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

Sho Amatsu[1,2#], Kazuki Saito[1], Shigeru Miyata[3], Masahiko Zuka[2], Hirofumi Nariya[4#], and Yukako Fujinaga[1#]

[1]Department of Bacteriology, Graduate School of Medical Sciences, Kanazawa University, Ishikawa, Japan

[2]Department of Forensic Medicine and Pathology, Graduate School of Medical Sciences, Kanazawa University, Ishikawa, Japan

[3]Department of Food and Nutritional Sciences, College of Bioscience and Biotechnology, Chubu University, Aichi, Japan

[4]Laboratory of Food Microbiology, Graduate School of Human Life Sciences Food and Nutritional Sciences, Jumonji University, Saitama, Japan

For correspondence: Sho Amatsu, amatsu@med.kanazawa-u.ac.jp; Hirofumi Nariya, nariya@jumonji-u.ac.jp; Yukako Fujinaga, yukafuji@med.kanazawa-u.ac.jp

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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.*

46 **Introduction**

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

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

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

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

97 **Materials and Methods**

98 ***Bacterial strains***

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

111 ***Conjugal transformation***

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

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

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

134 ***Heat-shock treatment***

135 500 μL of the group I cell cultures were transferred to 1.5 mL O-ring Seal Screw Cap
136 Tube (WATSON). The tightly capped tubes were incubated at 45°C for 15 min outside
137 an anaerobic chamber.

138 ***pXMTL plasmid construction***

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

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

161 ***Gene knockout***

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

182 ***Western blotting***

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

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

191 **Results and Discussion**

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

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

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

220 **Optimizing the transformation protocol**

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

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

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

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

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

254 developed a novel conjugal suicide vector (termed pXMTL) derived from pXM (13) and
255 pMTL83151 (16) (Fig. 4A). pXMTL contains a MCS, *catP*, replication origin of *E. coli*,
256 *traJ*, *mazF* and *xylR*. The plasmid is a xylose-inducible *mazF*-based suicide vector (Fig.
257 4A) and can be transferred to *Clostridium* spp. by conjugation. To delete the *bontA* gene
258 in *C. botulinum* 7I03-H, LHA and RHA were designed and cloned into the MCS of
259 pXMTL (Fig. 4). LHA contains 1,500 bp of sequence upstream from the second codon
260 of *bontA*. RHA contains the same length (1,500 bp) of sequence downstream from the
261 second to last codon (the codon before the stop codon). The first single crossover event
262 integrates the suicide plasmid into the bacterial chromosome, resulting in
263 chloramphenicol resistance (Fig. 4B). Due to lack of a replication origin of *Clostridium*
264 spp. (Fig. 4A), single crossover events will occur in Cm-resistant isolates. It is known
265 that the length of the homology arms is critical for the frequency of homologous
266 recombination in *C. difficile* (33). Our preliminary experiments obtained few or no first
267 recombinants using pXMTL-bontA with shorter homology arms (500 or 1,000 bp, data
268 not shown). pXMTL with 1,500 bp arms resulted in N- and C-crossover events in strain
269 7I03-H (Fig. 5A). The first recombinants were induced by xylose to undergo second
270 crossover events, yielding knockout (KO) and revertant (RT) cells (Fig. 4B, 5). As a result,
271 8 of 15 clones (53.3%) were *bontA*-KO cells (Fig. 5A). The precise genome editing and
272 the loss of BoNT/A expression were confirmed by Sanger DNA sequencing (Fig. 5B) and
273 western blotting (Fig. 5C), respectively. The ACE system also deleted the target genes
274 using pXMTL in *C. botulinum* 62A and *C. sporogenes* JCM 1416^{T} (data not shown). This
275 system provides a rapid and markerless gene mutagenesis method for group I *C.*
276 *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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299 **Contribution of authors**

