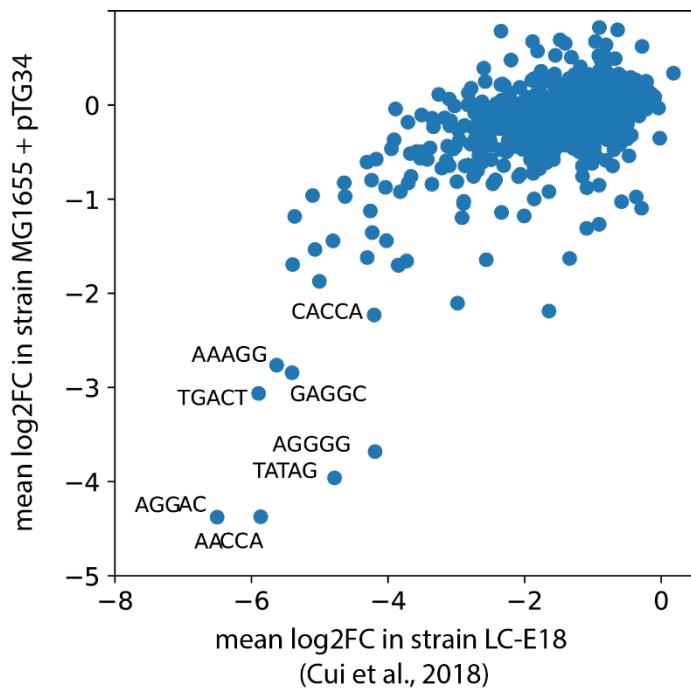
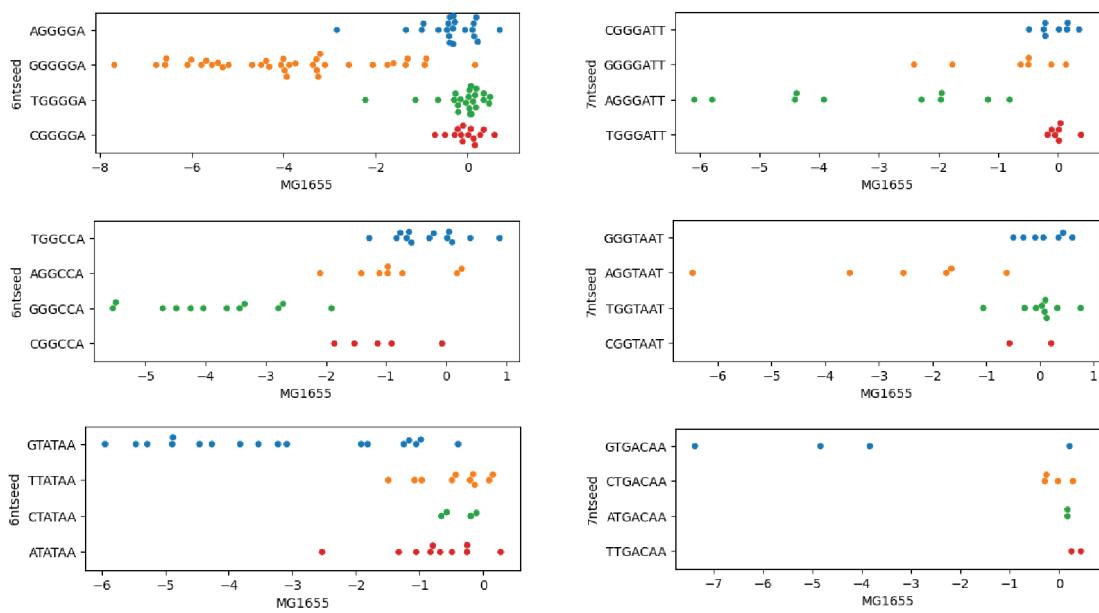


