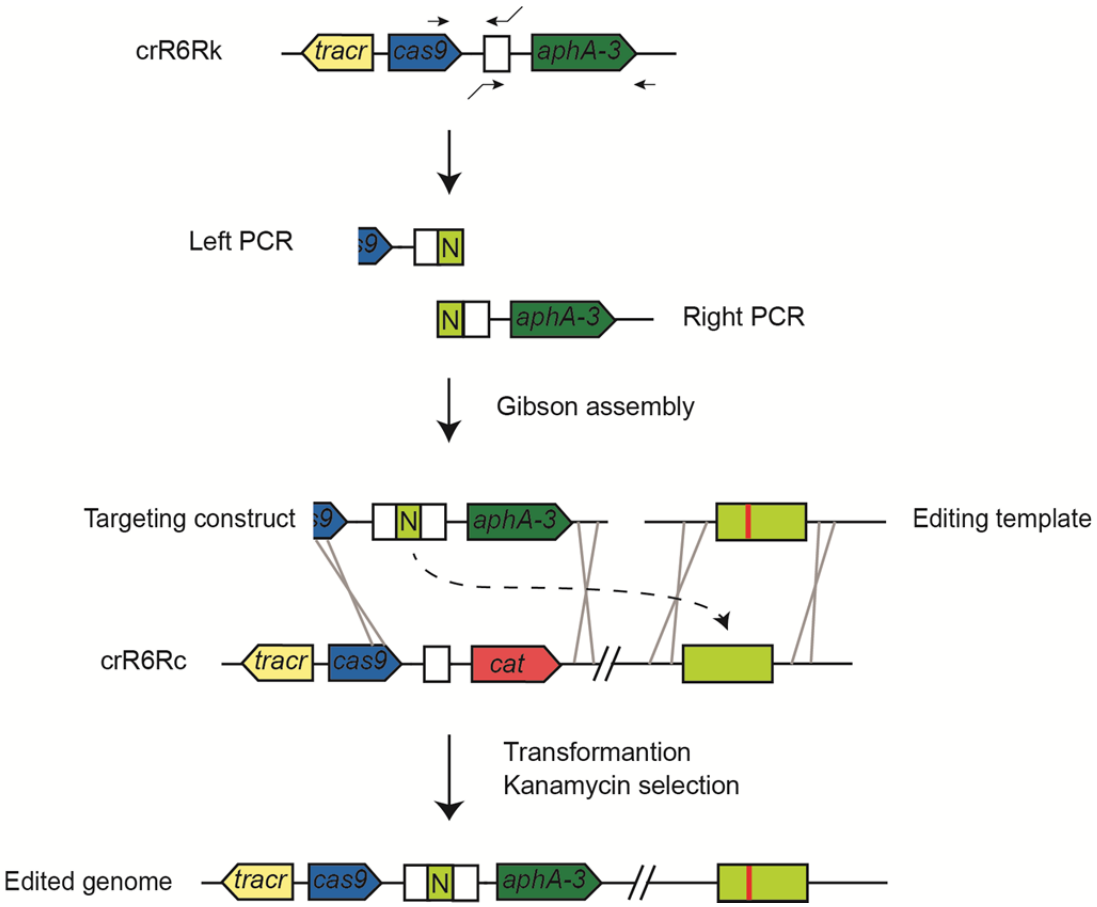
Supplementary Fig. 1. Transformation of crR6 genomic DNA leads to editing of the targeted locus **(a)** The *IS1167* element of *S. pneumoniae* R6 was replaced by the CRISPR01 locus of *S. pyogenes* SF370 to generate crR6 strain. This locus encodes for the Cas9 nuclease, a CRISPR array with six spacers, the tracrRNA that is required for crRNA biogenesis and Cas1, Cas2 and Csn2, proteins not necessary for targeting. Strain crR6M contains a minimal functional CRISPR system without *cas1*, *cas2* and *csn2*. The *aphA-3* gene encodes kanamycin resistance. Protospacers from the streptococcal bacteriophages ϕ8232.5 and ϕ370.1 were fused to a chloramphenicol resistance gene (*cat*) and integrated in the *srtA* gene of strain R6 to generate strains $R6^{8232.5}$ and $R6^{370.1}$. (**b**) Left panel: Transformation of crR6 and crR6M genomic DNA in $R6^{8232.5}$ and $R6^{370.1}$. As a control of cell competence a streptomycin resistant gene was also transformed. Right panel: PCR analysis of 8 $R6^{8232.5}$ transformants with crR6 genomic DNA. Primers that amplify the *srtA* locus were used for PCR. 7/8 genotyped colonies replaced the $R6^{8232.5}$ srtA locus by the WT locus from the crR6 genomic DNA.



