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

Masataka Goto[a], Tomoko Hanawa[b], Akio Abe[a] and Asaomi Kuwae[a#]

[a]Laboratory of Bacterial Infection, Graduate School of Infection Control Sciences, Kitasato University, Tokyo, Japan

[b]Department of Infectious Diseases, Kyorin University School of Medicine, Tokyo, Japan

Running Head: Reducing Conditions Downregulate *Bordetella* T3SS.

[#]**Address Correspondence to:** Asaomi Kuwae, kuwae@lisci.kitasato-u.ac.jp

ABSTRACT

Bordetella pertussis uses a type III secretion system (T3SS) to inject virulence proteins into host cells. Although the *B. pertussis* T3SS would play a role in host colonization, the efficient secretion of type III secreted proteins from *B. pertussis* has not been observed. To investigate the roles of type III secreted proteins during infection, we attempted to optimize culture conditions for the production and secretion of a type III secreted protein, BteA, in *B. pertussis*. We observed that *B. pertussis* efficiently secretes BteA in ascorbic acid-depleted (AsA^-) medium. When L2 cells, a rat lung epithelial cell line, were infected with *B. pertussis* cultured in the AsA^- medium, BteA-dependent cytotoxicity was observed. We also performed an immunofluorescence assay of L2 cells infected with *B. pertussis*. The clear fluorescence signals of Bsp22, a needle structure of T3SS, were detected on the bacterial surface of *B. pertussis* cultured in the AsA^- medium. Moreover, when *B. pertussis* was cultured in liquid medium containing other reducing agents such as 2-mercaptoethanol and dithioerythritol, the production of type III secreted proteins was repressed. These results suggest that in *B. pertussis*, the production and secretion of type III secreted proteins are downregulated under reducing conditions.

IMPORTANCE

The type III secretion system (T3SS) of *Bordetella pertussis* forms a needle-like structure that protrudes from the bacterial cell surface. *B. pertussis* uses T3SS to translocate virulence proteins called effectors into host cells. The culture conditions for effector production in *B. pertussis* have not been investigated. We attempted to optimize culture medium compositions for producing and secreting type III secreted proteins. We found that *B. pertussis* secretes type III secreted proteins in reducing agent-deprived liquid medium, and that BteA-secreting *B. pertussis* provokes cytotoxicity against cultured mammalian cells. These results suggest that redox signaling is involved in the regulation of *B. pertussis* T3SS.

47

48 **Introduction**

49 The genus *Bordetella* is made up of Gram-negative bacteria and has been subdivided into
50 16 subspecies to date (1). *Bordetella pertussis* is a causative agent of whooping cough, also
51 known as pertussis. *B. pertussis* strictly adapts to humans, and it lacks an environmental
52 reservoir (2). A variety of virulence factors have been identified in *Bordetella*, e.g.,
53 filamentous hemagglutinin (3), adenyl cyclase toxin (4) and pertactin (5). These virulence
54 factors are regulated by a *Bordetella* two-component regulatory system, BvgAS. In addition
55 to these virulence factors, the type III secretion system (T3SS) of *B. pertussis* is also
56 regulated by BvgAS (6, 7).

57 A T3SS that forms a needle-like structure is conserved among many Gram-negative bacteria.
58 In order to subvert hosts, bacteria use a T3SS to inject type III secreted proteins into host
59 cells (8). Such type III secreted proteins are called effectors. Three types of effectors have
60 been identified in *Bordetella*: BteA ((9) [also referred to as BopC (10)], BopN (11, 12), and
61 BspR (13) [also referred to as BtrA (14, 15)]. BteA is 658 amino acids in length and is
62 localized to lipid rafts in host cells via its N-terminal region (16). BteA induces necrotic cell
63 death in several types of cultured mammalian cells (9, 10), and BteA translocation into host
64 cells is promoted by the functioning of BopN (11). BspR is a negative regulator for type III
65 secreted proteins (13) and is translocated into nuclei of cultured mammalian cells (17). BspR
66 has been suggested to be an anti-sigma factor against BtrS that functions as a positive
67 regulator for type III secreted proteins (15). Some type III secreted proteins are called
68 translocators rather than effectors. Three types of translocators have been identified in
69 *Bordetella*, i.e., BopB (18), BopD (19) and Bsp22 (20), and they function as the path for
70 effectors delivered into host cells. The complex of BopB and BopD forms the pores on the
71 host membrane (19), and Bsp22 forms a filamentous-like structure and is associated with
72 the T3SS needle and with a pore-forming component, BopD (21).

73 The optimal culture conditions for the production and secretion of BteA have not been
74 established. To investigate the roles of BteA and other type III secreted proteins during
75 *B. pertussis* infection, we investigated culture conditions for the maximal production and
76 secretion of type III secreted proteins in *B. pertussis*.
77
78 **MATERIALS AND METHODS**
79 **Bacterial strains**
80 The strains used in this study are listed in Table 1. Tohama I (22) was used as the wild-type
81 strain. BP344, BP348, BP350, and BP351 were used as clinical isolates and were a kind gift
82 from Dr. K. Kamachi (National Institue of Infectious Diseases) (23). *Escherichia coli* DH10B
83 and Sm10λ*pir* were used for DNA cloning and conjugation, respectively (Table 1).
84 *B. pertussis* was grown on Bordet-Gengou agar plates at 37ºC for 5 days. Fresh colonies of
85 *B. pertussis* on each Bordet-Gengou agar plate were suspended in Stainer-Scholt (SS)
86 medium [Table 2, (24)]. In this study, we used a liquid medium described as a cyclodextrin-
87 containing medium as the SS medium (25). Low casamino acids (LCA) medium was
88 prepared by removing the agar from cyclodextrin solid medium (CSM) contents [Table 2,
89 (26)]. *B. pertussis* was cultured in SS medium with a starting A_{600} of 0.23 under static
90 conditions at 37ºC. The liquid cultivation period was 22 hr for the protein preparation, mRNA
91 preparation, and infection.
92
93 **Generation of a Tohama I nalidixic acid-resistant mutant**
94 For the generation of a Tohama I nalidixic acid-resistant mutant, Tohama I cultured in SS
95 medium for 24 hr as described above was spread on 20 sets of Bordet-Gengou agar plates
96 containing nalidixic acid at the final concentration of 30 μg/ml. After 5 days, surviving
97 colonies were designated as MGKU1. We checked whether the MGKU1 cells showed
98 hemolysis on a Bordet-Gengou agar plate, and we observed that the amounts of the

