Transcriptional Downregulation of a Type III Secretion System under Reducing Conditions in *Bordetella pertussis*

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Running Head: Reducing Conditions Downregulate *Bordetella* T3SS.

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21 **ABSTRACT**

22 *Bordetella pertussis* uses a type III secretion system (T3SS) to inject virulence
23 proteins into host cells. Although the *B. pertussis* T3SS was presumed to be involved
24 in host colonization, the efficient secretion of type III secreted proteins from
25 *B. pertussis* has not been observed. To investigate the roles of type III secreted
26 proteins during infection, we attempted to optimize culture conditions for the
27 production and secretion of a type III secreted protein, BteA, in *B. pertussis*. We
28 observed that *B. pertussis* efficiently secretes BteA in ascorbic acid-depleted (AsA^-)
29 medium. When L2 cells, a rat lung epithelial cell line, were infected with *B. pertussis*
30 cultured in the AsA^- medium, BteA-dependent cytotoxicity was observed. We also
31 performed an immunofluorescence assay of L2 cells infected with *B. pertussis*. The
32 clear fluorescence signals of Bsp22, a needle structure of T3SS, were detected on
33 the bacterial surface of *B. pertussis* cultured in the AsA^- medium. Since ascorbic
34 acid is known as a reducing agent, we cultured *B. pertussis* in liquid medium
35 containing other reducing agents such as 2-mercaptoethanol and dithioerythritol.
36 Under these reducing conditions, the production of type III secreted proteins was
37 repressed. These results suggest that in *B. pertussis*, the production and secretion of
38 type III secreted proteins are downregulated under reducing conditions.

39

40 **IMPORTANCE**

41 The type III secretion system (T3SS) of *Bordetella pertussis* forms a needle-like
42 structure that protrudes from the bacterial cell surface. *B. pertussis* uses T3SS to
43 translocate virulence proteins called effectors into host cells. The culture conditions
44 for effector production in *B. pertussis* have not been investigated. We attempted to

45 optimize culture medium compositions for producing and secreting type III secreted
46 proteins. We found that *B. pertussis* secretes type III secreted proteins in reducing
47 agent-deprived liquid medium, and that BteA-secreting *B. pertussis* provokes
48 cytotoxicity against cultured mammalian cells. These results suggest that redox
49 signaling is involved in the regulation of *B. pertussis* T3SS.
50

51 **Introduction**

52 The genus *Bordetella* is made up of Gram-negative bacteria and has been
53 subdivided into 16 subspecies to date (1). *Bordetella pertussis* is a causative agent
54 of whooping cough, also known as pertussis. *B. pertussis* strictly adapts to humans,
55 and it lacks an environmental reservoir (2). A variety of virulence factors have been
56 identified in *Bordetella*, e.g., filamentous hemagglutinin (3), adenyl cyclase toxin (4)
57 and pertactin (5). These virulence factors are regulated by a *Bordetella* two-
58 component regulatory system, BvgAS. In addition to these virulence factors, the type
59 III secretion system (T3SS) of *B. pertussis* is also regulated by BvgAS (6, 7).
60 A T3SS that forms a needle-like structure is conserved among many Gram-negative
61 bacteria. In order to subvert hosts, bacteria use a T3SS to inject type III secreted
62 proteins into host cells (8). Such type III secreted proteins are called effectors. Three
63 types of effectors have been identified in *Bordetella*: BteA ((9) [also referred to as
64 BopC (10)], BopN (11, 12), and BspR (13) [also referred to as BtrA (14, 15)]. BteA is
65 658 amino acids in length and is localized to lipid rafts in host cells via its N-terminal
66 region (16). BteA induces necrotic cell death in several types of cultured mammalian
67 cells (9, 10), and BteA translocation into host cells is promoted by the functioning of
68 BopN (11). BspR is a negative regulator for type III secreted proteins (13) and is

69 translocated into nuclei of cultured mammalian cells (17). BspR has been suggested
70 to be an anti-sigma factor against BtrS that functions as a positive regulator for type
71 III secreted proteins (15). Some type III secreted proteins are called translocators
72 rather than effectors. Three types of translocators have been identified in *Bordetella*,
73 i.e., BopB (18), BopD (19) and Bsp22 (20), and they function as the path for effectors
74 delivered into host cells. The complex of BopB and BopD forms the pores on the
75 host membrane (19), and Bsp22 forms a filamentous-like structure and is associated
76 with the T3SS needle and with a pore-forming component, BopD (21).

77 The optimal culture conditions for the production and secretion of BteA have
78 not been established. To investigate the roles of BteA and other type III secreted
79 proteins during *B. pertussis* infection, we investigated culture conditions for the
80 maximal production and secretion of type III secreted proteins in *B. pertussis* strain
81 Tohama I.

82

83 **MATERIALS AND METHODS**

84 **Bacterial strains**

85 The strains used in this study are listed in Table 1. *B. pertussis* Tohama I (22) was
86 used as the wild-type strain. BP344, BP348, BP350, and BP351 were used as
87 clinical isolates and were a kind gift from Dr. K. Kamachi (National Institute of
88 Infectious Diseases) (23). *Escherichia coli* DH10B and Sm10λ*pir* were used for DNA
89 cloning and conjugation, respectively (Table 1). *B. pertussis* was grown on Bordet-
90 Gengou agar plates at 37°C for 5 days. Bordet-Gengou agar plates were prepared
91 as follows. We mixed 15 g of Bordet-Gengou agar base (BD #248200), 5 mL of
92 glycerol, 100 mL of defibrinated horse blood (Nihon Bio-Supp Center), and 400 mL

93 of distilled water. The mixture was autoclaved and poured onto plastic plates. Fresh
94 colonies of *B. pertussis* on each Bordet-Gengou agar plate were suspended in
95 Stainer-Scholt (SS) medium [Table 2, (24)]. In this study, we used a liquid medium
96 described as a cyclodextrin-containing medium as the SS medium (25). Low
97 casamino acids (LCA) medium was prepared by removing the agar from cyclodextrin
98 solid medium (CSM) contents [Table 2, (26)]. *B. pertussis* was cultured in SS
99 medium with a starting A_{600} of 0.23 under static conditions at 37ºC. To measure the
100 bacterial density, a spectrophotometer (Ultrospec 2100 *pro*, Amersham) was used.
101 The final A_{600} of *B. pertussis* Tohama I culture after 22 hr was ca 0.67, and the
102 culture contained ca 1.0 x 10^9 cfu/mL. The liquid cultivation period was 22 hr for the
103 protein preparation, mRNA preparation, and infection.
104
105 **Generation of a Tohama I nalidixic acid-resistant mutant**
106 For the generation of a Tohama I nalidixic acid-resistant mutant, Tohama I cultured
107 in SS medium for 24 hr as described above was spread on 20 sets of Bordet-
108 Gengou agar plates containing nalidixic acid at the final concentration of 30 μg/ml.
109 After 5 days, surviving colonies were designated as MGKU1. The growth rate, colony
110 size and hemolysis on the BG plates of the resulting nalidixic acid-resistant strain
111 were almost equivalent to those of the wild-type strain, and we observed that the
112 amounts of the production and secretion of type III secreted proteins in MGKU1 were
113 not significantly different from those of Tohama I wild-type (data not shown).
114
115 **Cell culture**

116 L2 cells, a rat lung epithelial cell line (ACTT CCL-149), were maintained in F-12K
117 (Invitrogen, Tokyo). The cell culture medium was supplemented with 10% fetal calf
118 serum (FCS). L2 cells were grown at 37ºC under a 5% CO_2 atmosphere.
119
120 **Construction of gene-disrupted mutants**
121 The primers used in this study are listed in Table 3. To construct the *bspR* mutant,
122 the 4.0 kb DNA fragment encoding *bspR* and its flanking regions was amplified by
123 polymerase chain reaction (PCR) with primers B1-bspR and B2-bspR using
124 *B. pertussis* Tohama I genomic DNA as the template. The resulting PCR product
125 was cloned into pDONR201 to obtain pMGKU401, by means of adapter PCR and
126 site-specific recombination techniques using a Gateway cloning system (Invitrogen).
127 An inverse PCR was carried out with the set of primers R1-bspR and R2-bspR using
128 circular pMGKU401 as the template.
129 The resulting PCR product was digested with BamHI and self-ligated to obtain
130 pMGKU402. This plasmid contained the BamHI site in addition to a 474-bp in-frame
131 deletion from 175 bp downstream of the 5' end of the *bspR* gene to 52 bp upstream
132 of the 3' end of the gene. This plasmid, pMGKU402, was mixed with a positive
133 suicide vector, pABB-CRS2 (27), to obtain pMGKU403 using the Gateway cloning
134 system. This plasmid, pMGKU403, was introduced into *E. coli* Sm10λ*pir* (18) and
135 transconjugated into MGKU1 as described previously (28). The resulting mutant
136 strain was designated as MGKU4 (BspR-deficient strain). Similarly, pABB-Δ*bscN*
137 (18) and pABB-*ΔbopC* (10) were separately introduced into *E. coli* Sm10λ*pir* and
138 were transconjugated into MGKU1. The resulting mutant strains were designated as
139 MGKU2 (a T3SS-inactive strain) and MGKU3 (a BteA-deficient strain), respectively.

