Development and Optimization of Genetic Manipulation Systems in Group I *Clostridium botulinum*

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Abstract

Clostridium botulinum causes the disease botulism, and nontoxigenic *Clostridium sporogenes* is closely related to group I *C. botulinum.* Despite its pathogenicity, *C. botulinum* remains poorly characterized. Genetic manipulation is critical for understanding bacterial physiology and disease. We compared the conjugal transformation efficiencies of seven strains, including group I *C. botulinum* (strains 62A, 7I03-H, Okra, Osaka05, 111) and *C. sporogenes* (strains JCM 1416^{T}, ATCC 15579), and our results showed that few or no transformants were obtained in certain strains. In the present study, we demonstrate that our optimized protocol increases the efficiency of DNA transfer from *E. coli* donor cells to recipient strains. In addition, we developed a novel conjugal suicide vector pXMTL that contains xylose-inducible *mazF* as a counter-selection marker, and can be transferred into *Clostridium* spp. by conjugation. The allele-coupled exchange (ACE) system using pXMTL provides a rapid method for precise, markerless and scarless genome editing in group I *C. botulinum* and *C. sporogenes*.

Importance

Group I *C. botulinum* and *C. sporogenes* exhibit low transformation efficiencies, and few or no transformants are yielded by some strains. In this study, we optimized the conjugation protocol to improve transformation efficiency. In addition, we developed a novel suicide vector pXMTL harboring a xylose-inducible *mazF* marker, and can be transferred into *Clostridium* spp. by conjugation. The combination of pXMTL and the optimized conjugation protocol provides a powerful tool for genetic manipulation of group I *C. botulinum* and *C. sporogens.*

47 **Introduction**

48 *Clostridium botulinum* and related species cause a severe paralytic illness, botulism. *C.*
49 *botulinum* is a gram-positive, spore-forming, obligate anaerobe, and is divided into four
50 genetically and physiologically distinct groups (I to IV). The organism produces
51 botulinum neurotoxin (BoNT) that has been traditionally classified into seven serotypes
52 (A to G) (1). Serotypes A, B, E and F toxins produced by group I and II *C. botulinum* are
53 mainly associated with human botulism, and those of serotypes C and D produced by
54 group III *C. botulinum* are mainly associated with animal botulism. No botulism cases
55 related to the serotype G toxin produced by group IV *C. botulinum* (also known as
56 *Clostridium argentinense*) have been reported (1). *Clostridium sporogenes* is closely
57 related to group I *C. botulinum* genetically and physiologically, whereas most strains of
58 them do not produce BoNT. The organism is the only gut symbiont known to produce
59 indolepropionic acid (IPA) (2), and obtains its energy by reductive Stickland metabolism
60 in the gut (3). Development and expansion of genetic manipulation in these organisms
61 would provide windows into mechanisms of intoxication/infection and gut microbiota-
62 dependent modulation of energy metabolism. However, Group I *C. botulinum* and *C.*
63 *sporogenes* exhibit low transformation efficiency (Fig. 1) and inefficient homologous
64 recombination (4). A lack of suitable genome editing systems limits analysis using these
65 strains.

66 Three approaches to genome editing in *C. botulinum* and related species have been
67 developed to overcome such limitations (5). First, the ClosTron system disrupts the target
68 genes of *C. botulinum* and *C. sporogenes* by group II intron insertion into the genomic
69 target (4). Mutants can be selected using a retrotransposition-activated marker (RAM)
70 integrated into the genome. Second, in a CRISPR-Cas9-mediated system, target genes

71 bearing a protospacer adjacent motif (PAM) are cleaved. To achieve precise gene editing,
72 double-strand DNA breaks (DSBs) are repaired by homology-directed recombination
73 (HDR) using donor templates. These markerless and scarless gene editing systems were
74 developed in *C. sporogenes* (6–8), group I *C. botulinum* (8) and group II *C. botulinum*
75 (9). Last, the two-step allele-coupled exchange (ACE) system also enables markerless
76 and scarless gene editing at unlimited target sites by double crossover recombination
77 without DSBs of the bacterial genome. In the ACE systems of *Clostridium* species (spp.),
78 targeted mutants have been successfully obtained using counterselection markers: *e.g.*, an
79 orotate phosphoribosyltransferase gene (*pyrE*) from *C. sporogenes* for *C. difficile* and
80 *Clostridium acetobutylicum* (5, 10), a cytosine deaminase gene (*codA*) from *Escherichia*
81 *coli* for *C. difficile* (11) and a *mazF* gene (mRNA interferase) from *E. coli* for *C. difficile*
82 (12). However, the *pyrE* and *codA* homolog genes are carried by *C. botulinum*, *C.*
83 *sporogenes* and *Clostridium perfringens* (10, 11), indicating that these genes are not
84 applicable for these organisms. The *mazF* gene is one of the suitable counterselection
85 markers, whereas a lactose-inducible promoter P_{bgaL} is slightly 'leaky' in *C.*
86 *acetobutylicum* (12). Recently, we developed a xylose-inducible *mazF*-based suicide
87 vector pXM (13). This plasmid can be introduced into *C. perfringens* by electroporation
88 transformation, in which the xylose-inducible promoter tightly regulates MazF
89 expression. However, DNA transfer efficiencies by electroporation or conjugation
90 methods vary between species and strains. Conjugative transfer of clostridial shuttle
91 vectors from *E. coli* CA434 donor cells to *Clostridium* spp. is highly efficient for *C.*
92 *difficile* and other clostridial recipients (5, 14). In the present study, we developed a novel
93 conjugal *mazF*-based suicide vector pXMTL that contains a xylose-inducible promoter.
94 In addition, we optimized the conjugal transformation protocol for group I *C. botulinum*

