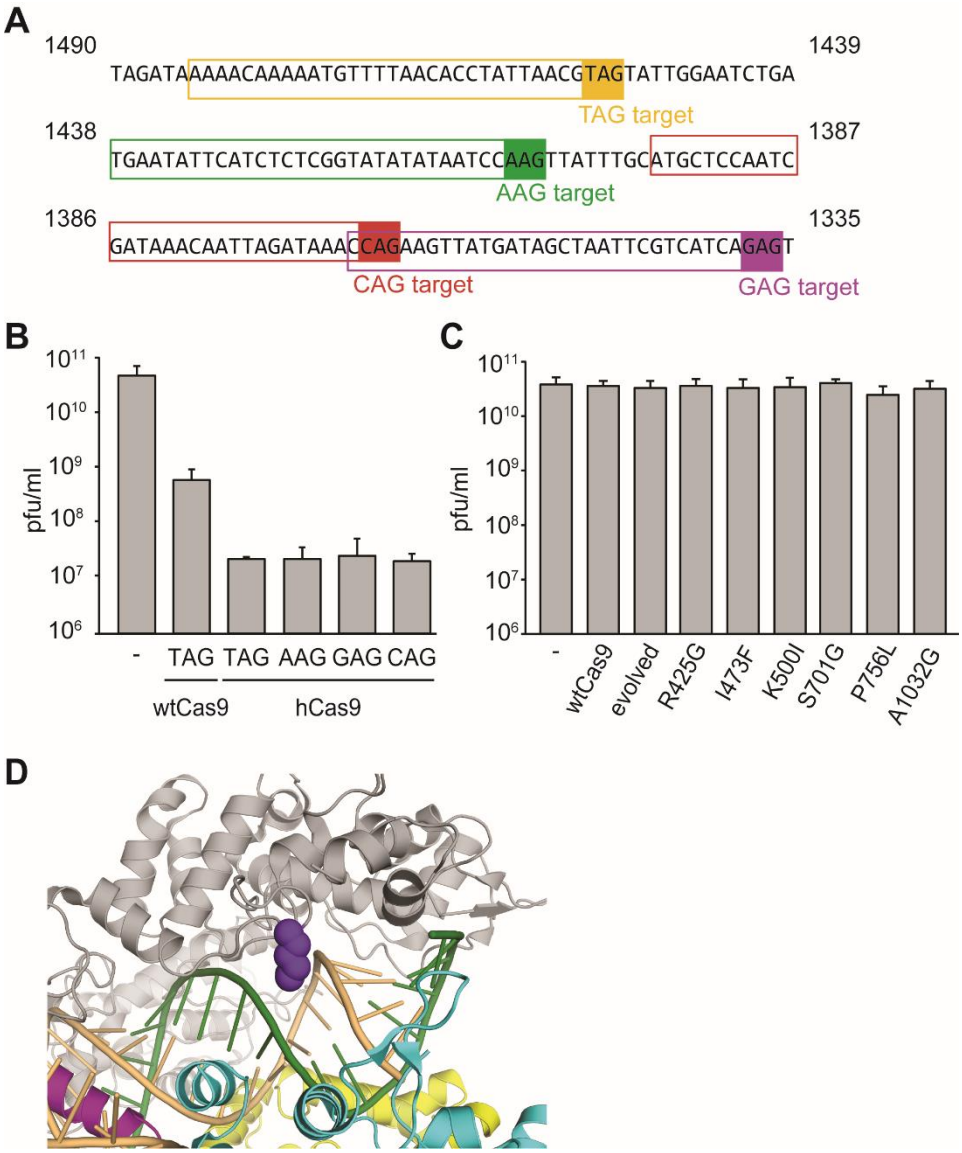
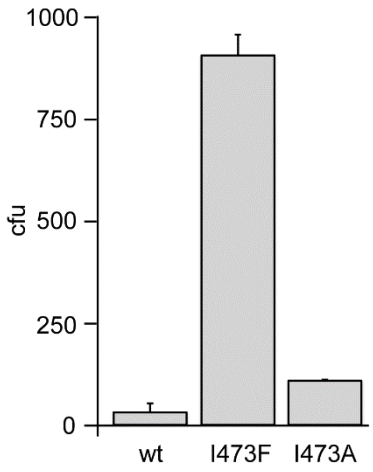
SUPPLEMENTARY FIGURES



