

Appendix

High-efficiency Delivery of CRISPR-Cas9 by engineered probiotics enables precise microbiome editing

Authors: Kevin Neil¹, Nancy Allard^{1§}, Patricia Roy^{1§}, Frédéric Grenier¹, Alfredo Menendez², Vincent Burrus¹, and Sébastien Rodrigue¹.

Affiliations:

¹ Département de biologie, Université de Sherbrooke, Sherbrooke, Québec, Canada, J1K 2R1.

² Département de microbiologie et infectiologie, Université de Sherbrooke, Sherbrooke, Québec, Canada, J1E 4K8.

§ These authors contributed equally to this work.

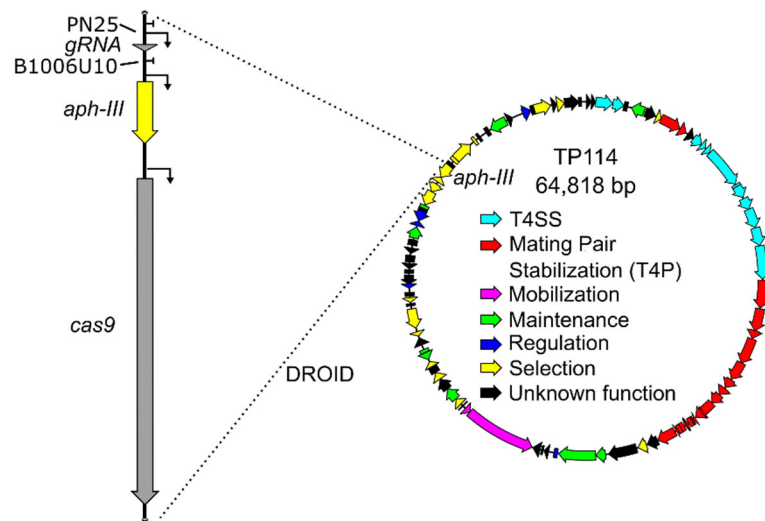
*Correspondence to: Sebastien.Rodrigue@USherbrooke.ca

This Appendix includes:

Appendix Figures S1-S6

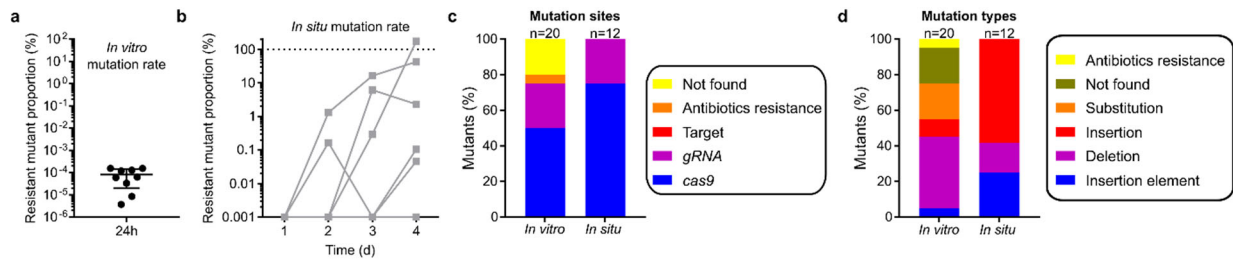
Appendix Table S1

Appendix References



Appendix Figure S1. Map of the COP genetic construct.

Schematic representation of the Kill module insertion in TP114 by DROID (Neil *et al*, 2019), which replaced the endogenous *aph-III* gene. The general function of the genes on TP114 is color coded.