95 and *C. sporogenes*, resulting in higher transformation efficiencies than the original
96 protocol. The ACE system using pXMTL provides a rapid method for precise, markerless
97 and scarless genome editing in group I *C. botulinum* and *C. sporogenes*.

98 **Materials and Methods**

99 ***Bacterial strains***

100 *C. botulinum* strain 62A (group I, serotype A1-HA), 7I03-H (group I, serotype A2-
101 OrfX), Okra (group I, serotype B1-HA), Osaka05 (group I, serotype B6-HA), 111 (group
102 I, serotype B2-HA/X-OrfX) and *C. sporogenes* strain JCM 1416^{T} (group I; NT, no toxin;
103 RIKEN BRC) and ATCC 15579 (group I, NT, American Type Culture Collection) were
104 cultured in TPGY broth (5% Difco™ Tryptone Peptone (Thermo Fisher Scientific), 0.5%
105 Bacto™ Peptone (Thermo Fisher Scientific), 0.5% Yeast Extract (Merck), 0.1% Glucose
106 (FUJIFILM Wako Chemicals), 0.1% Sodium Thioglycolate (FUJIFILM Wako
107 Chemicals) or on TPGY agar plates (1.5% agar) at 37°C in an anaerobic chamber
108 (HIRASAWA) or anaeropack system (Sugiyama-gen), unless otherwise described. All
109 bacterial experiments using *C. botulinum* were conducted in the Fujinaga Lab, approved
110 by the biosafety committee of Kanazawa University and the Minister of Health, Labour
111 and Welfare of Japan.

112 ***Conjugal transformation***

113 Conjugal transformation was performed according to a standard conjugation protocol
114 (original protocol) (7, 14). Chemically competent cells (15) of *E. coli* CA434 (14) were
115 transformed with pMTL83153 (16) harboring a codon-optimized *mCherry* (GeneScript)
116 gene (pMTL83153-mCherry-opt) using the heat shock method at 42°C for 1 min. The

117 cells were cultured in Luria-Bertani (LB) broth with 30 μg/mL kanamycin and 30 μg/mL
118 chloramphenicol (Kan/Cm) at 37°C under aerobic conditions. 1 mL of overnight cultures
119 of CA434 with pMTL83153-mCherry-opt was pelleted by centrifugation and washed
120 with 1 mL of Nutrient Broth (0.5% Bacto™ Peptone, 0.3% Yeast Extract, 0.5% NaCl).
121 The pellets were resuspended in 200 μL of an overnight culture of *C. botulinum* or *C.*
122 *sporogenes* and 50 μL (5 × 10 μL) of the suspensions were spotted onto TPGY agar plates.
123 After anaerobic culture at 37°C for 12-24 hr, the cells were harvested in 1 mL of TPGY
124 broth. 100 μL/plate of the conjugated cell suspensions were inoculated onto TPGY agar
125 plates with 15 μg/mL thiamphenicol and 250 μg/mL D-cycloserine (Tm/Cs). The plates
126 were incubated at 37°C for 1-3 days.

127 For some experiments, this original protocol was adopted as follows. (I) The pre-
128 culture of CA434 cells harboring pMTL83153-mCherry-opt was harvested at the early-
129 log phase (OD600 ~ 0.1-0.2), mid-log phase (OD600 ~ 0.5-1.0) or stationary phase
130 (overnight culture; 12-16 hr). (II) CA434 cells were cultured in LB, Terrific Broth (TB),
131 or SOC media. (III) CA434 cell pellets were suspended in group I cell cultures and spotted
132 onto TPGY, BHIS (1 × Pearlcore Brain Heart Infusion agar (Eiken Chemical), 0.5%
133 Yeast Extract (Merck), 0.2% Fructose (FUJIFILM Wako Chemicals), 0.1% L-Cysteine
134 (Peptide Institute)) or LB agar plates.

135 ***Heat-shock treatment***

136 500 μL of the group I cell cultures were transferred to 1.5 mL O-ring Seal Screw Cap
137 Tube (WATSON). The tightly capped tubes were incubated at 45°C for 15 min outside
138 an anaerobic chamber.