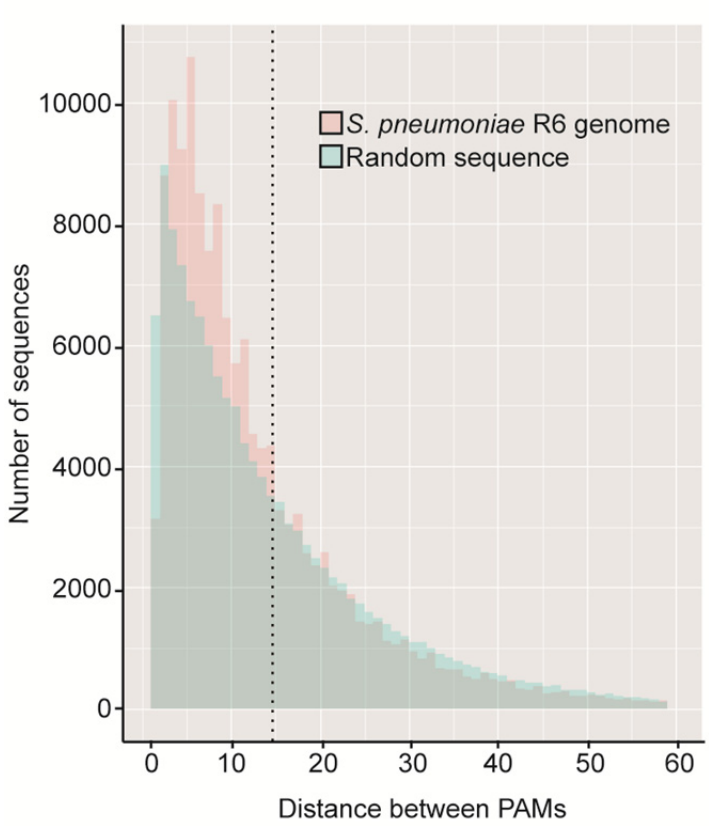
Supplementary Fig. 2. Chromatograms of DNA sequences of edited cells obtained in this study. In all cases the wild-type and mutant protospacer and PAM sequences (or their reverse complement) are indicated. When relevant, the amino acid sequence encoded by the protospacer is provided. For each editing experiment, all strains for which PCR and restriction analysis corroborated the introduction of the desired modification were sequenced. A representative chromatogram is shown. **(a)** Chromatogram for the introduction of a PAM mutation into the R6$^{8232.5}$ target (Fig. 1d). **(b)** Chromatograms for the introduction of the R>A and NE>AA mutations into β-galactosidase (*bgaA*) (Fig. 3c). **(c)** Chromatogram for the introduction of a 6664 bp deletion within *bgaA* ORF (Figs. 3c and 3f). The dotted line indicates the limits of the deletion. **(d)** Chromatogram for the introduction of a 729 bp deletion within *srtA* ORF (Fig. 3f). The dotted line indicates the limits of the deletion. **(e)** Chromatograms for the generation of a premature stop codon within *ermAM* (Supplementary Fig. 6). **(f)** *rpsL* editing in *E. coli* (Fig. 5).



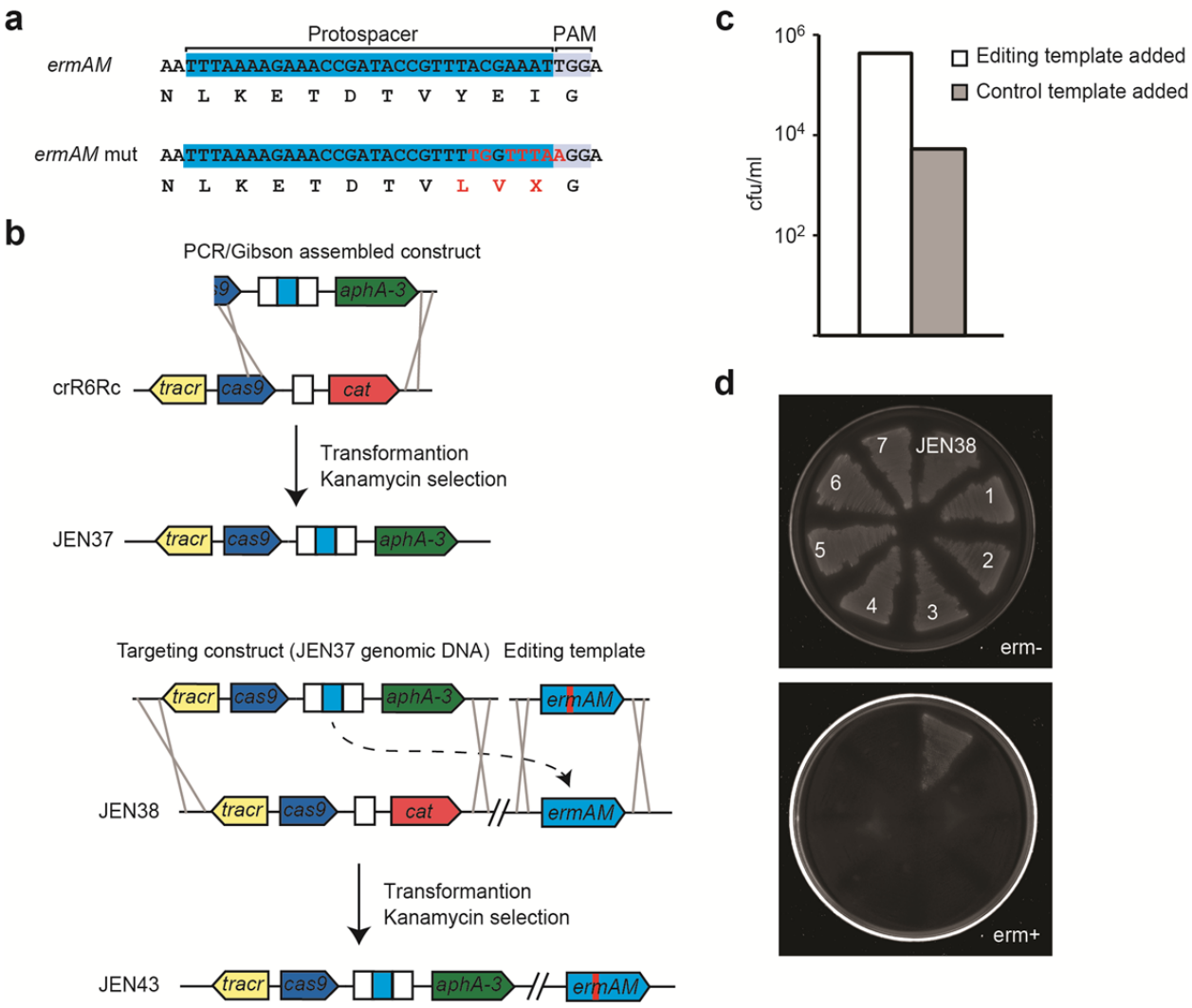
Supplementary Fig. 3. CRISPR immunity against random *S. pneumoniae* targets containing different PAMs. **(a)** Position of the 10 random targets on the *S. pneumoniae* R6 genome. The chosen targets have different PAMs and are on both strands. **(b)** Spacers corresponding to the targets were cloned in a minimal CRISPR array on plasmid pLZ12 and transformed into strain crR6Rc, which supplies the processing and targeting machinery in trans. **(c)** Transformation efficiency of the different plasmids in strain R6 and crR6Rc. No colonies were recovered for the transformation of pDB99-108 (T1-T10) in crR6Rc. The dashed line represents limit of detection of the assay.