99 production and secretion of type III secreted proteins in MGKU1 were not significantly
100 different from those of Tohama I wild-type (data not shown).
101
102 **Cell culture**
103 L2 cells, a rat lung epithelial cell line (ACTT CCL-149), were maintained in F-12K (Invitrogen,
104 Tokyo). The cell culture medium was supplemented with 10% fetal calf serum (FCS). L2
105 cells were grown at 37ºC under a 5% CO_2 atmosphere.
106
107 **Construction of gene-disrupted mutants**
108 The primers used in this study are listed in Table 3. To construct the *bspR* mutant, the 4.0 kb
109 DNA fragment encoding *bspR* and its flanking regions was amplified by polymerase chain
110 reaction (PCR) with primers B1-bspR and B2-bspR using *B. pertussis* Tohama I genomic
111 DNA as the template. The resulting PCR product was cloned into pDONR201 to obtain
112 pMGKU401, by means of adapter PCR and site-specific recombination techniques using a
113 Gateway cloning system (Invitrogen). An inverse PCR was carried out with the set of primers
114 R1-bspR and R2-bspR using circular pMGKU401 as the template.
115 The resulting PCR product was digested with BamHI and self-ligated to obtain pMGKU402.
116 This plasmid contained the BamHI site in addition to a 474-bp in-frame deletion from 175 bp
117 downstream of the 5' end of the *bspR* gene to 52 bp upstream of the 3' end of the gene. This
118 plasmid, pMGKU402, was mixed with a positive suicide vector, pABB-CRS2 (27), to obtain
119 pMGKU403 using the Gateway cloning system. This plasmid, pMGKU403, was introduced
120 into *E. coli* Sm10λ*pir* (18) and transconjugated into MGKU1 as described previously (28).
121 The resulting mutant strain was designated as MGKU4 (BspR-deficient strain). Similarly,
122 pABB-Δ*bscN* (18) and pABB-*ΔbopC* (10) were separately introduced into *E. coli* Sm10λ*pir*
123 and were transconjugated into MGKU1. The resulting mutant strains were designated as
124 MGKU2 (a T3SS-inactive strain) and MGKU3 (a BteA-deficient strain), respectively.

125

126 **Preparation of proteins from culture supernatants and whole bacterial cell lysates**

127 Secreted proteins released into bacterial culture supernatants and whole bacterial cell
128 lysates were prepared by trichloroacetic acid precipitation. The culture supernatants were
129 filtered, and bacterial pellets were resuspended in distilled water. Trichloroacetic acid was
130 then added to each sample at the final concentration of 10%. The samples were incubated
131 on ice for 15 min, then centrifuged for 5 min. The resulting precipitated proteins were
132 neutralized by 2 M Tris-base and dissolved in the sodium dodecyl sulfate-polyacrylamide gel
133 electrophoresis (SDS-PAGE) sample buffer. The protein samples were separated by SDS-
134 PAGE and analyzed by western blotting.

135

136 **Antibodies**

137 Anti-BteA, anti-BopB, and anti-Bsp22 antibodies were purified from rabbit serum in our
138 previous study (10, 18, 29). To detect filamentous hemagglutinin (FHA) signals, we used
139 mouse anti-FHA serum (30). Mouse anti-CyaA monoclonal antibody was purchased from
140 Santa Cruz Biotechnology (Santa Cruz, CA).

141

142 **Quantitative reverse transcription-PCR (qRT-PCR)**

143 The amounts of mRNA were measured by qRT-PCR. Bacterial total RNA was prepared
144 using a Trizol Max Bacterial RNA Isolation Kit (Invitrogen), an RNeasy Mini Kit (Qiagen,
145 Hilden, Germany) and an RNase-free DNase-free Kit (Qiagen). The RNA sample was
146 reverse transcribed with Transcriptor Universal cDNA Master (Roche Diagnostics,
147 Indianapolis, IN) and a T100 Thermal Cycler (Bio-Rad, Hercules, CA). The resulting cDNA
148 was amplified by FastStart Essential DNA Probes Master (Roche) using the following primer
149 pairs: 5-recA and 3-recA for *recA*; 5-bteA and 3-bteA for *bteA*; 5-bopB and 3-bopB for *bopB*
150 (Table 3). The results were calculated as described in the Roche manual. The amount of

151 *recA* mRNA was used as an internal control. The mRNA amounts are presented herein as
152 relative to the amounts in the Tohama I cultured in SS medium, which was defined as 1.
153
154 **LDH Assays**
155 To examine the release of lactate dehydrogenase (LDH) from *B. pertussis*-infected cells,
156 5.0×10^4 cells/well of L2 cells seeded in 24-well plates were infected with bacteria at the
157 multiplicity of infection (MOI) of 125, 250, or 500. The plates were centrifuged at 900 *g* for 5
158 min and incubated for 3 hr at 37ºC under a 5% CO_2 atmosphere. The amounts of LDH were
159 measured spectrophotometrically using a Cyto-Tox 96 Non-radioactive Cytotoxicity Assay
160 Kit (Promega, Madison, WI). The LDH (%) level is shown as a ratio, with the value obtained
161 from the well treated with 0.1% TritonX-100 set as 100%.
162
163 **Immunofluorescent staining**
164 For the immunofluorescent staining assay, 2.5×10^5 cells/well of L2 cells were seeded on
165 coverslips in six-well plates and incubated overnight, and then infected with *B. pertussis* at
166 an MOI of 125. The plates were centrifuged at 900 *g* for 5 min and incubated for 3 hr at 37ºC
167 under a 5% CO_2 atmosphere. The infected L2 cells were then immunostained as described
168 previously (12). Briefly, the cells were treated with 4% paraformaldehyde, 50 mM NH_4Cl,
169 0.2% Triton-X, and 4% bovine serum albumin (BSA). After blocking, the cells were stained
170 by anti-Bsp22 antibody (29). As a secondary antibody, Alexa Fluor 488 goat anti-rabbit IgG
171 (Invitrogen) was used. F-actin was stained with Rhodamine Phalloidin (Molecular Probes,
172 Eugene, OR). Bsp22 signals per one cell were counted under a fluorescence microscope.
173
174 **Statistical analyses**

175 The statistical analyses were performed using the nonparametric unpaired *t*-test with a one-
176 tailed p-value with Prism ver. 5.0f software (Graphpad, La Jolla, CA). Values of $p<0.05$ were
177 considered significant.
178
179 **RESULTS**
180 **Type III secreted proteins are secreted from *B. pertussis* cultured in LCA medium**
181 Cyclodextrin solid medium (CSM) is reported as a synthetic medium for clinical isolation of
182 *B. pertussis* (26). We sought to determine whether *B. pertussis* grown on this agar secretes
183 type III effectors, and we prepared a liquid medium, designated "low casamino acids (LCA)"
184 medium, by removing the agar from CSM contents (Table 2). *B. pertussis* was cultured in SS
185 or LCA medium, and then the whole cell lysates (WCL) and supernatant fractions (Sup)
186 samples were subjected to western blotting with antibodies against BteA (an effector, a type
187 III secreted protein) and BopB (a translocator, a type III secreted protein).
188 BteA and BopB signals were detected in WCL and Sup samples of LCA medium (Fig.
189 1). As reported previously (10), BteA forms SDS-resistant multimers, and the signals were
190 also detected around 200 kDa. In contrast, the BteA signal was faintly detected in the WCL
191 samples of SS medium, and no BteA signal was detected in the Sup samples of SS medium
192 (Fig. 1). BopB signals were not detected in WCL or Sup samples of SS medium (Fig. 1).
193 These results suggest that *B. pertussis* secretes type III secreted proteins in LCA medium.
194
195 **Ascorbic acid downregulates the production and secretion of type III secreted**
196 **proteins in *B. pertussis***
197 Although the ingredients of the LCA and SS media are the same, the amounts of ascorbic
198 acid and casamino acids in LCA medium are lower than those in SS medium (Table 2). To
199 determine which substance is involved in the production and secretion of type III secreted
200 protein, we cultured *B. pertussis* in SS medium, ascorbic acid-deprived SS medium

