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Stabilizing genetically unstable simple sequence repeats in the *Campylobacter jejuni* genome by multiplex genome editing: a reliable approach for delineating multiple phase-variable genes

Short title: Genetic engineering of multiple hypermutable simple sequence repeats

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Abstract

Hypermutability of simple sequence repeats (SSR) through DNA slippage is a major mechanism of phase variation in *Campylobacter jejuni*. The presence of multiple SSR-mediated phase-variable genes encoding enzymes that modify surface structures, including capsular polysaccharide (CPS) and lipooligosaccharide (LOS), generates high levels of structural variants within bacterial populations, thereby promoting adaptation to selective pressures in host environments. Therefore, the phenotypic diversity generated by phase variation can limit the reproducibility of results with *C. jejuni*; therefore, researchers need to genetically control the mutability of multiple SSRs. Here, we show that natural "cotransformation" is an effective method for *C. jejuni* genome editing. Cotransformation is a trait of naturally competent bacteria that causes uptake and integration of multiple different DNA fragments, which has been recently adapted to multiplex genome editing by natural transformation (MuGENT), a method for introducing multiple scarless mutations into the genomes of these bacteria. We found that the cotransformation frequencies of antibiotic resistance gene-marked DNA fragments and unmarked DNA fragments reached ~40% in *C. jejuni*. To examine the feasibility of MuGENT in *C. jejuni*, we "locked" either different polyG SSR tracts in strain NCTC11168 (which are located in the biosynthetic CPS and LOS gene clusters) into either the ON or OFF configurations by interrupting the continuous runs of G residues without changing the encoded amino acids. This approach, termed "MuGENT-SSR," enabled the generation of all eight edits within 2 weeks and the identification of a phase-locked strain with a highly stable type of Penner serotyping, a CPS-based serotyping scheme. Furthermore, extensive genome editing of this strain by MuGENT-SSR identified a phase-variable gene that determines the Penner serotype of

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NCTC11168. Thus, MuGENT-SSR provides a platform for genetic and phenotypic engineering of genetically unstable *C. jejuni*, making it a reliable approach for elucidating the mechanisms underlying phase-variable expression of specific phenotypes.

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Author summary

Campylobacter jejuni is the leading bacterial cause of food-borne gastroenteritis in developed countries and occasionally progresses to the autoimmune disease Guillain–Barré syndrome. The genetically and phenotypically unstable features of this bacterial species limit research and development efforts. A relatively large number of hypermutable simple sequence repeat (SSR) tracts in the *C. jejuni* genome markedly decreases its phenotypic stability through reversible changes in the ON or OFF expression states of the genes in which they reside, a phenomenon called phase variation. Thus, controlling SSR-mediated phase variation can be important for achieving stable and reproducible research on *C. jejuni*. In this study, we developed a feasible and effective approach to genetically manipulate multiple SSR tracts in the *C. jejuni* genome using natural cotransformation, a trait of naturally transformable bacterial species that causes the uptake and integration of multiple different DNA molecules. This approach will greatly help to improve the genetic and phenotypic stability of *C. jejuni* to enable diverse applications in research and development.

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Introduction

Simple sequence repeats (SSR) or microsatellites in bacterial genomes are highly mutable because of the potential for DNA-strand slippage during DNA replication [1]. Mispairing DNA by slippage results in the insertion or deletion of one or more repeats within a repetitive DNA element. When an SSR tract is present within an open reading frame (ORF), promoter, or other regulatory sequences, its hypermutability mediates reversible and frequent changes in specific phenotypes through transcriptional or translational genetic switches between ON and OFF states, which is called "phase variation" [2,3]. In several bacterial species, including *Campylobacter jejuni*, the presence of multiple SSR-mediated phase-variable genes (also called contingency genes) per genome generates high levels of phenotypic variants within bacterial populations [4,5].

C. jejuni is the leading bacterial cause of food-borne gastroenteritis in developed countries, primarily depending on its ability to colonize the caeca of chickens and to survive in the food chain by attaching to undercooked chicken meat [6,7]. *C. jejuni* readily colonizes the intestinal mucosa of a wide variety of wild and domestic birds and other animals. Infections in poultry are usually asymptomatic, while human infection can cause significant inflammation and bloody diarrhea, occasionally progressing to the autoimmune disease, Guillain–Barré syndrome [8]. As a commensal bacterium of poultry and a human pathogen, *C. jejuni* needs to rapidly adapt to differences in host environments, such as changing nutrient compositions and immune systems. Additional selective pressures are caused by transmission through genetically and immunologically variable host populations and exposure to bacteriophages. *C. jejuni* likely utilizes phase variation as a major mechanism for adapting to these selective pressures.

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A relatively large number of SSR tracts consisting of seven or more G or C bases was unexpectedly found in the AT-rich genomes of *C. jejuni*. These polyG/C SSR tracts are mainly involved in phase variation in this species, and a genomic analysis of four *C. jejuni* strains indicated the presence of 12 to 29 tracts per genome [9]. *C. jejuni* NCTC11168 encodes 29 polyG/C tracts, 23 of which are in protein-coding regions [10], suggesting that phase variation in this strain is regulated mainly at the translational level. The majority of these loci are clustered on the genomic regions predicted to encode enzymes involved in modifying surface structures, including lipooligosaccharide (LOS), capsular polysaccharide (CPS), and flagella, but a few are located on genes encoding cell-surface proteins or restriction enzymes [5]. The ability to change the antigenicity of surface structures by phase variation strongly suggests that this phenotypic diversity could affect colonization or pathogenesis in host organisms. Consistently, it has been reported that phase-variable expression of specific genes affects pathogenesis in humans and colonization in chickens [11–15]. Comprehensive studies of multiple phase-variable genes in *C. jejuni* have also demonstrated that culturing or passage through animal and human hosts results in significant phase changes in multiple SSR-containing genes, particularly those involved in surface-structure modifications, which in some cases, are coupled with enhanced colonization and pathogenesis [9,16–20].

A limitation of studying the contributions of multiple SSR-mediated phase-variable genes to specific phenotypes is that researchers currently lack genetic tools for efficiently “locking” SSR into either ON or OFF states at multiple loci in the *C. jejuni* genome. For example, the use of a multiplexed SSR-editing technology enables phenotype stabilization and therefore promotes reproducible research activities in this

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111 area. Such phenotypic engineering would also be applicable for stably producing phase-
112 variable surface antigenic determinants, which can be used for vaccine development and
113 raising serotyping antisera. To date, multiplex automated genome engineering (MAGE)
114 using oligonucleotides, a highly efficient λRed recombinase, and clustered regularly
115 interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein (Cas)
116 systems have been developed for targeted genome engineering in bacteria [21,22].
117 However, these methods require not only engineered plasmids or chromosomes for the
118 respective genome-editing systems (i.e., expressing proteins and/or RNAs involved in
119 the λRed and CRISPR/Cas systems) but also, in the case of CRISPR/Cas, the selection
120 of edited genomic sites. Natural competence for transformation is shared by diverse
121 bacterial species [23,24]. This process involves the uptake of exogenous DNA, followed
122 by integration of the imported DNA into the genome by homologous recombination.
123 Because natural transformation does not require any special factors and is dependent on
124 the recipient bacterium and donor DNA molecules, it has long been used for genetic
125 engineering in naturally competent bacterial species. *C. jejuni* was shown to be
126 naturally transformable over 30 years ago [25]. However, natural transformation has not
127 been conventionally used as a genetic engineering method in this bacterium because it
128 can be highly transformed with DNA prepared from *C. jejuni*, but not with DNA
129 propagated in *Escherichia coli* or amplified by the polymerase chain reaction (PCR).
130 Recently, Beauchamp *et al*. demonstrated that methylation at the RAATTY sequence of
131 *E. coli*-derived plasmid DNA or PCR-amplified DNA can efficiently transform *C. jejuni*
132 [26]. In a study of other transformable species conducted by Dalia *et al*., the authors
133 established the multiplex genome editing by natural transformation (MuGENT) system,
134 which is based on “cotransformation,” a trait that causes the uptake and integration of

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multiple different DNA molecules [27–29]. These two pioneering studies have provided great potential for performing multiplexed gene modifications in *C. jejuni* and motivated us to examine this feasibility. Here, we optimized natural transformation and cotransformation using PCR-amplified donor DNA fragments and demonstrated its utility as a method for multiplex genome editing in naturally competent *C. jejuni*.