140

141 **Preparation of proteins from culture supernatants and whole bacterial cell**

142 **lysates**

143 Secreted proteins released into bacterial culture supernatants and whole bacterial

144 cell lysates were prepared by trichloroacetic acid precipitation. In the case of the

145 supernatant fraction (Sup) samples, the culture supernatants were filtered with a

146 0.22 μm filter (Millipore #SLGVR33RB). Next, 2 μL of 5% deoxycholic acid and 100

147 μL of 100% trichloroacetic acid were added to the 1 mL of filtered supernatants. After

148 being incubated on ice for 15 min, the samples were centrifuged at 21130 ×*g* for 5

149 min. The resulting precipitated proteins were neutralized with 2 μL of 2 M Tris-base

150 and dissolved in 13 μL of 2×SDS-PAGE sample buffer. In the case of the whole cell

151 lysate (WCL) samples, bacterial pellets collected from 1 mL of bacterial cultures

152 were resuspended in distilled water. 100 μL of 100% trichloroacetic acid was added

153 to 1 mL of the bacterial suspension. After being incubated on ice for 15 min, the

154 samples were centrifuged at 21130 ×*g* for 5 min. The resulting precipitated proteins

155 were neutralized with 5 μL of 2 M Tris-base and dissolved in 95 μL of 2×SDS-PAGE

156 sample buffer. When the A_{600} of bacterial culture was 0.67, 0.5 μL of WCL and 5 μL

157 of Sup samples were loaded on the SDS-PAGE gels. The loaded sample amounts

158 were adjusted by the A_{600} of each bacterial culture in order to load samples prepared

159 from the same number of bacteria. The protein samples were separated by SDS-

160 PAGE and analyzed by western blotting.

161

162 **Antibodies**

163 Anti-BteA, anti-BopB, and anti-Bsp22 antibodies were purified from rabbit serum in
164 our previous study (10, 18, 29). To detect filamentous hemagglutinin (FHA) signals,
165 we used mouse anti-FHA serum (30). Mouse anti-CyaA monoclonal antibody was
166 purchased from Santa Cruz Biotechnology (Santa Cruz, CA). To prepare the anti-
167 BtrS antibody, the peptide corresponding to the C-terminus region of BtrS
168 (CALREALRERGYDSVP) were conjugated with hemocyanin from keyhole limpets
169 (Sigma) by using 3-maleimidobenzoic acid N-hydroxysuccinimide ester (Sigma).
170 These cross-linked peptides were used to immunize rabbits, and resulting anti-sera
171 were incubated with peptide immobilized on epoxy-activated sepharose 6B
172 (Amersham) to obtain specific Ig-fractions.
173
174 **Quantitative reverse transcription-PCR (qRT-PCR)**
175 The amounts of mRNA were measured by qRT-PCR. Bacterial total RNA was
176 prepared using a Trizol Max Bacterial RNA Isolation Kit (Invitrogen), an RNeasy Mini
177 Kit (Qiagen, Hilden, Germany) and an RNase-free DNase-free Kit (Qiagen). The
178 RNA sample was reverse transcribed with Transcriptor Universal cDNA Master
179 (Roche Diagnostics, Indianapolis, IN) and a T100 Thermal Cycler (Bio-Rad, Hercules,
180 CA). The resulting cDNA was amplified by FastStart Essential DNA Probes Master
181 (Roche) using the following primer pairs: 5-recA and 3-recA for *recA*; 5-bteA and 3-
182 bteA for *bteA*; 5-bopB and 3-bopB for *bopB* (Table 3). The results were calculated as
183 described in the Roche manual. The amount of *recA* mRNA was used as an internal
184 control. The mRNA amounts are presented herein as relative to the amounts in the
185 Tohama I cultured in SS medium, which was defined as 1.
186

187 **LDH Assays**

188 To examine the release of lactate dehydrogenase (LDH) from *B. pertussis*-infected
189 cells, 5.0×10^4 cells/well of L2 cells seeded in 24-well plates were infected with
190 bacteria at the multiplicity of infection (MOI) of 125, 250, or 500. The plates were
191 centrifuged at 900 *g* for 5 min and incubated for 3 hr at 37ºC under a 5% CO_2
192 atmosphere. The amounts of LDH were measured spectrophotometrically using a
193 Cyto-Tox 96 Non-radioactive Cytotoxicity Assay Kit (Promega, Madison, WI). We
194 added 50 μL of 10 %Triton X-100 to the 950 μL of extracellular medium of L2 cells in
195 24-well plate. We then mixed the extracellular medium by pipetting. We subtracted
196 the LDH value obtained from extracellular medium of uninfected cells from the value
197 obtained from the Triton X-100-treated cells and used the resulting value as 100%.
198

199 **Immunofluorescent staining**

200 For the immunofluorescent staining assay, 2.5×10^5 cells/well of L2 cells were
201 seeded on coverslips in six-well plates and incubated overnight, and then infected
202 with *B. pertussis* at an MOI of 125. The plates were centrifuged at 900 *g* for 5 min
203 and incubated for 3 hr at 37ºC under a 5% CO_2 atmosphere. The infected L2 cells
204 were then immunostained as described previously (12). Briefly, the cells were
205 treated with 4% paraformaldehyde, 50 mM NH_4Cl, 0.2% Triton-X, and 4% bovine
206 serum albumin (BSA). After blocking, the cells were stained by anti-Bsp22 antibody
207 (29). As a secondary antibody, Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen) was
208 used. F-actin was stained with Rhodamine Phalloidin (Molecular Probes, Eugene,
209 OR). Bsp22 signals per one cell were counted under a fluorescence microscope.
210

211 **Statistical analyses**

212 The statistical analyses were performed using the nonparametric unpaired *t*-test with
213 a one-tailed p-value with Prism ver. 5.0f software (Graphpad, La Jolla, CA). Values
214 of $p<0.05$ were considered significant.

215

216 **RESULTS**

217 **Type III secreted proteins are secreted from *B. pertussis* cultured in LCA**
218 **medium**

219 Cyclodextrin solid medium (CSM) is reported as a synthetic medium for clinical
220 isolation of *B. pertussis* (26). We sought to determine whether *B. pertussis* grown on
221 this agar secretes type III effectors, and we prepared a liquid medium, designated
222 "low casamino acids (LCA)" medium, by removing the agar from CSM contents
223 (Table 2). *B. pertussis* was cultured in SS or LCA medium, and then the whole cell
224 lysates (WCL) and supernatant fractions (Sup) samples were subjected to western
225 blotting with antibodies against BteA (an effector, a type III secreted protein) and
226 BopB (a translocator, a type III secreted protein).

227 BteA and BopB signals were detected in WCL and Sup of the wild-type
228 Tohama I cultured in LCA medium (Fig. 1). As reported previously, BteA forms SDS-
229 resistant multimers, and in our present study we also detected the signals at around
230 200 kDa. In contrast, the BteA and BopB signals were faintly detected in the WCL
231 samples of SS medium, and no BteA and BopB signals were detected in the Sup
232 samples of MGKU2 (a T3SS-inactive strain) or the wild-type cultured in SS medium
233 (Fig. 1). These results suggest that *B. pertussis* secretes type III secreted proteins in
234 LCA medium.

235

236 **Ascorbic acid downregulates the production and secretion of type III secreted**

237 **proteins in *B. pertussis***

238 Although the ingredients of the LCA and SS media are the same, the amounts of

239 ascorbic acid and casamino acids in LCA medium are lower than those in SS

240 medium (Table 2). To determine which substance is involved in the production and

241 secretion of type III secreted protein, we cultured *B. pertussis* in SS medium,

242 ascorbic acid-deprived SS medium (SS_AsA$^-$), casamino acid-deprived SS medium

243 (SS_CaA$^-$), or both ascorbic acid- and casamino acid-deprived medium

244 (SS_AsA$^-$_CaA$^-$). WCL and Sup samples were subjected to western blotting with

245 antibodies against BteA and BopB (Fig. 2).