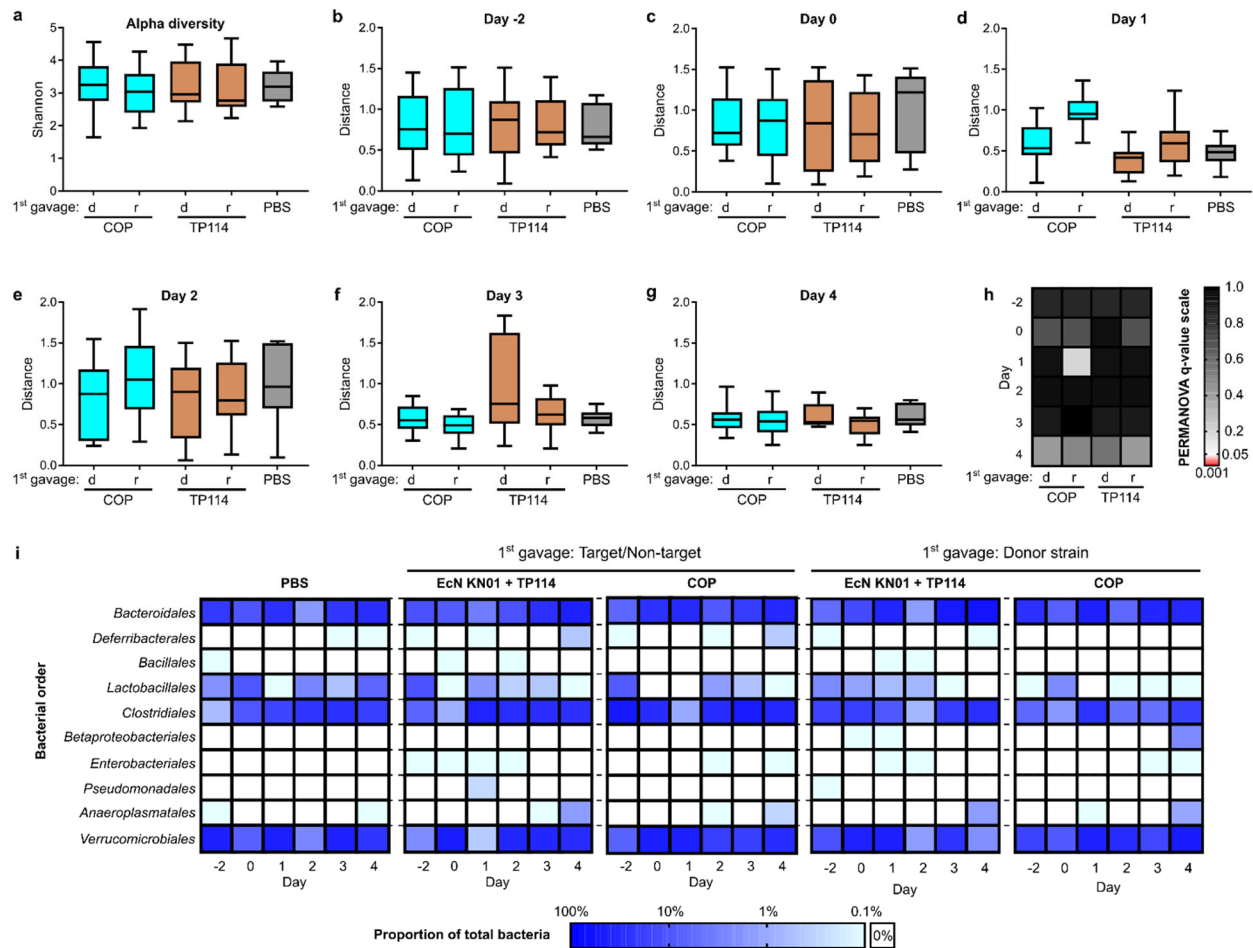


Appendix Figure S2. Analysis of COP treatment escapers.

a Frequency of transconjugants (escapers) following a 2-hour conjugation assay in broth between the COP strain and the target bacteria KN02 (n=9). Line and error bars represent the average and standard deviation of the data respectively.

b Proportion of the KN02 target bacterium population from Figure 1h that acquired TP114::Kill1 but survived (n=8). Escapers from both *in vitro* and *in situ* experiments were sequenced and the integrity of the CRISPR-*cas9* module and targeted *cat* gene sequences were analyzed. The dotted line highlights the mutant proportion at which the target bacteria population can be considered to be composed entirely of escaper mutants.