201 (SS_AsA$^-$), casamino acid-deprived SS medium (SS_CaA$^-$), or both ascorbic acid- and
202 casamino acid-deprived medium (SS_AsA$^-$_CaA$^-$). WCL and Sup samples were subjected
203 to western blotting with antibodies against BteA and BopB (Fig. 2).
204 The BteA multimer signal intensities in the WCL samples of the SS_AsA$^-$, SS_CaA$^-$ and
205 SS_AsA$^-$_CaA$^-$ media were stronger than that in the SS medium (Fig. 2). A faint BteA signal
206 intensity in the Sup samples of SS was detected (Fig. 2). The BteA signal intensities in the
207 Sup samples were evident in the SS_AsA$^-$ and SS_AsA$^-$_CaA$^-$ media (Fig. 2). Again, BopB
208 signal intensities in both WCL and Sup samples were evident in the SS_AsA$^-$ and
209 SS_AsA$^-$_CaA$^-$ media (Fig. 2).
210 We also investigated the potential involvement of ascorbic acid or casamino acids in
211 Bvg-regulated virulence factors, e.g., FHA and CyaA. WCL and Sup samples were subjected
212 to western blotting with antibodies against FHA and CyaA (Fig. 2). The results revealed that
213 there were no significant differences in the FHA or CyaA signal intensities in the WCL and
214 Sup samples among the media used here (Fig. 2). Collectively, these results suggest that
215 ascorbic acid has a specific influence on the production and secretion of BteA and BopB in
216 *B. pertussis*.
217 Standard SS medium contains ascorbic acid at a final concentration of 2270 μM. To further
218 explore whether ascorbic acid affects the production and secretion of type III secreted
219 proteins, we cultured *B. pertussis* in SS medium in the presence of ascorbic acid at the final
220 concentrations of 91, 454, or 2270 μM. WCL and Sup samples were prepared from each
221 bacterial culture and analyzed by western blotting (Fig. 3). The BteA signal was detected in
222 both WCL and Sup samples of SS medium at 0–454 μM ascorbic acid, but the BteA signal of
223 the SS medium at 2270 μM ascorbic acid was faint or absent (Fig. 3). The BopB signals
224 showed a similar pattern (Fig. 3). These results indicate that *B. pertussis* produces and
225 secretes type III secreted proteins under low ascorbic acid concentrations.

226

227 **BteA and BopB are secreted from other *B. pertussis* clinical isolates cultured in**
228 **SS_AsA⁻ medium**
229 To determine whether *B. pertussis* clinical isolates secrete type III secreted proteins under
230 low ascorbic acid concentrations, we cultured four strains of *B. pertussis* (Table 1) in SS
231 medium or ascorbic acid-deprived medium (SS_AsA⁻). The prepared WCL and Sup samples
232 were analyzed by western blotting (Fig. 4). BteA signals in the Sup samples of BP350 and
233 BP351 were evident in the SS_AsA⁻ medium (Fig. 4, AsA⁻). Again, BopB signals in the Sup
234 samples of BP350 and BP351 were evident in SS_AsA⁻ medium. These results suggest that
235 some clinical strains, e.g., BP350 and BP351, also secrete type III secreted proteins under
236 low ascorbic acid concentrations.
237
238 **Translocator BopB is upregulated at the transcriptional level in *B. pertussis* cultured**
239 **in SS_AsA⁻ medium**
240 To investigate the gene expressions of type III secreted proteins under ascorbic acid-starved
241 conditions, we prepared total RNA from *B. pertussis* cultured in SS or SS_AsA⁻ medium. The
242 cDNA samples reverse-transcribed from the total RNA samples were subjected to a
243 quantitative RT-PCR analysis to quantify the relative amounts of *bteA* and *bopB* mRNA. The
244 results demonstrated that the relative amount of *bteA* mRNA of Tohama I in SS_AsA⁻
245 medium was increased compared to that in SS medium (Fig. 5A). However, the relative
246 amount of *bteA* mRNA of BP350 in the SS_AsA⁻ medium was not significantly different from
247 that in the SS medium (Fig. 5A). The relative amounts of *bopB* mRNA of Tohama I and
248 BP350 in SS_AsA⁻ medium were increased compared to those in SS medium (Fig. 5B).
249 These results suggest that BopB is upregulated at the transcriptional level in Tohama
250 I and BP350 under low ascorbic acid concentrations. BteA is also upregulated at the
251 transcriptional level in Tohama I under low ascorbic acid concentrations but not in BP350.
252

253 ***B. pertussis* induces cytotoxicity against cultured mammalian cells**

254 A *B. pertussis* BspR-deficient strain was reported to induce cytotoxicity against cultured
255 mammalian cells (15). In the present study, we identified better culture conditions for the
256 production and secretion of BteA in *B. pertussis* than those provided with standard SS
257 medium. We thus investigated whether wild-type *B. pertussis* provokes cytotoxicity against
258 cultured mammalian cells under these conditions. L2 cells, a rat lung epithelial cell line, were
259 infected in SS medium comprised of one-twentieth of ascorbic acid (SS_1/20AsA), SS
260 medium comprised of one-twentieth of ascorbic acid and one-half of casamino acids
261 (SS_1/20AsA_1/2CaA), or SS medium comprised of one-twentieth of ascorbic acid and no
262 casamino acids (SS_1/20AsA_CaA$^-$) at an MOI of 500 for 3 hr. The amounts of lactate
263 dehydrogenase (LDH) released into the extracellular medium were then measured.
264 The results revealed that released LDH was detected from L2 cells infected with Tohama I in
265 the SS_1/20AsA or SS_1/20AsA_1/2CaA medium (Fig. 6). The L2 cells incubated in the
266 SS_AsA$^-$ or SS_1/20AsA_CaA$^-$ medium were injured by the medium itself. Therefore, these
267 media were not useful for this assay. Taken together, these results demonstrate that we
268 successfully observed cytotoxicity of mammalian cells induced by wild-type *B. pertussis*.
269