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

304 **Abbreviations**

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

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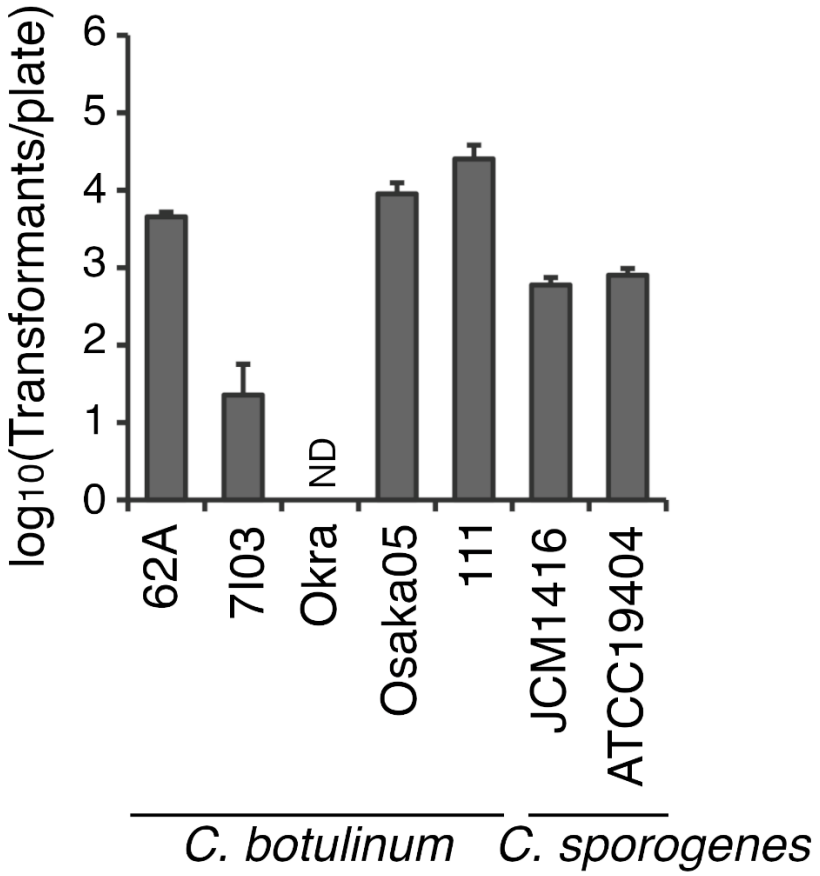