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Results and discussion

Optimization of donor DNA for the natural transformation of *C. jejuni*

Data from a previous study by Beauchamp *et al*. demonstrated that DNA methylated at the RA^{m6}ATTY sequence is an efficient substrate for *C. jejuni* transformation [26]. To optimize the natural transformation of this bacterium, we investigated in greater detail the characteristics of DNA substrate generated using PCR products. For this purpose, we amplified various DNA fragments of the *rpsL*^{K88R} allele, which confers streptomycin resistance (Sm^{R}) [30]. These *rpsL*^{K88R} marker fragments had different lengths of regions homologous to the recombination target sequence (50–2,000 base pairs [bp]) and different numbers of the EcoRI methyltransferase-recognition sequence (GAATTC), which was endogenously present or exogenously added to the PCR primers (Fig 1, n = 1 to 3). Each 0.5 pmol of the amplified DNA was treated with EcoRI methyltransferase and then used in transformation assays to transform the NCTC11168 and 81-176 strains, which are representative strains used for genetic studies of *C. jejuni* [5,31,32] (Table 1). We did not obtain transformants using fragments with short regions of homology (50 or 100 bp), even though these DNA fragments had two methylated GAATTC sites (Fig 1, *rpsL*^{K88R}-1 and *rpsL*^{K88R}-2). In contrast, the NCTC11168 and 81-176 strains were transformed at substantially higher frequencies using fragments with longer regions of homology (500 bp; Fig 1, *rpsL*^{K88R}-5). The highest transformation frequencies were obtained when 1,000- to 2,000-bp homologies were present (Fig 1, *rpsL*^{K88R}-8 and *rpsL*^{K88R}-9). The length of a homologous sequence is a major determinant of the efficiency of RecA-dependent recombination [33]. In the Δ*recA* background, no transformants were detected using a fragment with 2,000 homologous bp (Fig 1, *rpsL*^{K88R}-9), suggesting that transformation in *C. jejuni* is mediated by RecA. We also

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164 confirmed that the lack of the GAATTC sequence or the lack of GAATTC methylation
165 markedly decreased the transformation frequency (Fig 1, *rpsL*^{K88R}-3 and unmethylated
166 *rpsL*^{K88R}-5). However, increasing the number of methylated GAATTC sites did
167 not substantially increase the transformation frequencies (Fig 1, *rpsL*^{K88R}-4 and
168 *rpsL*^{K88R}-5 or *rpsL*^{K88R}-6, *rpsL*^{K88R}-7, and *rpsL*^{K88R}-8). These results suggest that one
169 methylated GAATTC sequence is sufficient for transformation, consistent with previous
170 results obtained using plasmid DNA as a donor [26]. In summary, to maximize the
171 efficiency of *C. jejuni* transformation, the donor DNA should contain two key structural
172 elements: (1) a ≥1,000-bp region of homology and (2) at least one methylated GAATTC
173 site.
174
175 **Fig 1. Effects of homology length and methylation on natural transformation.** *C.*
176 *jejuni* cells were transformed using *rpsL*^{K88R} marker fragments with different lengths of
177 homologous regions compared to the recombination target sequence (50–2,000 bp,
178 indicated in parentheses) and different numbers of EcoRI methyltransferase-recognition
179 sequences (RIs). Each 0.5 pmol of amplified DNA was treated with EcoRI
180 methyltransferase and then used in the transformation assays. The transformation
181 frequency was defined as the number of Sm^{R} colony-forming units (CFUs) divided by
182 the total number of CFUs. Data from three independent transformations are presented as
183 the means ± standard deviations. As controls, we used unmethylated DNA (indicated by
184 "Unmethylation of *rpsL*^{K88R}-5") or assayed for transformation in the absence of donor
185 DNA (indicated by "No addition of DNA"). "ND" indicates not done. The following
186 strains were used: NCTC11168 WT, NCTC11168; NCTC11168 Δ*recA*, SYC1004; 81-
187 176 WT, 81-176; 81-176 Δ*recA*, and SYC2004. The following DNA fragments were

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188 used (S2 Table): *rpsL*^{K88R}-1 and *rpsL*^{K88R}-2 (for both strains); *rpsL*^{K88R}-3 and *rpsL*^{K88R}-
189 3-1 (for NCTC11168) and *rpsL*^{K88R}-3-2 (for 81-176); *rpsL*^{K88R}-4 and *rpsL*^{K88R}-4-1 (for
190 NCTC11168) and *rpsL*^{K88R}-4-2 (for 81-176); *rpsL*^{K88R}-5 and *rpsL*^{K88R}-5-1 (for
191 NCTC11168) and *rpsL*^{K88R}-5-2 (for 81-176); *rpsL*^{K88R}-6 and *rpsL*^{K88R}-6-1 (for
192 NCTC11168) and *rpsL*^{K88R}-6-2 (for 81-176); *rpsL*^{K88R}-7 and *rpsL*^{K88R}-7-1 (for
193 NCTC11168) and *rpsL*^{K88R}-7-2 (for 81-176); *rpsL*^{K88R}-8 and *rpsL*^{K88R}-8-1 (for
194 NCTC11168) and *rpsL*^{K88R}-8-2 (for 81-176); and *rpsL*^{K88R}-9 and *rpsL*^{K88R}-9-1 (for
195 NCTC11168) and *rpsL*^{K88R}-9-2 (for 81-176).
196
197 **Table 1 Strains and plasmids used in this study**

Strain or plasmid	Characteristics	Source or reference
Strain		
NCTC11168	*C. jejuni* human origin, serotype B (antigenic factor HS2)	[31]
SYC1001	NCTC11168 Δ*flaA*::*cat*	This study
SYC1002	NCTC11168 Δ*flaA*::*kan*	This study
SYC1003	NCTC11168 *rpsL*^{K88R}	This study
SYC1004	NCTC11168 Δ*recA*::*cat*	This study
SYC1006	NCTC11168 *cj1426*::*astA* Δ*flaA*::*kan*	This study
SYC1007	NCTC11168 *cj1426*^{ON}::*astA* Δ*flaA*::*cat*	This study
SYC1008	NCTC11168 *cj1426*^{OFF}::*astA* Δ*flaA*::*kan*	This study
SYC1P000K	NCTC11168 Δ*flaA*::*kan* *cj1139*^{OFF} *cj1144*^{OFF} *cj1420*^{OFF} *cj1421*^{OFF} *cj1422*^{OFF} *cj1426*^{OFF} *cj1429*^{OFF} *cj1437*^{OFF}	This study
SYC1P000	NCTC11168 *cj1139*^{OFF} *cj1144*^{OFF} *cj1420*^{OFF} *cj1421*^{OFF} *cj1422*^{OFF} *cj1426*^{OFF} *cj1429*^{OFF} *cj1437*^{OFF}	This study
SYC1P001C	NCTC11168 Δ*flaA*::*cat* *cj1139*^{OFF} *cj1144*^{OFF} *cj1420*^{OFF} *cj1421*^{OFF} *cj1422*^{OFF} *cj1426*^{OFF} *cj1429*^{OFF} *cj1437*^{ON}	This study
SYC1P004K	NCTC11168 Δ*flaA*::*kan* *cj1139*^{OFF} *cj1144*^{OFF}	This study