270 **The T3SS of *B. pertussis* cultured in SS_AsA$^-$ medium is active during infection**
271 **against cultured mammalian cells**

272 As described above, we observed cytotoxicity of *B. pertussis* against cultured cells. In order
273 to confirm that *B. pertussis* indeed secretes type III secreted proteins during infection, we
274 infected L2 cells with *B. pertussis* in SS or SS_1/20AsA_1/2CaA medium at an MOI of 125
275 for 3 hr. After infection, Bsp22 (a translocator, and a component of the filamentous-like
276 structure) and F-actin were stained with anti-Bsp22 and rhodamine phalloidin, respectively.
277 The results showed that the amounts of Bsp22 signals (Fig. 7A, green) on L2 cells infected
278 with Tohama I and BP350 cultured in SS_1/20AsA_1/2CaA medium were greater than those

279 in SS medium (Fig. 7). Thus, we demonstrated that *B. pertussis* T3SS is activated during the
280 infection of cultured mammalian cells under appropriate conditions.
281
282 **The cytotoxicity induced by wild-type *B. pertussis* depends on the T3SS**
283 It has been reported that *B. bronchiseptica* induces BteA-dependent necrotic cell death
284 against cultured mammalian cells (10). To investigate whether the cytotoxicity provoked by
285 wild-type *B. pertussis* also depends on BteA function, we infected L2 cells with MGKU1 (a
286 Tohama I nalidixic acid-resistant strain), MGKU2 (a T3SS-inactive strain), MGKU3 (a BteA-
287 deficient strain), or MGKU4 (a BspR-deficient strain that allows the constitutive activation of
288 T3SS) in SS_1/20AsA_1/2CaA medium at an MOI of 125 or 250 for 3 hr. The amounts of
289 LDH released into extracellular medium were measured.
290 As a result, LDH was not detected in the medium of L2 cells infected with MGKU2 (Fig.
291 8A,B). The amount of LDH detected in the medium of L2 cells infected with MGKU3 was
292 significantly lower than that in the medium of L2 cells infected with MGKU1 (Fig. 8A,B). The
293 amount of LDH detected in the medium of L2 cells infected with MGKU4 was significantly
294 greater than that in the medium of L2 cells infected with MGKU1 at an MOI of 125 (Fig. 8A),
295 but not at an MOI of 250 (Fig. 8B).
296 WCL and Sup samples were prepared from each strain and separated by SDS-PAGE, then
297 analyzed by western blotting with anti-BteA antibody (Fig. 8C). BteA signals of the WCL
298 samples were detected in MGKU1, MGKU2, and MGKU4, but not in MGKU3 (Fig. 8C). BteA
299 signals of the Sup samples were detected in MGKU1 and MGKU4, but not in MGKU2 or
300 MGKU3 (Fig. 8C). These results suggest that the cytotoxicity induced by wild-type
301 *B. pertussis* (Fig. 6) is dependent on T3SS activity and is partially dependent on BteA.
302
303 **The secretion of type III secreted proteins in *B. pertussis* is downregulated under**
304 **reducing conditions**

305 Ascorbic acid is known as a reducing agent. To determine whether the secretion of type III
306 secreted proteins is downregulated under reducing conditions, we cultured Tohama I in
307 ascorbic acid-deprived SS medium (SS_AsA$^-$, Fig. 9, “none”) and in SS_AsA$^-$ medium
308 containing the reducing agents ascorbic acid (AsA), 2-mercaptoethanol (2-ME) or
309 dithioerythritol (DTE), respectively. The prepared WCL and Sup samples were analyzed by
310 western blotting with anti-BteA, anti-BopB, or anti-FHA antibodies (Fig. 9). The BteA and
311 BopB signal intensities of both WCL and Sup samples were decreased in the presence of
312 each of the reducing agents (Fig. 9). In contrast, the FHA signal intensities of both WCL and
313 Sup samples were not affected by the reducing agents. The growth of bacteria cultured in
314 SS medium with reducing agents was not significantly different from that of the bacteria
315 cultured in SS medium (data not shown). These results suggest that the production and
316 secretion of type III secreted proteins in *B. pertussis* are downregulated under reducing
317 conditions.
318
319 **DISCUSSION**
320 In this study, *B. pertussis* increased the production and secretion of type III secreted proteins,
321 BteA and BopB, when cultured in ascorbic acid-deprived SS medium (SS_AsA$^-$). We
322 successfully produced cytotoxicity against mammalian cells by the infection of wild-type
323 Tohama I cultured in SS 1/20AsA_1/2CaA medium. *B. pertussis* decreased the production
324 and secretion of type III secreted proteins when cultured in SS_AsA$^-$ medium containing
325 reducing agents such as 2-ME or DTT. These results suggest that redox signaling is
326 involved in regulation of the T3SS in *B. pertussis*.
327 It has been reported that no secretion of the type III secreted proteins BteA and Bsp22
328 was detected from Tohama I (31), and that the secretion of the type III secreted proteins
329 BteA and BopD was detected from a non-vaccine-type strain, BP157. However, BP157
330 showed no cytotoxicity against L2 cells, J774 mouse macrophage-like cells, and HeLa cells

331 (32). In another report, glutamate limitation upregulates production of the type III secreted
332 proteins (33). However, the requirement of a type III secretion system (T3SS) for virulence in
333 *B. pertussis* thus remains unclear. On the other hand, a BscN-deficient mutant in which the
334 T3SS was inactive colonized mouse lungs to a significantly lower degree compared to the
335 wild-type (31). In addition, cytotoxicity against HeLa cells was observed by infection with a
336 BspR (a negative regulator for type III secreted proteins)-deficient *B. pertussis* strain (15).
337 These results suggest that the T3SS has a significant role in the virulence of *B. pertussis*.
338 The production and the relative amounts of *bopB* mRNA of Tohama I cultured in SS
339 AsA^{-} medium were significantly increased compared to those of Tohama cultured in SS
340 medium, but this was not the case for BteA mRNA (Figs. 2, 5). It was reported that *bteA* and
341 *bopB* genes are upregulated by BtrS (a sigma factor for type III secreted proteins) and
342 downregulated by BspR (13, 15). It has also been reported that the amount of *bteA* mRNA
343 was increased 2-fold in a BspR-deficient strain compared to the wild-type, whereas the
344 amount of *bopB* mRNA was increased by 18-fold compared to the wild-type by RNA-seq
345 (15). The *bopB* gene is localized in the T3SS apparatus locus (*bsc* locus) adjacent to the
346 *btr*S gene. Since the *bteA* gene is separated from the *bsc* locus by >2.5 Mb (9), BtrS would
347 preferentially regulate transcription of the *bopB* gene over that of the *bteA* gene.
348 The cytotoxicity against L2 cells was observed by the infection of Tohama I cultured in
349 SS_1/20AsA _1/2CaA medium (Fig. 6). Before infection, we exchanged Ham's F-12K
350 medium for the SS_1/20AsA _1/2CaA medium. When L2 cells were infected with Tohama I
351 in Ham's F-12K medium, the cytotoxicity against L2 cells was not observed (data not shown).
352 We therefore speculate that a substance which inhibits the secretion of type III secreted
353 proteins from Tohama I was present in the Ham's F-12K medium, or a substance that is
354 necessary for the secretion of type III secreted proteins from Tohama I was absent in the
355 Ham's F-12K medium. We found that infection of the human alveolar epithelium cell line
356 A549 with wild-type *B. pertussis* cultured in SS_1/20AsA _1/2CaA medium did not result in

357 cytotoxicity (data not shown). For the observation of cytotoxicity against A549 cells, it is
358 necessary to optimize the conditions for the production and secretion of type III secreted
359 proteins in *B. pertussis*.