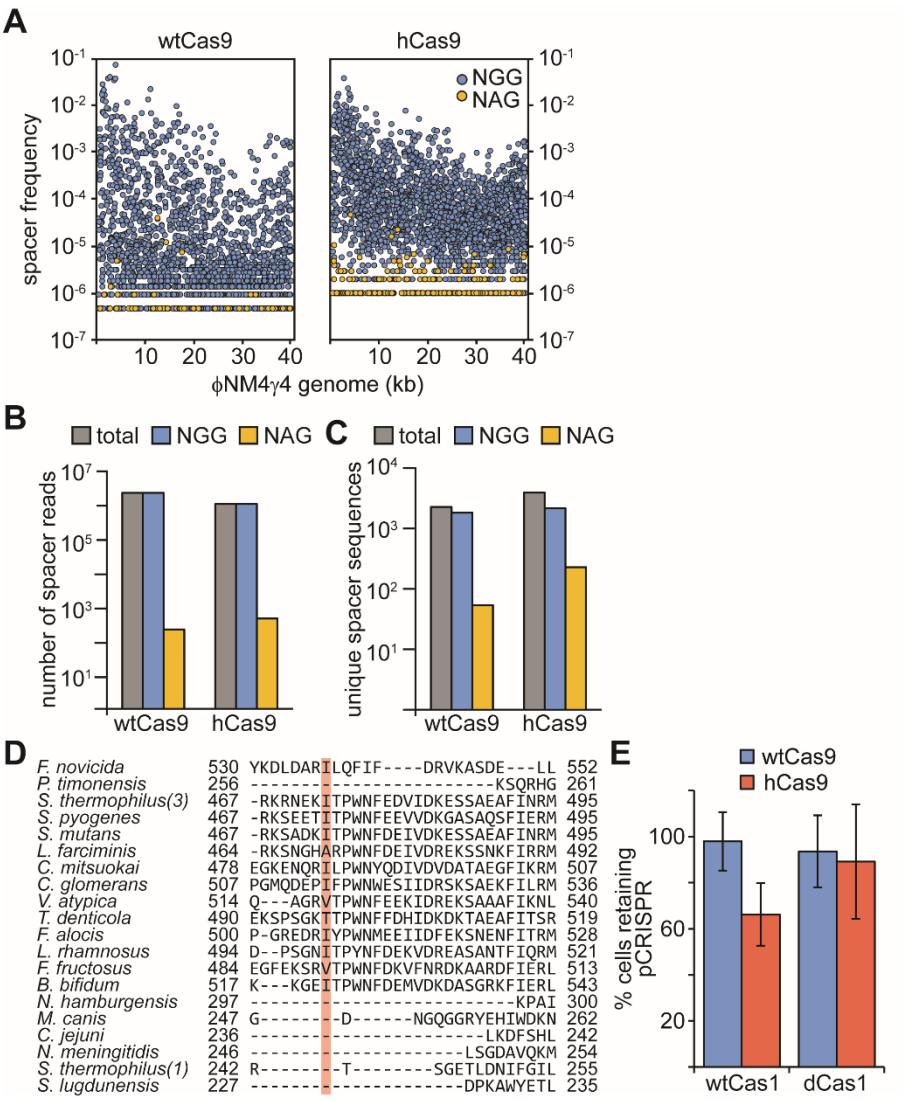
Supplemental figure S1. Protection of host cells by hCas9 programmed against different NAG-flanked targets. Related to Fig. 1.
(**A**) The ability of hCas9 to target protospacers with different PAM was tested by measuring phage propagation in cells harboring CRISPR-Cas systems containing either wtCas9 or hCas9 and programmed to target the sequences shown, which are followed by TAG, AAG, GAG or CAG PAMs.
(**B**) Phage propagation was measured as the number of plaque forming units (pfu) per ml of stock, on cells targeting the TAG, AAG, GAG, and CAG-adjacent protospacers and hCas9.
(**C**) Measurement of pfu formation on staphylococci carrying plasmids with different *cas9* mutations after infection with ϕ85, a phage lacking the target recognized in ϕNM4γ4.