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	cj1420^{OFF} *cj1421*^{OFF} *cj1422*^{OFF} *cj1426*^{ON} *cj1429*^{OFF} *cj1437*^{OFF}	
SYC1P005C	NCTC11168 Δ*flaA*::*cat* *cj1139*^{OFF} *cj1144*^{OFF} *cj1420*^{OFF} *cj1421*^{OFF} *cj1422*^{OFF} *cj1426*^{ON} *cj1429*^{OFF} *cj1437*^{ON}	This study
SYC1P032K	NCTC11168 Δ*flaA*::*kan* *cj1139*^{OFF} *cj1144*^{OFF} *cj1420*^{ON} *cj1421*^{OFF} *cj1422*^{OFF} *cj1426*^{OFF} *cj1429*^{OFF} *cj1437*^{OFF}	This study
SYC1P033C	NCTC11168 Δ*flaA*::*cat* *cj1139*^{OFF} *cj1144*^{OFF} *cj1420*^{ON} *cj1421*^{OFF} *cj1422*^{OFF} *cj1426*^{OFF} *cj1429*^{OFF} *cj1437*^{ON}	This study
SYC1P036C	NCTC11168 Δ*flaA*::*cat* *cj1139*^{OFF} *cj1144*^{OFF} *cj1420*^{ON} *cj1421*^{OFF} *cj1422*^{OFF} *cj1426*^{ON} *cj1429*^{OFF} *cj1437*^{OFF}	This study
SYC1P037C	NCTC11168 Δ*flaA*::*cat* *cj1139*^{OFF} *cj1144*^{OFF} *cj1420*^{ON} *cj1421*^{OFF} *cj1422*^{OFF} *cj1426*^{ON} *cj1429*^{OFF} *cj1437*^{ON}	This study
SYC1P037	NCTC11168 *cj1139*^{OFF} *cj1144*^{OFF} *cj1420*^{ON} *cj1421*^{OFF} *cj1422*^{OFF} *cj1426*^{ON} *cj1429*^{OFF} *cj1437*^{ON}	This study
SYC1P255K	NCTC11168 Δ*flaA*::*kan* *cj1139*^{ON} *cj1144*^{ON} *cj1420*^{ON} *cj1421*^{ON} *cj1422*^{ON} *cj1426*^{ON} *cj1429*^{ON} *cj1437*^{ON}	This study
SYC1P255	NCTC11168 *cj1139*^{ON} *cj1144*^{ON} *cj1420*^{ON} *cj1421*^{ON} *cj1422*^{ON} *cj1426*^{ON} *cj1429*^{ON} *cj1437*^{ON}	This study
81-176	*C. jejuni* raw milk origin, serotype R (antigenic factors HS23/HS36)	[32]
SYC2001	81-176 Δ*flaA*::*cat*	This study
SYC2002	81-176 Δ*flaA*::*kan*	This study
SYC2003	81-176 *rpsL*^{K88R}	This study
SYC2004	81-176 Δ*recA*::*cat*	This study
Plasmid		
pUCFa	Cloning vector, *bla*	Fasmac
pSYC-*cat*	pUCFa *cat* from *Campylobacter coli*	This study
pSYC-*kan*	pUCFa *kan* from *C. coli*	This study

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Occurrence of natural cotransformation in *C. jejuni*

Using *Vibrio cholerae* and *Streptococcus pneumoniae*, Dalia *et al*. developed the MuGENT system using cotransformation, a trait of several naturally competent species [27–29]. During MuGENT, a bacterial culture is incubated with two types of donor DNA fragments: (1) a selected fragment that introduces an antibiotic-resistance gene into the genome and (2) unselected fragments that introduce scarless or transgene-free edits of interest at one or more loci. In *V. cholerae*, the frequencies of cotransformation of these distinct genetic markers can be made to exceed 60% by increasing the length of homology and the concentration of the unselected fragment [27]. To assess natural cotransformation in *C. jejuni*, we used two strains (SYC1003 and SYC2003) as recipients, which harbor the *rpsL*^{K88R} mutations in the NCTC11168 and 81-176 genomes, respectively, and therefore are resistant to Sm (Fig 2). We also used a Δ*flaA*::*kan* fragment with 1,000-bp regions of homology to replace the flagellin gene with a kanamycin-resistance (Km^{R}) marker (selected) and *rpsL*$^{+}$ fragments with homologous regions of different sizes (1,000 bp: *rpsL*$^{+}$-8, 2,000 b: *rpsL*$^{+}$-9) to revert to the wild-type Sm-sensitive (Sm^{S}) phenotype (unselected). After transforming SYC1003 and SYC2003 with equimolar concentrations of the selected and unselected methylated DNA (mDNA) fragments, we selected Km^{R}-transformants, 100 of which were then subjected to Sm-sensitivity testing to evaluate cotransformation (Fig 2). Using unselected mDNA with a 1,000-bp region of homology resulted in cotransformation frequencies of 24% and 16% in SYC1003 and SYC2003, respectively, and acquisition of the Km^{R} and Sm^{S} phenotypes (Fig 2). Furthermore, increasing the homology of unselected mDNA (2,000 bp) slightly increased the cotransformation frequencies of

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222 both strains (Fig 2). Thus, we recommend the use of unselected mDNA with 2,000-bp
223 homology for efficient *C. jejuni* cotransformation.
224
225 **Fig 2. Evaluation of natural *C. jejuni* cotransformation.** Natural cotransformation of
226 *C. jejuni* was examined using two different PCR fragments, namely Δ*flaA*::*kan* and *rpsL*
227 $^{+}$ (S2 Table). The former fragments (Δ*flaA*::*kan*-1 for NCTC11168, or Δ*flaA*::*kan*-2 for
228 81-176) had 1,000 bp regions of homology (selected), whereas the latter fragments
229 (*rpsL*$^{+}$-8-1 and *rpsL*$^{+}$-9-1 for NCTC11168, or *rpsL*$^{+}$-8-2 and *rpsL*$^{+}$-9-2 for 81-176) had
230 1,000-b or 2,000-b regions of homology (unselected). "Me" indicates a methylated site.
231 Equimolar quantities (0.5 pmol) of selected and unselected fragments were
232 independently methylated (indicated by the labeling "Selected mDNA" and "Unselected
233 mDNA," respectively), mixed, and then purified. After transforming strains carrying the
234 *rpsL*^{K88R} allele (SY1003 and SY2003) with these mDNA fragments, the bacteria were
235 spread onto brain heart infusion (BHI) agar plates containing Km. One hundred Km^{R}-
236 transformants were replica-plated onto BHI agar plates containing Sm. The
237 cotransformation frequency (%) was calculated as follows: 100 × number of Sm^{S} CFUs
238 per 100 Km^{R} CFUs. Data from three independent transformations are presented as the
239 means ± standard deviations.
240
241 Natural cotransformation is thought to reflect the nature of competent bacterial cells,
242 among which only a subpopulation of cells in a culture is transformable [28]. We
243 demonstrated for the first time that *C. jejuni* cells are capable of cotransformation. It
244 will be important to elucidate whether *C. jejuni* cells show variable competence within a
245 population.

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246

247 **Establishment of MuGENT in *C. jejuni***

248 MuGENT provides methods for simultaneously generating multiple scarless
249 mutations and can therefore be broadly applied in diverse research and biotechnology
250 applications [27]. We wanted to test whether natural cotransformation could be used for
251 multiplex genome editing of *C. jejuni*. Fig 3 presents a schematic representation of our
252 strategy. Briefly, multiple unselected mDNA fragments (used for introducing genome
253 edits of interest) were mixed at equimolar concentrations with a selected mDNA
254 fragment, and the resulting mixture was used to cotransform *C. jejuni* cells. Because
255 MuGENT often requires multiple cycles of cotransformation in order to complete the
256 genome editing, different selectable markers should be used for each cycle [27]. We
257 swapped different resistance markers at the *flaA* gene at every MuGENT cycle. This
258 also enabled easy removal of the marker genes and reversion to the wild-type allele by
259 transformation with *flaA*$^{+}$ mDNA and subsequent selection of motile clones. Genome
260 editing was verified by multiplex allele-specific colony (MASC) PCR [34] and
261 nucleotide sequencing.

262

263 **Fig 3. Overview of MuGENT in *C. jejuni*.** Multiple unselected mDNA fragments
264 (used for introducing genome edits of interest) were mixed at equimolar concentrations
265 with a selected mDNA fragment, in order to cotransform *C. jejuni* cells. During
266 MuGENT, we swapped different selective markers at the *flaA* gene during every cycle,
267 resulting in the cells becoming nonmotile (Mot^{-}). This also enabled easy removal of
268 marker genes and reversion to the wild-type allele by transformation with a *flaA*$^{+}$

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mDNA and subsequent selection of motile (Mot^+) clones. Genome edits in the transformants were verified by MASC PCR and nucleotide sequencing.

To demonstrate the efficacy of MuGENT in *C. jejuni*, we targeted the biosynthetic genes, CPS and LOS. These cell-surface molecules are known to play key roles in interactions that affect bacteriophage infectivity, chick colonization, invasion of human epithelial cells, and host immune responses [34–40]. CPS is also the primary antigenic determinant of the Penner serotyping scheme, a passive slide hemagglutination [41], although other surface molecules, including LOS, may contribute to serotype specificity [42]. Because the CPS and LOS gene clusters contain multiple phase-variable genes that are interrupted by polyG tracts, *C. jejuni* cells can generate structural variations of CPS and LOS, which aids in evading killing by host immune systems or predation by bacteriophages [12,43–46]. In addition, phase-variable expression of CPS and LOS markedly decreases the phenotypic stability of *C. jejuni* cells and, thus, may limit related research, including basic studies, epidemiological surveillance, and vaccine development. For example, the NCTC11168 strain (serotype B, antigenic factor HS2) did not provide reproducible results for Penner serotyping, which frequently changed between typeable and untypeable during subcultures (S1 Fig). Although this unstable phenotype may be attributable to ON/OFF switching of the CPS expression due to phase variation [41,47,48], the mechanism whereby phase variation regulates CPS gene expression to determine the Penner serotype remains unknown.