246 The BteA multimer signal intensities in the WCL samples of the SS_AsA$^-$, SS_CaA$^-$

247 and SS_AsA$^-$_CaA$^-$ media were stronger than that in the SS medium (Fig. 2). A faint

248 BteA signal intensity in the Sup samples of SS was detected (Fig. 2). The BteA

249 signal intensities in the Sup samples were evident in the SS_AsA$^-$ and

250 SS_AsA$^-$_CaA$^-$ media (Fig. 2). Again, BopB signal intensities in both WCL and Sup

251 samples were evident in the SS_AsA$^-$ and SS_AsA$^-$_CaA$^-$ media (Fig. 2).

252 We also investigated the potential involvement of ascorbic acid or casamino

253 acids in Bvg-regulated virulence factors, e.g., FHA and CyaA. WCL and Sup

254 samples were subjected to western blotting with antibodies against FHA and CyaA

255 (Fig. 2). The results revealed that there were no significant differences in the FHA or

256 CyaA signal intensities in the WCL and Sup samples among the media used here

257 (Fig. 2). Collectively, these results suggest that ascorbic acid has a specific influence

258 on the production and secretion of BteA and BopB in *B. pertussis*.

259 Standard SS medium contains ascorbic acid at a final concentration of 2270 μM. To
260 further explore whether ascorbic acid affects the production and secretion of type III
261 secreted proteins, we cultured *B. pertussis* in SS medium in the presence of ascorbic
262 acid at the final concentrations of 91, 454, or 2270 μM. WCL and Sup samples were
263 prepared from each bacterial culture and analyzed by western blotting (Fig. 3). The
264 BteA signal was detected in both WCL and Sup samples of SS medium at 0–454 μM
265 ascorbic acid, but the BteA signal of the SS medium at 2270 μM ascorbic acid was
266 faint or absent (Fig. 3). The BopB signals showed a similar pattern (Fig. 3). These
267 results indicate that *B. pertussis* produces and secretes type III secreted proteins
268 under low ascorbic acid concentrations.

269

270 **BteA and BopB are secreted from other *B. pertussis* clinical isolates cultured**
271 **in SS_AsA⁻ medium**

272 To determine whether *B. pertussis* clinical isolates secrete type III secreted proteins
273 under low ascorbic acid concentrations, we cultured four strains of *B. pertussis*
274 (Table 1) in SS medium or ascorbic acid-deprived medium (SS_AsA⁻). The prepared
275 WCL and Sup samples were analyzed by western blotting (Fig. 4). BteA signals in
276 the Sup samples of BP350 and BP351 were evident in the SS_AsA⁻ medium (Fig. 4,
277 AsA⁻). Again, BopB signals in the Sup samples of BP350 and BP351 were evident in
278 SS_AsA⁻ medium. These results suggest that some clinical strains, e.g., BP350 and
279 BP351, also secrete type III secreted proteins under low ascorbic acid
280 concentrations.

281

282 **Translocator BopB is upregulated at the transcriptional level in *B. pertussis***
283 **cultured in SS_AsA⁻ medium**
284 To investigate the gene expressions of type III secreted proteins under ascorbic
285 acid-starved conditions, we prepared total RNA from *B. pertussis* cultured in SS or
286 SS_AsA⁻ medium. The cDNA samples reverse-transcribed from the total RNA
287 samples were subjected to a quantitative RT-PCR analysis to quantify the relative
288 amounts of *bteA* and *bopB* mRNA. The results demonstrated that the relative
289 amount of *bteA* mRNA of Tohama I in SS_AsA⁻ medium was increased compared to
290 that in SS medium (Fig. 5A). However, the relative amount of *bteA* mRNA of BP350
291 in the SS_AsA⁻ medium was not significantly different from that in the SS medium
292 (Fig. 5A). The relative amounts of *bopB* mRNA of Tohama I and BP350 in SS_AsA⁻
293 medium were increased compared to those in SS medium (Fig. 5B).
294 These results suggest that BopB is upregulated at the transcriptional level in
295 Tohama I and BP350 under low ascorbic acid concentrations. BteA is also
296 upregulated at the transcriptional level in Tohama I under low ascorbic acid
297 concentrations but not in BP350.
298
299 ***B. pertussis* induces cytotoxicity against cultured mammalian cells**
300 A *B. pertussis* BspR-deficient strain was reported to induce cytotoxicity against
301 cultured mammalian cells (15). In the present study, we identified better culture
302 conditions for the production and secretion of BteA in *B. pertussis* than those
303 provided with standard SS medium. We thus investigated whether wild-type
304 *B. pertussis* provokes cytotoxicity against cultured mammalian cells under these
305 conditions. L2 cells, a rat lung epithelial cell line, were infected at an MOI of 500 for 3

306 hr in several modified SS media shown in Fig. 6. The amounts of ascorbic acid
307 and/or casamino acids was reduced to one-twentieth of and/or to one-half of those in
308 SS medium. We also used the SS media without ascorbic acid and/or casamino
309 acids.

310 The amounts of lactate dehydrogenase (LDH) released into the extracellular
311 medium were then measured.
312 The results revealed that released LDH was detected from L2 cells infected with
313 Tohama I in the SS_1/20AsA or SS_1/20AsA_1/2CaA medium (Fig. 6). The
314 SS_1/20AsA medium and SS_1/20AsA_1/2CaA medium showed no cytotoxicity. On
315 the other hand, the L2 cells incubated in the SS_AsA$^-$ or SS_1/20AsA_CaA$^-$
316 medium were injured by the medium itself. Therefore, these media were not useful
317 for this assay. Taken together, these results demonstrate that we successfully
318 observed cytotoxicity of mammalian cells induced by wild-type *B. pertussis*.
319

320 **The T3SS of *B. pertussis* cultured in SS_AsA$^-$ medium is active during**
321 **infection against cultured mammalian cells**
322 As described above, we observed cytotoxicity of *B. pertussis* against cultured cells.
323 In order to confirm that *B. pertussis* indeed secretes type III secreted proteins during
324 infection, we infected L2 cells with *B. pertussis* in SS or SS_1/20AsA_1/2CaA
325 medium at an MOI of 125 for 3 hr. After infection, Bsp22 (a translocator, and a
326 component of the filamentous-like structure) and F-actin were stained with anti-
327 Bsp22 and rhodamine phalloidin, respectively.
328 The results showed that the amounts of Bsp22 signals (Fig. 7A, green) on L2 cells
329 infected with Tohama I and BP350 cultured in SS_1/20AsA_1/2CaA medium were

330 greater than those in SS medium (Fig. 7). Thus, we demonstrated that *B. pertussis*
331 T3SS is activated during the infection of cultured mammalian cells under appropriate
332 conditions.
333
334 **The cytotoxicity induced by wild-type *B. pertussis* depends on the T3SS**
335 It has been reported that *B. bronchiseptica* induces BteA-dependent necrotic cell
336 death against cultured mammalian cells (10). To investigate whether the cytotoxicity
337 provoked by wild-type *B. pertussis* also depends on BteA function, we infected L2
338 cells with MGKU1 (a Tohama I nalidixic acid-resistant strain), MGKU2 (a T3SS-
339 inactive strain), MGKU3 (a BteA-deficient strain), or MGKU4 (a BspR-deficient strain
340 that allows the constitutive activation of T3SS) in SS_1/20AsA_1/2CaA medium at
341 an MOI of 125 or 250 for 3 hr. The amounts of LDH released into extracellular
342 medium were measured.
343 As a result, LDH was not detected in the medium of L2 cells infected with MGKU2
344 (Fig. 8A,B). The amount of LDH detected in the medium of L2 cells infected with
345 MGKU3 was significantly lower than that in the medium of L2 cells infected with
346 MGKU1 (Fig. 8A,B). The amount of LDH detected in the medium of L2 cells infected
347 with MGKU4 was significantly greater than that in the medium of L2 cells infected
348 with MGKU1 at an MOI of 125 (Fig. 8A), but not at an MOI of 250 (Fig. 8B).
349 WCL and Sup samples were prepared from each strain and separated by SDS-
350 PAGE, then analyzed by western blotting with anti-BteA antibody (Fig. 8C). BteA
351 signals of the WCL samples were detected in MGKU1, MGKU2, and MGKU4, but
352 not in MGKU3 (Fig. 8C). BteA signals of the Sup samples were detected in MGKU1
353 and MGKU4, but not in MGKU2 or MGKU3 (Fig. 8C). These results suggest that the