421

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

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

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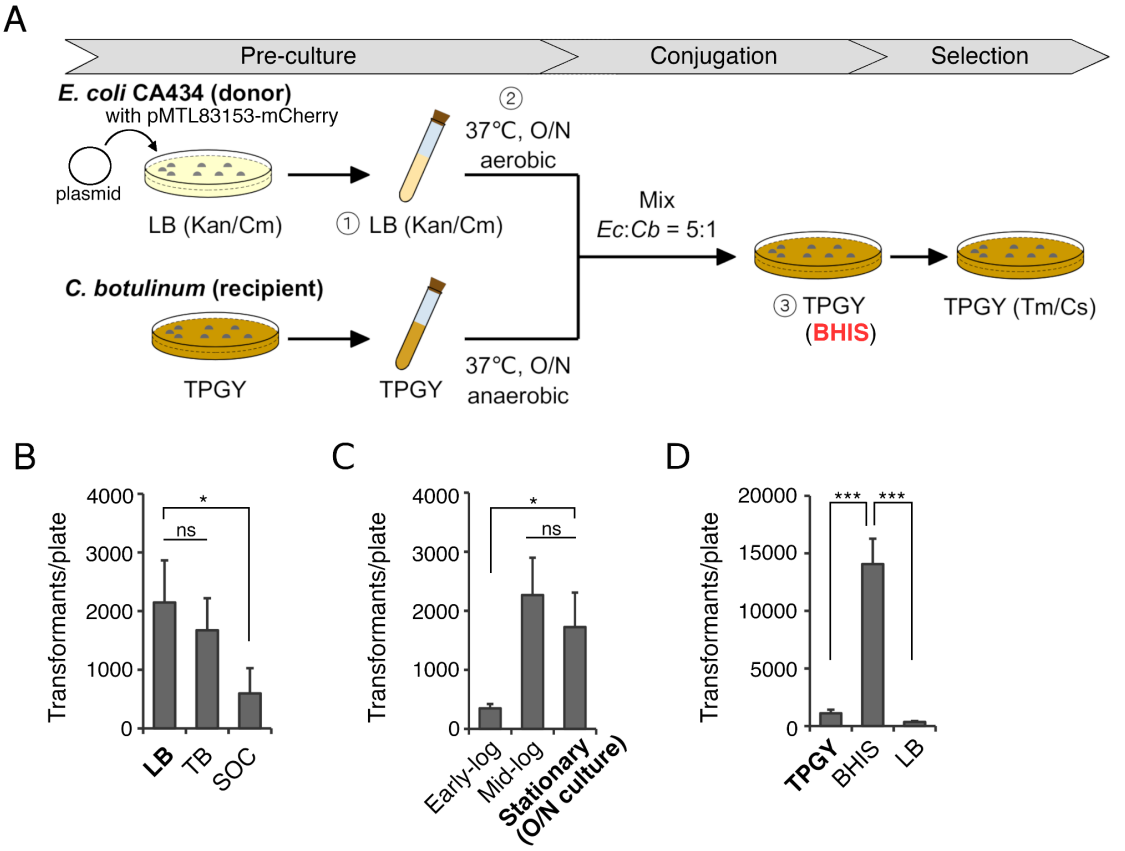
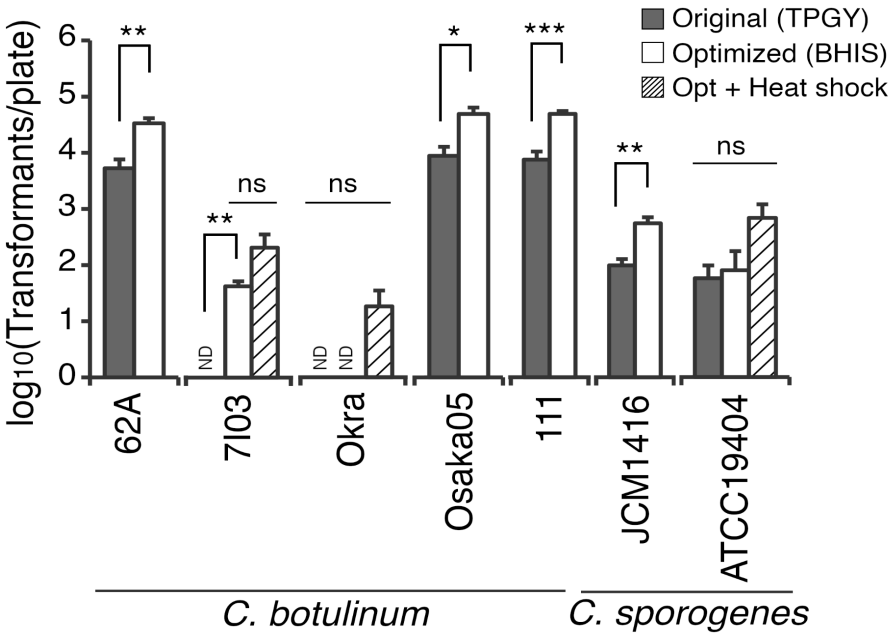


FIG 2 Optimization of conjugation protocol for group I *C. botulinum*.

(**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.



440

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

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

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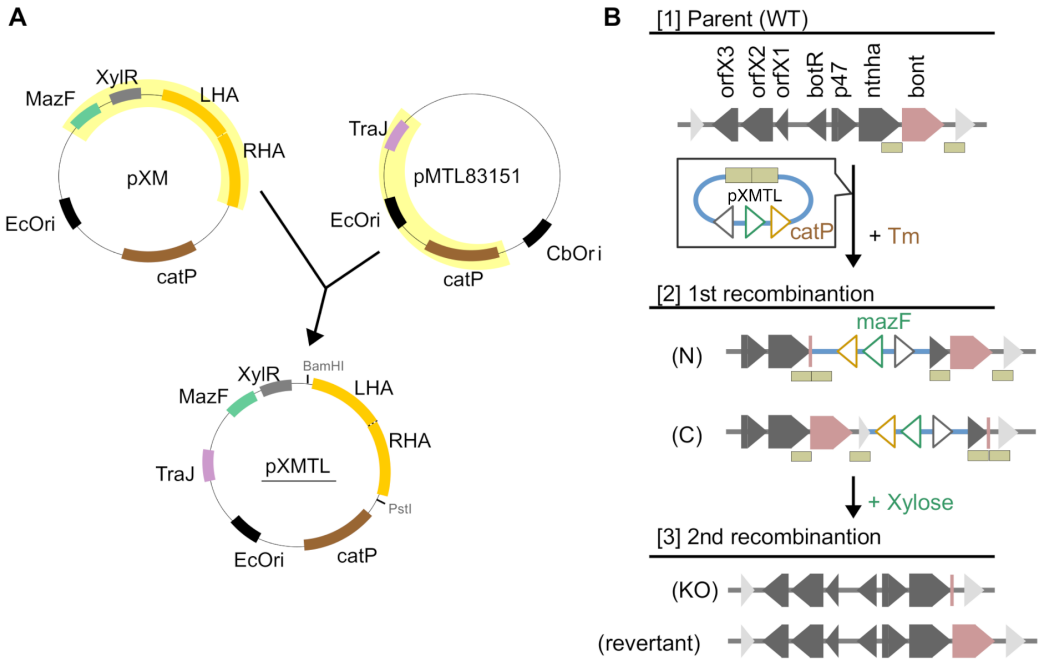