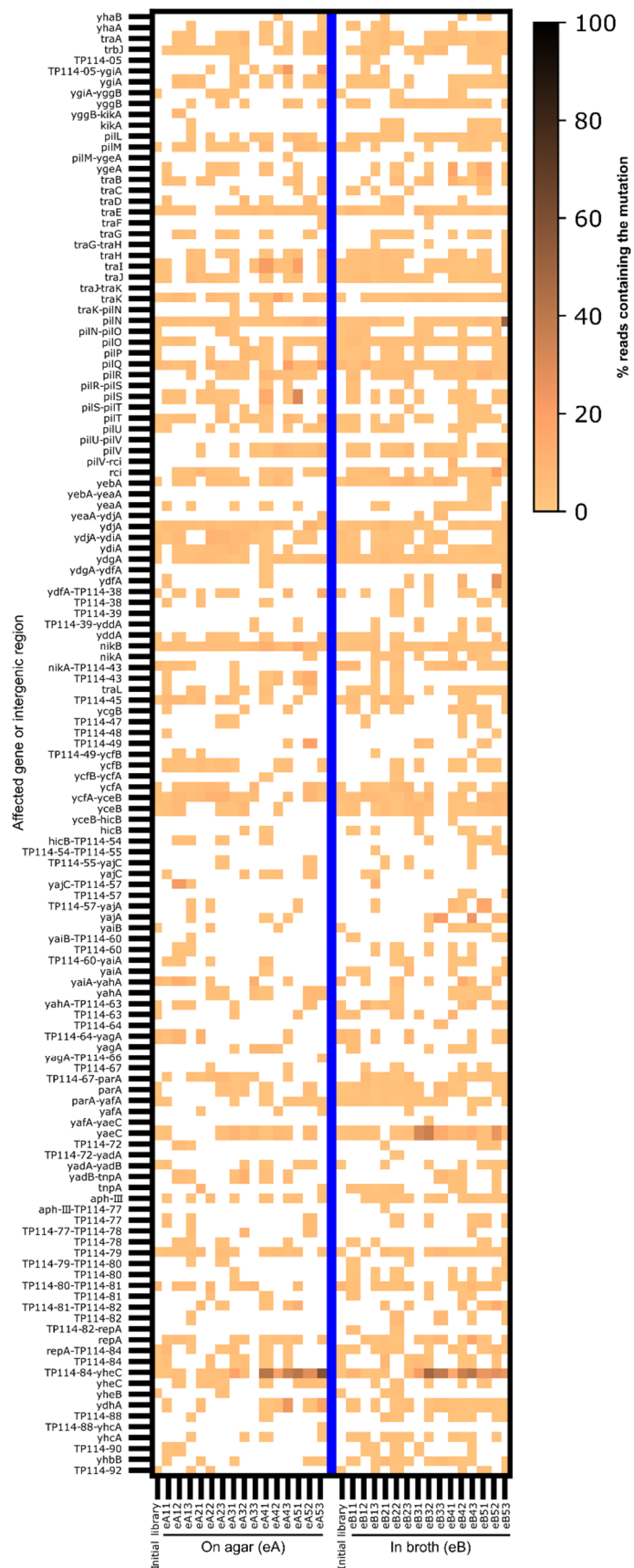
c-d Localization of deleterious mutations (**c**) and mutation types (**d**) for escapers recovered in both conditions.



Appendix Figure S3. COP treatment has no major effect on microbiota composition.