354 cytotoxicity induced by wild-type *B. pertussis* (Fig. 6) is dependent on T3SS activity
355 and is partially dependent on BteA.
356
357 **The secretion of type III secreted proteins in *B. pertussis* is downregulated**
358 **under reducing conditions**
359 Ascorbic acid is known as a reducing agent. To determine whether the secretion of
360 type III secreted proteins is downregulated under reducing conditions, we cultured
361 Tohama I in ascorbic acid-deprived SS medium (SS_AsA$^-$, Fig. 9, "none") and in
362 SS_AsA$^-$ medium containing the reducing agents ascorbic acid (AsA), 2-
363 mercaptoethanol (2-ME) or dithioerythritol (DTE), respectively. The prepared WCL
364 and Sup samples were analyzed by western blotting with anti-BteA, anti-BopB, or
365 anti-FHA antibodies (Fig. 9). The BteA and BopB signal intensities of both WCL and
366 Sup samples were decreased in the presence of each of the reducing agents (Fig. 9).
367 In contrast, the FHA signal intensities of both WCL and Sup samples were not
368 affected by the reducing agents. The growth of bacteria cultured in SS medium with
369 reducing agents was not significantly different from that of the bacteria cultured in SS
370 medium (data not shown). These results suggest that the production and secretion of
371 type III secreted proteins in *B. pertussis* are downregulated under reducing
372 conditions.
373
374 **DISCUSSION**
375 In this study, *B. pertussis* increased the production and secretion of type III secreted
376 proteins, BteA and BopB, when cultured in ascorbic acid-deprived SS medium
377 (SS_AsA$^-$). We successfully produced cytotoxicity against mammalian cells by the

378 infection of wild-type Tohama I cultured in SS 1/20AsA_1/2CaA medium.
379 *B. pertussis* decreased the production and secretion of type III secreted proteins
380 when cultured in SS_AsA⁻ medium containing reducing agents such as 2-ME or DTT.
381 These results suggest that redox signaling is involved in regulation of the T3SS in
382 *B. pertussis*.
383 It has been reported that no secretion of the type III secreted proteins BteA
384 and Bsp22 was detected from Tohama I (31), and that the secretion of the type III
385 secreted proteins BteA and BopD was detected from a non-vaccine-type strain,
386 BP157. However, BP157 showed no cytotoxicity against L2 cells, J774 mouse
387 macrophage-like cells, and HeLa cells (32). In another report, glutamate limitation
388 upregulates production of the type III secreted proteins (33). However, the
389 requirement of a type III secretion system (T3SS) for virulence in *B. pertussis* thus
390 remains unclear. On the other hand, a BscN-deficient mutant in which the T3SS was
391 inactive colonized mouse lungs to a significantly lower degree compared to the wild-
392 type (31). In addition, cytotoxicity against HeLa cells was observed by infection with
393 a BspR (a negative regulator for type III secreted proteins)-deficient *B. pertussis*
394 strain (15). These results suggest that the T3SS has a significant role in the
395 virulence of *B. pertussis*.
396 The production and the relative amounts of *bopB* mRNA of Tohama I cultured
397 in SS AsA⁻ medium were significantly increased compared to those of Tohama
398 cultured in SS medium, but this was not the case for BteA mRNA (Figs. 2, 5). It was
399 reported that *bteA* and *bopB* genes are upregulated by BtrS (a sigma factor for type
400 III secreted proteins) and downregulated by BspR (13, 15). It has also been reported
401 that the amount of *bteA* mRNA was increased 2-fold in a BspR-deficient strain

402 compared to the wild-type, whereas the amount of *bopB* mRNA was increased by
403 18-fold compared to the wild-type by RNA-seq (15). The *bopB* gene is localized in
404 the T3SS apparatus locus (*bsc* locus) adjacent to the *btr*S gene. Since the *bteA*
405 gene is separated from the *bsc* locus by >2.5 Mb (9), BtrS would preferentially
406 regulate transcription of the *bopB* gene over that of the *bteA* gene.
407 The cytotoxicity against L2 cells was observed by the infection of Tohama I
408 cultured in SS_1/20AsA _1/2CaA medium (Fig. 6). Before infection, we exchanged
409 Ham's F-12K medium for the SS_1/20AsA _1/2CaA medium. When L2 cells were
410 infected with Tohama I in Ham's F-12K medium, the cytotoxicity against L2 cells was
411 not observed (Fig. S1). In order to establish oxidizing conditions in the tissue culture
412 media, we used several oxidizing agents, i.e., hydrogen peroxide (H_2O_2), potassium
413 ferricyanide(III) ($K_3[Fe(CN)_6]$), methyl 3-nitro-2-pyridinesulfenate (Npys-OMe), and
414 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB). We also used another tissue culture
415 medium, i.e., Minimum Essential Medium Eagle (MEM), because MEM contains no
416 reducing agents, while F-12K, which is the medium for the L2 cells culture, contains
417 a reducing agent, i.e., cysteine. Even when L2 cells were infected in F-12K medium
418 containing the oxidizing agent or MEM, *B. pertussis* showed no cytotoxicity (Fig. S1).
419 We therefore speculate that a substance which inhibits the secretion of type III
420 secreted proteins from Tohama I was present in the Ham's F-12K medium, or a
421 substance that is necessary for the secretion of type III secreted proteins from
422 Tohama I was absent in the Ham's F-12K medium. We found that infection of the
423 human alveolar epithelium cell line A549 with wild-type *B. pertussis* cultured in
424 SS_1/20AsA _1/2CaA medium did not result in cytotoxicity (data not shown). For the
425 observation of cytotoxicity against A549 cells, it is necessary to optimize the

426 conditions for the production and secretion of type III secreted proteins in
427 *B. pertussis*.
428 A previous study showed that cytotoxicity against the cell line J774A.1 was
429 achieved by infection with wild-type *B. pertussis* 18323, but not by infection with a
430 CyaA-deficient strain (34). This finding suggested that CyaA has the potential to
431 provoke the cytotoxicity. However, we confirmed herein that the production and
432 secretion of CyaA in a BteA-deficient strain (MGKU3) were not significantly different
433 from those of the wild-type (data not shown). In our results, the amounts of LDH
434 released from L2 cells infected with MGKU3 were greater than those of the T3SS-
435 inactive strain (MGKU2), and no LDH release was detected from L2 cells infected
436 with MGKU2 (Fig. 8). These results raised the possibility that an unknown type III
437 effector other than BteA is involved in the cytotoxicity of *B. pertussis*.
438 Although we observed no significant difference in the growth rate of Tohama I
439 between SS medium and SS_AsA$^-$ medium (data not shown), the production and
440 secretion of type III secreted proteins of Tohama I cultured in the SS_AsA$^-$ medium
441 were increased compared to those in the SS medium (Fig. 2). It had not been
442 determined whether the expression of type III secreted proteins is regulated by
443 reducing agents at the molecular level, and in this study, the production and
444 secretion of CyaA and FHA in Tohama I cultured in SS medium were not significantly
445 different from those in SS_AsA$^-$ medium (Fig. 2). It was reported that there is no
446 difference in Prn production by Tohama I cultured in modified SS medium compared
447 to culture in modified SS medium containing dithiothreitol (DTT), a reducing agent
448 (35). These results suggest that BvgAS-regulated virulence factors other than type III
449 secreted proteins are not affected by reducing agents.

450 Ascorbic acid, urea, and glutathione (GSH) function as reducing agents in the
451 epithelial lining fluid (ELF) of the human airway. The concentrations of ascorbic acid
452 and urea in ELF in the upper respiratory tract are not significantly different from
453 those in the lower respiratory tract. However, the concentration of GSH in the ELF in
454 the upper respiratory tract is 100-fold lower than that in the lower respiratory tract.
455 One of the possibilities is that GSH forms GS-SG (an oxidized from of GSH) in the
456 ELF in the upper respiratory tract (36). It may thus be considered that the upper
457 respiratory tract is more oxidized than the lower respiratory tract. It was reported that
458 the transcription of genes encoding type III secreted proteins of *B. pertussis*
459 recovered from mouse nasal lavage was higher than that from bronchoalveolar
460 lavage (37). We thus speculate that the T3SS is important for *B. pertussis* to
461 colonize the upper respiratory tract of the host.
462 In this study, the production and secretion of BopB by Tohama I cultured in the
463 SS_AsA$^-$ medium were significantly increased compared to those in SS medium (Fig.
464 2). Bacteria use redox-sensing proteins to resist oxidative stress (38). For example,
465 OxyR (a member of the LysR family of transcriptional regulators) of *Escherichia coli*
466 is converted to an active mode by the oxidation of intramolecular cysteine residues,
467 which then increases the expression of antioxidants genes, e.g., hydroperoxidase I
468 [*katG*, (39)]. Vfr (a member of the CRP/FNR superfamily) of *Pseudomonas* forms an
469 active mode by retaining intramolecular cysteine residues as reducing states, and
470 then increases the expression of type III secreted proteins (40). Btr is a regulator
471 protein produced by *B. pertussis* and is homologous to Vfr (41). A cysteine cluster is
472 located in the N-terminal domain of Btr, and a helix-turn-helix DNA binding domain is
473 located in the C-terminal domain of Btr. Therefore, when *B. pertussis* is cultured in