451

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

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

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

464

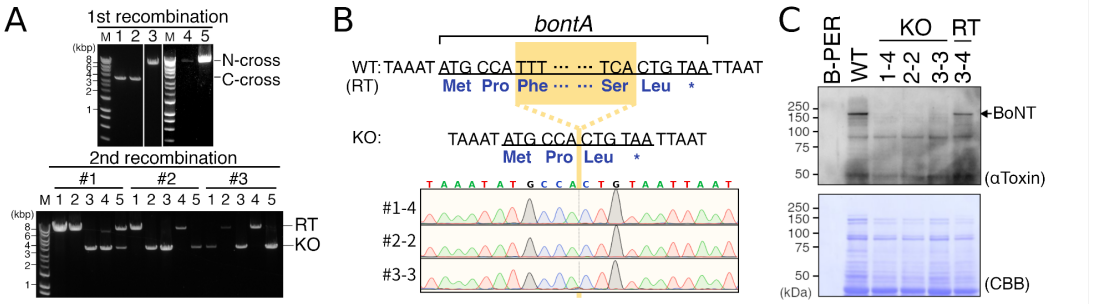

FIG 5 *bontA* gene knockout in *C. botulinum* 7I03-H.

(**A**) PCR analysis of the first (upper panel) and second (lower panel) recombinants of *C. botulinum* 7I03-H.

(**B**) DNA sequence of interest of *bontA*-KO (#1-4, #2-2, #3-3) cells was confirmed by Sanger DNA sequencing. Sequence chromatograms were visualized using SnapGene Viewer 6.0.2.

(**C**) Protein expression of BoNT/A in WT, *bontA*-KO (#1-4, #2-2, #3-3) and RT (#3-4) cells was confirmed by western blotting.

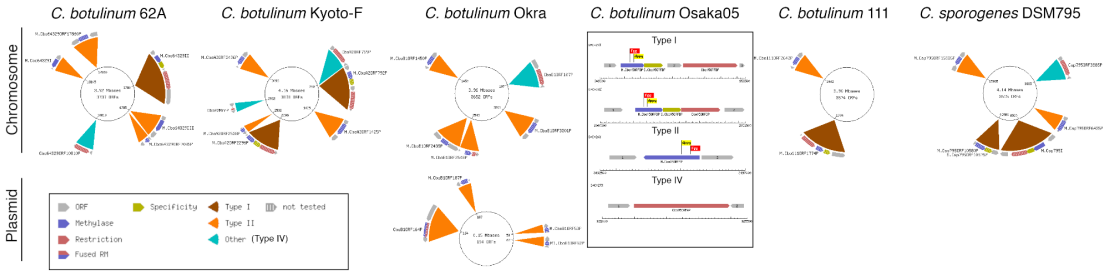

Fig. S1 Restriction and modification systems in group I *C. botulinum* and *C. sporogenes*. Restriction modification (RM) systems of strain 62A, Kyoto-F (genetically similar to 7I03-H), Okra, Osaka05, 111 and DSM 795^{T} (JCM 1416^{T}) listed in the REBASE database (27). Brown, orange and cyan colors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