360 A previous study showed that cytotoxicity against the cell line J774A.1 was achieved
361 by infection with wild-type *B. pertussis* 18323, but not by infection with a CyaA-deficient
362 strain (34). This finding suggested that CyaA has the potential to provoke the cytotoxicity.
363 However, we confirmed herein that the production and secretion of CyaA in a BteA-deficient
364 strain (MGKU3) were not significantly different from those of the wild-type (data not shown).
365 In our results, the amounts of LDH released from L2 cells infected with MGKU3 were greater
366 than those of the T3SS-inactive strain (MGKU2), and no LDH release was detected from L2
367 cells infected with MGKU2 (Fig. 8). These results raised the possibility that an unknown type
368 III effector other than BteA is involved in the cytotoxicity of *B. pertussis*.

369 Although we observed no significant difference in the growth rate of Tohama I
370 between SS medium and SS_AsA$^-$ medium (data not shown), the production and secretion
371 of type III secreted proteins of Tohama I cultured in the SS_AsA$^-$ medium were increased
372 compared to those in the SS medium (Fig. 2). It had not been determined whether the
373 expression of type III secreted proteins is regulated by reducing agents at the molecular
374 level, and in this study, the production and secretion of CyaA and FHA in Tohama I cultured
375 in SS medium were not significantly different from those in SS_AsA$^-$ medium (Fig. 2). It was
376 reported that there is no difference in Prn production by Tohama I cultured in modified SS
377 medium compared to culture in modified SS medium containing dithiothreitol (DTT), a
378 reducing agent (35). These results suggest that BvgAS-regulated virulence factors other
379 than type III secreted proteins are not affected by reducing agents.

380 Ascorbic acid, urea, and glutathione (GSH) function as reducing agents in the
381 epithelial lining fluid (ELF) of the human airway. The concentrations of ascorbic acid and
382 urea in ELF in the upper respiratory tract are not significantly different from those in the

383 lower respiratory tract. However, the concentration of GSH in the ELF in the upper
384 respiratory tract is 100-fold lower than that in the lower respiratory tract. One of the
385 possibilities is that GSH forms GS-SG (an oxidized from of GSH) in the ELF in the upper
386 respiratory tract (36). It may thus be considered that the upper respiratory tract is more
387 oxidized than the lower respiratory tract. It was reported that the transcription of genes
388 encoding type III secreted proteins of *B. pertussis* recovered from mouse nasal lavage was
389 higher than that from bronchoalveolar lavage (37). We thus speculate that the T3SS is
390 important for *B. pertussis* to colonize the upper respiratory tract of the host.
391 In this study, the production and secretion of BopB by Tohama I cultured in the
392 SS_AsA$^-$ medium were significantly increased compared to those in SS medium (Fig. 2).
393 Bacteria use redox-sensing proteins to resist oxidative stress (38). For example, OxyR (a
394 member of the LysR family of transcriptional regulators) of *Escherichia coli* is converted to
395 an active mode by the oxidation of intramolecular cysteine residues, which then increases
396 the expression of antioxidants genes, e.g., hydroperoxidase I [*katG*, (39)]. Vfr (a member of
397 the CRP/FNR superfamily) of *Pseudomonas* forms an active mode by retaining
398 intramolecular cysteine residues as reducing states, and then increases the expression of
399 type III secreted proteins (40). Btr is a regulator protein produced by *B. pertussis* and is
400 homologous to Vfr (41). A cysteine cluster is located in the N-terminal domain of Btr, and a
401 helix-turn-helix DNA binding domain is located in the C-terminal domain of Btr. Therefore,
402 when *B. pertussis* is cultured in reducing agent-deprived SS medium, Btr might be oxidized
403 and change its conformation, thereby increasing the expression of type III secreted proteins.
404 Although the signal pathway that *B. pertussis* uses to increase the production of type III
405 secreted proteins is unclear, it is possible that Btr regulates gene expressions for type III
406 secreted proteins.
407
408

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541

542 **Figure legends**

543 **Fig. 1.** BteA and BopB production by *B. pertussis* cultured in LCA medium. Tohama I was
544 cultured in SS or LCA medium. Whole cell lysates (WCL) and supernatant fractions (Sup)
545 samples were separated by SDS-PAGE and analyzed by western blotting with antibodies
546 against BteA (*upper panel*) or BopB (*lower panel*). BteA forms SDS-resistant multimers (10).
547 NS: nonspecific signals. Loaded WCL and Sup samples were prepared from an equal
548 volume of bacterial culture. Experiments were performed at least three times, and
549 representative data are shown.

550

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

559

560 **Fig. 3.** Effects of ascorbic acid on type III secreted proteins. Whole cell lysates and secreted
561 proteins prepared from Tohama I cultured in SS medium in the presence of ascorbic acid at
562 the final concentration of 91, 454 or 2270 μM were separated by SDS-PAGE and then
563 analyzed by western blotting with antibodies against BteA or BopB. NS: nonspecific signals.
564 Loaded WCL and Sup samples were prepared from an equal volume of bacterial culture.
565 Experiments were performed at least three times, and representative data are shown.
566
567 **Fig. 4.** Effects of ascorbic acid on the secretion of BteA and BopB in clinical isolates cultured
568 in SS_AsA$^-$ medium. The *B. pertussis* clinical isolates BP344, BP348, BP350, and BP351
569 were cultured in SS (+) or SS_AsA$^-$ (–) medium. WCL and Sup samples were separated by
570 SDS-PAGE and analyzed by western blotting with antibodies against BteA (*upper panel*) or
571 BopB (*lower panel*). NS: nonspecific signals. Loaded WCL and Sup samples were prepared
572 from an equal volume of bacterial culture. Experiments were performed at least three times,
573 and representative data are shown.
574
575 **Fig. 5.** Results of the RT-PCR analysis for mRNA levels of *bteA* and *bopB* from *B. pertussis*
576 cultured under ascorbic acid-starved conditions. Total RNA was prepared from Tohama I or
577 BP350 cultured in SS or SS_AsA$^-$ medium and subjected to a quantitative RT-PCR analysis.
578 The histograms show the relative amounts of *bteA* (**A**) and *bopB* (**B**) mRNA normalized by
579 the housekeeping gene, *recA* mRNA. Error bars: the standard error of the mean (SEM) from
580 triplicate experiments. *$p<0.05$; N.S.: no significant difference. Experiments were performed
581 at least three times, and representative data are shown.
582
583 **Fig. 6.** Cytotoxic activity of *B. pertussis* cultured in SS_AsA$^-$ medium. L2 cells were infected
584 with Tohama I cultured in SS medium, SS_AsA$^-$ medium, SS medium containing one-
585 twentieth of ascorbic acid (SS_1/20AsA), SS medium containing one-twentieth of ascorbic