474 reducing agent-deprived SS medium, Btr might be oxidized and change its
475 conformation, thereby increasing the expression of type III secreted proteins.
476 Although the signal pathway that *B. pertussis* uses to increase the production of type
477 III secreted proteins is unclear, it is possible that Btr regulates gene expressions for
478 type III secreted proteins.
479
480

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622

623 **Figure legends**

624 **Fig. 1.** BteA and BopB production by *B. pertussis* Tohama I cultured in LCA medium.
625 Tohama I and MGKU2 (a T3SS-inactive strain) were cultured in SS or LCA medium.
626 Whole cell lysates (WCL) and supernatant fraction (Sup) samples were separated by
627 SDS-PAGE and analyzed by western blotting with antibodies against BteA (*upper*
628 *panel*), BopB (*middle panel*) or BtrS (*lower panel*). BtrS, an RNA polymerase sigma
629 factor (15), was used as the unsecreted protein control. BteA forms SDS-resistant
630 multimers (10). NS: nonspecific signals. Loaded WCL and Sup samples were
631 prepared from an equal volume of bacterial culture. Experiments were performed at
632 least three times, and representative data are shown.

633

634 **Fig. 2.** Effects of ascorbic acid and casamino acids on Bvg-regulated virulence
635 factors. Tohama I was cultured in SS medium or ascorbic acid-deprived SS medium
636 (SS_AsA^{-}), casamino acid-deprived SS medium (SS_CaA^{-}) or both ascorbic acid-
637 and casamino acid-deprived SS medium (SS AsA^{-}_CaA^{-}). WCL and Sup samples
638 were separated by SDS-PAGE and analyzed by western blotting with antibodies

639 against BteA, BopB, FHA, or CyaA. NS: nonspecific signals. Loaded WCL and Sup
640 samples were prepared from an equal volume of bacterial culture. Experiments were
641 performed at least three times, and representative data are shown.
642
643 **Fig. 3.** Effects of ascorbic acid on type III secreted proteins. Whole cell lysates and
644 secreted proteins prepared from Tohama I cultured in SS medium in the presence of
645 ascorbic acid at the final concentration of 91, 454 or 2270 μM were separated by
646 SDS-PAGE and then analyzed by western blotting with antibodies against BteA or
647 BopB. NS: nonspecific signals. Loaded WCL and Sup samples were prepared from
648 an equal volume of bacterial culture. Experiments were performed at least three
649 times, and representative data are shown.
650
651 **Fig. 4.** Effects of ascorbic acid on the secretion of BteA and BopB in clinical isolates
652 cultured in SS_AsA$^-$ medium. The *B. pertussis* clinical isolates BP344, BP348,
653 BP350, and BP351 were cultured in SS (+) or SS_AsA$^-$ (–) medium. WCL and Sup
654 samples were separated by SDS-PAGE and analyzed by western blotting with
655 antibodies against BteA (*upper panel*) or BopB (*lower panel*). NS: nonspecific signals.
656 Loaded WCL and Sup samples were prepared from an equal volume of bacterial
657 culture. Experiments were performed at least three times, and representative data
658 are shown.
659
660 **Fig. 5.** Results of the RT-PCR analysis for mRNA levels of *bteA* and *bopB* from
661 *B. pertussis* cultured under ascorbic acid-starved conditions. Total RNA was
662 prepared from Tohama I or BP350 cultured in SS or SS_AsA$^-$ medium and

663 subjected to a quantitative RT-PCR analysis. The histograms show the relative
664 amounts of *bteA* (**A**) and *bopB* (**B**) mRNA normalized by the housekeeping gene,
665 *recA* mRNA. Error bars: the standard error of the mean (SEM) from triplicate
666 experiments. *$p<0.05$; N.S.: no significant difference. Experiments were performed at
667 least three times, and representative data are shown.
668
669 **Fig. 6.** Cytotoxic activity of *B. pertussis* cultured in SS_AsA$^-$ medium. L2 cells were
670 infected with Tohama I cultured in SS medium, SS_AsA$^-$ medium, SS medium
671 containing one-twentieth of ascorbic acid (SS_1/20AsA), SS medium containing one-
672 twentieth of ascorbic acid and one-half of casamino acids (SS_1/20AsA_1/2CaA), or
673 SS medium containing one-twentieth of ascorbic acid and no casamino acids
674 (SS_1/20AsA_CaA$^-$) at an MOI of 500 for 3 hr. +, 1/2 and 1/20 show the amounts of
675 indicated substances that are equal to, one-half of, and one-twentieth of those in SS
676 medium, respectively. The amounts of LDH released into the extracellular medium
677 from infected cells are shown, and the relative cytotoxicity (%) was determined as
678 described in the Materials and Methods section. Error bars: the SEM from triplicate
679 experiments. n.a.: No available result. Experiments were performed at least three
680 times, and representative data are shown.
681
682 **Fig. 7.** Immunofluorescent staining of Bsp22 on L2 cells infected with *B. pertussis*.
683 **A:** L2 cells were infected with Tohama I or BP350 in the indicated media at an MOI
684 of 125 for 3 hr. +, 1/2 and 1/20 indicate the amounts of indicated substances that are
685 equal to, one-half of, and one-twentieth of those in SS medium, respectively. After
686 fixation, cells were stained with anti-Bsp22 antibody (*green*) and rhodamine

687 phalloidin (*red*). *Arrowheads* indicate the Bsp22 (*green*) signal. **B:** Bsp22 signals per
688 one cell were counted under a fluorescent microscope. At least 120 cells were
689 randomly chosen. Error bars: the SEM from triplicate experiments. *$p<0.05$; N.S.: no
690 significant difference. Experiments were performed at least three times, and
691 representative data are shown.
692
693 **Fig. 8.** Cytotoxic activities of *B. pertussis* mutants. The results of LDH assays at an
694 MOI of 125 (**A**) or 250 (**B**) are shown as a histogram. Error bars: the SEM from
695 triplicate experiments. *$p<0.05$. Experiments were performed at least three times,
696 and representative data are shown. **C:** MGKU1 (Tohama I nalidixic acid-resistant
697 strain, 1: WT Nal^r), MGKU2 (T3SS-inactive strain, 2: ΔT3SS), MGKU3 (BteA-
698 deficient strain, 3: ΔBteA), or MGKU4 (BspR-deficient strain, 4: ΔBspR) was cultured
699 in SS_1/20AsA_1/2CaA medium. WCL and Sup samples were separated by SDS-
700 PAGE and analyzed by western blotting with anti-BteA antibody. We loaded a 3-fold
701 smaller amount of MGKU4 supernatant fraction on the SDS-PAGE gel to avoid
702 obtaining an excess signal intensity. NS: nonspecific signals. Loaded WCL and Sup
703 samples were prepared from an equal volume of bacterial culture. Experiments were
704 performed at least three times, and representative data are shown.
705
706 **Fig. 9.** Effects of reducing agents on the production and secretion of type III secreted
707 proteins in *B. pertussis*. Tohama I was cultured in SS_AsA^- medium or SS_AsA^-
708 medium containing ascorbic acid (AsA), 2-mercaptoethanol (2-ME), or dithioerythritol
709 (DTE) at final concentrations of 2.3, 2.1, or 3.0 mM, respectively. WCL and Sup
710 samples were separated by SDS-PAGE, then analyzed by western blotting with

711 antibodies against BteA, BopB, or FHA. Loaded WCL and Sup samples were

712 prepared from an equal volume of bacterial culture. NS: nonspecific signals.