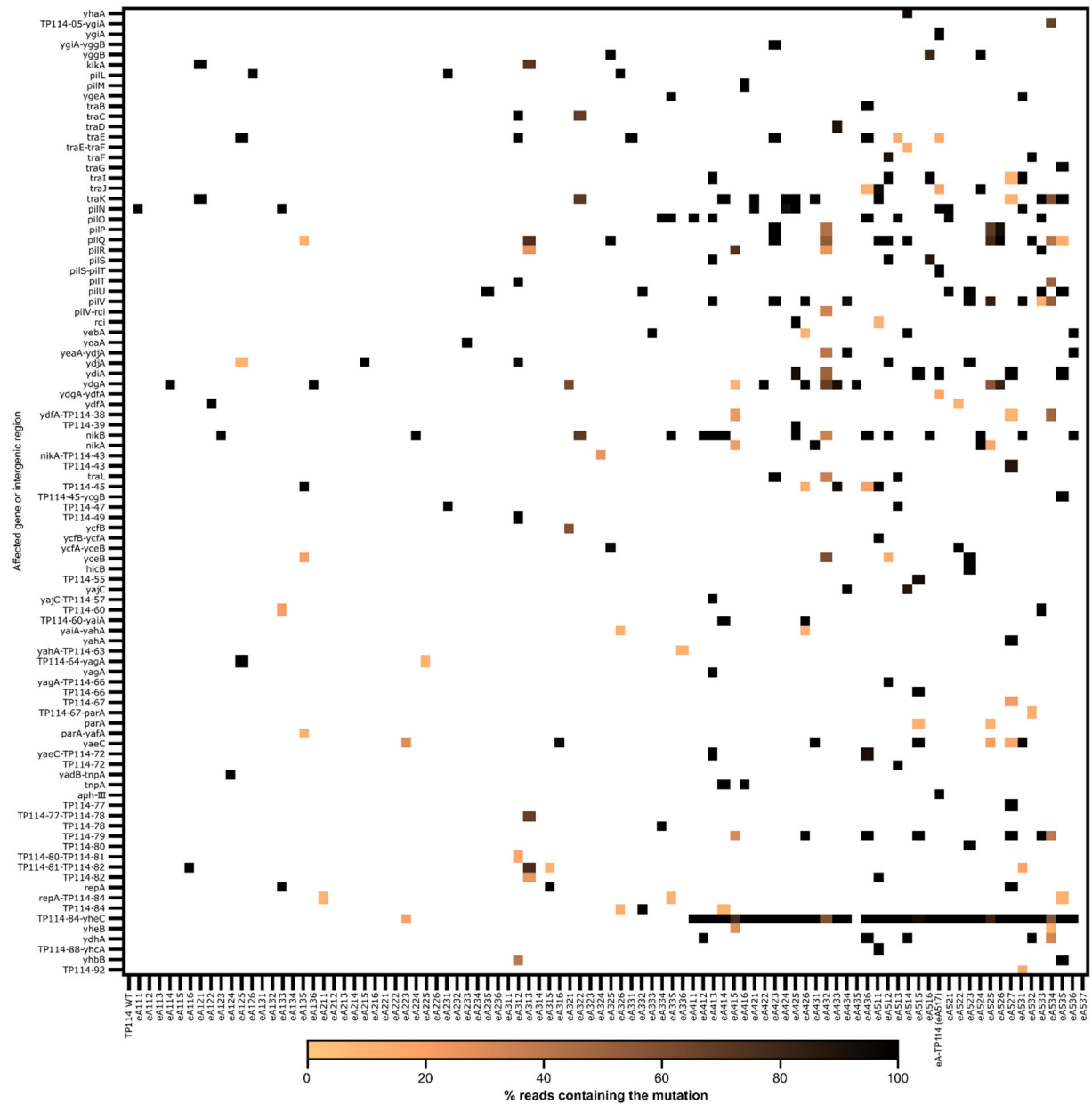
a-h Microbiota diversity for each treatment groups (see Figure 1 and Figure EV2) from day 1 to 4 evaluated using Shannon's index (**a**) or beta diversity calculated by weighted unifracs comparing each group to the PBS control at day -2 (**b**), 0 (**c**), 1 (**d**), 2 (**e**), 3 (**f**), and 4 (**g**). The corrected significance of beta diversity divergence to the PBS control is shown as a heatmap (**h**). Days are given relative to the first gavage on day 0. Day -2 represents the beginning of the streptomycin treatment. Mice groups are divided by treatment types (COP, TP114 or PBS) and strains used for the first gavage, i.e. donor (d), recipient (r) or phosphate buffered saline (PBS). All groups contained at least 4 mice. For all box and whiskers plots, medians are shown as a bar, boxes represent the 10-90% percentile of the data and whiskers extend to the minimal and maximal values.