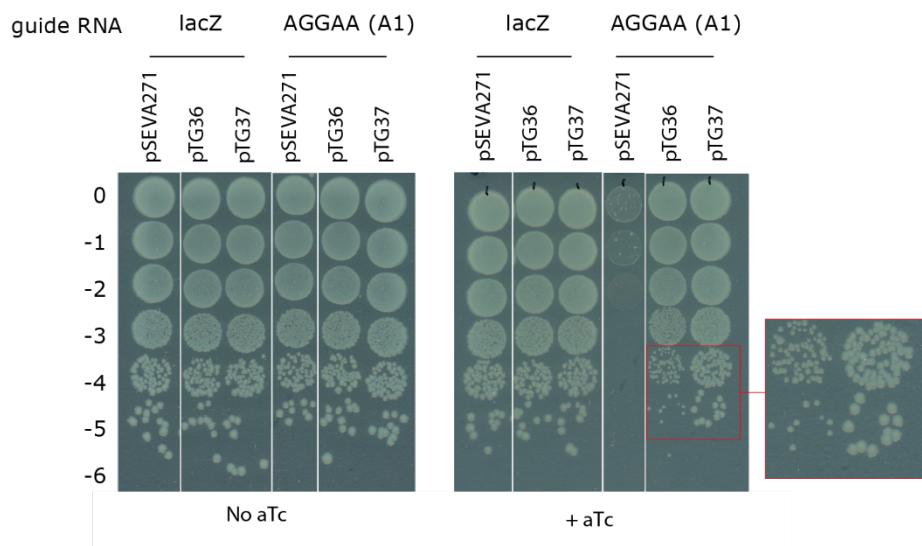
**Figure S1. Correlation between log2FC computed on independent biological replicates.**



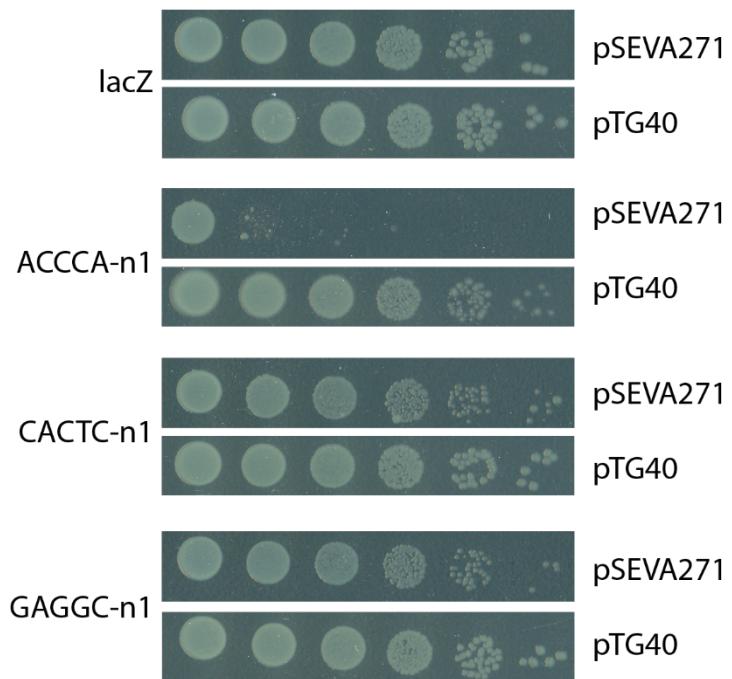
**Figure S2.** Comparison between the mean log<sub>2</sub>FC of guide RNAs sharing the same seed sequence in strain LC-E18 (Cui et al, Nature Communications, 2018) and MG1655 + pTG34 (this study).