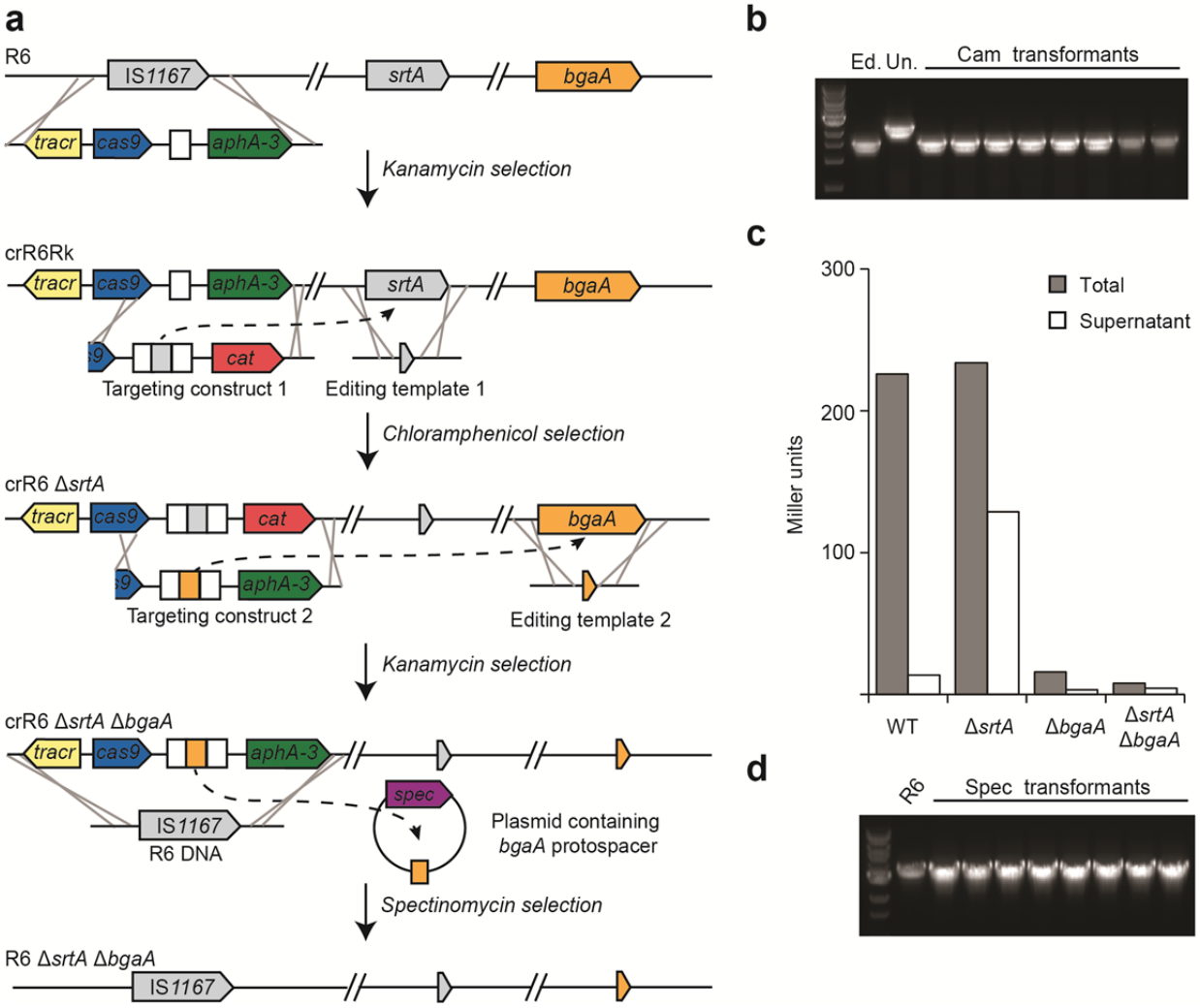
Supplementary Fig. 4. A general scheme for targeted genome editing. To facilitate targeted genome editing, crR6M was further engineered to contain tracrRNA, Cas9 and only one repeat of the CRISPR array followed by kanamycin resistance marker (*aphA-3*), generating strain crR6Rk. DNA from this strain is used as a template for PCR with primers designed to introduce a new spacer (green box designated with N). The left and right PCRs are assembled using the Gibson method to create the targeting construct. Both the targeting and editing constructs are then transformed into strain crR6Rc, which is a strain equivalent to crR6Rk but has the kanamycin resistance marker replaced by a chloramphenicol resistance marker (*cat*). About 90 % of the kanamycin-resistant transformants contain the desired mutation.