i Relative abundance of 10 bacterial orders by 16S ribotyping in mice groups treated with PBS, EcN KN01 + TP114 or the COP (n=4).



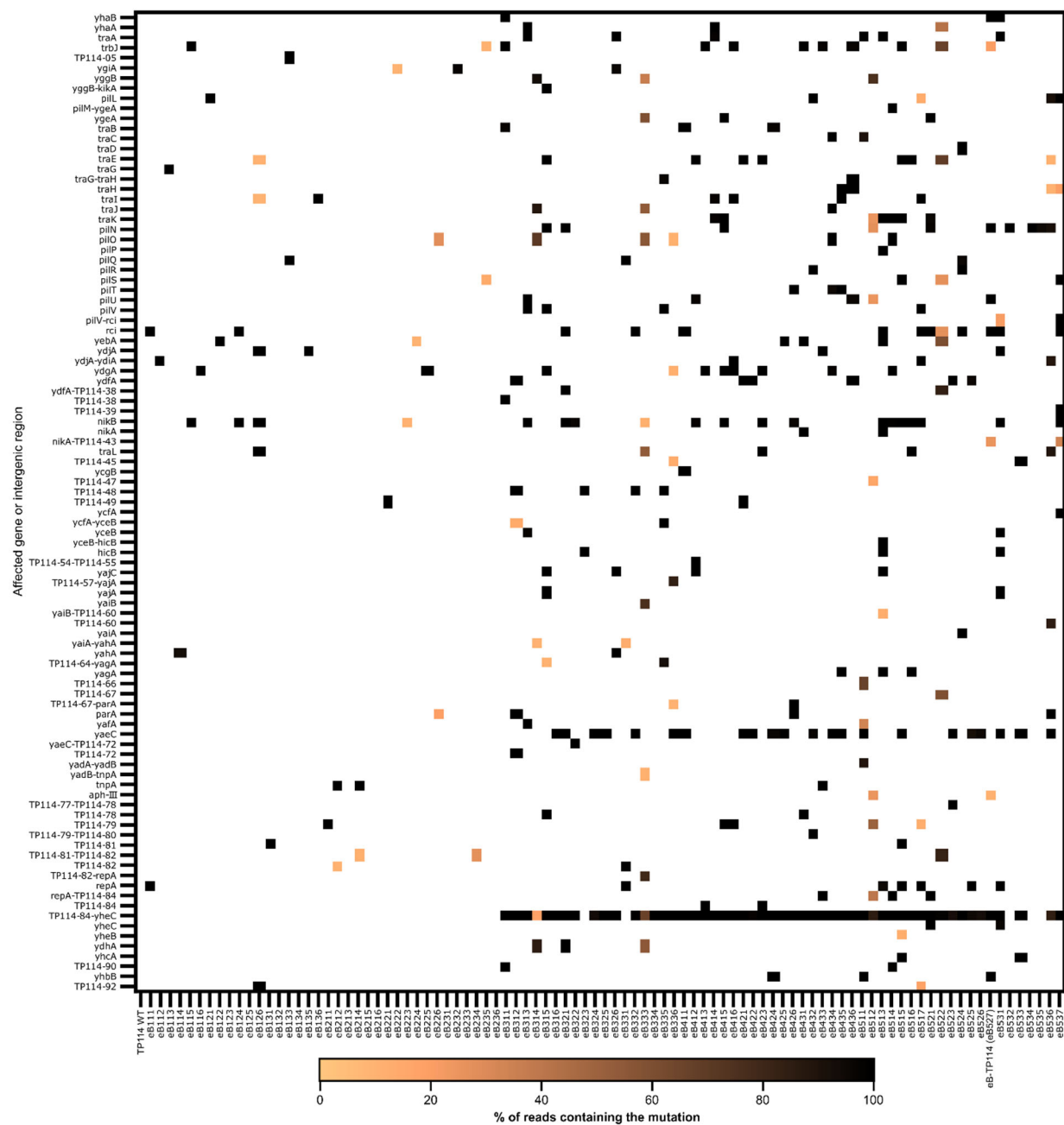
Appendix Figure S4. Accelerated laboratory evolution selected for mutations in specific regions of TP114.