(**D**) Location of residue K500 on the Cas9:single-guide RNA ribonucleoprotein (PDB 4UN3). Purple, K500; orange, sgRNA; green, target DNA (the GG PAM highlighted in red); grey, alpha-helical (REC) lobe; yellow, HNH domain; light blue, RuvC domain; blue, PAM-interacting CTD.



Supplemental figure S2. CRISPR-Cas immune response of cells expressing Cas9^{I473A}. Related to Fig. 2. Cultures harboring plasmids with *tracrRNA*, *cas1*, *cas2* and *csn2* genes, and either wild-type, I473F or I473A cas9 alleles, were infected with ΦNM4γ4 phage on top agar media and poured on plates. After 24 hours of incubation at 37 °C the CRISPR-surviving colonies were counted.

Supplemental figure S3. In vivo and in vitro targets. Related to Fig. 3.
(**A**) Region of the ΦNM4γ4 phage genome (nucleotides 1441 to 1490) containing the TAG- and TGG-flanked protospacers, yellow and blue respectively, used in Figs. 3A and 3B.
(**B**) Sequences of the dsDNA target oligonucleotides used in Fig. 3C. The protospacer sequence is the same, but it is flanked by either a TAG (yellow) or TGG (blue) PAM sequence. Radiolabel is at the 5' end (P). Grey and black arrowheads mark the cleavage sites of the RuvC and HNH domains, respectively.



Supplemental figure S4. Analysis of next-generation sequencing results. Related to Fig. 4.

(**A**) Data presented in Fig. 4B and in Supplementary Data File was plotted as the the number of reads for each spacer sequence across the phage genome, normalized by the total number of spacer reads obtained. Spacers matching protospacers with NGG PAMs are shown in blue, with NAG PAMs in yellow.

(**B**) Quantification of the data shown in panel **A**.

(**C**) Quantification of the data shown in Fig. 4B.

(**D**) Alignment of Cas9 protein sequences belonging to type II CRISPR-Cas systems. Highlighted in orange is the I473 residue. An equivalent residue is not found in some type II-B and II-C systems.

(**E**) Fraction (%) of staphylococci retaining the plasmid harboring wt*cas9* and h*cas9* after 10 days of culture; with one transfer (1:100 dilution into fresh media) per day. Cells were plated in solid media with and without chloramphenicol, an antibiotic that selects for cells harboring the pCRISPR plasmid. The fraction of staphylococci carrying this plasmid was obtained dividing the chloramphenicol-resistant cfu by the total cfu count.

SUPPLEMENTARY TABLES

Supplementary table S1. Related to Fig. 2. Spacer sequences acquired by wtCas9-expressing cells.