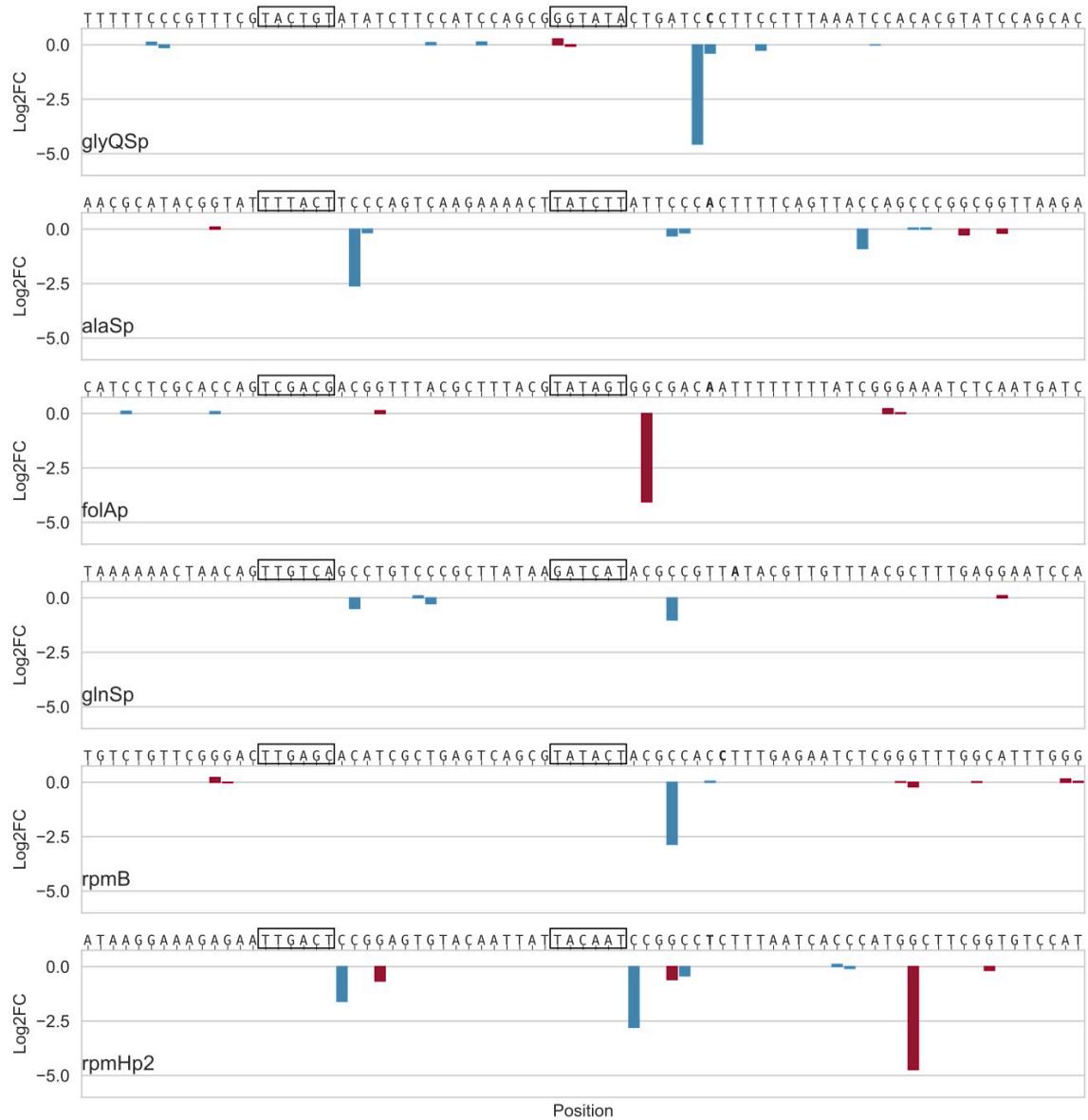
**Figure S3. Manually selected examples of sets of guides that are differently depleted depending on the nature of the 6<sup>th</sup> or 7<sup>th</sup> nucleotide.** The x axis shows the log<sub>2</sub>FC of individual guides in strain MG1655.



**Figure S4. The expression of the *glyQS* operon in trans rescues the toxicity of the AGGAA bad seed.** Plasmid pTG36 is a derivative of pSEVA271 carrying the *glyQS* operon under its native promoter. Plasmid pTG37 is a derivative of pTG36 in which the PAM site of a candidate off-target site for AGGAA guide RNA was mutated. These plasmids were introduced in strain LC-E18 which carries dCas9 under the control of a ptet promoter inducible by aTc. The control *lacZ* guide or the AGGAA-n1 guide RNAs are expressed from the psgRNA plasmid. Overnight cultures were serial diluted and spotted on plates with or without inducer.



**Figure S5. The expression of the rpmH-rnpA operon in trans rescues the toxicity of the CACTC and GAGGC toxic seed sequences.** The off-target positions in the promoter of rpmH are underlined on the sequence. Serial dilution and spotting of strain AV01, expressing dCas9 under the control of a ptet promoter, in the presence of plasmid pTG40 carrying the rpmH-rnpA operon or a control empty vector (pSEVA271). Experiments were done in the presence of the following guide RNAs: the control lacZ guide, ACCCA-n1, CACTC-n1 and GAGGC-n1. The CACTC and GAGGC guides produce a small colony phenotype in the presence of pSEVA271 that is rescued in the presence of pTG40.