Mutation heatmap showing the proportion of reads detecting a mutation in a specific gene or intergenic region in mutant libraries selected for conjugation on agar or in broth. Mutant libraries are named as either evolved on agar (eA) or in broth (eB), followed by two digits that identify the mutagenesis round and the library replicate, respectively (resulting in an eAXX or eBXX identifier). The blue line defines the limit between mutant libraires evolved on agar or in broth.



Appendix Figure S5. Isolated clones from mutagenesis rounds on agar present enriched mutations in specific regions of TP114.

Mutation heatmap showing the proportion of reads detecting a sequence variant in a specific gene or intergenic region in isolated clones. The clones obtained from evolution experiments selected on agar were named with the letters eA, followed by a three-digit suffix respectively corresponding to the mutagenesis round, the replicate, and the clone number (eAXXX).



Appendix Figure S6. Isolated clones from mutagenesis rounds in broth present enriched mutations in specific regions of TP114.

Mutation heatmap showing the proportion of reads detecting a sequence variant in a specific gene or intergenic region in isolated clones. The clones obtained from evolution experiments selected in broth were named with the letters eB, followed by a three-digit suffix respectively corresponding to the mutagenesis round, the replicate, and the clone number (eBXXX).

Appendix Table S1: List of strains and plasmids used in this study.