There are 29 polyG/C tracts in the NCTC11168 genome, eight of which are located within the CPS and LOS gene clusters [10]. Six of these phase-variable genes (*cj1420*, *cj1421*, *cj1422*, *cj1426*, *cj1429*, and *cj1437*) reside in the CPS cluster, whereas the other

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293 genes (*cj1139* and *cj1144*) reside in the LOS cluster, which theoretically gives rise to
294 ~2^8 different combinations of ON (1) /OFF (0) expression states or more specifically
295 "phasotypes" [4]. For example, a bacterium that has the following expression states —
296 *cj1139*^{ON} *cj1144*^{OFF} *cj1420*^{OFF} *cj1421*^{OFF} *cj1422*^{OFF} *cj1426*^{OFF} *cj1429*^{OFF} *cj1437* ^{OFF} (in
297 that order) — would have a binary phasotype coded as 1-0-0-0-0-0-0-0, which can be
298 converted to decimal 128 format. In this study, we defined one of the 2^8 phasotypes
299 generated by these eight phase-variable genes as a "Penner phasotype (PPT)." Using
300 MuGENT, we tried to "lock" all of the eight ORFs into ON or OFF states, where their
301 polyG tracts were altered to translate into the largest possible ORF (locked-"ON" states)
302 or a smaller incomplete ORF by frameshifting through -1 deletions from the ON states
303 (locked-"OFF" states). For example, *cj1139* encodes a glucosyltransferase that mediates
304 phase variation of LOS epitopes responsible for autoimmunity [13], with G8 being in an
305 ON state and G7 in an OFF state (Fig 4A). To prevent replicative slippage at the polyG
306 tracts, we interrupted the continuous run of G residues without changing the translated
307 amino acids by replacing the last G residue of every G triplet with a different nucleotide
308 (Fig 4A). As an example of phase-locked mutant construction, we introduced locked-
309 OFF mutations into *cj1139*, *cj1144*, *cj1420*, *cj1421*, *cj1422*, *cj1426*, *cj1429*, and *cj1437*
310 by repeating the MuGENT cycle to generate a "PPT0" strain. During MuGENT,
311 genome editing from 40 transformants per cycle was monitored by MASC PCR. After
312 the second cycle, we found that 100% of the population had at least one edit (Fig 4B
313 and S2 Fig). We accomplished all eight edits within the 4th cycle, which took less than
314 2 weeks to perform (Fig 4B and S2 Fig). Thus, MuGENT works effectively in *C. jejuni*
315 and is useful for rapidly introducing multiple mutations.
316

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Fig 4. Constructing a strain with all eight phase-variable genes locked into the OFF state by MuGENT against SSR (MuGENT-SSR) sites. The polyG tracts from eight genes (*cj1139*, *cj1144*, *cj1420*, *cj1421*, *cj1422*, *cj1426*, *cj1429*, and *cj1437*) located in the biosynthetic gene clusters for LOS and CPS in NCTC11168 were subjected to MuGENT-SSR to lock them all into OFF states. (A) Example of polyG editing (*cj1139*). The polyG tract was altered to translate into the largest possible ORF (G8: ON state, encoding 303 amino acids [aa]) or a smaller incomplete ORF by introducing frameshifts via -1 deletions (G7: OFF state, encoding 148 amino acids). To prevent replicative slippage at the polyG tract, we interrupted the continuous run of G residues without changing the translated amino acids by replacing the last G residue of every G triplet with a different nucleotide. We also used different nucleotides when locking the genes into ON and OFF states, so that these two different states could be swapped. (B) Distribution of genome edits in the population following successive cycles of MuGENT. This image was based on the MASC PCR data (S2 Fig).

Improving the phenotypic stability during Penner serotyping using MuGENT

Controlling SSR-mediated phase variation is important for achieving reproducible results with *C. jejuni*. Regarding the low reproducibility of Penner serotyping (S1 Fig), we hypothesized that this may be associated with the phase-variable expression of one or more genes that determine antigenicity. To narrow down candidate genes, we sequenced PPT from naturally occurring typeable and untypeable variants of NCTC11168, termed “PPT-Seq.” PPT-Seq analysis of 12 variants revealed six PPT subtypes, one of which was typeable (Fig 5 and S1 Fig, PPT37). We constructed a strain locked to PPT37 by MuGENT and confirmed that it was also typeable (Fig 5 and S1

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Fig). In contrast to the wild-type strain, the locked PPT37 mutant maintained typeability during at least five subcultures (S1 Fig). To evaluate the effect of polyG editing by MuGENT on phase variation, the *astA* gene (encoding arylsulfatase) [49] was fused in frame to the phase-variable gene *cj1426* (Fig 5), which encodes a methyltransferase that methylates the heptose of the CPS repeat unit of NCTC11168 [50], and to its phase-locked derivatives, such that changes in the repeat number would alter arylsulfatase expression [51]. Single colonies grown on BHI agar plates containing 5-bromo-4-chloro-3-indolyl sulfate (XS), a chromogenic substrate of arylsulfatase, were used to measure mutation rates, as described in the Methods section. With the wild-type *cj1426*::*astA* fusion, the ON-to-OFF mutation occurred at a rate of 2.8×10^{-2}, whereas the OFF-to-ON mutation rate was 2.3×10^{-2} (Table 2). In the locked-ON and locked-OFF constructs, however, switching to different phases was not detectable (Table 2). These results suggest that MuGENT can serve as a feasible and effective approach for stabilizing unstable phenotypes generated by phase variation with a defined set of multiple SSRs.

Fig 5. Penner serotyping and phasotyping of naturally occurring and phase-locked variants of NCTC11168. After naturally occurring variants (typeable or untypeable by Penner serotyping; SC1-1 to SC2-6, S1 Fig) were subjected to PPT-Seq, their PPTs were decoded (indicated by decimal). The numbers with in red boxes indicate the numbers of repeat polyG tracts in the ON configurations, whereas those in the gray boxes indicate those in the OFF configurations. The serotyping results for the phase-locked variants are shown in S3 Fig. The following phase-locked strains were used: Locked-0, SYC1P000; Locked-255, SYC1P255; Locked-37, SYC1P037; Locked-36, SYC1P036C; Locked-5,

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365 SYC1P005C; Locked-33, SYC1P033C; Locked-4, SYC1P004K; Locked-1,

366 SYC1P001C; Locked-32, SYC1P032K.

367

368 **Table 2 Effect of the polyG editing on phase variation of *cj1426***

Reporter gene[a]	Mutation rate[b]	
	ON to OFF	OFF to ON
cj1426::*astA*	2.8×10^{-2}	2.3×10^{-2}
cj1426^{ON}::*astA*	$<2.1 \times 10^{-3}$	ND[c]
cj1426^{OFF}::*astA*	ND	$<2.2 \times 10^{-3}$

369 [a]The following strains were used: *cj1426*::*astA*, SYC1006; *cj1426*^{ON}::*astA*, SYC1007;
370 and *cj1426*^{OFF}::*astA*, SYC1008.
371 [b]Mutation rates were calculated using the equation reported previously by Drake [52]
372 (see Methods section).
373 [c]Not done
374

375 **Using MuGENT-SSR to identify a phase-variable gene that determines the Penner**
376 **serotype of NCTC11168**

377 Phenotypic engineering using MuGENT-SSR combined with PPT-Seq identified
378 PPT37 as a PPT subtype typeable for Penner serotyping. In PPT37, *cj1420*, *cj1426*, and
379 *cj1437* were in the ON state, whereas the other five genes were in the OFF state. These
380 findings suggest that one or more genes of these "ON" genes may determine typeability.
381 To elucidate the responsible gene(s), we constructed strains locked to various PPT
382 subtypes by MuGENT-SSR and then performed serotyping. We demonstrated that
383 *cj1426* expression was indispensable and sufficient for serological typing, whereas
384 *cj1420* and *cj1437* expression were not (Fig 5 and S3 Fig). The CPS repeat unit of *C.*
385 *jejuni* NCTC11168 consists of β-D-ribose , β -D-N-acetylgalactosamine (GalfNAc), α-