**Figure S6. Log2FC of guide RNAs with off-target positions within the promoters of genes responsible for the toxicity of bad seeds.** For each PAM site in the promoters of the genes glyQSp, alaSp, folAp, glnSp, rpmB and rpmHp2, the median log2FC of the set of guides with a 5nt off-target to the position is shown. Off-targets are shown in blue or red depending on their orientation. The -35 and -10 boxes of the promoters are highlighted and the transcription start site is in bold case.

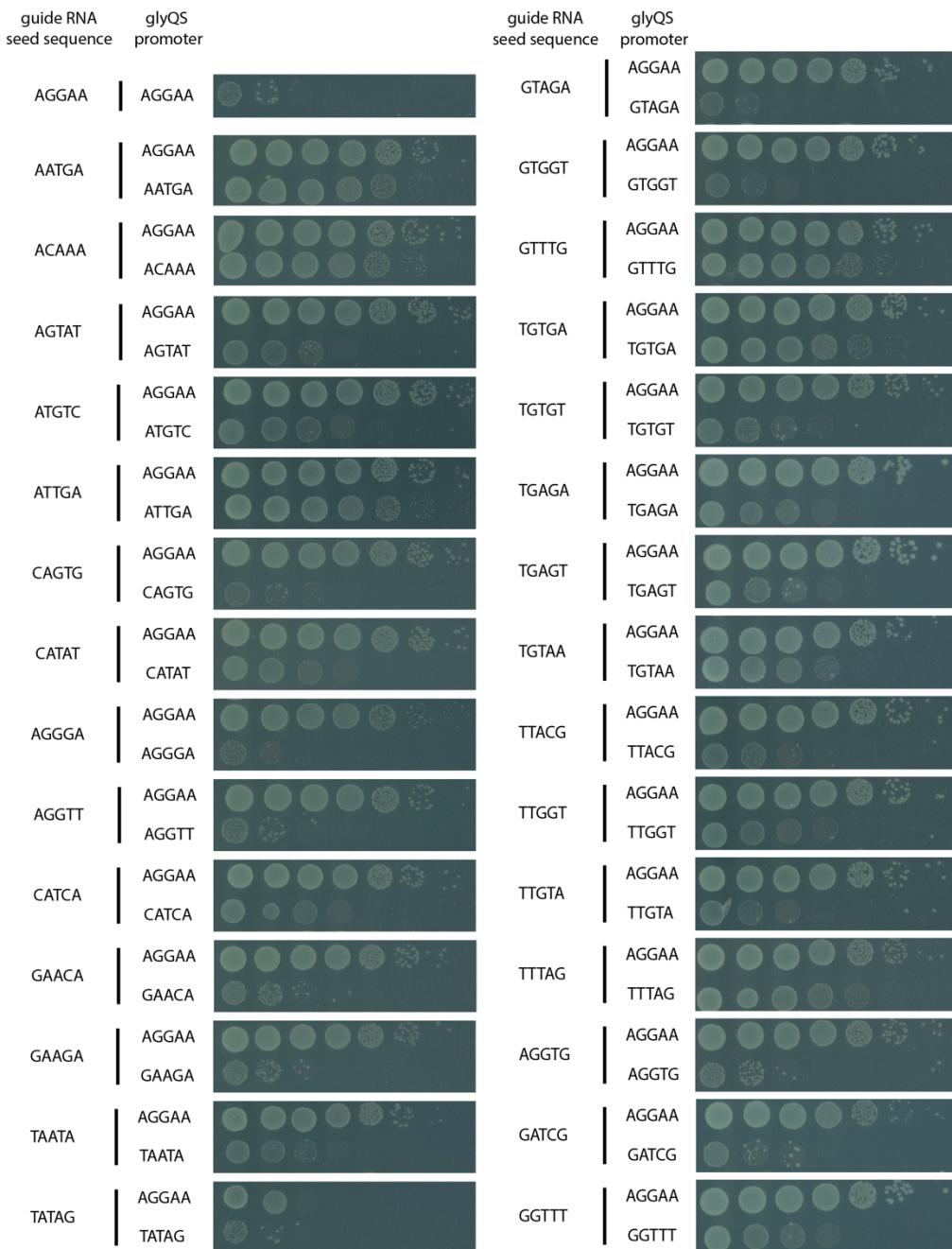