586 acid and one-half of casamino acids (SS_1/20AsA_1/2CaA), or SS medium containing one-
587 twentieth of ascorbic acid and no casamino acids (SS_1/20AsA_CaA^{-}) at an MOI of 500 for
588 3 hr. +, 1/2 and 1/20 show the amounts of indicated substances that are equal to, one-half of,
589 and one-twentieth of those in SS medium, respectively. The amounts of LDH released into
590 the extracellular medium from infected cells are shown, and the relative cytotoxicity (%) was
591 determined as described in the Materials and Methods section. Error bars: the SEM from
592 triplicate experiments. n.a.: No available result. Experiments were performed at least three
593 times, and representative data are shown.
594
595 **Fig. 7.** Immunofluorescent staining of Bsp22 on L2 cells infected with *B. pertussis*. **A:** L2
596 cells were infected with Tohama I or BP350 in the indicated media at an MOI of 125 for 3 hr.
597 +, 1/2 and 1/20 indicate the amounts of indicated substances that are equal to, one-half of,
598 and one-twentieth of those in SS medium, respectively. After fixation, cells were stained with
599 anti-Bsp22 antibody (*green*) and rhodamine phalloidin (*red*). *Arrowheads* indicate the Bsp22
600 (*green*) signal. **B:** Bsp22 signals per one cell were counted under a fluorescent microscope.
601 At least 120 cells were randomly chosen. Error bars: the SEM from triplicate experiments.
602 *$p<0.05$; N.S.: no significant difference. Experiments were performed at least three times,
603 and representative data are shown.
604
605 **Fig. 8.** Cytotoxic activities of *B. pertussis* mutants. The results of LDH assays at an MOI of
606 125 (**A**) or 250 (**B**) are shown as a histogram. Error bars: the SEM from triplicate
607 experiments. *$p<0.05$. Experiments were performed at least three times, and representative
608 data are shown. **C:** MGKU1 (Tohama I nalidixic acid-resistant strain, 1: WT Nal^{r}), MGKU2
609 (T3SS-inactive strain, 2: ΔT3SS), MGKU3 (BteA-deficient strain, 3: ΔBteA), or MGKU4
610 (BspR-deficient strain, 4: ΔBspR) was cultured in SS_1/20AsA_1/2CaA medium. WCL and
611 Sup samples were separated by SDS-PAGE and analyzed by western blotting with anti-BteA

612 antibody. We loaded a 3-fold smaller amount of MGKU4 supernatant fraction on the SDS-
613 PAGE gel to avoid obtaining an excess signal intensity. NS: nonspecific signals. Loaded
614 WCL and Sup samples were prepared from an equal volume of bacterial culture.
615 Experiments were performed at least three times, and representative data are shown.
616
617 **Fig. 9.** Effects of reducing agents on the production and secretion of type III secreted
618 proteins in *B. pertussis*. Tohama I was cultured in SS_AsA^{-} medium or SS_AsA^{-} medium
619 containing ascorbic acid (AsA), 2-mercaptoethanol (2-ME), or dithioerythritol (DTE) at final
620 concentrations of 2.3, 2.1, or 3.0 mM, respectively. WCL and Sup samples were separated
621 by SDS-PAGE, then analyzed by western-blotting with antibodies against BteA, BopB, or
622 FHA. Loaded WCL and Sup samples were prepared from an equal volume of bacterial
623 culture. NS: nonspecific signals. Experiments were performed at least three times, and
624 representative data are shown.
625

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.

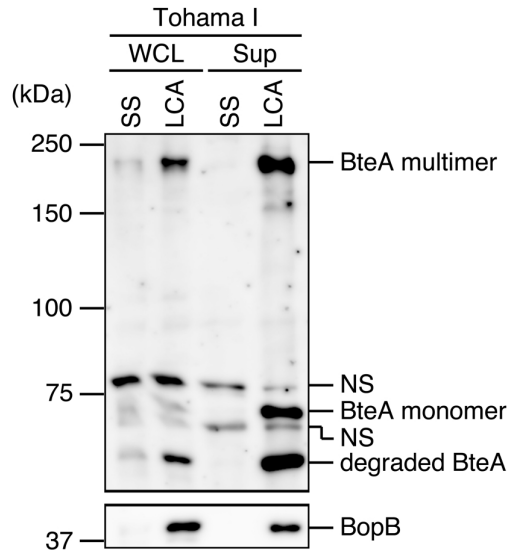


Fig. 1. BteA and BopB production by *B. pertussis* cultured in LCA medium. Tohama I was cultured in SS or LCA medium. Whole cell lysates (WCL) and supernatant fractions (Sup) samples were separated by SDS-PAGE and analyzed by western blotting with antibodies against BteA (*upper panel*) or BopB (*lower panel*). 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.

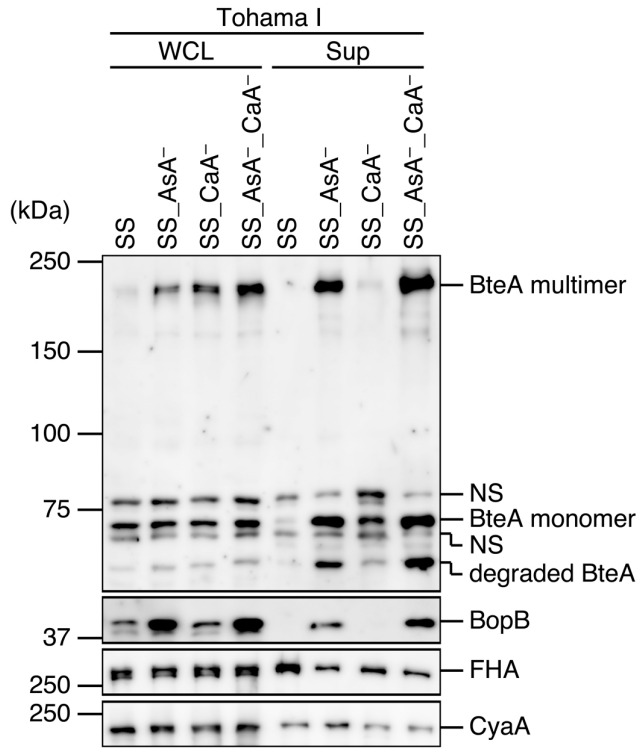


Fig. 2. Effects of ascorbic acid and casamino acids on Bvg-regulated virulence factors. Tohama I was cultured in SS medium or ascorbic acid-deprived SS medium (SS_AsA⁻), casamino acid-deprived SS medium (SS_CaA⁻) or both ascorbic acid- and casamino acid-deprived SS medium (SS AsA⁻_CaA⁻). WCL and Sup 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.