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386 D-glucuronic acid modified with 2-amino-2-deoxyglycerol at C-6, and 3,6-O-methyl-
387 D-glycero-α-L-gluco-heptose (Hep) [53] (S4 Fig). Examination of the CPS structure by
388 high-resolution magic-angle spinning nuclear magnetic resonance spectroscopy
389 revealed highly variable or no modification of the methyl, ethanolamine, aminoglycerol,
390 and phosphoramidate groups of the repeating unit [38,54]. In particular, changes in
391 methyl (Me) and phosphoramidate modifications were regulated by at least three phase-
392 variable genes located in the CPS cluster (*cj1421*, *cj1422*, and *cj1426*). The *cj1426* gene
393 encodes the methyltransferase involved in 6-O-Me modification of the Hep residue of
394 NCTC11168 CPS [50] (S4 Fig), suggesting that the presence of this modification may
395 be an antigenic determinant of the Penner B serotype (or antigenic factor HS2).
396 However, we observed that some PPT subtypes (including naturally occurring variants
397 PPT109, PPT111, PPT189, and PPT191, and a phase-locked variant PPT255), where
398 *cj1426* and some or all of the other phase-variable genes (including *cj1144*, *cj1421*,
399 *cj1422*, and *cj1429*) were simultaneously switched ON, were untypeable (Fig 5, S1 and
400 S3 Figs). Of these genes, the *cj1421* and *cj1422* genes have already been demonstrated
401 to encode O-Me-phosphoramidate (MeOPN) transferases that attach MeOPN to the
402 GalfNAc and Hep residues, respectively [38]. Thus, one or more CPS modifications
403 catalyzed by these gene products may interfere with the binding of specific antibodies to
404 the epitope (S4 Fig). To directly test this possibility, structural analysis using nuclear
405 magnetic resonance spectroscopy and deeper genetic and immunogenic studies are
406 required.

407 Similar results were reported previously regarding the phase-variable CPS of *C.*
408 *jejuni*. In a series of studies on the tropism of *Campylobacter* bacteriophages, Sørensen
409 *et al*. demonstrated that the MeOPN-modified GalfNAc of the NCTC11168 CPS acts as

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a receptor of the F336 phage, but that its receptor function was modulated by the presence or absence of other CPS modifications [37,45]. In addition, a study of *C. jejuni* 81-176 (Penner serotype R, antigenic factors HS23 and HS36) revealed that phase-variable MeOPN modifications at the three CPS galactose residues modulated serum resistance [44]. These previous findings and our current findings suggest that phase-variable changes in the CPS structure of *C. jejuni* occur at two levels: (1) phase variations of the receptors and epitopes themselves, and (2) interference with receptor and epitope functions by chemical modifications at different positions. These combinatorial effects may aid in rapidly avoiding killing by phages and the host immune system, while lowering the typeability of Penner serotyping.

Concluding remarks

Here, we demonstrate that MuGENT is applicable for genetic engineering of *C. jejuni*. In this genetically unstable species, MuGENT specifically provided a feasible and effective approach for editing multiple hypermutable SSRs. Specifically, MuGENT-SSR was performed to uncover the contributions of multiple phase-variable genes to specific phenotypes. By combining MuGENT-SSR with whole-genome SSR analysis [4,10] may enable comprehensive studies of numerous phase-variable genes (~30 polyG/C-containing genes per genome) in order to decode the “phasotypes” that determine specific phenotypes and more collective behaviors, such as colonization of animal hosts [9,16–20].

We also propose that MuGENT-SSR can be utilized to engineer strains suitable for serological typing and vaccination. In *C. jejuni* NCTC 11168, the majority of polyG/C SSRs are clustered in genomic regions encoding proteins involved in the biosynthesis of

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434 cell-surface antigenic determinants, including CPS and LOS [5]. Thus, phase variation
435 makes these antigens less desirable as serodeterminants and vaccine candidates. Penner
436 serotyping is often utilized to investigate epidemiological associations with Guillain–
437 Barré syndrome, but its low typeability is currently problematic [55]. The generation of
438 phase-locked strains by MuGENT-SSR may overcome these defects and stabilize their
439 antigenicity, thereby increasing the supply of stable serotyping antisera and vaccines.
440 Furthermore, PPT-decoding of Penner serotypes provides a reliable approach for
441 identifying antigenic determinant genes and improving or developing DNA-based
442 typing methods that do not rely on CPS expression [56].

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Methods

Bacterial culture conditions

C. jejuni strains used in this study (Table 1) were routinely cultured for 24–48 h at 42°C on BHI (Becton Dickinson) plates containing 1.3% agar (Kyokuto) under microaerophilic conditions using Mitsubishi Anaeropack MicroAero gas generator packs (Mitsubishi Gas). Motility was determined by culturing the cells on BHI plates containing 0.3% agar. For liquid culture, fresh single colonies grown on BHI agar plates were inoculated into 5 ml of BHI broth in 25 ml volumetric test tubes and then cultured overnight at 42°C with reciprocal shaking at 160 rpm in a Taitec Precyto MG-71M-A Obligatory Anaerobe Culture System (Taitec), which can efficiently create a microaerophilic atmosphere by actively aerating gases into each test tube. The aeration conditions in each test tube were set to 5% O_2, 10% CO_2, and 85% N_2 at a constant flow rate (10 ml/min). The antibiotics used were 25 μg/ml Cm, 50 μg/ml Km, and 10 μg/ml Sm. If necessary, XS (Sigma) was added to the BHI agar plates at a final concentration of 100 μg/ml.

DNA manipulation

PCR amplification of DNA was performed using a LifeECO thermal cycler (version 1.04, Bioer Technology) and Quick Taq® HS DyeMix DNA polymerase (Toyobo). Sanger sequencing was performed by Fasmac. PCR products were purified using a High Pure PCR Product Purification Kit (Roche). Customized oligonucleotide primers (S1 Table) were purchased from Fasmac and Hokkaido System Science. Chromosomal and plasmid DNA molecules were extracted with a DNeasy Blood and Tissue Kit (Qiagen) and a High Pure Plasmid Isolation Kit (Roche), respectively.

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467 **Construction of pSYC-*cat* and pSYC-*kan***

468 The pSYC-*cat* and pSYC-*kan* plasmids (Table 1) were constructed by Fasmac as
469 follows: DNA fragments containing the Cm-resistance gene (*cat*) (GenBank accession
470 number M35190.1, nucleotides 1–1,034) and the Km-resistance gene (*kan*) (GenBank
471 accession number M26832.1, nucleotides 1–1,426) from *C. coli* [57,58] were synthesized
472 and cloned into the EcoRV site of pUCFa (Fasmac) to generate pSYC-*cat* and pSYC-*kan*,
473 respectively.

474

475 **Natural transformation of *C. jejuni* cells using PCR products**

476 Genetically engineered *C. jejuni* strains were constructed by performing natural
477 transformation [26] optimized to use PCR products as donor DNA. Briefly, the
478 procedures include three processes: (1) PCR amplification of donor DNA with
479 methylation sites, (2) methylation of the amplified DNA, and (3) transformation of *C.*
480 *jejuni* with methylated DNA.

481

482 **PCR amplification of donor DNA with methylation sites.** The donor DNA fragments
483 used for natural transformation were amplified using specific primers and templates. The
484 typical amplified fragment contained an antibiotic-resistance gene (or mutation) with 5′-
485 and 3′-flanking regions homologous to the target site and an EcoRI-recognition sequence
486 (GAATTC) on both sides. DNA was amplified via splicing by overlap extension PCR,
487 which includes a two-step amplification process [59,60]. The first step independently
488 generated two or three fragments with overlapping sequences. In the second step, the
489 splicing by overlap extension PCR fragments amplified in the first step are ligated. The
490 thermal cycling program was as follows: 94°C for 5 min, followed by 30 cycles of 94°C

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for 30 s, 55°C for 30 s, and 68°C for 1 min per kb. S2 Table shows the specific combinations of template DNA and primer pairs used for PCR amplification.

Methylation of donor DNA. DNA methylation was performed using a 25 µl mixture containing 20 nM DNA, EcoRI methyltransferase reaction buffer (New England Biolabs; 50 mM Tris-HCl, 50 mM NaCl, 10 mM ethylenediaminetetraacetic acid, pH 8.0), 80 µM *S*-adenosylmethionine (New England Biolabs), and 40 units of EcoRI methyltransferase (New England Biolabs). After incubation for 2.5 h at 37°C, the reaction was terminated by incubation for 20 min at 65°C. Methylated DNA was purified using the High Pure PCR Product Purification Kit and dissolved in 100 µl H_2O.