*yfcZp*

**-35**                    **-10**                    **+1**  
TTAACAACTGGAGCAGCACTTCCGGAAGCGTAAAATATCCAC**A**ACGTTATCTGGCACCTGGGTGC  
AATTGTTGACCTCGTCGTGAAAGGCCTTCGCATTTATAGGTGTTGCAAATAGACCGTGGAACCCACG

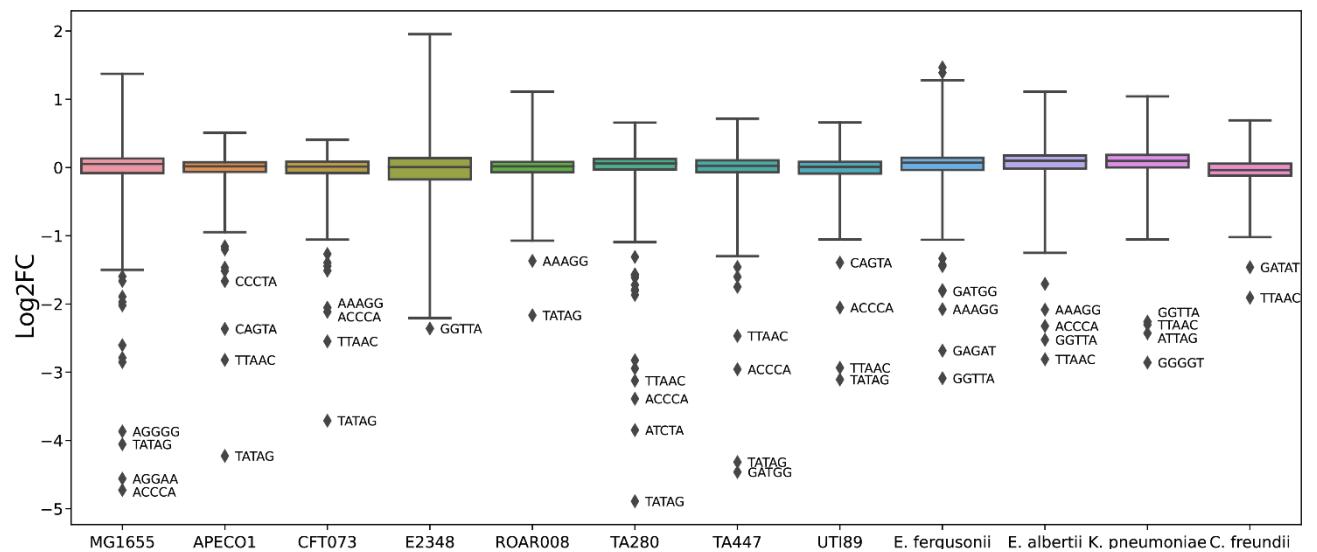
*hdhAp*

**-35**                    **-10**                    **+1**  
ATTGCAGCGAAATAATCCTCTCTTATCTGCTATACCTGGTAGT**G**TCCCTTCCTCAAGGTTAATG  
TAACGTCGCTTATTAGGAGAGAAATAGACGATATGGACCATCACAGGAAAGGAGTTCCAATTAC