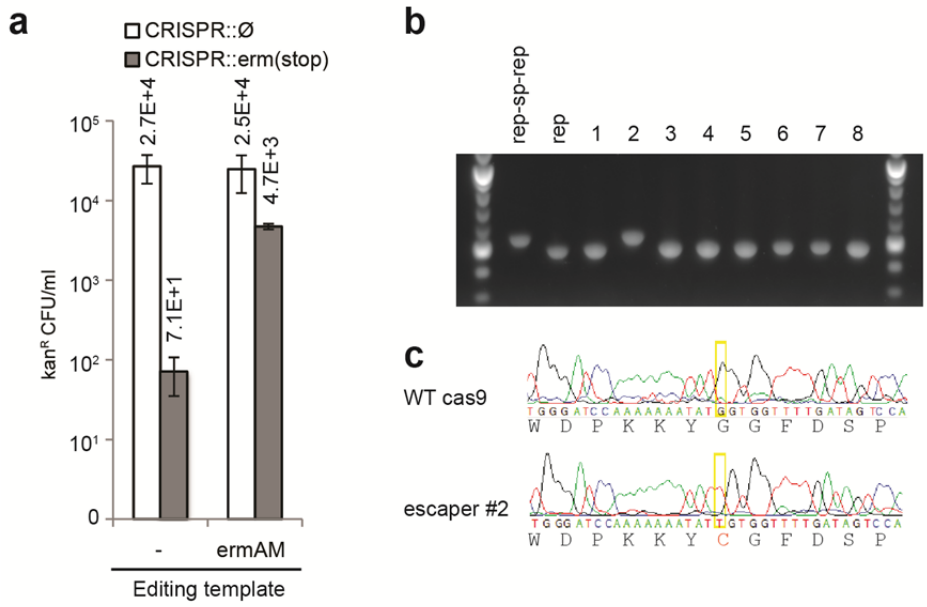
Supplementary Fig. 5. Distribution of distances between PAMs**.** NGG and CCN are considered to be valid PAMs. Data is shown for the *S. pneumoniae* R6 genome as well as for a random sequence of the same length and with the same GC-content (39.7 %). The dotted line represents the average distance (12) between PAMs in the R6 genome.



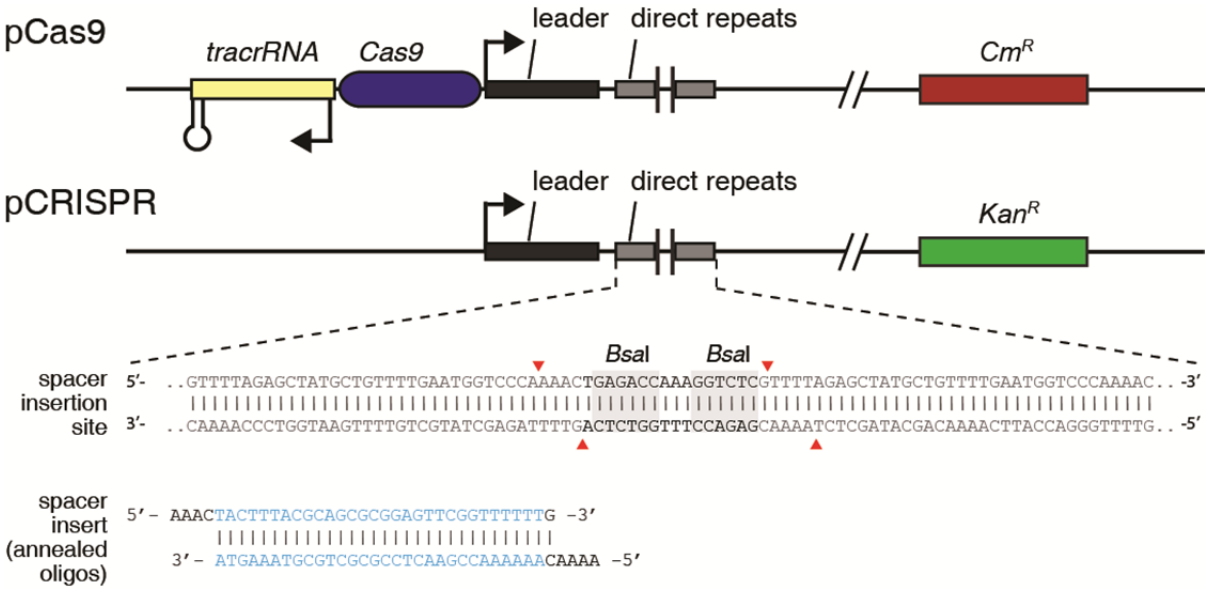
Supplementary Fig. 6. CRISPR-mediated editing of the *ermAM* locus using genomic DNA as targeting construct. To use genomic DNA as targeting construct it is necessary to avoid CRISPR autoimmunity, and therefore a spacer against a sequence not present in the chromosome must be used (in this case the *ermAM* erythromycin resistance gene). **(a)** Nucleotide and amino acid sequences of the wild-type and mutated (red letters) *ermAM* gene. The protospacer and PAM sequences are shown. **(b)** A schematic for CRISPR-mediated editing of the *ermAM* locus using genomic DNA. A construct carrying an *ermAM*-targeting spacer (blue box) is made by PCR and Gibson assembly, and transformed into strain crR6Rc, generating strain JEN37. The genomic DNA of JEN37 is then used as a targeting construct, and is co-transformed with the editing template into JEN38, a strain in which the *srtA* gene was replaced by a wild-type copy of *ermAM*. Kanamycin-resistant transformants contain the edited genotype (JEN43). **(c)** Number of kanamycin-resistant cells obtained after co-transformation of targeting and editing or control templates. In the presence of the control template 5.4×10^3 cfu/ml were obtained, and 4.3×10^5 cfu/ml when the editing template was used. This difference indicates an editing efficiency of about 99 % $[(4.3\times10^5\text{-}5.4\times10^3)/4.3\times10^5]$. **(d)** To check for the presence of edited cells seven kanamycin-resistant clones and JEN38 were streaked on agar plates with (erm+) or without (erm–) erythromycin. Only the positive control displayed resistance to erythromycin. The *ermAM* mut genotype of one of these transformants was also verified by DNA sequencing (Supplementary Fig. 1E).