139 ***pXMTL plasmid construction***

140 The DNA sequence of plasmids pXM (13) and pMTL83151 (16) was amplified by
141 PCR using primers (pXM: GTTTTAAcacagaaaggatgattgttatg/
142 GTTGGGTCGtgcagggggcccgatcg, and pMTL: CCCTGCAcgacccaacactctcctac/
143 TTCTGTGttaaaacaaggatttttccttg). The sequence containing *mazF*, *xylR* and multiple
144 cloning site (MCS) of pXM and the sequence containing the *traJ*, replication origin of *E.*
145 *coli* and *catP* of pMTL83151 were ligated using Gibson Assembly kit (New England
146 Biolabs) (Fig. 4A). The ligated plasmid (termed pXMTL) was transformed into
147 chemically competent *E. coli* DH5α cells (TaKaRa Bio). Cm-resistance and xylose-
148 inducible *mazF* expression of the plasmid were confirmed by culturing the transformants
149 on LB agar plates with Cm (LB-Cm) and 1% xylose (LB-Cm/Xyl), respectively.

150 For gene knockout of *bontA*, each 1,500 bp fragment upstream from the second codon
151 of *bontA* (termed LHA: left-hand arm) and downstream from the second to last codon of
152 that (termed RHA: right-hand arm) were amplified from genomic DNA extracted from *C.*
153 *botulinum* 7I03-H or 62A with primers (ΔbontA_LHA-BamHI_for:
154 ataGGATCCgaaattctaatatatcatctgataac, ΔbontA_LHA-SOE_rev:
155 AATTACAGtggcatatttaacacctc, ΔbontA/7I03_RHA-SOE_for:
156 ATATGCCActgtaattaatctcaaactatatgaac, ΔbontA/7I03_RHA-PstI_rev:
157 cttCTGCAGaagcataccacaatgcatatc, ΔbontA/62A_RHA-SOE_for:
158 ATATGCCActgtaattaatctcaaactacatg, ΔbontA/62A_RHA-PstI_rev:
159 cttCTGCAGtcaataggtttggacgttctatag). The LHA-RHA overlapping sequences were
160 amplified using splicing by overlap extension PCR (SOE-PCR) and cloned into the
161 *Bam*HI/*Pst*I site of pXMTL (pXMTL-bontA).

162 ***Gene knockout***

163 For the first recombination, conjugal transformation was performed using the
164 optimized protocol. Briefly, CA434 cells harboring pXMTL-bontA were grown to mid-
165 log or stationary phases in LB-Kan/Cm medium. The CA434 cells and an overnight
166 culture of *C. botulinum* were mated on BHIS agar plates. Conjugants were selected on
167 TPGY agar plates with 7.5 μg/mL thiamphenicol and 250 μg/mL D-cycloserine
168 (Tm7.5/Cs) for strain 7I03-H or Tm/Cs for strain 62A. Resultant colonies of
169 transformants were suspended in 100 μL of TE (10 mM Tris pH 8.0, 1 mM EDTA) and
170 microwaved at 700 W for 2 min. To check the first single crossover recombination, PCR
171 was performed with 1 μL of the cell suspensions and specific primers (pXMTL-IN_for:
172 ACAGCCATAACCCTTTAAATTATACATTC, ΔbontA/7I03-check_rev:
173 TTGCCATATACCACACTTTC, ΔbontA/62A-check_rev:
174 TATTGTTCTTATCTGTACGAAAC). Clones replicated on TPGY-Tm7.5/Cs or TPGY-
175 Tm/Cs agar plates were cultured in TPGY broth without antibiotics, and the cultures were
176 inoculated onto TPGY agar plates with 1% xylose (TPGY-Xyl). The second double
177 crossover recombination was checked by colony PCR with specific primers
178 (ΔbontA/7I03-check_for: AAAATCAATATTAGCTCAAGAAAC, ΔbontA/7I03-
179 check_rev, ΔbontA/62A-check_for: ATCCAAATATATCTATGTGTATCTC,
180 ΔbontA/62A-check_rev) and by direct Sanger DNA sequencing. Clones replicated on
181 TPGY-Xyl agar plates were cultured in TPGY broth, and stored in 18% (w/v) glycerol at
182 -80°C.

183 ***Western blotting***

184 *C. botulinum* 7I03-H cells were cultured in 1 mL of TPGY broth, pelleted by
185 centrifugation, and lysed in 100 μL of B-PER™ Complete Bacterial Protein Extraction

186 Reagent (Thermo Fisher Scientific). After heating at 95°C for 5 min, the proteins were
187 separated by SDS-PAGE and transferred to PVDF membranes (Bio-Rad). The
188 membranes were blocked with 5% skim milk/PBS-T, and incubated with a rabbit anti-
189 A16S (62A) polyclonal antibody, followed by an HRP-conjugated anti-rabbit IgG
190 antibody. Chemiluminescence of the membranes was detected using ECL-select (Cytiva)
191 and LAS4000 mini (Cytiva).