Strain	Sequence	PAM	Location on φNM4γ4	Strand
RH71	ataaataaaaaagttactactcacacacta	agg	258	-
RH64	cgaactaggaagaaaaatcgccatcaattca	agg	453	-
RH69	aatagagatactttatctaacatgatacac	ggg	805	+
RH51	tgatacacgggagaacaaaaccatcctacc	cgg	827	+
RH99	tgatacacgggagaacaaaaccatcctacc	cgg	827	+
RH47	gagaacaaaaccatcctacccggtaataaa	tgg	837	+
RH107	tttattttgcgttagaattgacacctcaaga	agg	873	+
RH127	tttattttgcgttagaattgacacctcaaga	agg	873	+
RH57-2	tttattttgcgttagaattgacacctcaaga	agg	873	+
RH57-1	tttagcgatattaattatgctcgtaagaat	cgg	1241	+
RH63	agtattggaatctgatgaatattcatctct	cgg	1423	-
RH40	aaaaatgttttaacacctattaacgtagtat	tgg	1448	-
RH85	aatattcatcagattccaatactacgttaat	agg	1461	+
RH36	ttcttcgcctctatatgtgttttctggtgt	tgg	2810	-
RH109	acaaatttttcttcgcctctatatgtgttttc	tgg	2816	-
RH10	ccaatttagaaatattaatcagagtgcctgt	tgg	2981	-
RH42	agaaaatttatacattgattattcaccaac	agg	2983	+
RH7	gctaagactgtgaagcataatactgctact	agg	3087	-
RH33	gctaagactgtgaagcataatactgctact	agg	3087	-
RH8	ttttaagctattcatttaaaaggtcatat	ggg	3400	+
RH42	gtgttctcttcaatccattcatctattgct	tgg	3502	-
RH85	atgaatggattgaagagaacacagacgaac	agg	3540	+
RH120	ggagtaactaatatctgaattgttatcagt	tgg	3650	-
RH97	attagttactccacaaatagaaatagagct	agg	3698	+
RH86	ccacaaatagaaatagagctagggagtttaa	cgg	3709	+
RH83	tagtttttgagtatgcttacttttcttg	tgg	3822	-
RH32	acgaaagcgtctttatctcttgtagcaaacg	tgg	3934	-
RH30	aaataagtctaaaaaaccaacgtttaatgat	tgg	4197	+
RH52	aaataagtctaaaaaaccaacgtttaatgat	tgg	4197	+
RH55-2	gaacgaattgtcagtatgtacagattaat	agg	4241	+
RH55-1	aagaagaatacaaattccactttgttattac	agg	4283	+
RH11	gcattacggacgtagtagaagcaattagaaa	tgg	4577	+
RH26-1	aaaaacaattgattgaattagttactcgatt	agg	4866	+
RH44	tagcttagattttgataccaatgatcttat	tgg	4917	+
RH77	tagcttagattttgataccaatgatcttat	tgg	4917	+
RH25	cggatttttcatttattaaaccttacaaaa	agg	5009	+
RH115	tggatatgacgaccaagatttagcgtttta	agg	5166	+
RH71	ataacgacggtacttattccgtcgttgctac	tgg	5238	+
RH36	taatacaggtttttacaaaagctttaccat	agg	5991	+
RH16-1	ctttaaatgttttaaaagaatagcatcatt	tgg	6436	+

Supplementary table S2. Related to Fig. 2. Spacer sequences acquired by hCas9-expressing cells.