Natural transformation. Overnight cultures of *C. jejuni* cells were diluted 1/50 in 5 ml BHI broth and grown until they reached an optical density and 600 nm (OD_{600}) of approximately 0.15. Each culture (900 µl) was mixed with methylated DNA (100 µl) and then statically incubated overnight in a 25 ml volumetric plastic test tube. On the following day, 10-fold serial dilutions of the culture were spread onto BHI agar plates, with or without antibiotics. The transformation frequency was defined as the number of antibiotic-resistant CFUs divided by the total number of CFUs.

Evaluation of natural cotransformation in *C. jejuni*

Natural cotransformation in *C. jejuni* was examined using two different PCR fragments, Δ*flaA*::*kan* and *rpsL*$^+$ (see S2 Table). The former fragment (Δ*flaA*::*kan*-1 or Δ*flaA*::*kan*-2) had 1,000-bp regions of homology, whereas the latter had 1,000-bp or 2,000-bp regions of homology (*rpsL*$^+$-8-1, *rpsL*$^+$-9-1, *rpsL*$^+$-8-2, or *rpsL*$^+$-9-2). The Δ*flaA*::*kan* and *rpsL*$^+$

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fragments were independently methylated and then mixed for purification as described above. After transformation of SY1003 and SY2003 with these methylated DNA fragments, the reactions were spread onto BHI agar plates containing Km. One hundred Km^R transformants were replica plated onto BHI agar plates containing Sm. The cotransformation frequency (%) was calculated as follows: 100 × number of Sm^S CFUs in 100 Km^R CFUs.

MuGENT-SSR

MuGENT-SSR was performed with *C. jejuni* cells, as follows. After independently methylating multiple unselected fragments, including eight fragments used for editing of the eight polyG sites in the biosynthetic LOS and CPS gene clusters in NCTC11168, as well as a single selected fragment (either Δ*flaA*::*kan*-1 or Δ*flaA*::*cat*-1), a mixture of these fragments was prepared as described above. Unselected fragments had 1,500–2,000-bp regions of homology, whereas the selected fragment had a 1,000-bp region of homology (S2 and S3 Tables). After transformation with the DNA mixture, the bacteria were plated onto BHI agar plates containing the antibiotic corresponding to the resistance protein encoded in the selected fragment. Colonies on selective agar plates were used for MASC PCR (described below). The remaining colonies (3,000–5,000 CFUs) were suspended in BHI broth and diluted in 5 ml BHI broth to an OD_{600} of 0.05. After the cells were grown to an OD_{600} of 0.15, the next cycle of MuGENT was performed using unselected fragments and a selected fragment with a different resistance gene from that used in the previous cycle. MuGENT cycles were repeated until editing was completed. Genome editing was ultimately verified by nucleotide sequencing using specific primers (see the PPT-Seq section below). If necessary, the Δ*flaA* mutation maintained during MuGENT

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539 was reverted to the wild-type allele by transformation using the *flaA*$^{+}$ fragment with a
540 1,000-bp region of homology. After the transformation, colonies showing motility (and
541 sensitivity to the antibiotic used for selection) were chosen for further study.
542
543 **MASC PCR**
544 During each cycle of MuGENT, 40 colonies were inoculated in 100 µl of BHI broth
545 in a 96-wel plate and incubated overnight. Each PCR mixture (20 µl) contained 2 µl of
546 the bacterial culture, 2 µL of 2.5 µM primer mix (one of four primer mixes, including Mix
547 ON1, Mix ON2, Mix OFF1, and Mix OFF2; see S4 Table), 6 µl of H_2O, and 10 µl of
548 Quick Taq® HS DyeMix. The following thermal cycling program was used for MASC
549 PCR: 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 62.6°C for 30 s, and 68°C
550 for 3 min. If necessary, several single colonies were isolated from positive colonies (to
551 eliminate contamination by unedited clones) and further assessed for genome editing by
552 MASC PCR.
553
554 **PPT-Seq**
555 The eight phase-variable genes located in the LOS- and CPS-biosynthetic gene clusters
556 in NCTC11168 were amplified by PCR, and the repeat numbers of the polyG SSR tracts
557 were determined by nucleotide sequencing. S5 Table shows the specific combinations of
558 target genes and primers used for PPT-Seq.
559
560 **Penner serotyping**
561 A single colony was streaked on the surface of a horse blood agar plate (Kyokuto) and
562 incubated for 48 h at 42°C to determine the Penner serotype [61]. After the resulting

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563 colonies grown on the plate were suspended in 250 µL of 0.9% NaCl, serotyping was
564 performed using *Campylobacter* antisera (Denka), including a commercial 25 antisera set,
565 and a reagent for preparing sensitized blood cells (Denka) according to the manufacturer's
566 instructions. Phenotypic stability was assessed by repeating the subculture cycle and
567 serotyping. Serotyping was performed using six single colonies for each cycle.
568
569 **Measurement of mutation rates**
570 The mutation rates of the phase-variable *cj1426* gene and its phase-locked variants
571 were determined as described previously [62], with some modifications. *C. jejuni* strains
572 carrying *astA* to *cj1426* translational fusions were streaked on BHI agar plates containing
573 XS. During growth on the medium, blue colonies were in the *cj1426*-ON phase, whereas
574 white colonies were in the *cj1426*-OFF phase. Single blue or white colonies were picked
575 using a micropipette tip were resuspended in 500 µL BHI, after which 250 µl of 10^4-, 10^5-,
576 and 10^6-fold dilutions were spread onto BHI agar plates containing XS. The number of
577 variant colonies that switched to different phases and the total numbers of colonies were
578 counted. Ten independent single colonies were examined for each strain and phase. To
579 estimate the mutation rate, the total number of colonies was averaged for all single
580 colonies tested, and the median value for the frequency of variants per colony was
581 calculated. The mutation rate was calculated using the following equation: $\mu =$
582 $0.4343f/log(N\mu)$, where μ is the mutation rate, f is the median frequency, and N is
583 the average population size [52]. The μ value was determined by solving the equation
584 using the Goal Seek function in Microsoft Excel.

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Supporting information captions

S1 Fig. Penner serotyping of naturally occurring and phase-locked NCTC11168 variants. Phenotypic stability was examined during successive subcultures, which highlights only the results obtained using anti-serotype B antiserum. Red-numbered colonies were typeable, and black-numbered colonies were untypeable.

S2 Fig. MASC PCR of transformants following successive cycles of MuGENT-SSR.

S3 Fig. Penner serotyping of phase-locked NCTC11168 variants.

S4 Fig. Putative modification patterns of the CPS repeat unit in naturally occurring and phase-locked NCTC11168 variants. Figures were modified from illustrations published in Sternberg et al, J Mol Biol 2013 (425) 186-197.

S1 Table. Primers used in this study.

S2 Table. Specific combinations of template DNA and primers used to amplify donor DNA templates.

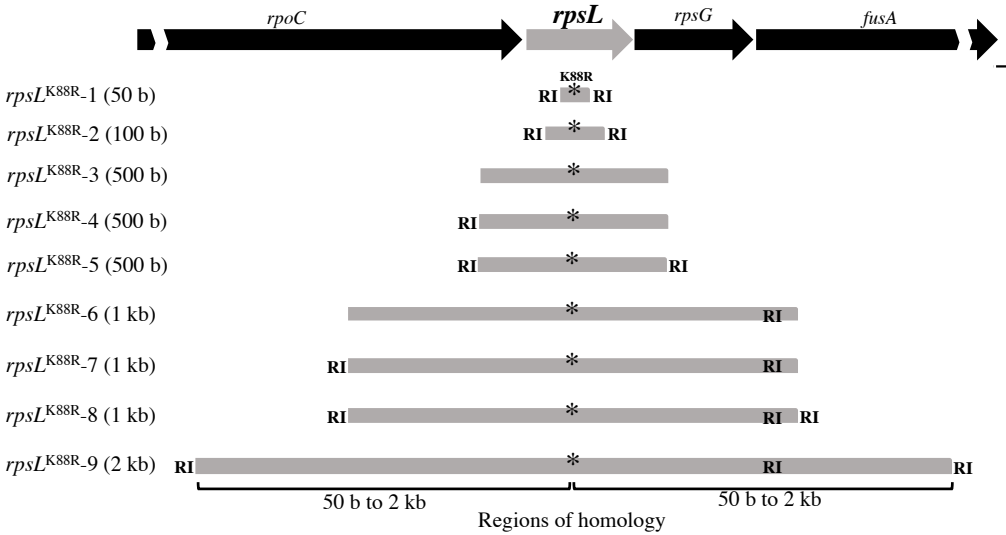
S3 Table. Specific combinations of donor DNA molecules and recipient strains used for natural transformation.