192 **Results and Discussion**

193 **Transformation efficiency of group I *C. botulinum* and *C. sporogenes***

194 We first assessed the conjugal transformation efficiency of group I *C. botulinum* strains
195 (62A, 7I03-H, Okra, Osaka05, 111) and *C. sporogenes* strains (JCM 1416^T, ATCC
196 15579) using pMTL83153-mCherry-opt. These cells were conjugated with *E. coli* CA434
197 cells harboring a plasmid by the original conjugation protocol (7, 14). Strains 62A, 7I03-
198 H, Osaka05, 111, JCM 1416^T and ATCC 15579 exhibited transformation efficiencies of
199 approximately 4.5×10^3, 23, 9.0×10^3, 2.5×10^4, 6.0×10^2 and 8.0×10^2 transformants
200 per plate, respectively (Fig. 1). No transformants were obtained in strain Okra (Fig. 1).
201 The *C. botulinum* strains produce BoNTs (62A, serotype A1 (17); 7I03-H, serotype A2
202 (18); Okra, serotype B1 (19); Osaka05, serotype B6 (20); 111, serotype B2 and X) (21,
203 22), but the *C. sporogenes* strains do not. The genes encoding BoNT are located on
204 chromosomes or plasmids (23): the chromosome in strains 62A and 7I03-H, the plasmid
205 in strains Okra and Osaka05, both in strain 111. There are two types of *bont* gene clusters
206 (23): the *ha*-type gene cluster in strains 62A, Okra and Osaka05; the *orfX*-type gene
207 cluster in strain 7I03-H; both in strain 111. Strain 62A is a long-standing laboratory strain.
208 Strain Okra was isolated from foodborne botulism cases, and strains 7I03-H (24),

209 Osaka05 (18) and 111 (25) were related to infant botulism cases. According to core gene
210 phylogenetic analysis, strains 62A, 7I03-H (genetically similar to strain Kyoto-F) and
211 Okra belong to the group I *C. botulinum* clade. In contrast, strains Osaka05 and ATCC
212 15579 belong to the *C. sporogenes* clade (26). These data suggest that transformation
213 efficiencies are not associated with these strains' phylogenetic or pathogenic
214 characteristics. It has been reported that CLSPO_c06750-knockout cells of *C. sporogenes*
215 NCIMB 10969 (JCM 1416^{T}), which is a mutant deficient in the Type IV restriction
216 system, has a 10-fold higher transformation efficiency than wild-type (WT) (7); *i.e.,*
217 overcoming the restriction barrier increases transformation efficiency. According to the
218 REBASE database (27), these strains used in this study employ diverse restriction-
219 modification (RM) systems (Fig. S1). These defense systems against foreign DNA might
220 affect the transformation efficiencies of these strains.

221 **Optimizing the transformation protocol**

222 To expand the range of strains capable of being transformed, we optimized the conjugal
223 transformation method (Fig. 2A). First, we assessed the effect of the culture media for the
224 donor CA434 cells on the efficiencies. Donor cells cultured in LB medium tended to yield
225 higher efficiency of DNA transfer to *C. botulinum* 62A compared to TB medium,
226 although the difference was not statistically significant (Fig. 2B). Second, we assessed
227 the effect of growth phase of the CA434 cells. Donor cells in the mid-log and stationary
228 (overnight culture) phases yielded higher efficiency than those in the early-log phase (Fig.
229 2C). Lastly, the mating was conducted on TPGY, BHIS or LB agar plates. It is known
230 that culture media during mating influence the transformation efficiency of *C. difficile*
231 (28). Consistent with this, mating conducted on BHIS agar plates exhibited a 10-fold

232 higher transformation efficiency of *C. botulinum* 62A than on TPGY agar plates (Fig.
233 2D). Collectively, the transformation protocol is optimized as below: CA434 cells are
234 pre-cultured in (I) LB medium, and harvested at (II) the stationary phase (overnight
235 culture). The cells were mated on (III) BHIS agar plates. This optimized protocol confers
236 higher transformation efficiencies for strains 62A, 7I03-H, Osaka05, 111 and JCM 1416^T
237 than the original protocol: 6.3-fold (62A), 5.6-fold (Osaka05), 6.5-fold (111) and 4.2-fold
238 (JCM 1416^T) (Fig. 3).

239 It is known that exposing recipient cells to heat shock at 52°C for 5 min before mixing
240 with donor cells significantly increases the transformation efficiency of *C. difficile* (28)
241 and other bacteria (29–31). Consistent with this, pre-treatment by heat shock at 45°C for
242 15 min tended to increase the transformation efficiency of certain strains of group I *C.*
243 *botulinum*, albeit not significantly (Fig. 3).

244 **Gene knockout in *C. botulinum* 7I03-H**

245 We established a markerless mutant generation system by ACE using pXM in *C.*
246 *perfringens* (13). pXM is a *mazF*-based suicide vector that is counter-selectable for xylose
247 sensitivity and is introduced into *C. perfringens* strains by electroporation (13). Two
248 methods are routinely pursued to enable DNA transfer to clostridial recipients:
249 electroporation and conjugation. Plasmids are capable of being introduced into *C.*
250 *botulinum* Hall (group I, A1-HA) by electroporation at ~10^3 transformants/μg of DNA
251 (32). Consistent with this study, we obtained a similar efficiency with strain 62A by
252 electroporation, whereas no transformants were obtained with strain 7I03-H (data not
253 shown). pMTL80000 vectors are transferred at high efficiency by conjugation from *E.*
254 *coli* CA434 (HB101 carrying R702) donor strain to *Clostridium* spp. (14, 16). We