Strain	Sequence	PAM	Location on φNM4γ4	Strand
RH213	aatagagatactttatctaacatgatacac	ggg	805	+
RH214	tgatacacgggagaacaaaaccatcctacc	cgg	827	+
RH177	gagaacaaaaccatcctacccggtaataaa	tgg	837	+
RH193	agtattggaatctgatgaatattcatctct	cgg	1423	-
RH216	aaaaatgttttaacacctattaacgtagtat	tgg	1448	-
RH206	aatattcatcagattccaatactacgttaat	agg	1461	+
RH166	ttcttcgcctctatatgtgttttctggtgt	tgg	2810	-
RH199	aaataagtctaaaaaaccaacgtttaatgat	tgg	4197	+
RH174	aataagatcattggtatcaaaatctaagct	agg	4889	-
RH195	cggatttttcatttattaaaccttacaaaa	agg	5009	+
RH210	cggatttttcatttattaaaccttacaaaa	agg	5009	+
RH187	cgacataacgctaatacatgtttgtcatag	tgg	5695	-
RH205	taatacaggtttttacaaaagctttaccat	agg	5991	+
RH211	tttttatttaagtattcgataatttctttata	ggg	7355	-
RH202	tgtatgtcgctttgatacgatccatcaacat	tgg	8123	-
RH175	attagacttttactttccattacttaaatca	tgg	9043	+
RH215	attagacttttactttccattacttaaatca	tgg	9043	+
RH164	ctaatactgttttaattaagttatcgatatc	cgg	9097	-
RH185	atttatatccgatcttatacgaagtaaaga	agg	13617	+
RH208	gcaaagttgagcgatcagtctgatttgatg	agg	13783	+
RH200	ggaatatgatagcaattcaattgcacagta	tgg	13911	+
RH203	aaaatgcaagaattaaactacccaccatat	agg	14402	-
RH169	gataaaatcaaacaacttcacgacgcaataa	cgg	15028	+
RH198	gataaaatcaaacaacttcacgacgcaataa	cgg	15028	+
RH197	cgagtccaacacgtcatcaaattcttttat	agg	16180	-
RH168	atatacacacatactaaacctgaacgatta	agg	16252	+
RH209	tatgtgactctattagagcctcaatatgctt	agg	16314	-
RH178	taagaatatagatccctataatgttatttttgt	tgg	16769	+
RH189	gaatatagatccctataatgttatttttgt	tgg	16769	+
RH176	ctcatcaatatcattctgattggttatttt	ggg	17669	-
RH179	attgaaaaagatacgtatgcacattacaca	agg	18135	+
RH204	ctaagatagctaaagcaatacgtgatgatgt	cgg	18192	+
RH196	gaacacgtgatactcatcgtcatttagatg	ggg	18365	+
RH180	ctaatcctttcgaatgataacgatctaattc	agg	19067	-
RH173	tttgatgaaattttagttgttcagatgtagt	agg	21085	-
RH192	taaactactacgacttaagcaggtgccata	tgg	21278	+
RH212	taaactactacgacttaagcaggtgccata	tgg	21278	+
RH201	aaaaataaggcaactgacagctagatattt	agg	23282	+
RH165	tccattttgctgttgattcttctatgctatc	cgg	37541	-
RH170	cctacgaatatgaacgacacaaatgattta	ggg	38151	+

Supplementary table S3. Related to Experimental Procedures. Oligonucleotides used in this study.

Name	Sequence
H024	aaacaaaaacaaaaatgttttaacacctattaacgg
H025	aaaaccgttaataggtgttaaaacatttttgttttt
H029	aaacaaaaatgttttaacacctattaacgtagtatg
H030	aaaacatactacgttaataggtgttaaaacattttt
H050	aaaacaaaaagcgcaagaagaaatcaaccagcgca
H052	aaaacttttttacaaattgagttatgttcatataa
H101	gctattttgagaggacaagaagacttttatcc
H102	ggataaaagtcttcttgtcctctcaaaatagc
H103	ggaagtctgaagaaacatttaccccatgg
H104	ccatggggtaaatgtttcttcagacttcc
H105	gacaaactttgatataaatcttccaaatgaaaaagtactacc
H106	ggtagtacttttttcatttggaagatttatatcaaagtttgtc
H107	ccatgatgatggtttgacatttaaagaagac
H108	gtcttctttaaatgtcaaaccatcatcatgg
H109	gggcggcataagctagaaaatatcg
H110	cgatattttctagcttatgccgccc
H111	gcaagaaataggcaaaggaaccgc
H112	gcggttcctttgcctatttcttgc
H207	ggaagtctgaagaaacagctaccccatgg
H208	ccatggggtagctgtttcttcagacttcc
H293	gcaaaaatggataagaaatactcaataggc
H294	tattgagtatttcttatccatttttgcctcc
H295	aacacgcattgatttgagtcagc
H296	tcctagctgactcaaatcaatgcg
H372	nnnnnactaggggcttttcaagactg
H373	nnnnnactgaagaaatcaaccagcgc
H374	nnnnnctgaggggcttttcaagactg
H375	nnnnnctggaagaaatcaaccagcgc
H376	nnnnntgaaggggcttttcaagactg
H377	nnnnntgagaagaaatcaaccagcgc
H378	caggggcttttcaagactgnnnnnnnnnngagacaaatagtgcg
H379	cagtcttgaaaagcccctg
H546	aaactgaatattcatctctcggtatatataatccg
H547	aaaacggattatatataccgagagatgaatattca
H548	aaacccagaagttatgatagctaattcgtcatcag
H549	aaaactgatgacgaattagctatcataacttctgg
H550	aaacatgctccaatcgataaacaattagataaacg
H551	aaaacgtttatctaattgtttatcgattggagcat
L400	cgaaatttttagacaaaaatagtc
L2 Target	gagtggaaggatgccagtgataagtggaatgccatgtgggctgtcaaaattgagc
L2 RC	gctcaattttgacagcccacatggcattccacttatcactggcatccttccactc
L2 AG PAM	gagtggaaggatgccagtgataagtggaatgccatgtaggctgtcaaaattgagc
L2 AG RC	gctcaattttgacagcctacatggcattccacttatcactggcatccttccactc
L2 crRNA	gugauaaguggaaugccaugguuuuagagcuaugcuguuuug
tracrRNA	ggacagcauagcaaguuaaaauaaggcuaguccguuaucaacuugaaaaaguggcaccgagucggugcuuuuu
L1 sgRNA	gacgcauaaagaugagacgcguuuuagagcuaugcuguuuuggaaacaaaacagcauagcaaguuaaaauaaggcuaguccguuaucaacuugaaaaaguggcaccgagucggugcuuuuuuuggauc