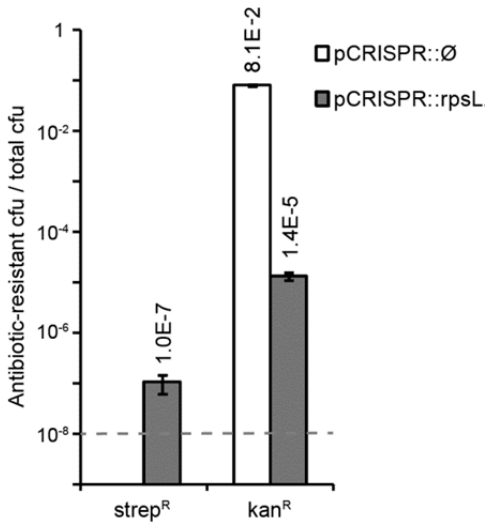
Supplementary Fig. 7. Sequential introduction of mutations by CRISPR-mediated genome editing. **(a)** A schematic for sequential introduction of mutations by CRISPR-mediated genome editing. First, R6 is engineered to generate crR6Rk. crR6Rk is co-transformed with a *srtA*-targeting construct fused to *cat* for chloramphenicol selection of edited cells, along with an editing construct for a Δ*srtA* in-frame deletion. Strain crR6 Δ*srtA* is generated by selection on chlramphenicol. Subsequently, the Δ*srtA* strain is co-transformed with a *bgaA*-targeting construct fused to *aphA-3* for kanamycin selection of edited cells, and an editing construct containing a Δ*bgaA* in-frame deletion. Finally, the engineered CRISPR locus can be erased from the chromosome by first co-transforming R6 DNA containing the wild-type *IS1167* locus and a plasmid carrying a *bgaA* protospacer (pDB97), and selection on spectinomycin. **(b)** PCR analysis for 8 chloramphenicol (Cam)-resistant transformants to detect the deletion in the *srtA* locus. **(c)** β-galactosidase activity as measured by Miller assay. In *S. pneumoniae*, this enzyme is anchored to the cell wall by sortase A. Deletion of the *srtA* gene results in the release of β-galactosidase into the supernatant. Δ*bgaA* mutants show no activity. **(d)** PCR analysis for 8 spectinomycin (Spec)-resistant transformants to detect the replacement of the CRISPR locus by wild-type *IS1167*.



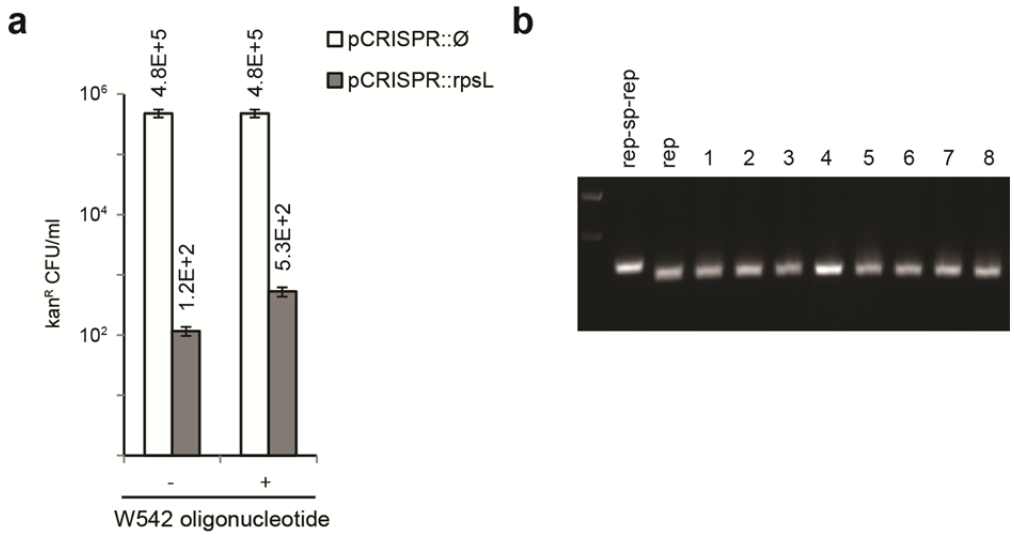
Supplementary Fig. 8. Background mutation frequency of CRISPR in *S. pneumoniae*. **(a)** Transformation of the CRISPR::Ø or CRISPR::erm(stop) targeting constructs in JEN53, with or without the ermAM editing template. The difference in kan^R CFU between CRISPR::Ø and CRISPR::erm(stop) indicates that Cas9 cleavage kills non-edited cells. Mutants that escape CRISPR interference in the absence of editing template are observed at a frequency of 3×10^{-3}. **(b)** PCR analysis of the CRISPR locus of escapers shows that 7/8 have a spacer deletion. **(c)** Escaper #2 carries a point mutation in *cas9*.