Strain or plasmid	Relevant phenotype or genotype*	Source/Reference
<i>Escherichia coli</i>		
AACSI01	Swine feces isolate serotype O25:H2, carries 4 natural plasmids	Kind gift from Dominic Poulin-Laprade. Cefotaxime resistant <i>E. coli</i> isolates from swine feces. Isolated from Canadian farms by Agriculture and Agri-food Canada
AACSI02	Swine feces isolate serotype O58:H21, carries 5 natural plasmids	
AACSI03	Swine feces isolate serotype O76:H7, carries 8 natural plasmids	
AACSI04	Swine feces isolate serotype O24:H4, carries 7 natural plasmids	
CP9	Extraintestinal pathogenic <i>E. coli</i> , isolated from a bacteremia, O4:K54:H5	Kind gift from Pr. Charles Dozois (Russo & Singh, 1993)
J96	Uropathogenic <i>E. coli</i> , O4:K6, isolated from a pyelonephritis patient	Kind gift from Pr. Charles Dozois (Klein & Gitai, 2013)
MT78	Avian pathogenic <i>E. coli</i> , O2:H ⁺	Kind gift from Pr. Charles Dozois (Matter et al., 2011)
RS218	Neo-natal meningitis causing <i>E. coli</i> , O18:H7:K1	Kind gift from Pr. Charles Dozois (Wijetunge et al., 2015)
KN01	Sm ^r , Sp ^r Nissle 1917	(Neil et al., 2020)
KN01 Δ dapA	Δ dapA KN01	
KN02	Sm ^r , Cm ^r Nissle 1917	
KN03	Sm ^r , Tc ^r Nissle 1917	
COP	KN01 + TP114::Kill1	This study
eB-COP	KN01 + eB-TP114::Kill1	This study
EC100Dpir ⁺	F- <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) ϕ 80 <i>dlacZ</i> Δ M15 Δ <i>lacX74</i> <i>recA1</i> <i>endA1</i> <i>araD139</i> Δ (<i>ara</i> , <i>leu</i>)7697 <i>galU</i> <i>galK</i> λ - <i>rpsL</i> <i>nupG</i> <i>pir</i> ⁺ (DHFR)	#ECP09500 (Lucigen)
MFDpir ⁺	Δ dapA, RP4 mobilization machinery inserted in chromosome	(Ferrières et al., 2010)
MG1655Nx ^R	K-12 F- λ - <i>ilvG</i> - <i>rfb</i> -50 <i>rph</i> -1 Nx ^r	(Carraro et al., 2014)
Nissle 1917	Wildtype probiotic strain	DSM-6601 (DSMZ)
<i>Citrobacter rodentium</i>		
DBS100	Model strain closely related to human EPEC and EHEC strains	Kind gift from Pr. Alfredo Menendez (Popov et al., 2019)
KN04	Sm ^R , Cm ^R DBS100 derivative	This study
Conjugative plasmids		
TP114	IncI ₂ , Km ^r	DSM-4246 (DSMZ) , Genbank accession: MF521836.1
eA-TP114 (eA517)	Evolved by accelerated laboratory evolution for 5 cycles on agar	This study
eB-TP114 (eB527)	Evolved by accelerated laboratory evolution for 5 cycles in broth	This study
Plasmids		
pBXB1	<i>oriV</i> _{pMB1} , <i>bxbl</i> integrase, <i>bla</i> (Ap ^r)	Genbank: MK756311(Neil et al., 2019)
pE-FLP	<i>oriV</i> _{pSC101ts} , <i>flp</i> , Ap ^r	Addgene #45978
pGRG36-SmCm	Tn7 machinery to insert the <i>aad7</i> and <i>cat</i> genes in the <i>glmS</i> terminator region	(Neil et al. 2020, McKenzie et al., 2006)
pKill1	<i>oriV</i> _{pSC101ts} , <i>attP</i> _{bxbl} , FRT, 1 gRNA vs <i>cat</i> , <i>aph-IIIa</i> (Km ^r), <i>cas9</i>	Genbank: MK756312(Neil et al., 2019)
MP6	Mutagenesis plasmid	(Badran & Liu, 2015)
pREC1	<i>oriV</i> _{R6K} , <i>attB</i> _{bxbl} , <i>tetB</i> (Tc ^r), FRT	Genbank: MK756315(Neil et al., 2019)
pSIM6	<i>oriV</i> _{pSC101ts} , Lambda Red recombinase, Ap ^r	(Datsenko & Wanner, 2000)
pT	<i>oriV</i> _{pMB1} , <i>sfGFP</i> , <i>araC</i> , <i>cat</i> .	IGEM : BBa_1746908 (Neil et al., 2019)
TP114::Kill1	TP114::tetB-Kill1 after FRT driven deletion of <i>tetB</i> and <i>oriV</i> _{pSC101ts}	(Neil et al., 2019)
TP114::tetB	TP114 Δ aph-III::tetB	(Neil et al., 2019)
TP114::tetB-Kill1	TP114::tetB with inserted Killing module 1	(Neil et al., 2019)
eB-TP114::Kill1	eB-TP114::tetB-Kill1 after FRT driven deletion of <i>tetB</i> and <i>oriV</i> _{pSC101ts}	This study
eB-TP114::tetB	eB-TP114 Δ aph-III::tetB	This study
eB-TP114::tetB-Kill1	eB-TP114::tetB with inserted Killing module 1	This study

*Antibiotic resistance: Ap^r: Ampicillin, Cm^r: Chloramphenicol, Km^r: Kanamycin, Nx^r: Nalidixic acid, Sp^r: Spectinomycin, Sm^r: Streptomycin, Su^r: Sulfamethoxazole, Tc^r: Tetracycline, and Tm^r: Trimethoprim.