SUPPLEMENTARY DATA FILE S1. Related to Experimental Procedures. Analysis of next generation sequencing data.

SUPPLEMENTARY EXPERIMENTAL PROCEDURES

Spacer acquisition assay during phage infection

Spacer acquisition assays of cells harboring the full CRISPR system of *Streptococcus pyogenes* were performed as described previously, both in liquid and on plate (Heler et al., 2015). For plate acquisition assays, overnight cultures were launched from single colonies and diluted to equal optical densities. CRISPR arrays were amplified by PCR with primer pairs L400-H050 or L400-H052 (Supplementary table S3).

Spacer acquisition assay in the absence of phage infection

Spacer acquisition assays were conducted by passaging cultures carrying the full *S. pyogenes* CRISPR system (expressing wtCas9 or hCas9) in the absence of phage for 10 days. Each day, the cultures were diluted 1:100 in fresh media with appropriate antibiotics. The pCRISPR plasmids had barcoded leader sequences. Spacer acquisition was quantified by PCR amplification of the CRISPR array followed by NGS.

Phage propagation assay

Overnight cultures were launched from single colonies. Serial dilutions of a stock of phage ϕNM4γ4 (Goldberg et al., 2014) or ϕ85 (Mazmanian et al., 2000) were spotted on fresh soft heart infusion agar (HIA) lawns of targeting cells containing chloramphenicol 10 μg ml^{-1} and 5 mM $CaCl_2$. Plates were incubated at 37 °C overnight and interference efficiency was measured in plaque forming units (pfu).

Bacterial growth curves

Overnight cultures were launched from single colonies and diluted 1:100 in BHI. After 1 hour of growth, optical density at 600 nm (OD_{600}) was measured for each culture, and samples were brought to equal cell densities and loaded into 96-well plates along with ϕNM4γ4 at MOI =1. Measurements were taken every 10 minutes for 24 hours.

Cas9 target cleavage assay

Cas9 was expressed and purified as previously described (Jinek et al., 2012). The I473F Cas9 expression vector was cloned by around-the-horn mutagenic PCR (Moore and Prevelige, 2002). crRNA and tracrRNA were transcribed using T7 RNA polymerase from single-stranded DNA templates and hybridized as previously described (Jinek et al., 2012; Sternberg et al., 2014). L2 oligonucleotides (Supplementary table S3) were hybridized to generate the two different target DNA duplexes and native PAGE-purified before 5' radiolabeling using [γ-^{32}P]-ATP (Perkin-Elmer) and T4 polynucleotide kinase (New England Biosciences).