Supplementary Fig. 9. The essential elements of the *S. pyogenes* CRISPR locus 1 are reconstituted in *E. coli* using pCas9. The plasmid contains tracrRNA, Cas9, as well as a leader sequence driving the crRNA array. The pCRISPR plasmids contains the leader and the array only. Spacers can be inserted into the crRNA array between *Bsa*I sites using annealed oligonucleotides. Oligonucleotide design is shown at bottom. pCas9 carries chloramphenicol resistance (CmR) and is based on the low-copy pACYC184 plasmid backbone. pCRISPR is based on the high-copy number pZE21 plasmid. Two plasmids are required because a pCRISPR plasmid containing a spacer targeting the *E. coli* chromosome cannot be constructed using this organism as a cloning host if Cas9 is also present (it will kill the host).



Supplementary Fig. 10. CRISPR-directed editing in *E.coli* MG1655. An oligonucleotide (W542) carrying a point mutation that both confers streptomycin resistance and abolishes CRISPR immunity, together with a plasmid targeting *rpsL* (pCRISPR::rpsL) or a control plasmid (pCRISPR::Ø) were co-transformed into wild-type *E.coli* strain MG1655 containing pCas9. Transformants were selected on media containing either streptomycin or kanamycin. Dashed line indicates limit of detection of the transformation assay.



Supplementary Fig. 11. Background mutation frequency of CRISPR in *E. coli* HME63. **(a)** Transformation of the pCRISPR::Ø or pCRISPR::rpsL plasmids into HME63 competent cells. Mutants that escape CRISPR interference are observed at a frequency of 2.6×10^{-4}. **(b)** Amplification of the CRISPR array of escapers showing that 8/8 have deleted the spacer.

Supplementary tables

Table S1. Relative abundance of PAM sequences in the crR6/R6 samples averaged over bases 1 and 5.

2nd position		3rd position: A		C		G		T		4th position
	A	AAA	1.04	ACA	1.12	AGA	0.73	ATA	1.10	A
		AAC	1.07	ACC	1.04	AGC	0.64	ATC	0.97	C
		AAG	1.00	ACG	1.09	AGG	0.61	ATG	1.07	G
		AAT	0.98	ACT	1.02	AGT	0.65	ATT	1.01	T
	C	CAA	1.05	CCA	1.05	CGA	0.99	CTA	1.07	A
		CAC	1.04	CCC	1.02	CGC	1.08	CTC	1.04	C
		CAG	1.08	CCG	1.08	CGG	0.81	CTG	1.05	G
		CAT	1.13	CCT	1.05	CGT	1.07	CTT	1.08	T
	G	GAA	0.97	GCA	1.05	**GGA**	**0.08**	GTA	0.99	A
		GAC	0.92	GCC	1.00	**GGC**	**0.05**	GTC	1.15	C
		GAG	0.96	GCG	0.98	**GGG**	**0.07**	GTG	0.98	G
		GAT	0.98	GCT	0.99	**GGT**	**0.06**	GTT	1.05	T
	T	TAA	1.08	TCA	1.16	TGA	1.05	TTA	1.14	A
		TAC	1.00	TCC	1.08	TGC	1.08	TTC	1.05	C
		TAG	1.02	TCG	1.11	TGG	0.77	TTG	1.01	G
		TAT	1.01	TCT	1.12	TGT	1.21	TTT	1.02	T

Table S2. Primers used in this study.

Primer	Sequence 5'-3'
B217	TCCTAGCAGGATTTCTGATATTACTGTCACGTTTTAGAGCTATGCTGTTTTGA
B218	GTGACAGTAATATCAGAAATCCTGCTAGGAGTTTTGGGACCATTCAAAACAGC
B229	GGGTTTCAAGTCTTTGTAGCAAGAG
B230	GCCAATGAACGGGAACCCTTGGTC
B250	NNNNGACGAGGCAATGGCTGAAATC
B251	NNNNTTATTTGGCTCATATTTGCTG
B255	CTTTACACCAATCGCTGCAACAGAC
B256	CAAAATTTCTAGTCTTCTTTGCCTTTCCCCATAAAACCCTCCTTA
B257	AGGGTTTTATGGGGAAAGGCAAAGAAGACTAGAAATTTTGATACC
B258	CTTACGGTGCATAAAGTCAATTTCC
B269	TGGCTCGATTTCAGCCATTGC
B270	CTTTGACGAGGCAATGGCTGAAATCGAGCCAANAAAGCGCAAG
B271	CTTTGACGAGGCAATGGCTGAAATCGAGCCAAANAAGCGCAAG
B272	CTTTGACGAGGCAATGGCTGAAATCGAGCCAAAANAGCGCAAG
B273	CTTTGACGAGGCAATGGCTGAAATCGAGCCAAAAANGCGCAAG
B274	CTTTGACGAGGCAATGGCTGAAATCGAGCCAAAAAANCGCAAG
B275	CTTTGACGAGGCAATGGCTGAAATCGAGCCAAAAAAGNGCAAG
B276	CTTTGACGAGGCAATGGCTGAAATCGAGCCAAAAAAGCNCAAGAAG
B277	CTTTGACGAGGCAATGGCTGAAATCGAGCCAAAAAAGCGNAAGAAG
B278	CTTTGACGAGGCAATGGCTGAAATCGAGCCAAAAAAGCGCNAGAAG
B279	GCGCTTTTTTGGCTCGATTTCAG
B280	CAATGGCTGAAATCGAGCCAAAAAAGCGCANGAAGAAATC
B281	CAATGGCTGAAATCGAGCCAAAAAAGCGCAANAAGAAATC
B282	CAATGGCTGAAATCGAGCCAAAAAAGCGCAAGNAGAAATC
B283	CAATGGCTGAAATCGAGCCAAAAAAGCGCAAGANGAAATC
B284	CAATGGCTGAAATCGAGCCAAAAAAGCGCAAGAANAAATC
B285	CAATGGCTGAAATCGAGCCAAAAAAGCGCAAGAAGNAATCAACC
B286	CAATGGCTGAAATCGAGCCAAAAAAGCGCAAGAAGANATCAACC
B287	CAATGGCTGAAATCGAGCCAAAAAAGCGCAAGAAGAANTCAACC
B288	CAATGGCTGAAATCGAGCCAAAAAAGCGCAAGAAGAAANCAACC
B289	CAATGGCTGAAATCGAGCCAAAAAAGCGCAAGAAGAAATNAACCAGC
B290	CAATGGCTGAAATCGAGCCAAAAAAGCGCAAGAAGAAATCNACCAGC
B296	gatccTCCATCCGTACAACCCACAACCCTGg
B297	aattcCAGGGTTGTGGGTTGTACGGATGGAg
B298	CATGGATCCTATTTCTTAATAACTAAAAATATGG
B299	CATGAATTCAACTCAACAAGTCTCAGTGTGCTG
B300	AAACATTTTTTCTCCATTTAGGAAAAAGGATGCTG
B301	AAAACAGCATCCTTTTTCCTAAATGGAGAAAAAAT