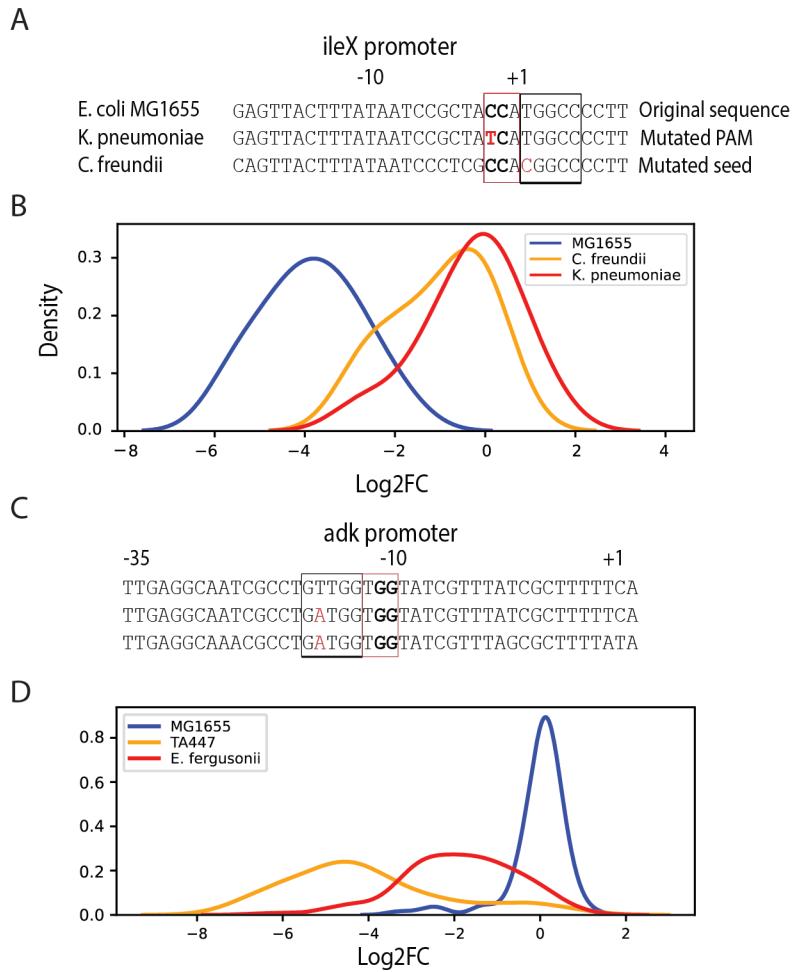
**Figure S7. Candidate off-target position for ACCCA guides in the promoter of *yfcZ* and AGGAA in the promoter of *hdhA*.**



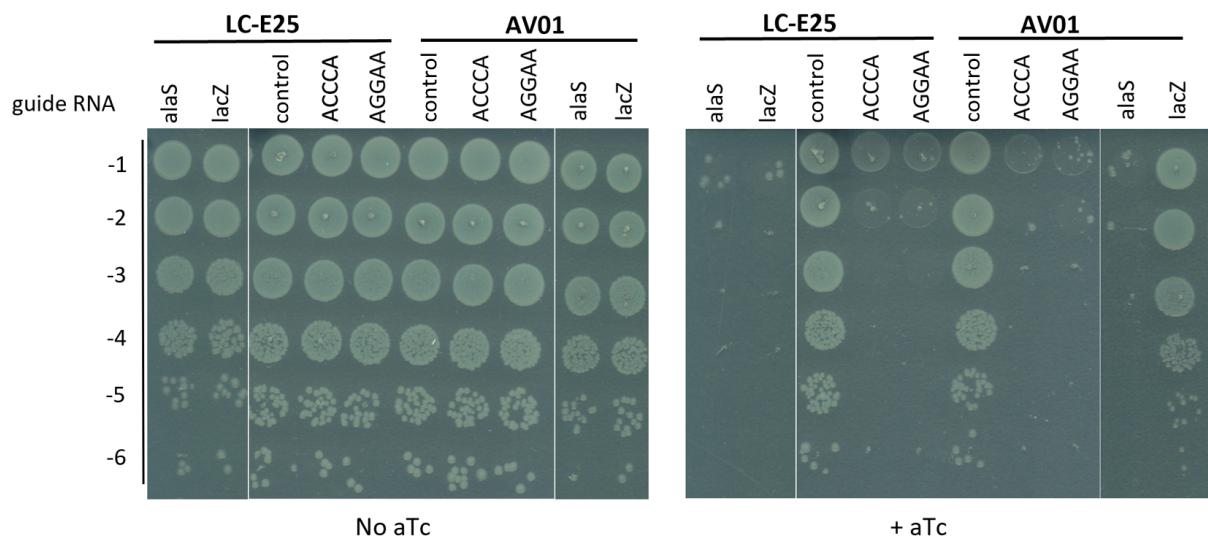
**Figure S8. Modification of the AGGAA toxic seed sequence.** The AGGAA sequence in the glyQS promoter sequence was modified to 29 unique sequences by oligonucleotide recombineering in strain LC-E18. A guide RNA with a seed sequence matching the sequence in the modified glyQS promoter was introduced in the modified strain or in LC-E18 (AGGAA sequence). Bacteria grown overnight were serial diluted and spotted on LB agar + chloramphenicol + aTc.