S4 Table. Primer mixes used for MASC PCR.

S5 Table. Specific combinations of target genes and primers in PPT-Seq.

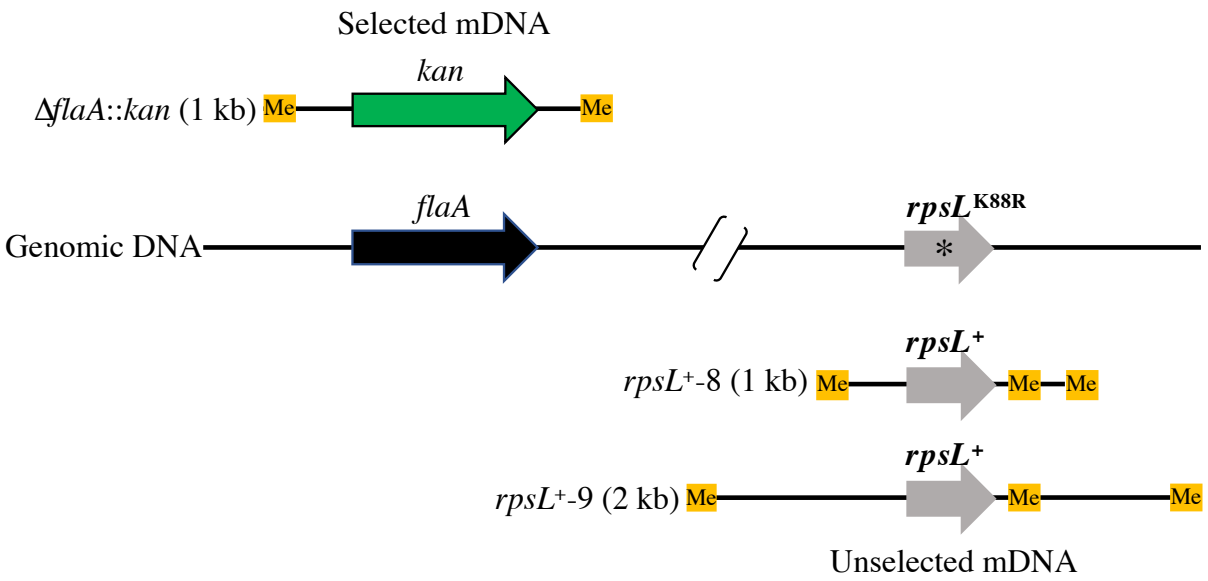
S6 Table. Primer sets used for allele-specific PCR and sequencing of *cj1426*::*astA* translational fusions.

Fig 1



	Transformation frequency (Sm^R CFUs/total CFUs)			
	NCTC11168		81-176	
	WT	Δ*recA*	WT	Δ*recA*
rpsL^{K88R}-1 (50 b)	$<1.5 \times 10^{-8}$	ND	$<2.1 \times 10^{-8}$	ND
rpsL^{K88R}-2 (100 b)	$<1.4 \times 10^{-8}$	ND	$<2.2 \times 10^{-8}$	ND
rpsL^{K88R}-3 (500 b)	$1.8 \pm 2 \times 10^{-8}$	ND	$1.4 \pm 1 \times 10^{-8}$	ND
rpsL^{K88R}-4 (500 b)	$1.2 \pm 1 \times 10^{-6}$	ND	$2.1 \pm 2 \times 10^{-5}$	ND
rpsL^{K88R}-5 (500 b)	$3.2 \pm 3 \times 10^{-6}$	ND	$3.1 \pm 1 \times 10^{-5}$	ND
rpsL^{K88R}-6 (1 kb)	$1.2 \pm 1 \times 10^{-5}$	ND	$4.0 \pm 1 \times 10^{-5}$	ND
rpsL^{K88R}-7 (1 kb)	$9.0 \pm 1 \times 10^{-6}$	ND	$1.3 \pm 1 \times 10^{-4}$	ND
rpsL^{K88R}-8 (1 kb)	$2.0 \pm 1 \times 10^{-5}$	ND	$1.3 \pm 1 \times 10^{-4}$	ND
rpsL^{K88R}-9 (2 kb)	$5.0 \pm 2 \times 10^{-5}$	$< 1.6 \times 10^{-7}$	$1.2 \pm 1 \times 10^{-4}$	$<1.4 \times 10^{-7}$
Unmethylation of *rpsL*^{K88R}-5	$2.7 \pm 1 \times 10^{-8}$	ND	$1.2 \pm 3 \times 10^{-8}$	ND
No addition of DNA	$<1.2 \times 10^{-8}$	ND	$<1.9 \times 10^{-8}$	ND

Fig 2



Cotransformation frequency (%: 100 × Sm^S CFUs per 100 Km^R CFUs)	
NCTC11168	81-176
24 ± 4	16 ± 3
41 ± 4	22 ± 3