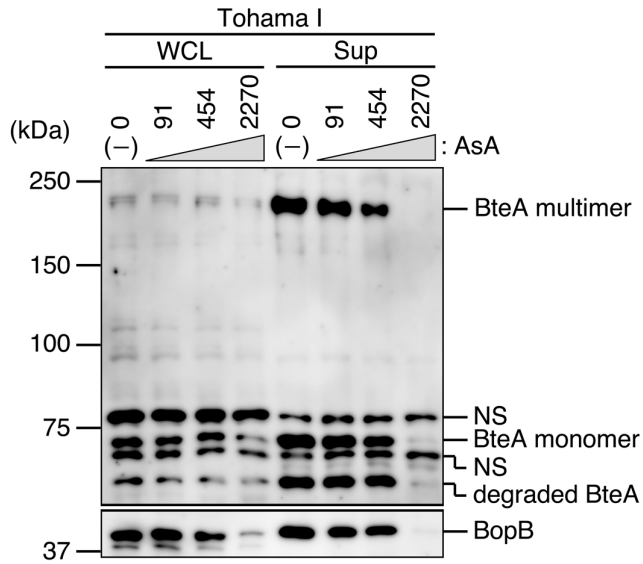


Fig. 3. Effects of ascorbic acid on type III secreted proteins. Whole cell lysates and secreted proteins prepared from Tohama I cultured in SS medium in the presence of ascorbic acid at the final concentration of 91, 454 or 2270 μM were separated by SDS-PAGE and then analyzed by western blotting with 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.

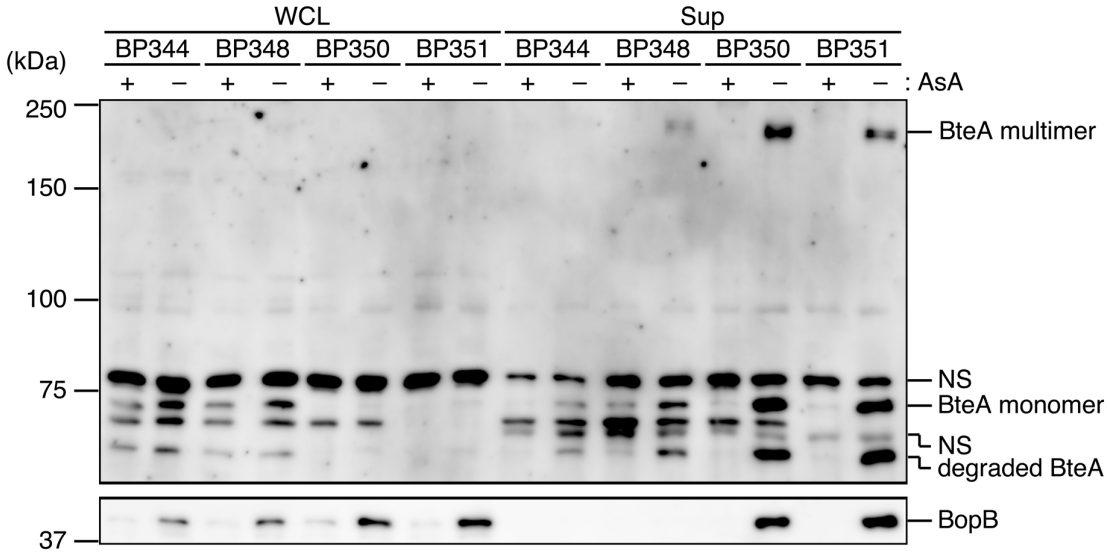


Fig. 4. Effects of ascorbic acid on the secretion of BteA and BopB in clinical isolates cultured in SS_AsA⁻ medium. The *B. pertussis* clinical isolates BP344, BP348, BP350, and BP351 were cultured in SS (+) or SS_AsA⁻ (–) medium. WCL and Sup samples were separated by SDS-PAGE and analyzed by western 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.

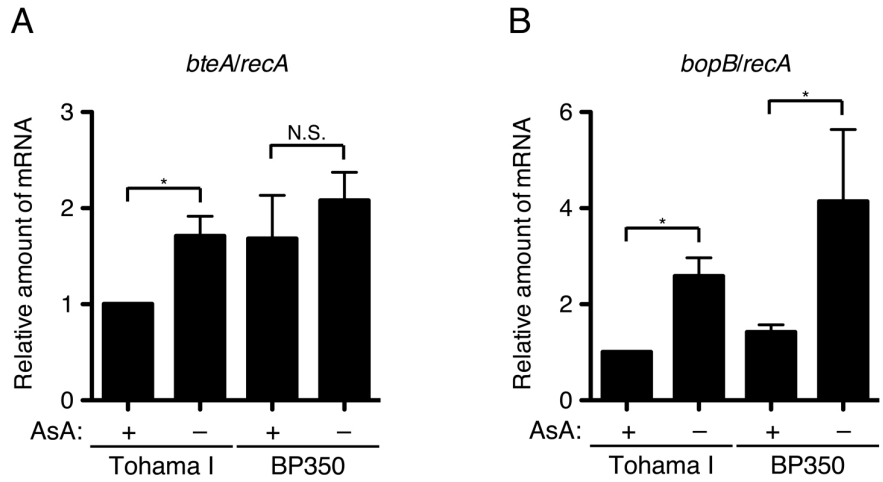


Fig. 5. Results of the RT-PCR analysis for mRNA levels of *bteA* and *bopB* from *B. pertussis* cultured under ascorbic acid-starved conditions. Total RNA was prepared from Tohama I or BP350 cultured in SS or SS_AsA^{-} medium and subjected to a quantitative RT-PCR analysis. The histograms show the relative 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.

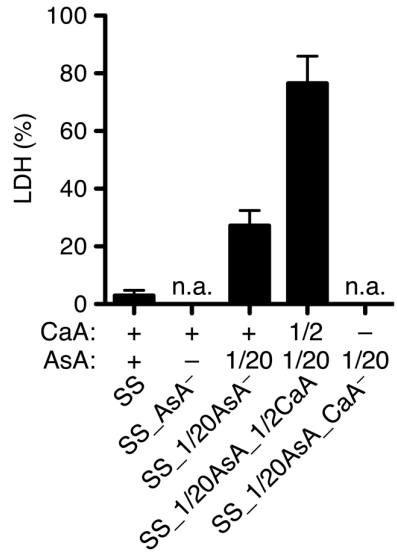


Fig. 6. Cytotoxic activity of *B. pertussis* cultured in SS_AsA^{-} medium. L2 cells were infected with Tohama I cultured in SS medium, SS_AsA^{-} medium, SS medium containing one-twentieth of ascorbic acid (SS_1/20AsA), SS medium containing one-twentieth of ascorbic acid and one-half of casamino acids (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.