Cleavage assays were carried out essentially as previously described (Sternberg et al., 2014). In brief, Cas9 and crRNA:tracrRNA were allowed to form an RNP complex before addition of target DNA. Final concentration of RNP was 100 nM and target was 1 nM. Reactions were incubated at room temperature, and aliquots were taken at 0.25, 0.5, 1, 2, 5, 10, 30, and 60 minutes and quenched by addition of an equal volume of 95% formamide and 50 mM EDTA. Samples were run on 10% urea-PAGE, visualized by phosphorimaging, and quantified using ImageQuant (GE Healthcare).

Plasmid construction

All cloning was performed using chemically competent *S. aureus* cells, as previously described (Goldberg et al., 2014). The sequences of all the oligonucleotides used in for plasmid construction are in Supplementary table S3. BsaI cloning was used to construct pRH065 and pRH079 by inserting TAG (annealed primers H024-H025 containing compatible BsaI overhangs) and NGG-adjacent (H029-H030) spacers targeting φNM4γ4 into pDB114 (Bikard et al., 2014). The mutant *cas9* library was constructed via 2-piece Gibson assembly (Gibson et al., 2009) by replacing wild-type *cas9* on pRH065 with error-prone *cas9* amplicons using primer pairs H294-H295 and H293-H296, respectively. The I473F mutation (codon ATT to TTT) was introduced on pRH065, pRH079, pWJ40 (Goldberg et al., 2014) and pDB114 by around-the-horn PCR (Moore and Prevelige, 2002) with primer pair H103-H104 to create plasmids pRH096, pRH176, pRH180 and pRH305. BsaI cloning was used to construct pRH306, pRH307 and pRH308 by inserting AAG (H546-H547), GAG (H548-H549) and CAG (H550-H551)-adjacent spacers targeting φNM4γ4 into pRH305. In addition, mutations R425G (AGA to GGA), I473A (ATT to GCT), K500I (AAA to ATA), S701G (AGT to GGT), P756L (CCA to CTA) and A1032G (GCA to GGA) were each introduced on both pRH065 and pWJ40 by around-the-horn PCR with primer pairs H101-H102, H207-H208, H105-H106, H107-H108, H109-H110 and H111-H112 respectively. The randomized pWJ40 and pRH180 leader-barcoded libraries used for MiSeq were each constructed by 2-piece Gibson assembly with primers pairs H378-H294 and H379-H293.

Plasmid loss assays

To assess plasmid loss, cultures carrying the full *S. pyogenes* CRISPR system (expressing wtCas9 or hCas9) were passaged in the absence of phage for 10 days. Each day, the cultures were diluted 1:100 in fresh media with no antibiotics. At the end of the experiment, dilutions of the cells were plated on plates without antibiotic (to count the total number of cells) and with antibiotic (to count the number of cells that still carried the pCRISPR plasmids).

Cas9 competition assays

Plasmids pWJ40 and pRH180 carrying the full *S. pyogenes* CRISPR system (expressing wtCas9 and hCas9, respectively) were transformed into *S. aureus* RN4220 (no antibiotic resistance) and OS2 (erythromycin resistance), respectively. Overnight cultures of RN4220:pWJ40 and OS2:pRH180 launched from single colonies were diluted 1:100 in BHI. After 1 hour of growth, optical density at 600 nm (OD_{600}) was measured for each culture, and samples were brought to equal cell densities. The two cultures were mixed in a 1:1 ratio and passaged for 5 days. Every day, the mixed culture was diluted 1:100 in fresh media and dilutions of the cells were plated on plates with chloramphenicol (to count the total number of cells) and plates with chloramphenicol and erythromycin (to count the number of cells that carried the hCas9 plasmid).

Protein sequence alignments

Amino acid sequences of Cas9 were obtained from the NCBI Protein database and aligned with Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). Alignments were visualized with Jalview (Waterhouse et al., 2009).

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