Fig 3

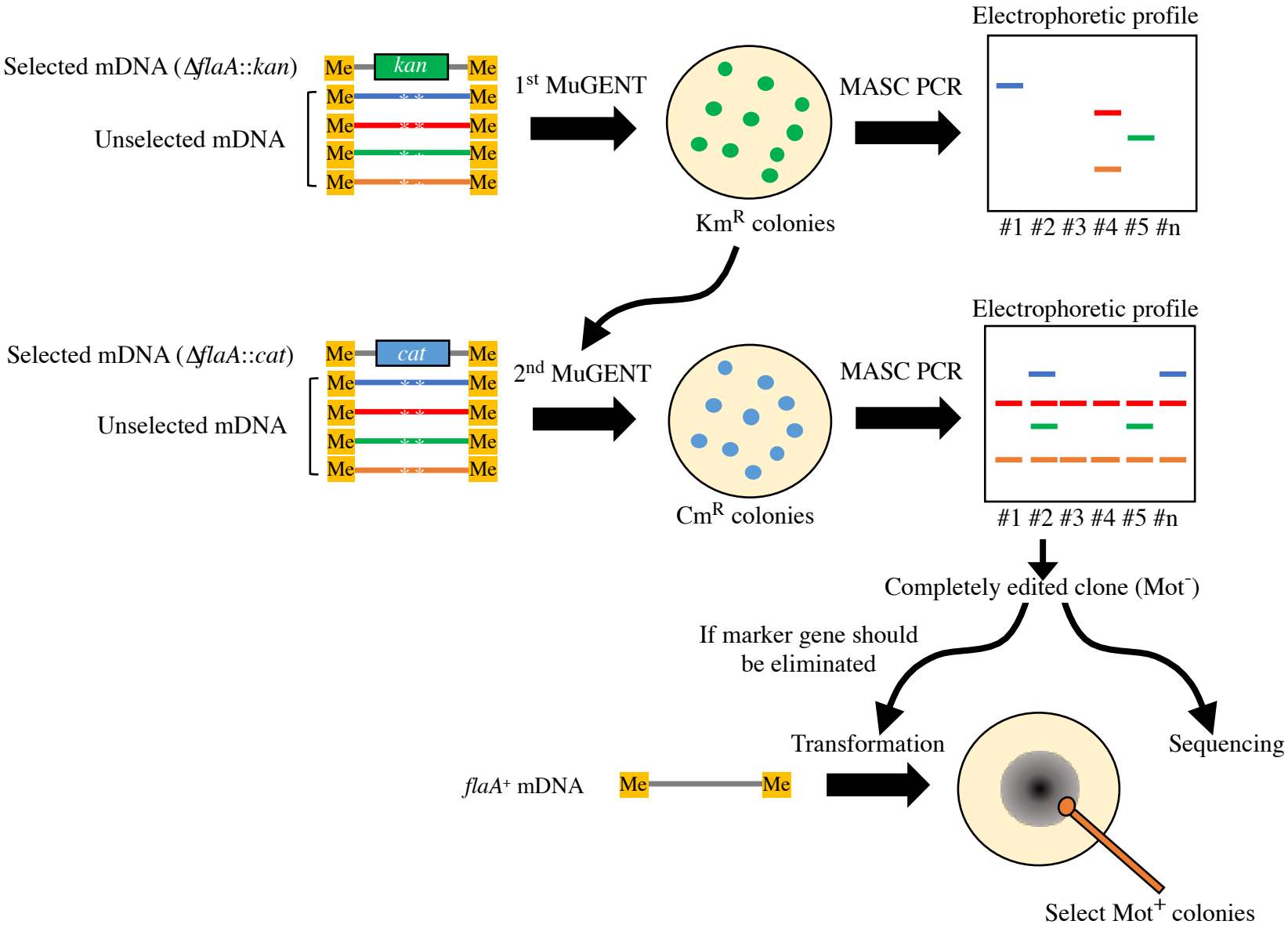
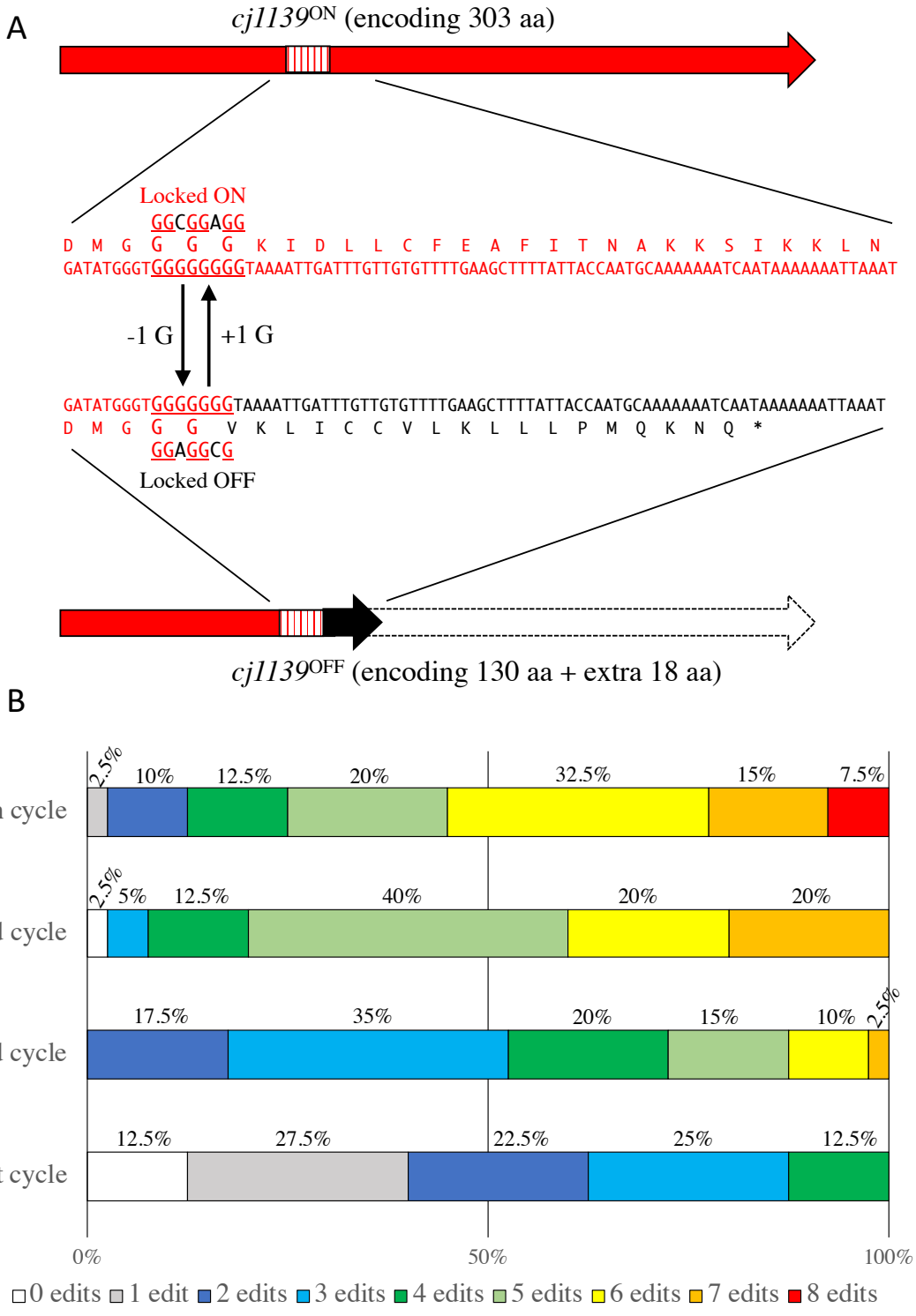

Selected mDNA (ΔflaA::kan)
Me
kan
Me
Unselected mDNA
1st MuGENT
KmR colonies
MASC PCR
Electrophoretic profile
#1 #2 #3 #4 #5 #n
Selected mDNA (ΔflaA::cat)
cat
2nd MuGENT
CmR colonies
Completely edited clone (Mot-)
If marker gene should be eliminated
Transformation
Sequencing
flaA+ mDNA
Select Mot+ colonies

Fig 4


A
cj1139ON (encoding 303 aa)
Locked ON
GGCGGAGG
D M G G G G K I D L L C F E A F I T N A K K S I K K L N
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-1 G
+1 G
GATATGGGTGGGGGGGTAAAATTGATTTGTTGTGTTTTGAAGCTTTTATTACCAATGCAAAAAAATCAATAAAAAAATTAAAT
D M G G G V K L I C C V L K L L L P M Q K N Q *
GGAGGCG
Locked OFF
cj1139OFF (encoding 130 aa + extra 18 aa)
B
4th cycle
3rd cycle
2nd cycle
1st cycle
2.5%
10%
12.5%
20%
32.5%
15%
7.5%
2.5%
5%
12.5%
40%
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17.5%
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10%
2.5%
12.5%
27.5%
22.5%
25%
12.5%
0%
50%
100%
0 edits
1 edit
2 edits
3 edits
4 edits
5 edits
6 edits
7 edits
8 edits

Fig 5

NCTC11168 variant	Typeability	Number of polyG tract repeats in phase-variable genes								PPT
		cj1139	*cj1144*	*cj1420*	*cj1421*	*cj1422*	*cj1426*	*cj1429*	*cj1437*	
		8	**7**	**9**	**9**	**9**	**10**	**10**	**9**	
SC1-1	No	9	**10**	**9**	10	**9**	**10**	11	**9**	109
SC1-2	No	9	**10**	**9**	10	**9**	**10**	**10**	**9**	111
SC1-3	No	**8**	9	**9**	**9**	**9**	**10**	11	**9**	189
SC1-4	**Yes**	9	9	**9**	10	10	**10**	11	**9**	**37**
SC1-5	No	**8**	9	**9**	**9**	**9**	**10**	**10**	**9**	191
SC1-6	No	9	**10**	**9**	10	**9**	**10**	11	**9**	109
SC2-1	**Yes**	9	9	**9**	10	10	**10**	9	**9**	**37**
SC2-2	**Yes**	9	9	**9**	10	10	**10**	11	**9**	**37**
SC2-3	**Yes**	9	9	**9**	10	10	**10**	11	**9**	**37**
SC2-4	No	9	9	8	10	10	9	11	**9**	1
SC2-5	No	9	9	8	10	10	11	11	**9**	1
SC2-6	**Yes**	9	9	**9**	10	10	**10**	11	**9**	**37**
Locked-0	No	Locked OFF	Locked OFF	Locked OFF	Locked OFF	Locked OFF	Locked OFF	Locked OFF	Locked OFF	0
Locked-255	No	**Locked ON**	**Locked ON**	**Locked ON**	**Locked ON**	**Locked ON**	**Locked ON**	**Locked ON**	**Locked ON**	255
Locked-37	**Yes**	Locked OFF	Locked OFF	**Locked ON**	Locked OFF	Locked OFF	**Locked ON**	Locked OFF	**Locked ON**	**37**
Locked-36	**Yes**	Locked OFF	Locked OFF	**Locked ON**	Locked OFF	Locked OFF	**Locked ON**	Locked OFF	Locked OFF	**36**
Locked-5	**Yes**	Locked OFF	Locked OFF	Locked OFF	Locked OFF	Locked OFF	**Locked ON**	Locked OFF	**Locked ON**	**5**
Locked-33	No	Locked OFF	Locked OFF	**Locked ON**	Locked OFF	Locked OFF	Locked OFF	Locked OFF	**Locked ON**	33
Locked-4	**Yes**	Locked OFF	Locked OFF	Locked OFF	Locked OFF	Locked OFF	**Locked ON**	Locked OFF	Locked OFF	**4**
Locked-1	No	Locked OFF	Locked OFF	Locked OFF	Locked OFF	Locked OFF	Locked OFF	Locked OFF	**Locked ON**	1
Locked-32	No	Locked OFF	Locked OFF	**Locked ON**	Locked OFF	Locked OFF	Locked OFF	Locked OFF	Locked OFF	32