255 developed a novel conjugal suicide vector (termed pXMTL) derived from pXM (13) and
256 pMTL83151 (16) (Fig. 4A). pXMTL contains a MCS, *catP*, replication origin of *E. coli*,
257 *traJ*, *mazF* and *xylR*. The plasmid is a xylose-inducible *mazF*-based suicide vector (Fig.
258 4A) and can be transferred to *Clostridium* spp. by conjugation. To delete the *bontA* gene
259 in *C. botulinum* 7I03-H, LHA and RHA were designed and cloned into the MCS of
260 pXMTL (Fig. 4). LHA contains 1,500 bp of sequence upstream from the second codon
261 of *bontA*. RHA contains the same length (1,500 bp) of sequence downstream from the
262 second to last codon (the codon before the stop codon). The first single crossover event
263 integrates the suicide plasmid into the bacterial chromosome, resulting in
264 chloramphenicol resistance (Fig. 4B). Due to lack of a replication origin of *Clostridium*
265 spp. (Fig. 4A), single crossover events will occur in Cm-resistant isolates. It is known
266 that the length of the homology arms is critical for the frequency of homologous
267 recombination in *C. difficile* (33). Our preliminary experiments obtained few or no first
268 recombinants using pXMTL-bontA with shorter homology arms (500 or 1,000 bp, data
269 not shown). pXMTL with 1,500 bp arms resulted in N- and C-crossover events in strain
270 7I03-H (Fig. 5A). The first recombinants were induced by xylose to undergo second
271 crossover events, yielding knockout (KO) and revertant (RT) cells (Fig. 4B, 5). As a result,
272 8 of 15 clones (53.3%) were *bontA*-KO cells (Fig. 5A). The precise genome editing and
273 the loss of BoNT/A expression were confirmed by Sanger DNA sequencing (Fig. 5B) and
274 western blotting (Fig. 5C), respectively. The ACE system also deleted the target genes
275 using pXMTL in *C. botulinum* 62A and *C. sporogenes* JCM 1416^{T} (data not shown). This
276 system provides a rapid and markerless gene mutagenesis method for group I *C.*
277 *botulinum* and *C. sporogenes*.

Conclusions

C. botulinum causes severe poisoning by producing BoNT (1), and *C. sporogenes*, a bacterium closely related to group I *C. botulinum*, is a key contributor to the production of bioactive metabolites in the human gut (2, 3). The ecologies of these organisms, including sporulation, germination, outgrowth and infection, remain elusive and functions of their genes are largely unknown. One reason might be because these bacteria exhibit low transformation efficiencies (Fig. 1). In the present study, we optimized the conjugal transformation protocol and yielded higher efficiencies than the original protocol. In addition, we developed a novel conjugal *mazF*-based suicide vector pXMTL harboring a xylose-inducible promoter, and provided a rapid method for precise, markerless and scarless genome editing by ACE. This system will facilitate the analysis of gene function in group I *C. botulinum* and *C. sporogenes*.

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Conflicts of interest

The authors declare no conflicts of interest.

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300 **Contribution of authors**

301 SA and YF conceived and designed the experiments. SA performed the experiments and
302 data analysis. KS and HN assisted in plasmid construction. SA and YF wrote the original
303 draft of the manuscript. All authors have discussed the results and approved the
304 manuscript.

305 **Abbreviations**

306 ACE, allele-coupled exchange; CRISPR, clustered regularly interspaced short
307 palindromic repeats; Cas9, CRISPR-associated protein 9; BoNT, botulinum neurotoxin;
308 HA, hemagglutinin; NT, no toxin; LB, Luria-Bertani; TB, Terrific broth; SOC, super
309 optimal broth with catabolite repression; TPGY, tryptone-peptone-glucose-yeast extract;
310 BHIS, brain heart infusion-supplemented; MCS, multiple cloning site; Kan, kanamycin;
311 Cm, chloramphenicol; Tm, thiamphenicol; Cs, D-cycloserine; Xyl, xylose; LHA, left-
312 hand arm; RHA, right-hand arm; SOE-PCR, splicing by overlap extension PCR; WT,
313 wild-type; RT, revertant; KO, knockout.