**Figure S9. Distribution of Log2-Fold Change for all 5-nt seeds.** Boxplot distribution of median Log2FC of all 5-nt seed sequences for the tested strains. Datapoints for the most depleted seeds for each strain are labelled.



**Figure S10. Candidate off-target positions for the GGCCA and GATGG toxic seed sequences.** (A) *ileX* promoter in *E. coli* MG1655, *K. pneumoniae* and *C. freundii*. (B) Distribution of the Log2FC of guide RNAs sharing the GGCCA seed sequence. (C) *adk* promoter in *E. coli* MG1655, *E. coli* TA447 and *E. fergusonii*. (D) Distribution of the Log2FC of guide RNAs sharing the GATGG seed sequence.



**Figure S11. The bad seed toxicity phenomenon can be observed with both Cas9 and dCas9.**  
 psgRNAc plasmids carrying various guide RNAs were electroporated in either strain AV01 (WT) which carries dCas9 under the control of a ptet promoter inducible by aTc or in the strain LC-E25 which carries the catalytically active Cas9 under control of ptet promoter. Cultures were recovered 1h after electroporation and serial diluted and spotted on plates with chloramphenicol and with or without inducer. The *alaS* and *lacZ* guides are control guides which do not carry a toxic seed sequence but have a perfect target in the chromosome of *E. coli*. Targeting the non-essential *lacZ* gene leads to cell death in the presence of Cas9 due to chromosome cleavage but not in the presence of dCas9. Targeting the essential *alaS* gene leads to cell death with both Cas9 and dCas9. Importantly, non-targeting guides carrying toxic seed sequences (AGGAA-n1 and ACCCA-n1) are toxic both with Cas9 and dCas9, showing that the bad seed phenomenon is not related to the catalytic activity of Cas9.

**Table S14. List of the top toxic seed sequences in strain LC-E18 and candidate off-target sites.**

	Seed	LC-E18 mean log2FC	Candidate off-target in the promoters of	Rational	Confirmed off- target
<b>1</b>	AGGAA	-6.50	<i>glyQS</i>	RNA-seq	<i>glyQS</i> (Fig. 2)
<b>2</b>	TGACT	-5.89	<i>rpoZ, alaS, prs</i>	Genome search	<i>alaS</i> (Fig. 3)
<b>3</b>	ACCCA	-5.86	<i>rpmH, lexA</i>	RNA-seq	<i>rpmH</i> (Fig. 2)
<b>4</b>	AAAGG	-5.63	<i>aspS, rpmB</i>	Genome search	<i>rpmB</i> (Fig. 3)
<b>5</b>	GAGGC	-5.40	<i>rpmH</i>	Found in promoter of <i>rpmH</i>	<i>rpmH</i> (Fig. S4)
<b>6</b>	CGGAA	-5.39	<i>glyQS</i>	4nt signal (see Fig1 & 2)	<i>glyQS</i> (Fig. 2)
<b>7</b>	ATATG	-5.36	<i>rplN, rpsG, ppa, rpsL</i>	Genome search	
<b>8</b>	AACTA	-5.10	<i>ftsL, ycaR-kdsB</i>	Genome search	
<b>9</b>	TGGAA	-5.06	<i>glyQS</i>	4nt signal (see Fig1 & 2)	<i>glyQS</i> (Fig. 2)
<b>10</b>	CACTC	-5.00	<i>rpmH</i>	Found in promoter of <i>rpmH</i>	<i>rpmH</i> (Fig. S4)
<b>11</b>	GTATA	-4.80	<i>ileS, rplY</i>	Genome search	
<b>12</b>	TATAG	-4.78	<i>dnaK, rnpB, folA</i>	Genome search	<i>folA</i> (Fig. 3)
<b>13</b>	GACTC	-4.64	<i>xseB, mreB</i>	Genome search	
<b>14</b>	GGGAC	-4.62	<i>glnS, prs, pssA</i>	Genome search	<i>glnS</i> (Fig. 3)