713 Experiments were performed at least three times, and representative data are shown.

714

Table 1. Bacterial strains and plasmids used in study

Strains or plasmids	Relevant genotype/description	Reference
Strains		
B. pertussis		
Tohama I	Clinical isolate	22
BP344	Clinical isolate	23
BP348	Clinical isolate	23
BP350	Clinical isolate	23
BP351	Clinical isolate	23
MGKU1	Tohama I nalidixic-acid resistant strain	This study
MGKU2	T3SS-inactive strain	This study
MGKU3	BteA-deficient strain	This study
MGKU4	BspR-deficient strain	This study
E. coli		
DH10B	Host strain for pDONR201	invitrogen
Sm10λ*pir*	Host strain for pABB-CRS2	18
Plasmids		
pDONR201	DNA cloning vector, Km^r	invitrogen
pABB-CRS2	Suicide vector for conjugation, Amp^r	27
pABB-Δ*bscN*	pABB-CRS2, Δ*bscN* gene	18
pABB-Δ*bopC*	pABB-CRS2, Δ*bopC* (Δ*bteA*) gene	10
pMGKU401	pDONR201, *bspR* gene	this study
pMGKU402	pDONR201, Δ*bspR* gene	this study
pMGKU403	pABB-CRS2, Δ*bspR* gene	this study

Table 2. Medium compositions

Basal medium (g/L)	SS	LCA
Sodium Gultamate	10.7	10.7
L-proline	0.24	0.24
NaCl	2.5	2.5
KCl	0.2	0.2
KH_2PO_4	0.5	0.5
$MgCl_2 \cdot 6H_2O$	0.1	0.1
$CaCl_2$	0.02	0.02
Tris	6.1	6.1
Casamino acids	10	0.5
Dimethyl-β-cyclodextrin	1	1

Supplement (g/L)	SS	LCA
L-cysteine•HCl	4	4
$FeSO_4 \cdot 7H_2O$	1	1
L-ascorbic acid	40	2
Niacin	0.4	0.4
L-glutathione reduced	15	15

Table 3. Primers used in this study.

Name	Sequence
B1-bspR	5'-AAAAAGCAGGCTCGCCGGTTTCCACCACGTAA-3'
B2-bspR	5'-AGAAAGCTGGGTGGCCAATATCCTGTCTCAGC-3'
R1-bspR	5'-CGCGGATCCCATACGGCGCAAGAATGGCT-3'
R2-bspR	5'-CGCGGATCCAAAGACATCAAGCTCCAAAG-3'
5-recA	5'-ATGAAGATCGGCGTGATGT-3'
3-recA	5'-TAGAACTTGAGCGCGTTGC-3'
5-bteA	5'-AATGGCCTTGGTGGGAAC-3'
3-bteA	5'-ATTTCAGCGCCGTGATCTT-3'
5-bopB	5'-GTCTGCTGACAGCGTTGG-3'
3-bopB	5'-GGGGTTCGATCAGCAAGAC-3'

Restriction enzyme recognition sites were underlined.

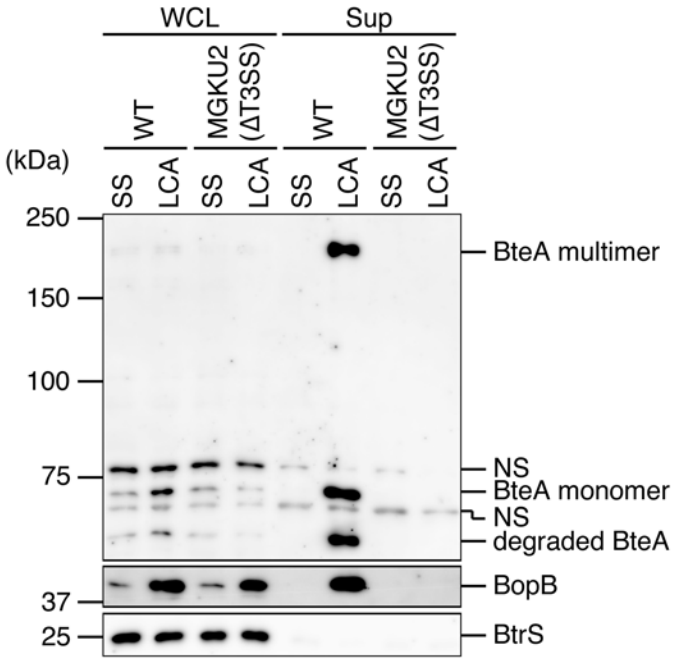


Fig. 1. BteA and BopB production by *B. pertussis* Tohama I cultured in LCA medium. Tohama I and MGKU2 (a T3SS-inactive strain) were cultured in SS or LCA medium. Whole cell lysates (WCL) and supernatant fraction (Sup) samples were separated by SDS-PAGE and analyzed by western blotting with antibodies against BteA (*upper panel*), BopB (*middle panel*) or BtrS (*lower panel*). BtrS, an RNA polymerase sigma factor (15), was used as the unsecreted protein control. BteA forms SDS-resistant multimers (10). NS: nonspecific signals Loaded WCL and Sup samples were prepared from an equal volume of bacterial culture. Experiments were performed at least three times, and representative data are shown.

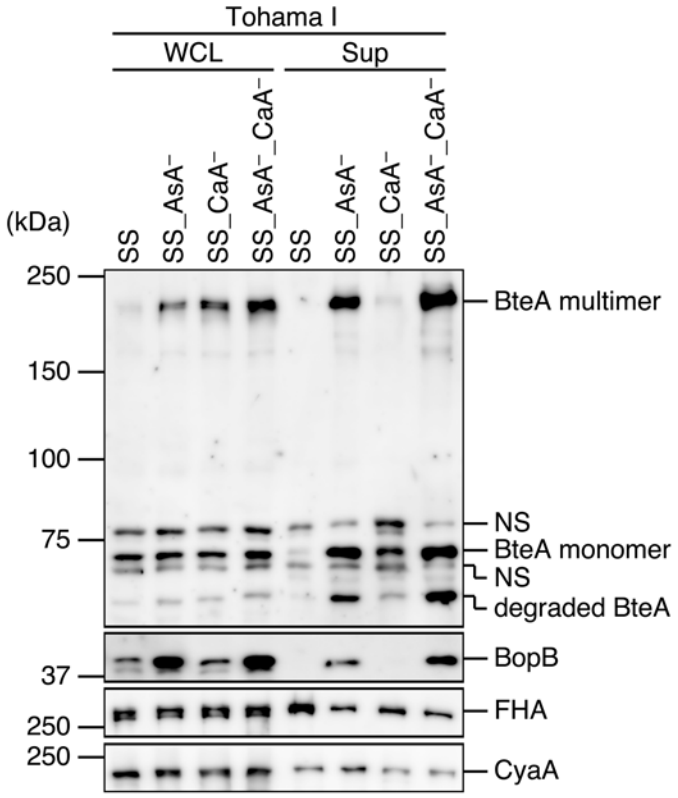


Fig. 2. Effects of ascorbic acid and casamino acids on Bvg-regulated virulence factors. Tohama I was cultured in SS medium or ascorbic acid-deprived SS medium (SS_AsA⁻), casamino acid-deprived SS medium (SS_CaA⁻) or both ascorbic acid- and casamino acid-deprived SS medium (SS AsA⁻_CaA⁻). WCL and Sup samples were separated by SDS-PAGE and analyzed by western blotting with antibodies against BteA, BopB, FHA, or CyaA. NS: nonspecific signals. Loaded WCL and Sup samples were prepared from an equal volume of bacterial culture. Experiments were performed at least three times, and representative data are shown.

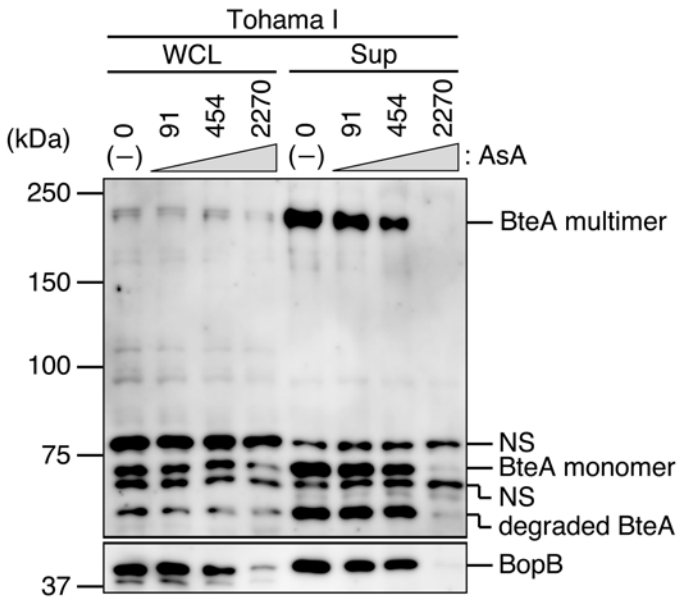


Fig. 3. Effects of ascorbic acid on type III secreted proteins. Whole cell lysates and secreted proteins prepared from Tohama I cultured in SS medium in the presence of ascorbic acid at the final concentration of 91, 454 or 2270 μM were separated by SDS-PAGE and then analyzed by western blotting with antibodies against BteA or BopB. NS: nonspecific signals. Loaded WCL and Sup samples were prepared from an equal volume of bacterial culture. Experiments were performed at least three times, and representative data are shown.