B302	AAACCTTAAATCAGTCACAAATAGCAGCAAAATTG
B303	AAAACAATTTTGCTGCTATTTGTGACTGATTTAAG
B304	AAACTTTTCATCATACGACCAATCTGCTTTATTTG
B305	AAAACAAATAAAGCAGATTGGTCGTATGATGAAAA
B306	AAACTCGTCCAGAAGTTATCGTAAAAGAAATCGAG
B307	AAAACTCGATTTCTTTTACGATAACTTCTGGACGA
B308	AAACAATCTCTCCAAGGTTTCCTTAAAAATCTCTG
B309	AAAACAGAGATTTTTAAGGAAACCTTGGAGAGATT
B310	AAACGCCATCGTCAGGAAGAAGCTATGCTTGAGTG
B311	AAAACACTCAAGCATAGCTTCTTCCTGACGATGGC
B312	AAACATCTCTATACTTATTGAAATTTCTTTGTATG
B313	AAAACATACAAAGAAATTTCAATAAGTATAGAGAT
B314	AAACTAGCTGTGATAGTCCGCAAAACCAGCCTTCG
B315	AAAACGAAGGCTGGTTTTGCGGACTATCACAGCTA
B316	AAACATCGGAAGGTCGAGCAAGTAATTATCTTTTG
B317	AAAACAAAAGATAATTACTTGCTCGACCTTCCGAT
B318	AAACAAGATGGTATCGCAAAGTAAGTGACAATAAG
B319	AAAACTTATTGTCACTTACTTTGCGATACCATCTT
B320	GAGACCTTTGAGCTTCCGAGACTGGTCTCAGTTTTGGGACCATTCAAAACAG
B321	TGAGACCAGTCTCGGAAGCTCAAAGGTCTCGTTTTAGAGCTATGCTGTTTTG
B352	aaacTACTTTACGCAGCGCGGAGTTCGGTTTTTTg
B353	aaaacAAAAAACCGAACTCCGCGCTGCGTAAAGTA
HC008_SP	ATGCCGGTACTGCCGGGCCTCTTGCGGGATTACGAAATCATCCTG
HC009_SP	GTGACTGGCGATGCTGTCGGAATGGACGATCACACTACTCTTCTT
HC010_SP	TTAAGAAATAATCTTCATCTAAAATATACTTCAGTCACCTCCTAGCTGAC
HC011_SP	ATTGATTTGAGTCAGCTAGGAGGTGACTGAAGTATATTTTAGATGAAG
HC014_SP	GAGACCTTTGAGCTTCCGAGACTGGTCTCAGTTTTGGGACCATTCAAAACAGCATAGCTCTAAAACCTCGTAGACTATTTTTGTC
HC015_SP	GAGACCAGTCTCGGAAGCTCAAAGGTCTCGTTTTAGAGCTATGCTGTTTTGAATGGTCCCAAAACTTCAGCACACTGAGACTTG
L403	AGTCATCCCAGCAACAAATGG
L409	CGTGGTAAATCGGATAACGTTCCAAGTGAAG
L422	Tgctcttcttcacaaacaaggg
L426	AAGCCAAAGTTTGGCACCACC
L430	GTAGCTTATTCAGTCCTAGTGG
L444	CGTTTGTTGAACTAATGGGTGCAAATTACGAATCTTCTCCTGACG
L445	CGTCAGGAGAAGATTCGTAATTTGCACCCATTAGTTCAACAAACG
L446	GATATTATGGAGCCTATTTTTGTGGGTTTTTAGGCATAAAACTATATG
L447	CATATAGTTTTATGCCTAAAAACCcACAAAAATAGGCTCCATAATATC
L448	ATTATTTCTTAATAACTAAAAATATGG
L457	CGTgtacaattgctagcgtacggc
L458	GCACCGGTGATCACTAGTCCTAGG