Appendix References

- Badran, A. H., & Liu, D. R. (2015). Development of potent in vivo mutagenesis plasmids with broad mutational spectra. *Nature Communications*, 6(8425), 1–10.
<https://doi.org/10.1038/ncomms9425>
- Carraro, N., Matteau, D., Luo, P., & Burrus, V. (2014). The Master Activator of IncA / C Conjugative Plasmids Stimulates Genomic Islands and Multidrug Resistance Dissemination. *PLoS Genetics*, 10(10), e1004714.
<https://doi.org/10.1371/journal.pgen.1004714>
- Datsenko, K. A., & Wanner, B. L. (2000). One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *PNAS*, 97(12), 6640–6645.
- Ferrières, L., Hémerly, G., Nham, T., Guérout, A. M., Mazel, D., Beloin, C., & Ghigo, J. M. (2010). Silent mischief: Bacteriophage Mu insertions contaminate products of *Escherichia coli* random mutagenesis performed using suicidal transposon delivery plasmids mobilized by broad-host-range RP4 conjugative machinery. *Journal of Bacteriology*, 192(24), 6418–6427. <https://doi.org/10.1128/JB.00621-10>
- Klein, E. A., & Gitai, Z. (2013). Draft genome sequence of uropathogenic *Escherichia coli* strain J96. *Genome Announcements*, 1(1), 6–7. <https://doi.org/10.1128/genomeA.00245-12>
- Matter, L. B., Barbieri, N. L., Nordhoff, M., Ewers, C., & Horn, F. (2011). Avian pathogenic *Escherichia coli* MT78 invades chicken fibroblasts. *Veterinary Microbiology*, 148(1), 51–59. <https://doi.org/10.1016/j.vetmic.2010.08.006>
- McKenzie, G. J., Craig, N. L., Grozdanov, L., Zähringer, U., Blum-Oehler, G., Brade, L., Henne, A., Knirel, Y., Schombel, U., Schulze, J., Sonnenborn, U., Gottschalk, G., Hacker, J., Rietschel, E., Dobrindt, U., Bumann, D., Hueck, C., Aebischer, T., Meyer, T., ... Maniatis, T. (2006). Fast, easy and efficient: site-specific insertion of transgenes into Enterobacterial chromosomes using Tn 7 without need for selection of the insertion event. *BMC Microbiology*, 6(39), 1–7. <https://doi.org/10.1186/1471-2180-6-39>
- Neil, K., Allard, N., Grenier, F., Burrus, V., & Rodrigue, S. (2020). Highly efficient gene transfer in the mouse gut microbiota is enabled by the IncI2 conjugative plasmid TP114. *Communications Biology*, 3(523), 1–9. <https://doi.org/10.1038/s42003-020-01253-0>
- Neil, K., Allard, N., Jordan, D., & Rodrigue, S. (2019). Assembly of large mobilizable genetic cargo by double recombinase operated insertion of DNA (DROID). *Plasmid*, 104(June),

102419. <https://doi.org/10.1016/j.plasmid.2019.102419>

- Popov, G., Fiebig-Comyn, A., Shideler, S., Coombes, B. K., & Savchenko, A. (2019). Complete Genome Sequence of *Citrobacter rodentium* Strain DBS100. *Microbiology Resource Announcements*, 8(24), 1–2. <https://doi.org/10.1128/mra.00421-19>
- Russo, T. A., & Singh, G. (1993). An extraintestinal, pathogenic isolate of *Escherichia coli* (O4/K54/H5) can produce a group 1 capsule which is divergently regulated from its constitutively produced group 2, K54 capsular polysaccharide. *Journal of Bacteriology*, 175(23), 7617–7623. <https://doi.org/10.1128/jb.175.23.7617-7623.1993>
- Wijetunge, D. S. S., Katani, R., Kapur, V., & Kariyawasama, S. (2015). Complete genome sequence of *Escherichia coli* strain RS218 (O18: H7: K1), associated with neonatal meningitis. *Genome Announcements*, 3(4), 5–6. <https://doi.org/10.1128/genomeA.00804-15>