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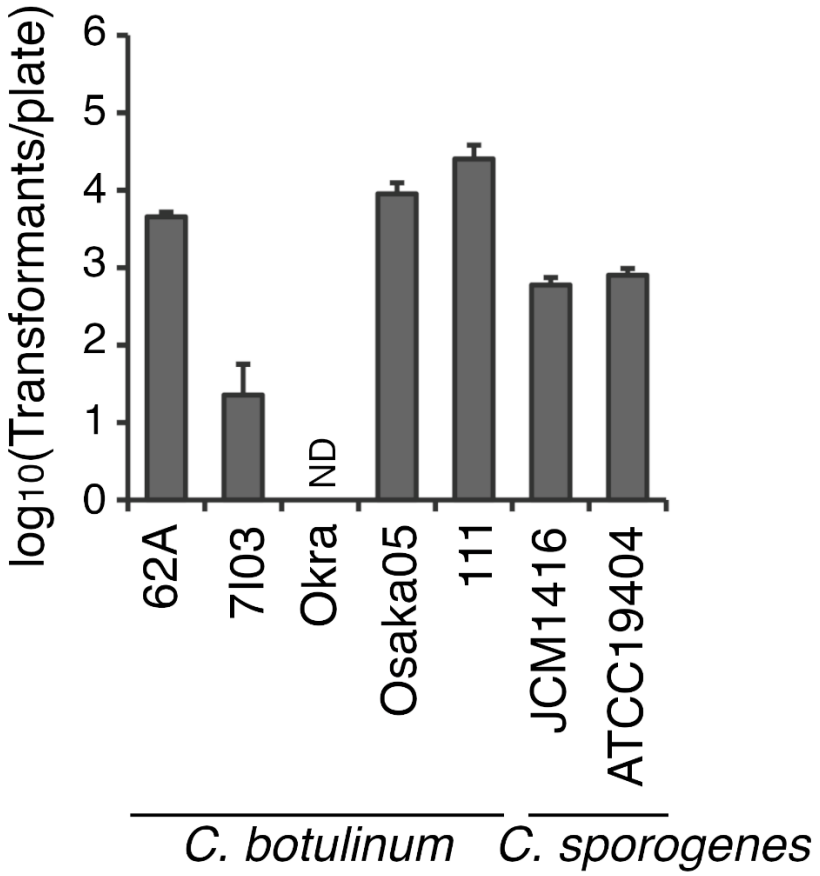

422

423 **FIG 1** Transformation efficiency of group I *C. botulinum* and *C. sporogenes* by the
424 original conjugation protocol.

425 According to the original conjugation protocol (7, 14), CA434 cells harboring
426 pMTL83153-mCherry-opt cultured overnight in LB medium were mated with *C.*
427 *botulinum* strains (62A, Okra, 7I03-H, 111 and Osaka05) and *C. sporogenes* strains (JCM
428 1416^{T} and ATCC 15579) on TPGY agar plates. The transformants were selected on
429 TPGY agar plates with thiamphenicol and D-cycloserine (Tm/Cs). Values are means ±
430 SD of triplicates. ND, not detected.

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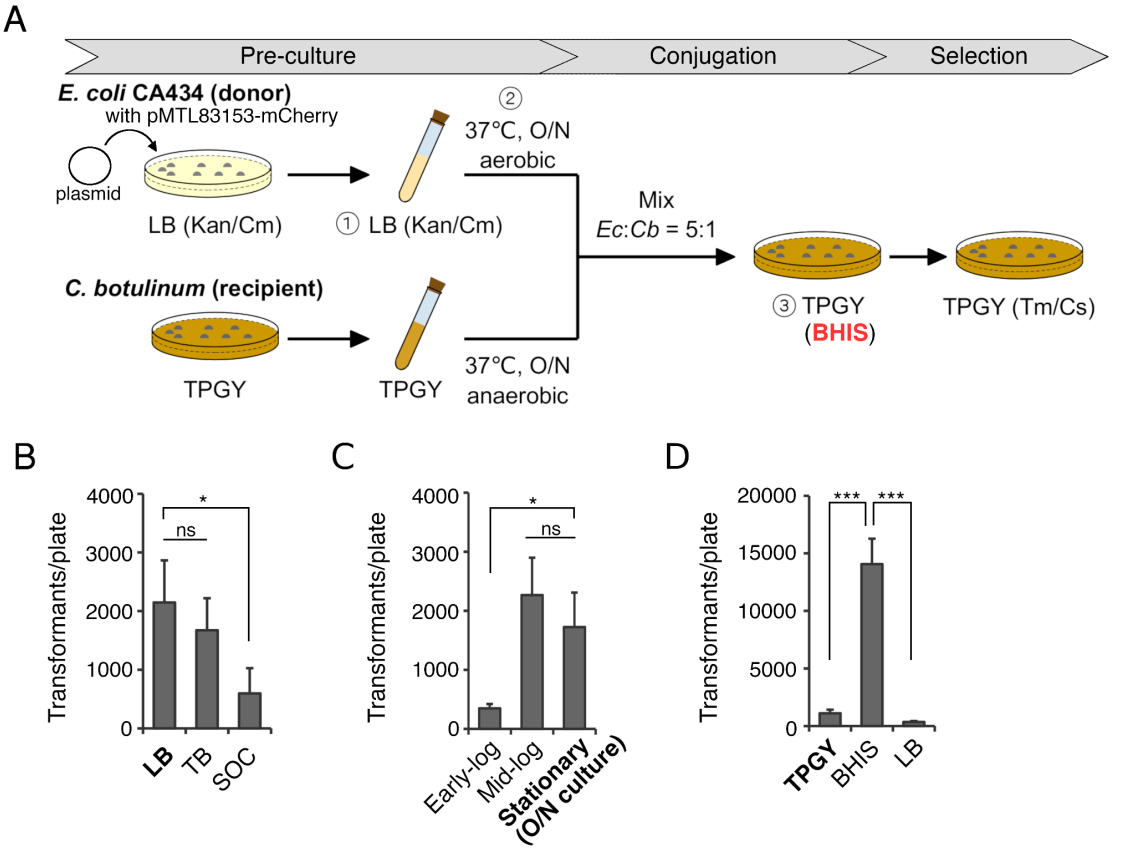
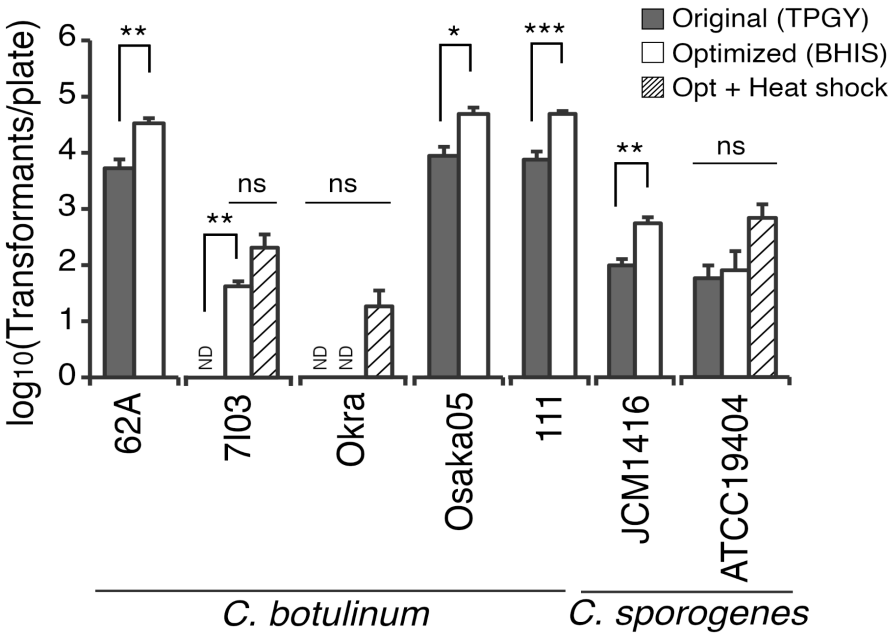