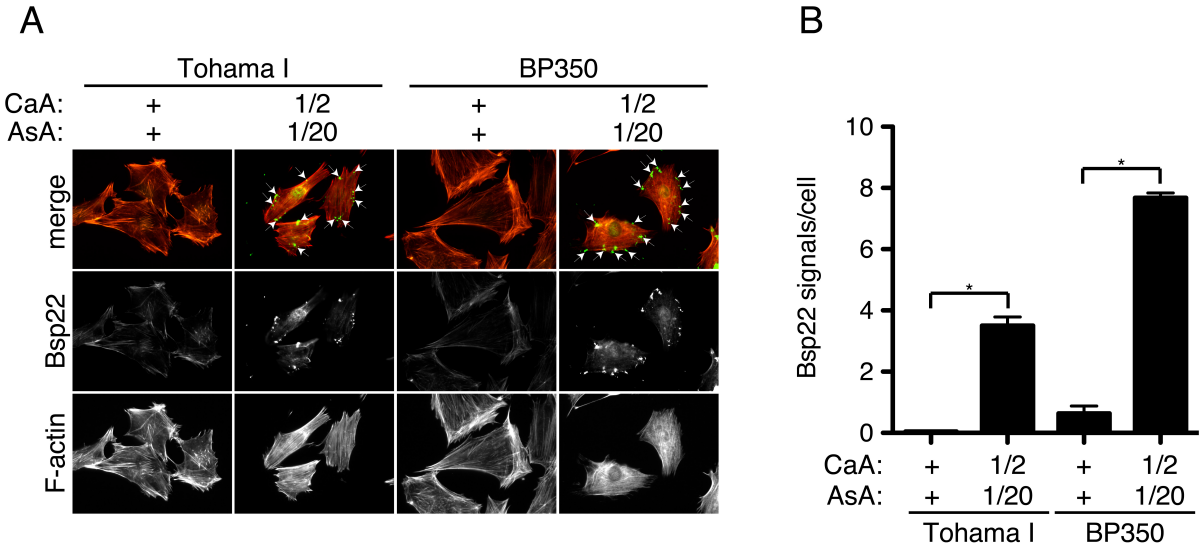
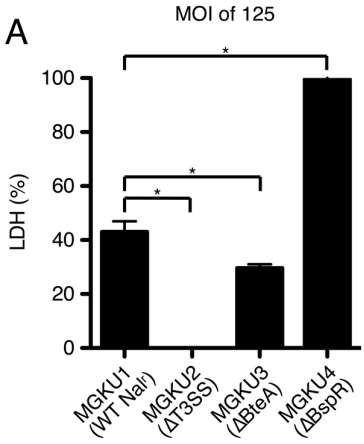
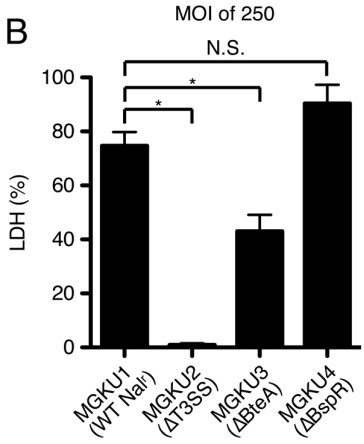


Fig. 7. Immunofluorescent staining of Bsp22 on L2 cells infected with *B. pertussis*. **A:** L2 cells were infected with Tohama I or BP350 in the indicated media at an MOI of 125 for 3 hr. +, 1/2 and 1/20 indicate the amounts of indicated substances that are equal to, one-half of, and one-twentieth of 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.





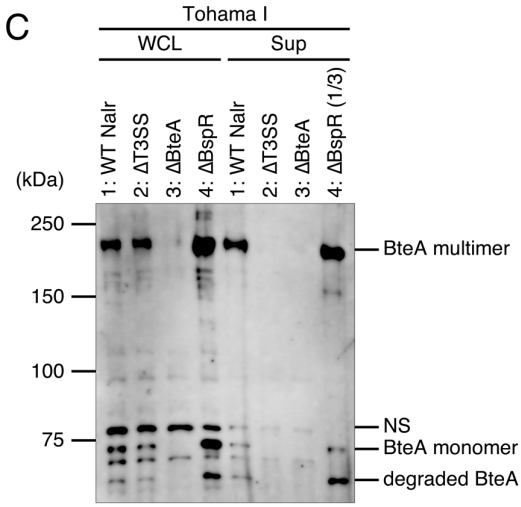


Fig. 8. Cytotoxic activities of *B. pertussis* mutants. The results of LDH assays at an MOI of 125 (**A**) or 250 (**B**) are shown as a histogram. Error bars: the SEM from triplicate experiments *p<0.05. Experiments were performed at least three times, and representative data are shown. **C:** MGKU1 (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.

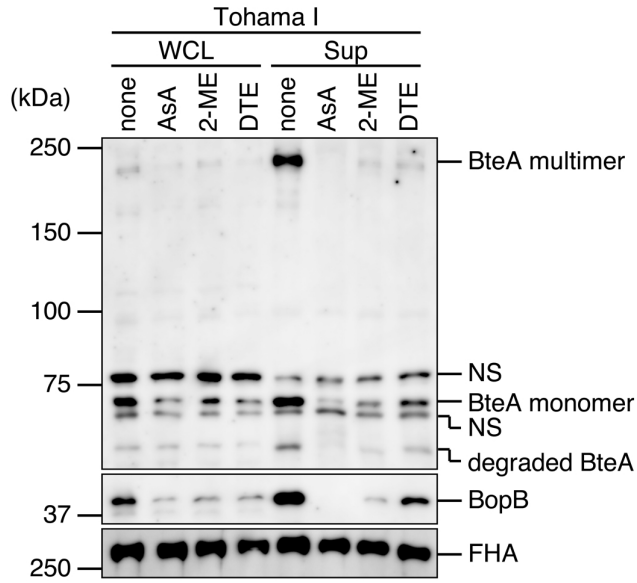


Fig. 9. Effects of reducing agents on the production and secretion of type III secreted proteins in *B. pertussis*. Tohama I was cultured in SS_AsA⁻ medium or SS_AsA⁻ medium containing ascorbic acid (AsA), 2-mercaptoethanol (2-ME), or dithioerythritol (DTE) at final concentrations of 2.3, 2.1, or 3.0 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.