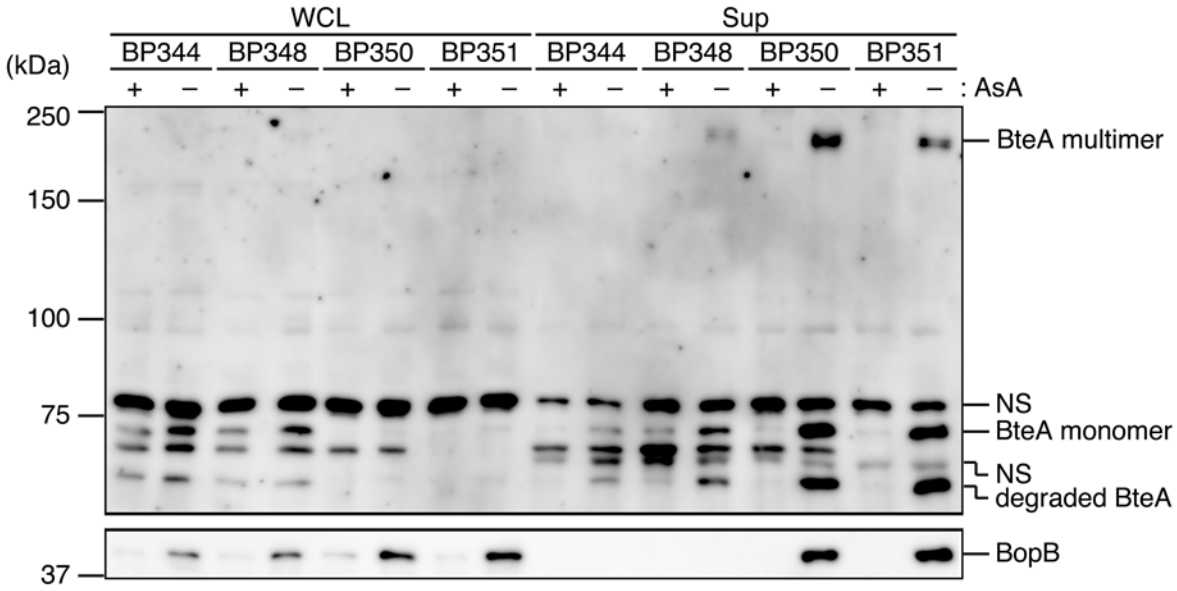


Fig. 4. Effects of ascorbic acid on the secretion of BteA and BopB in clinical isolates cultured in SS_AsA^{-} medium. The *B. pertussis* clinical isolates BP344, BP348, BP350, and BP351 were cultured in SS (+) or SS_AsA^{-} (–) medium. WCL and Sup samples were separated by SDS-PAGE and analyzed by western blotting with antibodies against BteA (*upper panel*) or BopB (*lower panel*). NS: nonspecific signals. Loaded WCL and Sup samples were prepared from an equal volume of bacterial culture. Experiments were performed at least three times, and representative data are shown.

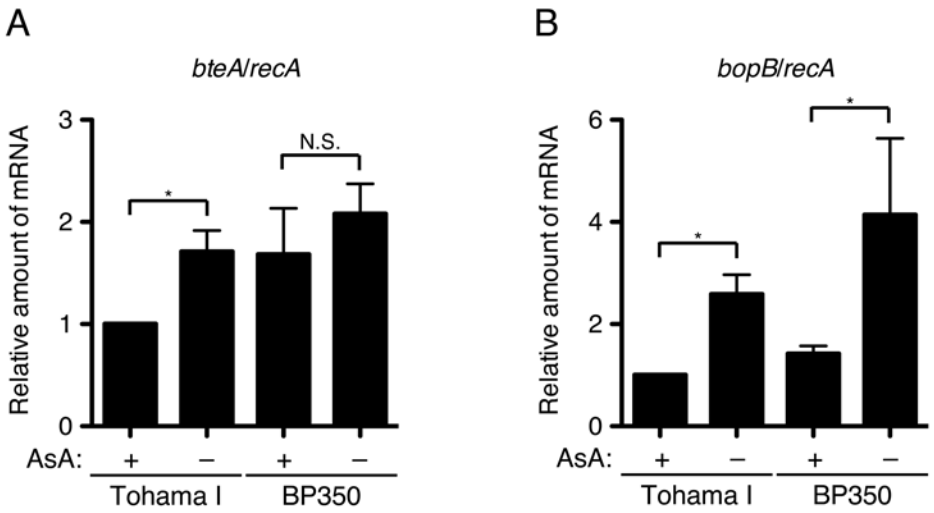


Fig. 5. Results of the RT-PCR analysis for mRNA levels of *bteA* and *bopB* from *B. pertussis* cultured under ascorbic acid-starved conditions. Total RNA was prepared from Tohama I or BP350 cultured in SS or SS_AsA^{-} medium and subjected to a quantitative RT-PCR analysis. The histograms show the relative amounts of *bteA* (**A**) and *bopB* (**B**) mRNA normalized by the housekeeping gene, *recA* mRNA. Error bars: the standard error of the mean (SEM) from triplicate experiments. *$p<0.05$; N.S.: no significant difference. Experiments were performed at least three times, and representative data are shown.

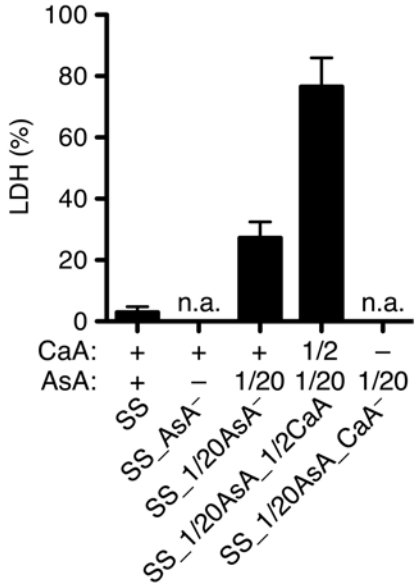


Fig. 6. Cytotoxic activity of *B. pertussis* cultured in SS_AsA^{-} medium. L2 cells were infected with Tohama I cultured in SS medium, SS_AsA^{-} medium, SS medium containing one-twentieth of ascorbic acid (SS_1/20AsA), SS medium containing one-twentieth of ascorbic acid and one-half of casamino acids (SS_1/20AsA_1/2CaA), or SS medium containing one-twentieth of ascorbic acid and no casamino acids (SS_1/20AsA_CaA^{-}) at an MOI of 500 for 3 hr. +, 1/2 and 1/20 show the amounts of indicated substances that are equal to, one-half of, and one-twentieth of those in SS medium, respectively. The amounts of LDH released into the extracellular medium from infected cells are shown, and the relative cytotoxicity (%) was determined as described in the Materials and Methods section. Error bars: the SEM from triplicate experiments. n.a.: No available result. Experiments were performed at least three times, and representative data are shown.