FIG 2 Optimization of conjugation protocol for group I *C. botulinum*.

(**A**) Schematic model of the standard (original) conjugation protocol.

(**B-D**) Transformation efficiency of *C. botulinum* 62A with pMTL83153-mCherry-opt containing a chloramphenicol (Cm) resistance gene. The conjugation protocol (**A**) was performed with three modifications: culture media of donor cells (**B**) and growth phases of donor cells (**C**) and culture media during mating (**D**). Bold font indicates the original protocol. Values are means ± SD of triplicates. ND, not detected. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$; by ANOVA with Tukey's post-hoc HSD test.



441

442 **FIG 3** Transformation efficiency of group I *C. botulinum* and *C. sporogenes* by the
443 optimized conjugation protocol.

444 Plasmid pMTL83153-mCherry-opt was transferred from CA434 cells to *C. botulinum*
445 strains (62A, Okra, 7I03-H, 111 and Osaka05) and *C. sporogenes* strains (JCM 1416^{T} and
446 ATCC 15579). Transformants were selected on TPGY-Tm/Cs agar plates. Gray, white,
447 and hatched bars represent transformation efficiencies by the original protocol, the
448 optimized protocol (BHIS), and the optimized protocol with heat shock, respectively.
449 Values are means ± SD of triplicates. ND, not detected. *$P < 0.05$, **$P < 0.01$, ***$P <$
450 0.001; by unpaired, two-tailed Student's *t*-test.

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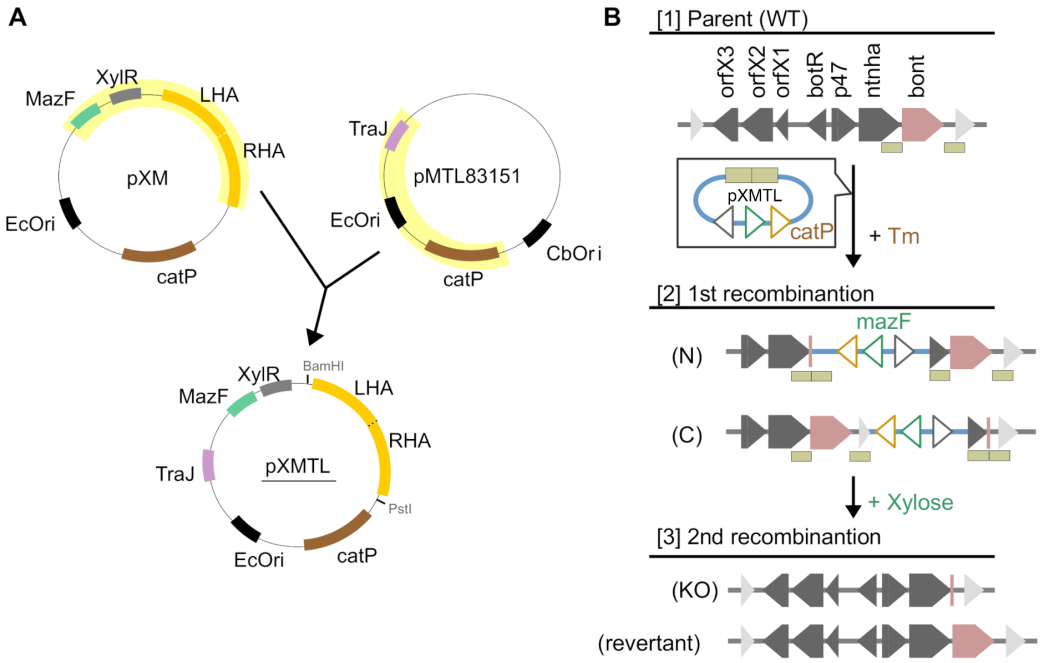