L459	cctaggactagtgatcaccggtGCAAATATGAGCCAAATAAATATAT
L461	GCCGTACGCTAGCAATTGTACACGTTTGTTGAACTAATGGGTGC
L481	TTCAAATTTTCCCATTTGATTCTCC
L488	CCATATTTTTAGTTATTAAGAAATAATACCAGCCATCAGTCACCTCC
W256	AGACGATTCAATAGACAATAAGG
W286	GTTTTGGGACCATTCAAAACAGCATAGCTCTAAAACCTCGTAGAC
W287	GCTATGCTGTTTTGAATGGTCCCAAAACcattattttaacacacgaggtg
W288	GCTATGCTGTTTTGAATGGTCCCAAAACGCACCCATTAGTTCAACAAACG
W326	AATTCTTTTCTTCATCATCGGTC
W327	AAGAAAGAATGAAGATTGTTCATG
W341	GGTACTAATCAAAATAGTGAGGAGG
W354	GTTTTTCAAAATCTGCGGTTGCG
W355	AAAAATTGAAAAAATGGTGGAAACAC
W356	ATTTCGTAAACGGTATCGGTTTCTTTTAAAGTTTTGGGACCATTCAAAACAGC
W357	TTTAAAAGAAACCGATACCGTTTACGAAATGTTTTAGAGCTATGCTGTTTTGA
W365	AAACGGTATCGGTTTCTTTTAAATTCAATTGTTTTGGGACCATTCAAAACAGC
W366	AATTGAATTTAAAAGAAACCGATACCGTTTGTTTTAGAGCTATGCTGTTTTGA
W370	GTTCCTTAAACCAAAACGGTATCGGTTTCTTTTAAATTC
W371	GAAACCGATACCGTTTTGGTTTAAGGAACAGGTAAAGGGCATTTAAC
W376	CGATTTCAGCCATTGCCTCGTC
W377	GCCTTTGACGAGGCAATGGCTGAAATCGNNNNNAAAAAGCGCAAGAAGAAATCAAC
W391	TCCGTACAACCCACAACCCTGCTAGTGAGCGTTTTGGGACCATTCAAAACAGC
W392	GCTCACTAGCAGGGTTGTGGGTTGTACGGAGTTTTAGAGCTATGCTGTTTTGA
W393	TTGTTGCCACTCTTCCTTCTTTC
W397	CAGGGTTGTGGGTTGTTGCGATGGAGTTAACTCCCATCTCC
W398	GGGAGTTAACTCCATCGCAACAACCCACAACCCTGCTAGTG
W403	GTGGTATCTATCGTGATGTGAC
W404	TTACCGAAACGGAATTTATCTGC
W405	AAAGCTAGAGTTCCGCAATTGG
W431	GTGGGTTGTACGGATTGAGTTAACTCCCATCTCCTTC
W432	GATGGGAGTTAACTCAATCCGTACAACCCACAACCCTG
W433	GCTTCACCTATTGCAGCACCAATTGACCACATGAAGATAG
W434	GTGGTCAATTGGTGCTGCAATAGGTGAAGCTAATGGTGATG
W463	CTGATTTGTATTAATTTTGAGACATTATGCTTCACCTTC
W464	GCATAATGTCTCAAAATTAATACAAATCAGTGAAATCATG
W465	GTTTTGGGACCATTCAAAACAGCATAGCTCTAAAACGTGACAGTAATATCAG
W466	GTTTTAGAGCTATGCTGTTTTGAATGGTCCCAAAACGCTCACTAGCAGGGTTG
W542	ATACTTTACGCAGCGCGGAGTTCGGTTTTgTAGGAGTGGTAGTATATACACGAGTACAT

Table S3. Design of targeting and editing constructs used in this study.

	Targeting Constructs					Editing Constructs						
Edition	**Template DNA**	**Left PCR**	**Right PCR**	**Spacer sequence**	**PAM**	**Template DNA**	**PCR A**	**PCR B**	**PCR C**	**SOEing PCR**	**Name of resulting strains**	**Primers used to verify edited genotype**
bgaA R>A	crR6Rk	W256/W391	W392/L403	GCTCACTAGCAGGGTTGTGGGTTGTACGGA	TGG	R6	W403/W397	W398/W404	N/A	W403/W404	JEN56	W403/W404
bgaA NE>AA	crR6Rk	W256/W391	W392/L403	GCTCACTAGCAGGGTTGTGGGTTGTACGGA	TGG	R6	W403/W431	W432/W433	W434/W404	W403/W404	JEN60	W403/W404
ΔbgaA	crR6Rk	W256/W391	W392/L403	GCTCACTAGCAGGGTTGTGGGTTGTACGGA	TGG	R6	B255/B256	B257/B258	N/A	B255/B258	JEN52	W393/W405
ΔsrtA	crR6Rc	W256/B218	B217/L403	TCCTAGCAGGATTTCTGATATTACTGTCAC	TGG	R6	B230/W463	W464/B229	N/A	B230/B229	JEN51	W422/W426
ermB Stop	crR6Rk	W256/W356	W357/L403	TTTAAAAGAAACCGATACCGTTTACGAAAT	TGG	JEN38	L422/W370	W371/L426	N/A	L422/L426	JEN43	L457/L458
ΔsrtA ΔbgaA	JEN51 (for Left PCR) and JEN52 (for Right PCR)	W256/W465	W466/W403	same as the ones used for ΔsrtA and ΔbgaA	TGG	same as the ones used for ΔsrtA and ΔbgaA					JEN64	same as the ones used for ΔsrtA and ΔbgaA