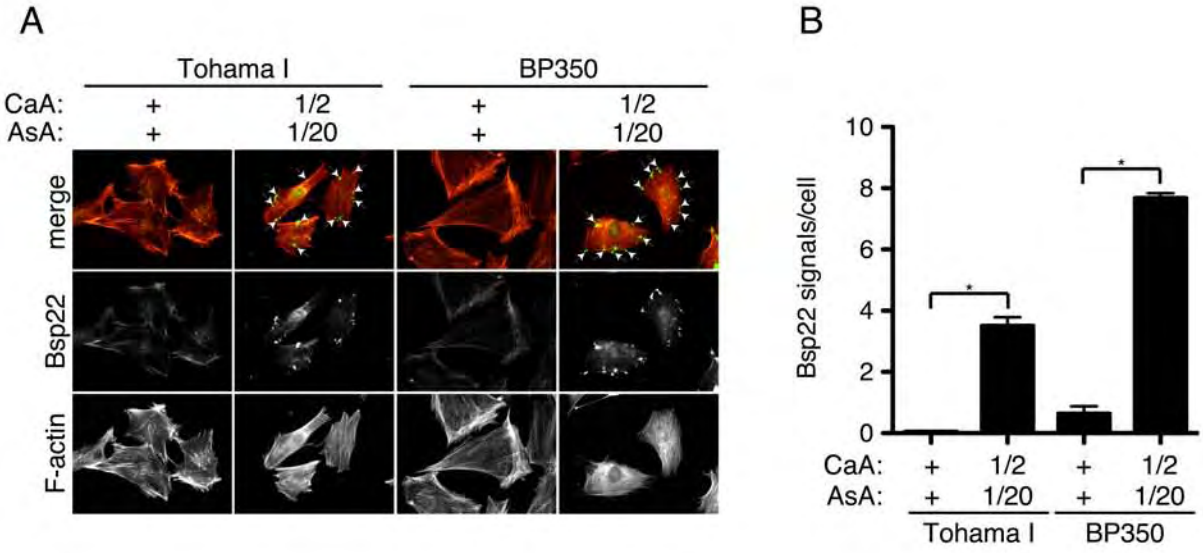
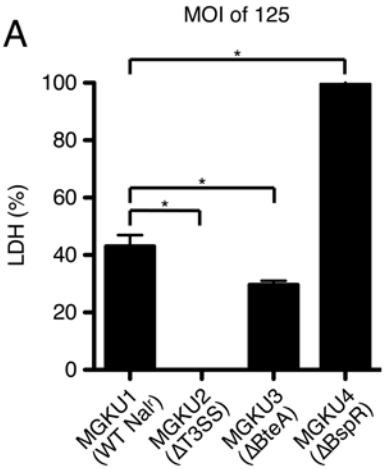
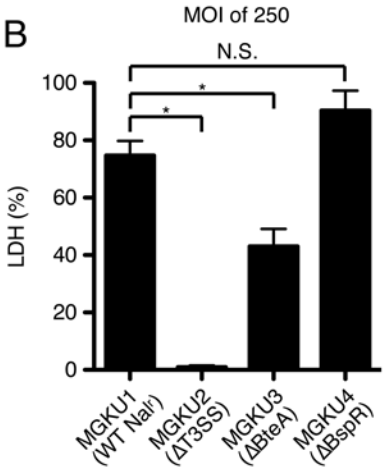


Fig. 7. Immunofluorescent staining of Bsp22 on L2 cells infected with *B. pertussis*. **A:** L2 cells were infected with Tohama I or BP350 in the indicated media at an MOI of 125 for 3 hr. +, 1/2 and 1/20 indicate the amounts of indicated substances that are equal to, one-half of, and one-twentieth of those in SS medium, respectively. After fixation, cells were stained with anti-Bsp22 antibody (*green*) and rhodamine phalloidin (*red*). *Arrowheads* indicate the Bsp22 (*green*) signal. **B:** Bsp22 signals per one cell were counted under a fluorescent microscope. At least 120 cells were randomly chosen. Error bars: the SEM from triplicate experiments. *$p<0.05$; N.S.: no significant difference. Experiments were performed at least three times, and representative data are shown.





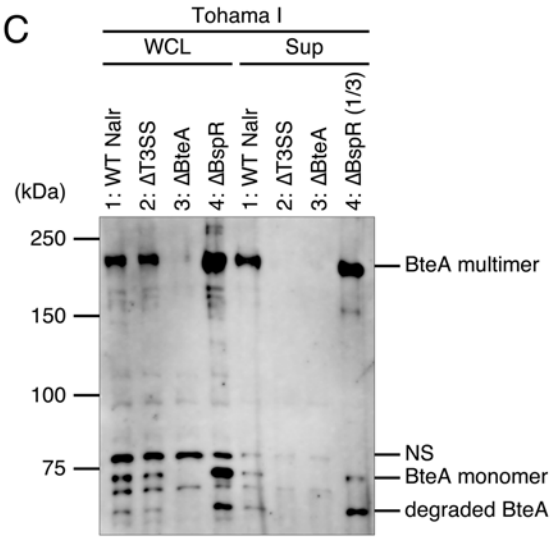


Fig. 8. Cytotoxic activities of *B. pertussis* mutants. The results of LDH assays at an MOI of 125 (**A**) or 250 (**B**) are shown as a histogram. Error bars: the SEM from triplicate experiments *$p<0.05$. Experiments were performed at least three times, and representative data are shown. **C:** MGKU1 (Tohama I nalidixic acid-resistant strain, 1: WT Nal^r), MGKU2 (T3SS-inactive strain, 2: ΔT3SS), MGKU3 (BteA-deficient strain, 3: ΔBteA), or MGKU4 (BspR-deficient strain, 4: ΔBspR) was cultured in SS_1/20AsA_1/2CaA medium. WCL and Sup samples were separated by SDS-PAGE and analyzed by western blotting with anti-BteA antibody. We loaded a 3-fold smaller amount of MGKU4 supernatant fraction on the SDS-PAGE gel to avoid obtaining an excess signal intensity. NS: nonspecific signals. Loaded WCL and Sup samples were prepared from an equal volume of bacterial culture. Experiments were performed at least three times, and representative data are shown.

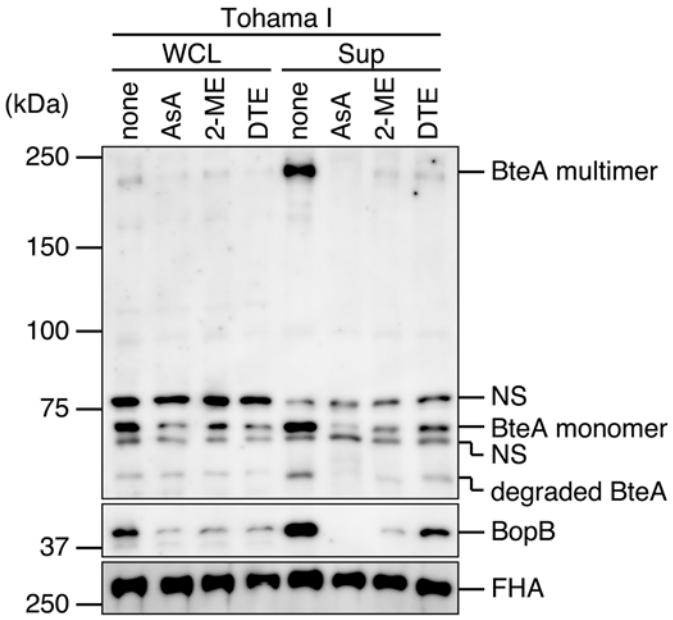


Fig. 9. Effects of reducing agents on the production and secretion of type III secreted proteins in *B. pertussis*. Tohama I was cultured in SS_AsA^{-} medium or SS_AsA^{-} medium containing ascorbic acid (AsA), 2-mercaptoethanol (2-ME), or dithioerythritol (DTE) at final concentrations of 2.3, 2.1, or 3.0 mM, respectively. WCL and Sup samples were separated by SDS-PAGE, then analyzed by western-blotting with antibodies against BteA, BopB, or FHA. Loaded WCL and Sup samples were prepared from an equal volume of bacterial culture. NS: nonspecific signals. Experiments were performed at least three times, and representative data are shown.

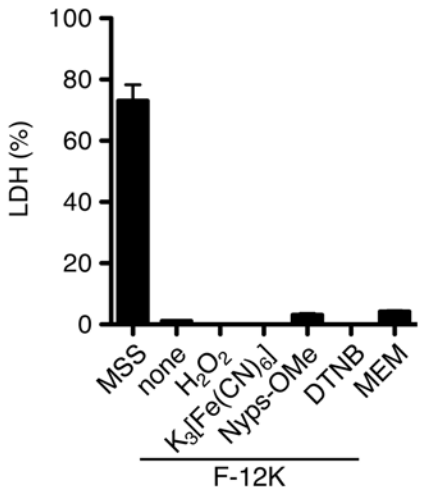


Fig. S1. Cytotoxic activity of *B. pertussis* Tohama I in F-12K medium containing oxidizing agents. L2 cells were infected with *B. pertussis* Tohama I in the modified SS medium (SS_1/20AsA_1/2CaA, MSS). The amounts of ascorbic acid and casamino acids in MSS are one-twentieth of, and one-half of those in SS medium, respectively. L2 cells were also infected in F-12K medium (none), F-12K medium containing hydrogen peroxide (H_2O_2), potassium ferricyanide(III) ($K_3[Fe(CN)_6]$), methyl 3-nitro-2-pyridinesulfenate (Npys-OMe) or 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB). The final concentrations of each oxidizing agent were 1 mM, 1 mM, 10 μM, or 2 mM, respectively. Minimum Essential Medium Eagle (MEM) was also used. The cells were infected at an MOI of 500 for 3 hr. The amounts of LDH released into the extracellular medium from infected cells are shown, and the relative cytotoxicity (%) was determined as described in the Materials and Methods section. Error bars are the SEM from triplicate experiments. Experiments were performed at least three times, and representative data are shown.