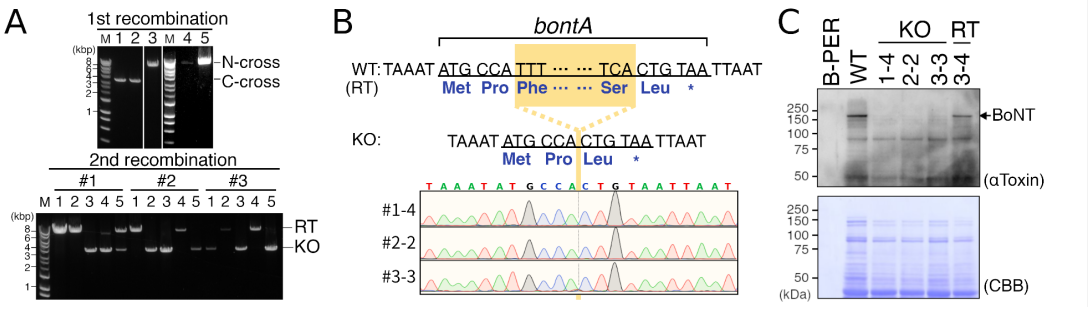
452

453 **FIG 4** Markerless mutagenesis by allele-coupled exchange (ACE) using a conjugal
454 suicide vector pXMTL.

455 (**A**) Map of the engineered plasmid pXMTL with homology arms. pXMTL harbors *mazF*,
456 *xylR* and MCS of pXM and *catP*, the replication origin of *E. coli* and *traJ* of
457 pMTL83151.

458 (**B**) Schematic protocol of ACE using pXMTL, which deletes the *bontA* gene on the
459 chromosome of *C. botulinum* 7I03-H. (1) Left homology arm (LHA) and right
460 homology arm (RHA) targeting *bontA* were cloned into MCS of pXMTL (pXMTL-
461 bontA). (2) At the first recombination step, the recombinant in which the pXMTL-
462 bontA plasmid is crossed is selected on TPGY-Tm7.5/Cs agar plates. (3) At the second
463 recombination step, the plasmid is cured by exposing 1% xylose, resulting in *bontA*-
464 KO mutants (KO) or wild-type revertants (RT).

465



466

467 **FIG 5** *bontA* gene knockout in *C. botulinum* 7I03-H.

468 (**A**) PCR analysis of the first (upper panel) and second (lower panel) recombinants of *C.*

469 *botulinum* 7I03-H.

470 (**B**) DNA sequence of interest of *bontA*-KO (#1-4, #2-2, #3-3) cells was confirmed by

471 Sanger DNA sequencing. Sequence chromatograms were visualized using SnapGene

472 Viewer 6.0.2.

473 (**C**) Protein expression of BoNT/A in WT, *bontA*-KO (#1-4, #2-2, #3-3) and RT (#3-4)

474 cells was confirmed by western blotting.

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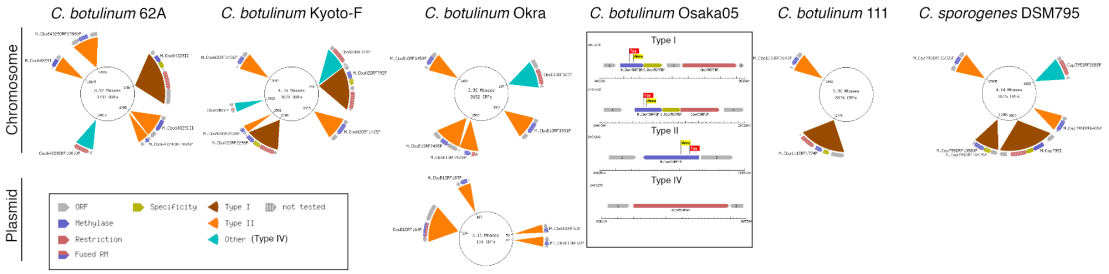

Fig. S1 Restriction and modification systems in group I *C. botulinum* and *C. sporogenes*. Restriction modification (RM) systems of strain 62A, Kyoto-F (genetically similar to 7I03-H), Okra, Osaka05, 111 and DSM 795^{T} (JCM 1416^{T}) listed in the REBASE database (27). Brown, orange and cyan colors indicate type I, II and IV RM systems, respectively.